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Proceedings (in part) of the Pertussis Toxin Conference held at the National Institutes of Health on September 20 and 21, 1984

PERTUSSIS TOXIN

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PREFACE

The contents of this volume are based, in part, on the proceedings of the Pertussis Toxin Conference held at the National Institutes of Health on September 20 and 21, 1984. This conference, sponsored jointly by the National Institute of Child Health and Human Development and by the National Heart, Lung and Blood Institute, was organized to bring together investigators sharing an interest in the action of pertussis toxin from the perspective of the toxin itself and from that of the toxin's multiple pharmacologic actions. It was timed to overlap with the Bacterial Vaccine Symposium (held at the National Institutes of Health on September 17–20, 1984) in the hope of including investigators interested in the potential role of pertussis toxin in the development of new acellular pertussis vaccines.

Research efforts leading to purification of pertussis toxin and to the elucidation of its multiple biologic activities originated, for the most part, in laboratories attempting to understand the mechanisms of *Bordetella pertussis* pathogenesis and immunity. As occurred with cholera toxin, pertussis toxin proved to be an invaluable probe in exploring the mechanisms for adenylate cyclase regulation. Studies with pertussis toxin have clearly established the role of the transducing protein N_i, first postulated by Rodbell and co-workers, in hormone-mediated regulation of adenylate cyclase. In addition, pertussis toxin-catalized ADP ribosylation of N_i served as an effective tool permitting purification, to homogeneity, of this regulatory element. Studies performed in several distinct systems confirm the role of pertussis toxin in blocking the action of hormones that modulate adenylate cyclase by inhibition of catalytic activity and also demonstrate an associated reduction in hormone-dependent GTPase activity. While many aspects of N_i function, at the molecular level, remain to be elucidated, data have been presented which indicate that the pertussis toxin-mediated modification of N_i leads to an uncoupling of hormone receptor from adenylate cyclase, probably by a mechanism involving blockade of high affinity receptor generation. N_i, however, may play a significant role in systems distinct from receptor-mediated inhibition of adenylate cyclase. Pertussis toxin treatment has been shown to enhance forskolin stimulation of adenylate cyclase and has been suggested as having effects on calcium mobilization and phospholipid turnover. Since N_i is present in membrane preparations at levels exceeding N_s, it is possible that N_i is coupled to second messenger systems distinct from cAMP.

Pertussis toxin, when administered *in vivo*, elicits a variety of responses including lymphocytosis, adjuvant activity (an IgE specific response), sensitization to histamine, and disruption of glucose regulation. While it is tempting to attribute these actions to toxin-catalyzed ADP-ribosylation of N_i, this extrapolation must be made with care. As shown in this volume by Ui and co-workers, pertussis toxin effects are not limited to actions mediated by ADP-ribosylation of N_i. Pertussis toxin B-oligomer (i.e., the subunits lacking ADP-ribosyltransferase activity) functions as an effective mitogen, and chemical modification of toxin subunits leads to selective loss of specific biologic activities, while others remain intact. Thus, interpretation of data generated after pertussis toxin treatment must take into consideration alternative mechanisms distinct from N_i modification.

Bordetella pertussis is the microbial agent responsible for the disease commonly known as whooping cough. Antibody response to toxin plays an important role in recovery from infection and in protection in disease. While the exact mechanisms are not understood it is likely that pertussis toxin plays a role in aiding the organism establish and maintain an infection. Since the net effect of pertussis toxin action is elevation of intracellular cAMP, the importance of cAMP in the regulation of the immune response must be considered. In this light, two papers have been included in this volume discussing the adenylate cyclase produced by *Bordetella pertussis*. This agent also functions to elevate the levels of cAMP in intoxicated cells, emphasizing the importance of cAMP regulation to the immune system.

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BIOLOGICAL ACTIVITIES OF PERTUSSIGEN (PERTUSSIS TOXIN)

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I. INTRODUCTION

The observations of Greenberg and Fleming in 1947 (1) and Parfentjev and Goodline in 1948 (2) marked the beginning of numerous investigations into the biological activities of pertussis vaccine. Greenberg and Fleming showed that pertussis vaccine enhanced the production of antibodies to antigens, whereas Parfentjev and Goodline described the increased susceptibility of pertussis vaccine-treated mice to histamine. Soon thereafter, the vaccine was found to increase the susceptibility of mice to anaphylaxis (3), serotonin, combinations of histamine and serotonin, endotoxin, and many other shock-enhancing agents (4). It also induced a hypersensitivity to cold stress, X-rays, and many other forms of shock. Interestingly, it had a profound potentiating action on the production of IgE isotype of antibodies to an antigen given with it (5). The vaccine also promoted the induction of experimental allergic encephalomyelitis (EAE) in mice (6) and rats (7); it induced a marked leukocytosis with a predominance of small lymphocytes (8); increased the production of insulin in rats and mice (9), and had other effects (4).

When we started to investigate *Bordetella pertussis* in 1953, our main concern was, as it still is for many workers, to develop a less reactogenic pertussis vaccine. To this end, we attempted to separate the mouse-protective antigen (MPA) from histamine-sensitizing factor (HSF), endotoxin

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(LPS) and dermonecrotic toxin (DNT). We soon learned that it was difficult, if not impossible, to separate the MPA from the HSF, although we could separate the MPA from the LPS, DNT, and many other antigens of the cell. When we finally obtained HSF in a reasonably pure form we called it pertussigen (4) to distinguish it from other toxins and antigens of B. pertussis (DNT, LPS, agglutinogens, filamentous hemagglutinin) as well as to emphasize that many of the descriptive names used for this factor (histamine-sensitizing factor, lymphocytosis-promoting factor, islet-activating factor, heat-labile adjuvant, late-appearing toxicity factor, etc.) were different names for the same substance. We thought of calling it pertussis toxin, but this term had already been used for the DNT (10, 11, 12). Pertussigen means pertussis-inducing substance, and thus is equivalent to the name pertussis toxin proposed by Pittman (13). Throughout this paper I will refer to this substance as Ptx, which can be translated as pertussigen or pertussis toxin, two terms that are synonymous, just as choleragen is to cholera toxin.

II. PURIFICATION AND ACTIVITIES OF PTX

The purification of Ptx took many years to accomplish. Before relatively simple methods were developed, we had to learn to keep Ptx in solution, to keep it from becoming attached to various surfaces and particles, and to keep it in an active form. The use of high salt-containing buffers, the use of supernatants of older cultures, and the development of a good synthetic medium that supported the growth of smooth cells, that produced significant amounts of Ptx (4), were factors that facilitated the development of the newer methods of purification. The most helpful findings were the development of a rapid semi-quantitative method to assay Ptx by its ability to agglutinate erythocytes in a typically granular fashion (14); the observation that Ptx could be adsorbed to and eluted from various particulate material, such as hydroxylapatite (4, 15) and Affi-Gel Blue (16), and the development of affinity columns made of either specific anti-Ptx antibodies (17) or with sialic acid containing proteins such as haptoglobulin (18) and fetuin (16). With this information at hand, simple methods to produce highly purified Ptx have now been developed (15-20). One of the simpliest methods is that recently published by Sekura et al. (16). In four steps, 1) adsorption of Ptx on Affi-Gel Blue and elution with high salt concentration; 2) adsorption

to and an elution from a fetuin-Sepharose column; 3) desalting by passing through a Sephadex G 25 column; and 4) precipitation with ammonium sulfate, this method yields a product of high purity.

When crystallized Ptx was tested for its biological effects, it was found to have most of the biological activities of pertussis vaccine, including the ability to protect mice from intracerebral (IC) (21) or respiratory tract infections with *B. pertussis* (22).

Small amounts of the purified toxin had remarkable *in vivo* biological activities (Table I). One of the most sensitive *in vivo* actions of Ptx was its ability to stimulate the IgE isotype of antibodies in mice to an antigen given at the time of Ptx administration. A dose of 0.1 ng induced a significant increase in the titer of IgE antibodies to hen's egg albumin in C57Bl mice, whereas it took 1 ng to increase the IgG_1 antibodies to the same antigen.

TABLE I. Some *in vivo* activities of Ptx[a]

Activity	Dose required in ng	Reference
Histamine-sensitization	0.5	21
Increase permeability of capillaries of striated muscle	0.5	21
Leukocytosis and lymphocytosis	8-40	21
Enhance insulin secretion	2	21
Immunopotentiating:		
IgE	0.1	21
IgG_1	10	21
Anaphylaxis	9.5	21
EAE (rats)	20	21
EAE	250	23
Lethal	546	21
Reduction in migration of peritoneal macrophages	80	24
Elevation of cerebellar cyclic GMP (rats)	25000	25
Protection of mice with detoxified Ptx	1700	21

[a]Unless otherwise indicated, all the activities were measured in the mouse.

In a dose of 0.5 ng Ptx increased the susceptibility of CFW female mice to histamine and increased the permeability of capillaries in striated muscle. A dose of 2 ng stimulated insulin secretion, 9.5 ng increased the susceptibility to anaphylaxis, 8-40 ng induced lymphocytosis, 20 ng increased the susceptibility of Lewis rats to hyperacute EAE, 250 ng facilitated the induction of EAE in SJL/BALB/c female mice (23), 80 ng reduced the migration of macrophages to the peritoneal cavity in rats (24), and 25000 ng increased the cerebellar cyclic GMP in rats (25). Ptx was lethal to mice at a dose of 546 ng (21). When detoxified with glutaraldehyde, a dose of 1700 ng protected them from an IC challenge with *B. pertussis* (21).

Many *in vitro* effects of Ptx on cells have recently been described. Some of these actions can be induced with picogram amounts. In Table II, some of these *in vitro* actions are listed. In rat pancreatic islet β-cells, a reversal of the epinephrine inhibition of glucose-induced insulin release can be demonstrated with 1 pg of Ptx per ml (26); the enhancement of insulin secretion and cAMP accumulation induced by glucose with 10 pg (27, 28); the induction

TABLE II. Some *in vitro* activities of Ptx on cells

Cell type	Biological action	Dose	Reference
Rat pancreatic islet B-cell	Reversal of epinephrine inhibition of glucose-induced insulin release	1 pg/ml	26
	Enhanced insulin secretion and cAMP accumulation induced by glucose	10 pg/ml	27
Rat C6 glioma cells	Enhanced cAMP accumulation and adenylate cyclase activity induced by isoproterenol or GTP	1 pg/ml	28
Rat heart cells	Enhanced cAMP accumulation induced by β-adrenergic or glucogon receptors	50 ng/ml	29

(continued)

TABLE II (continued)

Cell type	Biological action	Dose	Reference
Chinese hamster ovary cells	Clustered growth	30 pg/ml	30
Human lymphocytes	Inhibition of cAMP accumulation induced by isoproterenol or PGE_1	5 ng/$2X10^6$ cells	31
Neuroblastoma X glioma (NG 108) cells	Enhanced cAMP accumulation induced by PGE_1	350 pg/ml	32
Rat adipocytes	Stimulation of lipolysis	300 pg/10^6 cells	33
Mouse peritoneal macrophages	Inhibition of migration	1 ng/ml	24
Human and murine T-lymphocytes	Mitogenesis	250-500 ng/$5X10^5$ cells	34-35
Erythrocytes	Agglutination goose cells	8-17 ng/well	18
	chicken cells	125-166 ng/well	18

of clustered growth of Chinese hamster ovary cells with 30 pg of Ptx per $5x10^3$ cells (30); the enhancement of the accumulation of cAMP induced by prostaglandin E_1 (PGE_1) in the hybrid neuroblastoma X glioma (NG 108) cells with 350 pg per ml (32); the stimulation of lipolysis in rat adipocytes with 300 pg per 10^6 cells (33); the inhibition of migration of mouse peritoneal macrophages with 1000 pg (24); and mitogenesis to human and murine T-lymphocytes with 250-500 ng per $5x10^5$ cells (34, 35). I am certain that many other actions of Ptx on cells will be found as investigations in this field continue at an accelerated pace.

In the rest of this paper, I will briefly describe some of the most important findings regarding the histamine-sensitizing, the lymphocytosis-promoting, and the immunopotentiating actions of Ptx. I will not discuss its highly important action of insulin stimulation, its action on the N_i regulatory cell membrane protein and other biochemical and genetic aspects of this problem. These topics will be covered by others in this conference.

III. HISTAMINE-SENSITIZING ACTION OF PTX

In the book "Bordetella pertussis. Immunological and other Biological Activities" (4) Bergman and I described our observations on the histamine-sensitizing action of Ptx. Most of the work was done with impure soluble preparations of Ptx, but I feel certain that similar findings could be obtained with pure Ptx. I will only summarize here what I consider were the most important findings (4).

As was shown by Parfentjev and Goodline (2), the increased susceptibility of pertussis vaccine-treated mice to histamine was about 200-fold. This action has been induced in CFW female mice with as little as 0.5 ng of crystalline Ptx. Increased sensitivity to histamine was demonstrable as early as 90 min after administration of Ptx, and persisted undiminished for 21 days. After this day the sensitivity decreased, but it was still detectable 85 days later when the LD_{50} of histamine for normal mice was 15 mg and that for Ptx-treated animals only 3 mg. This long lasting effect of Ptx undoubtedly plays an important role in its biological activities. These data suggest Ptx attaches to cell receptors and remains there for a long time. The persistence of Ptx at a site of injection is indicated by the development of an immune inflammatory reaction in foot pads of mice 5-6 days after receiving Ptx into that site (36).

The phenomenon of histamine sensitivity can be observed only in relatively few strains of mice, among which the CFW female is one of the most sensitive. This susceptibility must be genetically controlled, but the exact gene or genes controlling it have not been determined. Most strains of mice do become highly sensitive to the combined challenge of histamine and serotonin (4). Of some 34 strains of mice tested by various workers, only the C_3H/H_2N was clearly resistant to a challenge with either histamine, serotonin, or a combination of the two amines. The resistance of most strains of mice to histamine is most likely due to efficient detoxification of this amine. Likewise resistance to

serotonin also may be due to efficient detoxification of this substance, but this hypothesis has not been experimentally tested.

The development of histamine sensitivity is subject to various factors. Stress induced by chemical, physical, or dietary factors makes the CFW mouse more resistant to sensitization (4). Very young mice (less than 5 weeks old) are less susceptible to histamine challenge than are adult mice (older than 5 weeks of age). It is thus meaningless to grade the challenge dose of histamine on a body weight basis. Male mice are less susceptible than female mice to sensitization by Ptx. The effect of Ptx on the SD_{50} of histamine shows an inverse quantitative relationship: as the dose of Ptx is increased, the SD_{50} of histamine decreases to a minimum point beyond which further increase of Ptx has no additional effect. This relationship may indicate that Ptx reacts with cell receptors and that when most of them are blocked no further action can be observed. With very small doses of Ptx, the LD_{50} of histamine was greater than 8 mg. As the Ptx dose was increased by 2-fold increments, the histamine LD_{50} decreased to 3.42, 1.3, 0.81, 0.48 mg in an almost straight line relationship. At doses of 2.5 μg or higher of the crude extract used, a plateau of sensitivity was reached beyond which further increases of Ptx caused no significant decrease in histamine LD_{50}. The lowest LD_{50} of histamine was at the highest doses of Ptx used (4).

The observations on the effect of stress on histamine sensitivity focused our attention on the role of the adrenal glands on this phenomenon. Adrenalectomy was known to render mice as susceptible to histamine as treatment with Ptx (4). Some workers thought that adrenal steroids were important, since large doses of steriods protected mice slightly from histamine death, but in fact it was epinephrine, a hormone produced by the medullary tissue of the adrenals, that was found to protect effectively adrenalectomized or Ptx-treated mice from histamine death (4). Protection of Ptx-treated mice was possible only when the amount of Ptx given to sensitize the mice was not excessive. At high doses of Ptx it appeared that epinephrine could not compensate for the effects mediated by pertussigen. These observations convinced me that Ptx interfered with a necessary function of epinephrine, a function which we thought was needed to maintain the blood volume, possibly by reducing or controlling permeability changes produced by histamine and other vasoactive amines. In the mouse, death from histamine, serotonin, histamine-serotonin, or anaphylaxis is due to loss of blood volume (4). We demonstrated this when mice dying from these forms of shock were protected simply by

restoring blood volume with physiological saline. Epinephrine must be capable of preventing some loss of fluid from the circulation and thus allowing the heart to continue to pump blood throughout the tissues. In normal mice, a histamine shock is thus non-fatal, while in the Ptx-treated animal it leads to death. It is not yet clear which adrenergic receptors are involved, and they may be different in different tissues. According to Wildt et al. (37), Ptx impairs the autonomic responsiveness within the cardiovascular system of the rat, since the α_2 adrenergic responses were reduced while the α_1 adrenergic response was increased. They also found cholinergic dysfunction in the blood vessels and heart. Similar conclusions were reached by Boyer et al. (38) who employed a purified Ptx preparation in the rat and found that the α_2 adrenergic receptors were blocked by this toxin. Wildt et al. (36) also found that Ptx decreased the blood pressure, and reduced total peripheral vascular resistance. In part these hemodynamic changes could have been due to the increased permeability of capillaries in skeletal muscle, as was demonstrated in mice treated with Ptx. We found that in the mouse the skeletal muscle was the only tissue that showed an increased capillary permeability after Ptx injection. It may well be that some agent released normally in this muscle has the ability to increase the permeability of capillaries, and that under the influence of Ptx this action is augmented because some adrenergic function is blocked.

Much still needs to be done before the histamine sensitization or shock enhancing effects of Ptx can be understood fully, but recent studies on the effect of Ptx on adrenergic receptors and on the ADP-ribosylation of the Ni membrane protein involved in the receptor-mediated inhibition of adenylate cyclase may well explain these phenomena.

IV. LYMPHOCYTOSIS PROMOTING ACTIVITY

The pronounced lymphocytosis occurring in children during attacks of pertussis was first noted by Frohlich in 1897 (39). Since then various workers have observed this phenomenon in children and in experimental animals receiving pertussis vaccine or Ptx (4).

Morse and co-workers (40) studied this action of Ptx in mice in detail and found that Ptx increased the total leukocyte count with a predominance of small lymphocytes. The leukocytosis was evident one day after injection of Ptx,

and it reached a peak 4 days later, after which it gradually decreased to normal. Ptx was most effective when given IV or IP and least effective when given SC.

The lymphocytosis induced by Ptx was followed by a marked decrease in the weight of the thymus and lymph nodes, and an increase in the spleen weight (the latter effect may have been influenced by the endotoxin in pertussis vaccine). Morse and co-workers showed that the lymphocytosis was not due to multiplication of lymphocytes, but rather to the prevention of the recirculation of these cells through the lymphoid tissue once released from these organs (40). Antibodies to Ptx prevented its lymphocytosis promoting action.

Recently, Spangrude *et al.* (41) have expanded these observations and showed that when lymphocytes are treated with Ptx *in vitro* their migration to lymph nodes *in vivo* is prevented in a dose- and time-dependent fashion. The toxin-induced alteration of lymphocytes was not lost after extended cultivation of these cells in Ptx-free medium, and Ptx had no effect on the viability or the ability of lymphocytes to respond to mitogenic agents. Spangrude *et al.* proposed "that the mechanism of lymphocyte extravasation involves a specific receptor-mediated binding event followed by an adenylate cyclase-dependent activation of cell motility."

V. IMMUNOPOTENTIATING ACTIVITIES OF PTX

A. IgE Stimulation

One of the most interesting actions of pertussis vaccine is its ability to stimulate antibody production. Because of this property, the vaccine has been used as an adjuvant in innumerable studies (4). As already mentioned, pertussis vaccine is also an effective adjuvant for the production of IgE isotype of antibodies (5). The active adjuvant in pertussis vaccine is Ptx (21). As little as 0.1 ng of Ptx given to a mouse at the time of immunization induces the production of IgE specific for the antigen given. In certain strains of mice receiving only one dose of antigen and Ptx, the antibody produced was almost exclusively of the IgE isotype (unpublished work), but when booster doses of antigen were administered other specific Ig isotypes were also increased to a greater extent than when Ptx was not given. Not much work has been done in this field with

purified Ptx. Iwata et al. (42) have studied the mechanism by which Ptx increases the IgE response, and Dr. Ishizaka will discuss their findings in this conference. I will only mention here that they found Ptx induced T-lymophocytes to produce an enzyme that glycosylated an IgE-binding factor. Glycosylation of this factor converted it from an inhibitor of IgE production into a stimulator. This work of Ishizaka and co-workers may well pave the way to elucidating the mechanism by which Ptx induces various other immunopotentiating actions.

B. Enhancement of Delayed Type Hypersensitivity (DTH)

Although the adjuvant action of pertussis vaccine on antibody response has been well documented (4), its action on DTH has not. Some have reported an actual inhibitory action of pertussis vaccine and Ptx on DTH (43). Using the mouse model, we have found Ptx extremely active in increasing the intensity and duration of DTH. In BALB/c mice sensitized with either hen's egg albumin or keyhole limpet hemocyanin emulsified in complete Freund's adjuvant (CFA), Ptx increased the ear swelling produced by challenge with the specific antigen (44). As little as 25 ng of Ptx given 1 day before or 3 days after sensitization increased the response significantly. The greatest increase in DTH was obtained when Ptx was given at the same time or 3 days after the injection of the antigen emulsion. DTH could be transferred to naive recipients with lymph node cells (LNC) or spleen cells from sensitized donors, but the transfer was most effective, and the reaction lasted longer when the naive recipients were also treated with Ptx at the time of the adoptive transfer. Cells from sensitized donors that had received Ptx were superior to cells from donors that had not received Ptx (45). The effect of Ptx was antigen specific and was mediated by Thy +, L3T4 +, and Ly 2 - T-lymphocytes.

Recently, Sewell et al. (46) found that the DTH response correlated with the production of gamma interferon (IFN-γ). Lymphoid cells from mice given Ptx at the time of immunization released appreciably greater quantities of IFN-γ during culture in the presence of the specific antigen. Cells from sensitized mice that did not receive Ptx produced little or no IFN-γ upon antigen stimulation. The effect of Ptx on antigen-driven IFN-γ production correlated with its effect on the capacity of the same cell populations to transfer DTH responses. After immunization with Ptx, IFN-γ was produced

by lymph node and spleen cells from 7 days onwards, and both cell types produced IFN-γ until at least 30 days after immunization. It is possible that the augmentation of antigen-specific IFN-γ production may contribute *in vivo* to the prolonged DTH reactions induced by Ptx. Ptx also promoted the development of DTH reactions induced in mice by T-cell clones or with anti-idiotypic sera (47).

C. Enhancement of Inflammation

Another expression of DTH may be the inflammation produced by CFA in mice and rats. As early as 1961 Levine and Wenk (48) found that pertussis vaccine greatly enhanced the inflammation and granulomatous response in the foot pads of rats receiving CFA. We recently observed a similar response produced by purified Ptx in mice (49) and rats (unpublished work). This enhancement of the foot pad swelling due to CFA lasted 5 weeks. Ptx was effective when given 3 days before to 3 days after the administration of CFA, but the maximum effect was observed when Ptx was given at the time CFA was injected or up to 3 days later. These results are similar to those obtained with DTH reactions or, as will be seen later, with the induction of experimental allergic encephalomyelitis (EAE). An antigen was required in the CFA to demonstrate this effect of Ptx, since incomplete Freund's adjuvant (IFA) alone was not sufficient. The Ptx effect was demonstrated when an antigen such as hen's egg albumin or keyhole limpet hemocyanin was added to IFA.

Histologically, no qualitative differences in the cellular infiltration were observed, but a striking quantitative difference between the Ptx-treated and the non-treated animals was noticed. This enhancement of inflammation appears to coincide with the increased production of IFN-γ by lymphoid cells obtained from Ptx-treated mice exposed to the specific antigen (46). The production of this lymphokine may well be responsible for the increased inflammation, as seems to be the case in the augmentation of DTH reactions (46).

D. Induction of Experimental Allergic Encephalomyelitis (EAE)

Another expression of DTH markedly enhanced by pertussis vaccine is the development of EAE in rats and mice. This action of pertussis vaccine was first observed in mice by Lee and Olitzky (6) and later in rats and mice by Levine and

co-workers (7). The adjuvant property of pertussis vaccine is due to Ptx (21, 50). In Lewis rats, as little as 20 ng given at the time of immunization with spinal cord homogenate induced the so-called hyperacute EAE in which the paralytic signs appeared early (9-13 days), and the perivascular infiltrates in the central nervous system (CNS) consisted of a mixture of polymorphonuclear leukocytes and monocytes. In addition, fibrin was deposited around the vessels in the CNS. In the Ptx-treated rat, a marked increase in the permeability of the blood-brain barrier occurred at the onset of the disease. It disappeared when the rats recovered 21 days after immunization (50). In mice, many attempts to induce EAE with the aid of pertussis vaccine have produced erratic results. However, extracts rich in Ptx were uniformly effective in promoting EAE (51). Purified Ptx used as an adjuvant promoted the development of EAE in SJL X BALB/c mice (23). The mice received IV 400 ng of Ptx and were immunized with an emulsion of spinal cord homogenate made in CFA containing 4 mg of *M. tuberculosis* H37 Ra per ml of the CFA. With this type of immunization, Ptx could be given IV 1 day before or 5 days after administration of the immunizing emulsion. Ptx given as late as 20 days after immunization still precipitated a mild form of EAE, which appeared 8-12 days later. When Ptx was given 5 days after immunization, a severe but non-fatal disease that persisted for the entire 74-days of observation was produced. Linthicum *et al.* (51) have shown that a marked increase in the permeability of blood-brain barrier occurs at the onset of EAE. Furthermore, an antiserotonin and antihistamine (H_1-blocker) drug (cyproheptadine) given from 6 to 18 days after sensitization prevented the development of clinical and histological signs of EAE in the mouse. This may indicate that humoral factors may be involved in the induction of EAE in the mouse and probably other animals as well.

We also found that some strains of mice previously considered genetically resistant, developed EAE when given 400 ng of Ptx at the time of immunization (52). Furthermore, adoptive transfer of the disease with LNC from sensitized donors was possible if donors, as well as recipients, were treated with Ptx at the time of sensitization of the donors and at the time of cell transfer to the recipients (53).

The induction of experimental allergic orchitis (EAO) has been induced in mice when large doses of pertussis vaccine were given at the time of immunization with the testicular antigen emulsified in CFA. However, erratic results were obtained with different lots of vaccine. The use of Ptx-rich extracts has made it possible to induce EAO

in mice with regularity (54). In this autoimmune disease, capillary permeability in the testes was increased at the time pathologic changes were seen (54).

VI. OTHER ACTIONS OF PTX

The *in vitro* action of Ptx on various cell lines listed in Table II are most likely the result of its ADP-ribosyltransferase activity. This enzyme catalyzes the transfer of ADP-ribose from NAD to the regulatory cell membrane protein N_i. Normally N_i suppresses adenylate cyclase, but when ADP-ribosylated, N_i loses this regulatory function. This leads to an enhanced receptor-mediated activation of membrane adenylate cyclase and a receptor-mediated accumulation of intracellular cAMP (55) that profoundly affects various cell functions. This is probably how Ptx markedly increases the insulin released by rat or mouse pancreatic islet β-cells as a result of a glucose load (55).

Ptx is mitogenic to T-cells *in vitro*, but this action requires the presence of an adherent cell type identified as a B-lymphocyte (56). Spleen cells from athymic nude mice did not respond to the mitogenic action of Ptx. The dose of Ptx required to induce mitogenic action of T-cells was high (250-500 ng per 5 X 10^5 cells) (34, 56), because doses of 1 pg are effective in some *in vitro* tests and as little as 0.1 ng is effective in some *in vivo* tests (see Tables I & II). The requirement of a B-lymphocyte in the mitogenic action of Ptx on T-lymphocytes is not exhibited by other mitogenic agents (57).

Levine and Sowinski (58) found that pertussis vaccine given to rats inhibited the migration of macrophages to an area of inflammation induced by heat injury in the brain. Similarly Meade *et al*. (34) showed that migration of macrophages to the peritoneal cavity was markedly inhibited in Ptx-treated mice that had received IP thioglycolate, phytohemagglutinin, or mineral oil as irritants. Ptx had no effect on viability, attachment, or superoxide production by the macrophages.

Sugimoto *et al*. (59) found that Ptx promoted the migration of lymphocytes into thymus epithelial cell layers in tissue cultures, and Ogawa *et al*. (60) found that Ptx enhanced the migration of human blood monocytes *in vitro*. The migration of the monocytes depended on a gradient of Ptx. On the other hand, Meade *et al*. (23) found that Ptx decreased the migration of peritoneal macrophages and RAW264 cells (a murine macrophage-like cell line) induced by

endotoxin-activated mouse serum; Ptx alone had no chemotactic activity. Thus, in these two studies, the human and mouse monocytes appeared to respond differently to Ptx.

As early as 1936, Regan and Tolstoouhov (61) observed that children with whooping cough were hypoglycemic. Later others observed hypoglycemia in animals receiving crude extracts of B. pertussis cells. Pittman et al. (62) showed that mice with infection of the respiratory-tract with B. pertussis also were hypoglycemic. This hypoglycemia caused by B. pertussis cells is most likely due to increased production of insulin by the pancreatic islets (9). This increased production of insulin stimulated by pertussis vaccine persisted for as long as 17 days in the mouse and 24 days in the rat. Gulbenkian et al. (9) also showed that pertussis vaccine reduced the hyperglycemia produced by epinephrine. In normal rats, epinephrine caused a slight fall in plasma insulin, while in the pertussis vaccine-treated rats it caused a marked rise in insulin. All of these changes have been induced by purified Ptx (63, 64).

Many other actions of Ptx have been reported and I am sure that in the near future many more will be found, especially on cells in vitro. Interested readers are referred to various reviews covering aspects of the biological activities of Ptx (4, 40, 57, 65-67).

VII. SUMMARY

Ptx is the most active toxin produced by Bordetella pertussis. In in vitro studies on various cell systems Ptx is active in picogram doses. In nanogram doses it induces hypersensitivity to histamine and to many shock-inducing agents; it stimulates antibody production of various Ig classes, but it preferentially increases the IgE isotype; it induces leukocytosis with a predominance of small lymphocytes; it increases insulin production; and it changes sugar and fat metabolism. It enhances the development of DTH, the production of autoimmune diseases, such as EAE and EAO, and enhances inflammatory responses having an immunological basis.

Ptx is also the main protective antigen for mice when challenged with B. pertussis either intracerebrally or by the respiratory tract. Ptx has been shown to have a ADP-ribosyltransferase activity and to affect the production of adenylate cyclase by ADP-ribosylating the N_i regulatory protein in cell membranes. Ptx affects epinephrine function, involving the adrenergic receptor. Some cholinergic

functions are also affected. These actions profoundly influence the production of insulin and adenylate cyclase, the permeability of capillaries, the susceptibility to shock, and probably many other functions of cells.

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ISLET-ACTIVATING PROTEIN, PERTUSSIS TOXIN: SUBUNIT STRUCTURE AND MECHANISM FOR ITS MULTIPLE BIOLOGICAL ACTIONS

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I. INTRODUCTION

Epinephrine, when given to rats in both the fed and fasted states gives rise to a marked hyperglycemia. If, however, the rats, prior to epinephrine administration, are given a single dose of pertussis vaccine, the catecholamine-induced hyperglycemia is blocked (1). The effect of pertussis vaccination in this regard is long-lasting and a single dose blocks the catecholamine response for several weeks. The failure of epinephrine to induce hyperglycemia in pertussis vaccine-treated animals was shown to result from an epinephrine-induced hyperinsulinemia which was unique to the vaccine-treated animals (2). Thus, in pertussis vaccine-treated animals the hyperinsulinemia induced by epinephrine effectively antagonized the hyperglycemic action of the catecholamine. In perfused pancreases from control rats, insulin secretion was inhibited by the action of epinephrine at α_2-adrenergic receptors (3); with pancreases obtained from pertussis vaccine-treated animals, epinephrine inhibition of insulin secretion was not observed, instead the catecholamine acted through β-adrenergic receptors to stimulate insulin secretion (4,5). These data suggested that pertussis vaccines contained a factor which abolished responsiveness at the α_2-

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receptor but still permitted action at the β-adrenergic receptor. This factor was successfully isolated and purified by us in 1978 from the supernatant of the culture medium of Bordetella pertussis, and named "islet-activating protein (IAP)" after its unique action to enhance insulin secretory responses of rodents (6,7).

IAP is a protein indistinguishable from pertussis toxin; it displays lymphocytosis-promoting, histamine-sensitizing, adjuvant and hemagglutinating activities in addition to the islet-activating activity. It was this preparation, however, that has exclusively contributed to recent developments in pertussis toxin research including analysis of the subunit structure found to be in comformity with the A-B model (8), discovery of an ADP-ribosyltransferase activity of the A-protomer as the basis of the toxin's action (9-11) and elucidation of the mechanism whereby receptor-mediated signal transduction is modified by bacterial toxins (12-22). This is the reason why we still use the term IAP in preference to pertussis toxin or pertussigen.

How can a single protein, IAP, exhibit such diverse biological activities? It appears to be due to the complicated subunit structure, especially to the subunit assembly of its B-oligomer moiety which is capable of binding to glycoproteins on the surface of a variety of mammalian cells. The binding of the B-oligomer has two effects; on the one hand it permits the A-protomer to enter the cell across the plasma membrane, while on the other hand it stimulates such cells as lymphocytes due to crosslinking of the membrane glycoproteins (23). These dual actions *in vitro* of the B-oligomer were differentially responsible for multiple biological actions *in vivo* as revealed by chemical modification of the toxin molecule.

II. THE A-B STRUCTURE OF ISLET-ACTIVATING PROTEIN (8)

IAP is a hexamer (Mr=117,000) composed of five dissimilar subunits, which were named in the order of their molecular sizes: S1 (Mr=28.000), S2 (23,000), S3 (22,000), S4 (11,700) and S5 (9,300). Exposure of IAP to 5 M urea at 4°C for 3-4 days gave four separate peaks upon the subsequent column chromatography with CM-Sepharose; the two were S1 and S5 and the other two were dimers (D1 and D2). These two dimers were further separated by exposure to 8 M urea for 16 h followed by DEAE-Sepharose column chromatography, to the constituent subunits: D1 to S2 and S4 and D2 to S3 and S4. Thus, these five subunits were separated from each other and purified to homogeneity as revealed by a sharp single peak on polyacrylamide gel electrophoresis. Based on the relative color

intensity of the individual subunit stained on SDS-polyacrylamide gel, the molecular ratio of these subunits in the IAP molecule was calculated as 1(S1)/1(S2)/1(S3)/2(S4)/1(S5).

Reconstitution of the original IAP molecule was next undertaken using these purified subunits. Combination of S2 with S4 and S3 with S4 in 2 M urea afforded D1 and D2, respectively. No dimer was formed from any other combination. Combination of D1 and D2 failed to form a tetramer, but the further addition of S5 to the mixture of D1 and D2 was, but the addition of S1 was not, effective in forming a pentamer, which exhibited no islet-activating activity when injected into rats. The subunit structure and the biological activity of IAP was then recovered by further combination of S1 with the pentamer.

The native IAP that had been exposed to urea under more mild conditions (5 M urea at 4°C for 6 h) was applied to a column of haptoglobin-Sepharose. A single sharp peak of the protein that passed through the column was identified as S1, while the pentamer was bound to the column and then eluted by 0.5 M NaCl/3 M KSCN again as a sharp peak. Thus, IAP was readily dissociated to S1 and the pentamer. S1 was enzymatically active as shown below. Hence, this subunit should be referred to as an A(Active)-protomer. The pentamer appeared to be a B(Binding)-oligomer, because the interaction of pertussis toxin with haptoglobin, a sialoprotein, was considered by Irons and MacLennan (24) to afford a model system for the toxin binding to the cell surface. We have thus proposed the A-B structure of IAP (8).

III. ADP-RIBOSYLTRANSFERASE ACTIVITY OF THE A-PROTOMER OF ISLET-ACTIVATING PROTEIN

When membranes from rat C6 glioma cells were incubated with [α-^{32}P]NAD and IAP, the ADP-ribosyl moiety of NAD was transferred to a membrane protein with Mr=41,000 (9,10). The A-protomer of IAP was as effective as the native IAP in this regard. This IAP substrate was later identified as the α-subunit of Ni that is involved in receptor-mediated inhibition of adenylate cyclase (17-22,25-28). The A_1 fragment of cholera toxin catalyzed ADP-ribosylation of other membrane proteins with Mr of 45,000 and 48,000/49,000 under the same conditions. The ADP-ribosyltransferase should catalyze hydrolysis of NAD to ADP-ribose and nicotinamide (NAD-glycohydrolase activity) in the absence of an acceptor protein for ADP-ribose. The A-protomer of IAP exhibited NAD-glycohydrolase activity only in the presence of dithiothreitol which cleaved disulfide bonds in the A-protomer peptide. No such NAD-glycohydrolase activity was observed with the native

IAP even in the presence of dithiothreitol. ADP-ribosyltransferase activity of IAP as well as its A-protomer as observed with C6 cell membranes was suppressed by adding oxidized glutathione to the incubation medium.

Thus, processing enzyme(s) are present in C6 cell membranes which are responsible for liberation of the A-protomer from IAP and for reductive cleavage of disulfide bonds in the A-protomer peptide. This processing is prerequisite for IAP to be capable of catalyzing ADP-ribosylation of the membrane Mr=41,000 protein. Such processing enzyme(s) are missing in membrane preparations from certain cell types such as rat heart cells (20) and pancreatic islets. We have to use the A-protomer preactivated by dithiothreitol in experiments with these cell membranes.

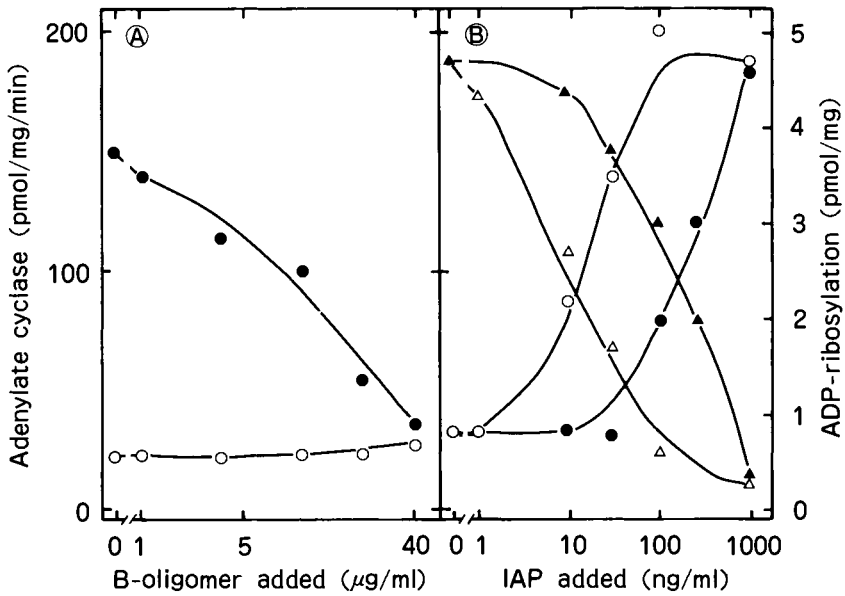

Fig. 1. Competitive inhibition by B-oligomer of the actions of IAP on intact C6 cells. Rat C6 glioma cells were exposed for 3 h to IAP (100 ng/ml in A) and/or its B-oligomer (10 μg/ml in B) at the final stage of cell culture. IAP was added (●) or not (○) in A, while the B-oligomer was added (●,▲) or not (○,△) in B. A batch of the membranes prepared from these cells was assayed for GTP-dependent adenylate cyclase activity (○,●). Another batch of the membranes was incubated with [α-^{32}P]NAD and the preactivated A-protomer of IAP to measure the incorporation of ADP-ribose into the residual Mr=41,000 protein (△,▲). From (23).

The IAP substrates so far discovered are the α-subunit (Mr=41,000) of Ni, the α-subunit (Mr=39,000) of transducin in the rod outer segment of vertebrate visual cells (29,30) and an Mr=39,000 protein in rat and bovine brain (unpublished). The function of the last protein is now under study. The physiological function of Ni (or transducin) to couple to receptors (or rhodopsin) and to the adenylate cyclase catalytic unit (or cGMP phosphodiesterase) was abolished by IAP-catalyzed ADP-ribosylation of these proteins.

IV. THE A-PROTOMER-TRANSPORTING ACTIVITY OF THE B-OLIGOMER OF ISLET-ACTIVATING PROTEIN

Evidence for the binding of IAP to the cell surface via the B-oligomer came from inhibition studies where the B-oligomer blocked the action of IAP on C6 cells in a competitive manner (Fig. 1). In cell membranes from C6 cells, pretreatment with IAP results in increased GTP-dependent adenylate cyclase activity (16), this results from the action of IAP to uncouple Ni from the catalytic unit (18,22,31). Progressive inhibition of the ability of IAP to mediate this action was observed when increasing concentrations of the B-oligomer were added together with IAP (Fig. 1A). The inhibition by the B-oligomer was competitive and was associated with

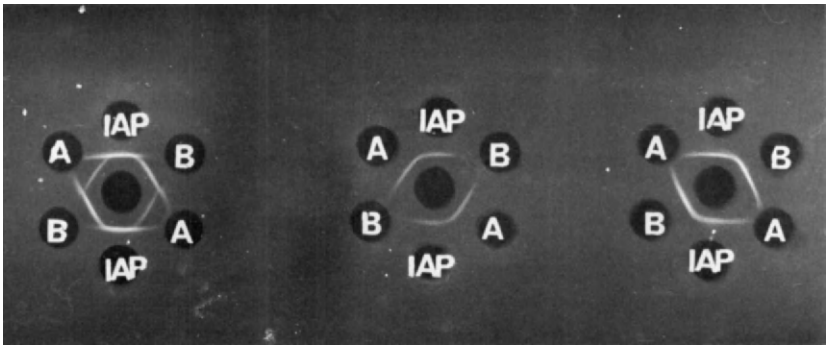

Fig. 2. Specific immunoprecipitation of IAP, its A-protomer and B-oligomer with their respective antibodies. Double immunodiffusion was carried out with 180 μg of anti-IAP (left) anti-A-protomer (center) or anti-B-oligomer (right) IgG in each center well. The contents of surrounding wells: IAP, 6 μg; A, 3.2 μg of A-protomer; B, 5.6 μg of B-oligomer. From (23).

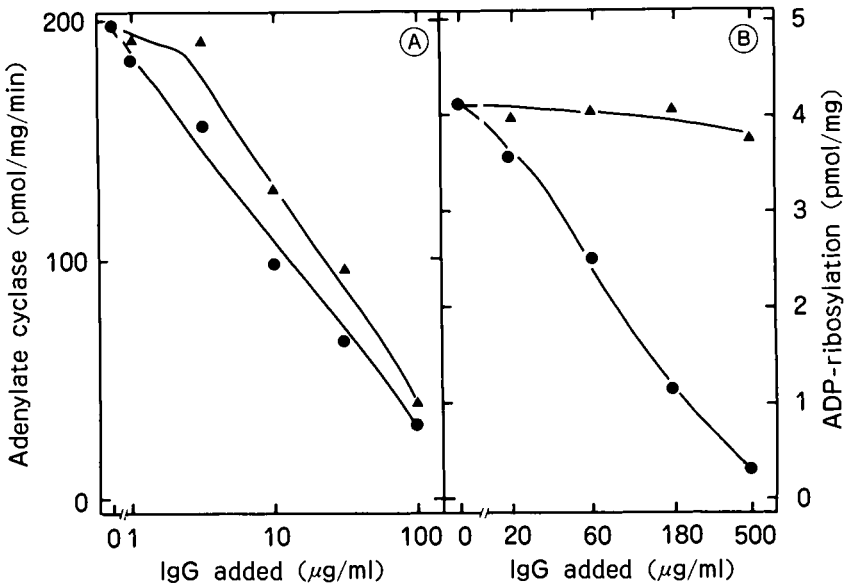

Fig. 3. Suppression of the IAP actions on intact cells and isolated membranes by the antibody against its A-protomer or B-oligomer. In A, anti-A-protomer (●) or anti-B-oligomer (▲) IgG was added to C6 cell cultures 15 min before the start of the usual 3-h IAP treatment. In B, membranes prepared from nontreated cells were submitted to the usual ADP-ribosylation reaction with IAP in the presence of anti-A-protomer (●) or anti-B-oligomer (▲) IgG. From (23).

just the same pattern of competitive inhibition observed for ADP-ribosylation of the membrane Mr=41,000 protein in the same cells (Fig. 1B).

Polyclonal antibodies were raised in rabbits using IAP, its A-protomer and its B-oligomer as antigens. The anti-IAP antibodies interacted with both the A-protomer and the B-oligomer, while the anti-A-protomer and anti-B-oligomer antibodies reacted selectively with the A-protomer and the B-oligomer, respectively (Fig. 2). The effect of IAP on intact C6 cells to enhance membrane GTP-dependent adenylate cyclase activity was suppressed by either anti-A-protomer or anti-B-oligomer antibodies (Fig. 3A). In contrast, ADP-ribosylation of the Mr=41,000 protein observed upon incubation of isolated membranes with IAP was prevented by the anti-A-protomer antibodies, but was not by the anti-B-oligomer anti-

bodies (Fig. 3B), clearly indicating that the B-oligomer played an indispensable role when IAP interacted with intact cells (23). No B-oligomer was needed for the direct interaction of IAP with the membrane target protein.

A definite lag time preceded the onset of the action of IAP on intact cells (32,33). The lag time was not shortened by increasing the concentration of IAP, suggesting that each IAP molecule experiences a similar interval between contact with the cell and generation of the biological action. Thus, the slow onset of the IAP action is likely to reflect the time for penetration of the A-protomer across the plasma membrane between binding of IAP via its B-oligomer to the cell surface and generation of the activated A-protomer, ADP-ribosyltransferase, inside the membrane.

V. MITOGENIC ACTION OF THE B-OLIGOMER OF ISLET-ACTIVATING PROTEIN

Incubation of mouse or rat splenic cells with IAP increased incorporation of [^{3}H]thymidine into DNA (23). This mitogenic action was reproduced by the purified B-oligomer of IAP (Fig. 4); it was due to binding of the B-oligomer to the cells. There was no effect seen in the splenic cells from

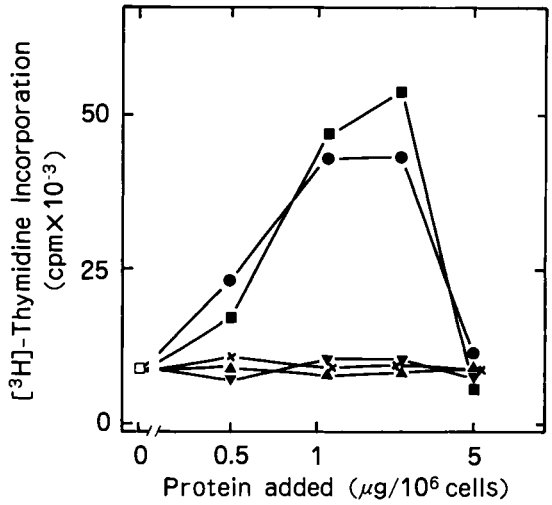

Fig. 4. Mitogenic action of IAP and its B-oligomer on mouse lymphocytes. Mouse splenic cells were cultured with IAP (●), B-oligomer (■), dimer-1 (▲), dimer-2 (▼) or C-subunit (S5) (x) for 48 h. From (23).

thymus-deficient nude mice. Thus, the B-oligomer of IAP is a potent T-cell mitogen. The B-oligomer was bound to cell surface via its two constituent dimers, D1 and D2, as evidenced by competitive inhibition of the B-oligomer-induced mitogenesis by D1 or D2. Neither D1 nor D2 was mitogenic by itself (Fig. 4). It is very likely, therefore, that the divalent binding of the B-oligomer to the cell surface via two dimers results in crosslinking of glycoproteins which is responsible for mitogenesis of T-lymphocytes.

Crosslinking of membrane proteins by concanavalin A, another T-cell mitogen, is known to result in stimulation of glucose oxidation in adipocytes. Glucose oxidation was stimulated by IAP, too, but not by D1 or D2, confirming our idea that crosslinking of membrane proteins is responsible for the mitogenic action of the B-oligomer. The B-oligomer exhibited no detectable activities in vivo when injected into rats, probably due to its instability in the blood circulation. Possible involvement of the B-oligomer-induced stimulation of lymphocytes (or other cells) in some of the biological activities in vivo of IAP was therefore studied by another

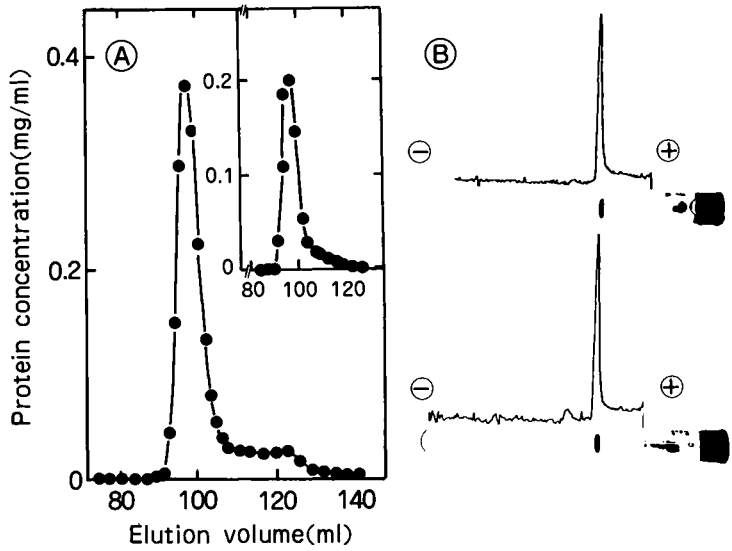

Fig. 5. Characterization of acetamidinated IAP. A: An elution profile of acetamidinated IAP from a Sephacryl S-200 column. The profile of the native IAP is shown in inset for comparison. B: The disc electrophoretogram and its densitometric pattern with the native (top) or acetamidinated (bottom) IAP. From (34).

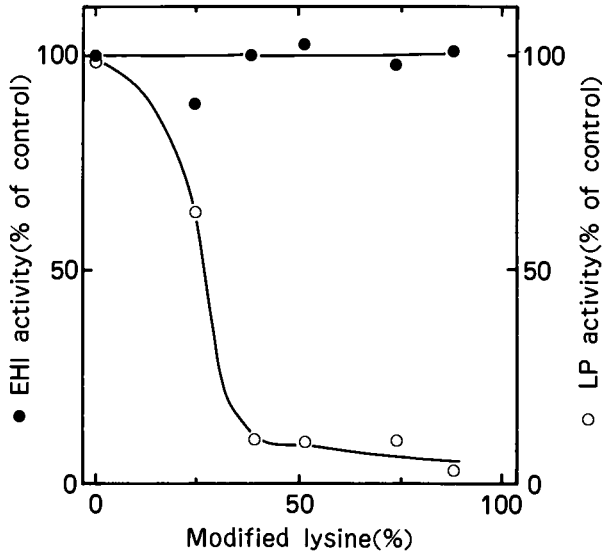

Fig. 6. Differential effects of acetamidination of IAP on its biological activities. Epinephrine-hyperglycemia inhibitory (EHI,●) and lymphocytosis-promoting (LP,○) activities of differently modified IAP (1 μg/100 g body wt) were assayed with rats. From (34).

approach in which mitogenecity was selectively blocked by chemical modification of the toxin molecule as will be described below.

VI. ACETAMIDINATION OF THE LYSINE RESIDUES OF ISLET-ACTIVATING PROTEIN: DIFFERENTIAL EFFECTS ON MULTIPLE BIOLOGICAL ACTIONS OF THE TOXIN

Chemical modification of free amino groups of the lysine residues in the IAP molecule exerted profound influences on the biological activities of the toxin. Acylated (acetylated, maleylated or succinylated) IAP exhibited no biological activity *in vivo*, because of destruction of the quarternary structure of the toxin molecule (34). Acetamidination was a more mild modification of the amino groups; the subunit assembly of IAP was maintained after the exhaustive (80-90%) acetamidination of lysine residues, as revealed by unaltered patterns of gel filtration and disc electrophoresis (Fig. 5).

Acetamidination of IAP was promising as a means of

differential modification of biological activities of the toxin. The IAP preparations acetamidinated in different degrees were injected into rats. Lymphocytosis-promoting action of the toxin decreased progressively as the degree of chemical modification of the lysine residues increased, while epinephrine-induced hyperglycemia was efficiently suppressed by the fully modified IAP as well as by the unmodified native IAP (Fig. 6). Differential mechanisms or differential target cells are thus suggested for these two activities of IAP (34). The suggestion led us to further studies on the effects of acetamidination on a number of biological activities of IAP. The activities of IAP have been divided into the following four classes based on their different susceptibilities to acetamidination (35).

A. Biological Activities of Islet-activating Protein Observed after Acetamidination of the Lysine Residues: "Class-1" Activities

Upon direct addition of the toxin to membranes prepared

TABLE I. Classification of biological activities of IAP

Class	Biological activities	Attenuation by acetamidination
1	ADP-ribosylation of the membrane protein	No
	Potentiation of adenylate cyclase	No
	Stimulation of insulin secretion	No
	Stimulation of lipolysis	No
	Inhibition of epinephrine hyperglycemia	No
	Potentiation of hyperinsulinemia	No
	Hypotensive activity	No
	Positive inotropic activity	No
2	Mitogenic activity	Yes
	Stimulation of glucose oxidation	Yes
	Promotion of lymphocytosis	Yes
	Increase in vascular permeability	Yes
	Histamine-sensitizing activity	Yes
	Adjuvant activity	Yes
3	Inhibition of histamine release	Yes
4	Hemagglutinin	No

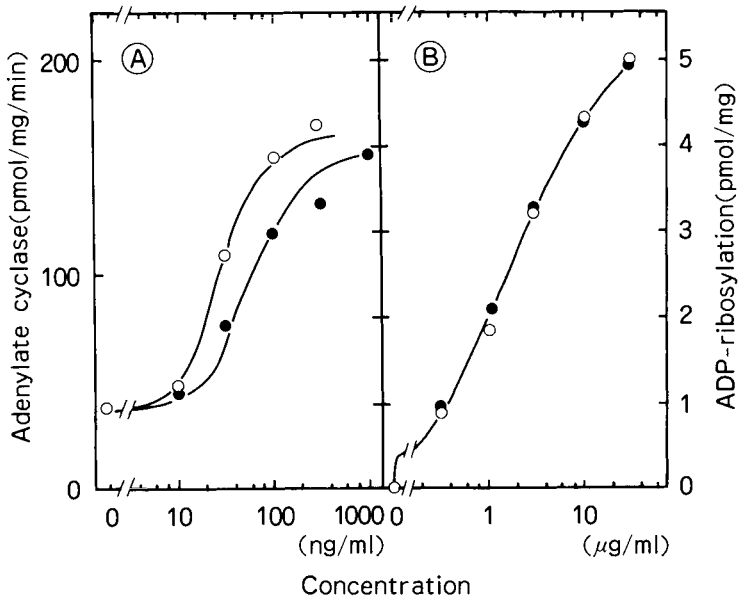

Fig. 7. Effects of IAP (○) and acetamidinated IAP (●) on intact C6 cells (A) or on isolated C6 cell membranes (B). GTP-dependent adenylate cyclase of membranes prepared from cells that had been exposed to the toxin (A) or the ADP-ribosylation of a membrane Mr=41,000 protein by direct addition of the toxin (B) was measured. From (34,35).

from rat C6 glioma cells, acetamidinated IAP was as effective as the native IAP in ADP-ribosylation of the membrane Mr=41,000 protein, the α-subunit of Ni (Fig. 7B). Thus, the free amino groups of the lysine residues are not required for (i) liberation of the A-protomer from the holotoxin, (ii) activation of the A-protomer resulting from reductive cleavage of disulfide bonds in the A-protomer peptide and (iii) ADP-ribosyltransferase activity of the thus activated A-protomer, all of which should occur upon direct exposure of C6 cell membranes to IAP. Fig. 7A shows that acetamidinated IAP was also effective in enhancing GTP-dependent membrane adenylate cyclase when it was added to intact C6 cells, though its potency was slightly less than the potency of the native IAP. Thus, acetamidination of IAP did not interfere with the function of the B-oligomer to bind to the cell surface thereby permitting the internalization of the A-protomer across the plasma membrane.

Acetamidinated IAP was almost as effective as the native

IAP in vitro in stimulating glycerol release from adipocytes (Fig. 8A) and in reversing α-adrenergic inhibition of insulin secretion from pancreatic islets (Fig. 8B). Adenosine is generated during incubation of adipocytes. Lipolysis is inhibited by this endogenously generated adenosine due to A_1-receptor-mediated (and Ni-mediated) inhibition of adenylate cyclase, which is readily reversed by the IAP-catalyzed ADP-ribosylation of Ni. This action of IAP is reflected in apparently spontaneous stimulation of glycerol release as observed in Fig. 8A (23). Thus the effect of IAP, in both glycerol and insulin release, results from reversal of Ni action in inhibiting the catalytic activity of adenylate cyclase. The observation that acetamidination does not adversely affect these activities of the toxin indicates that this chemical modification does not effect entry of the toxin

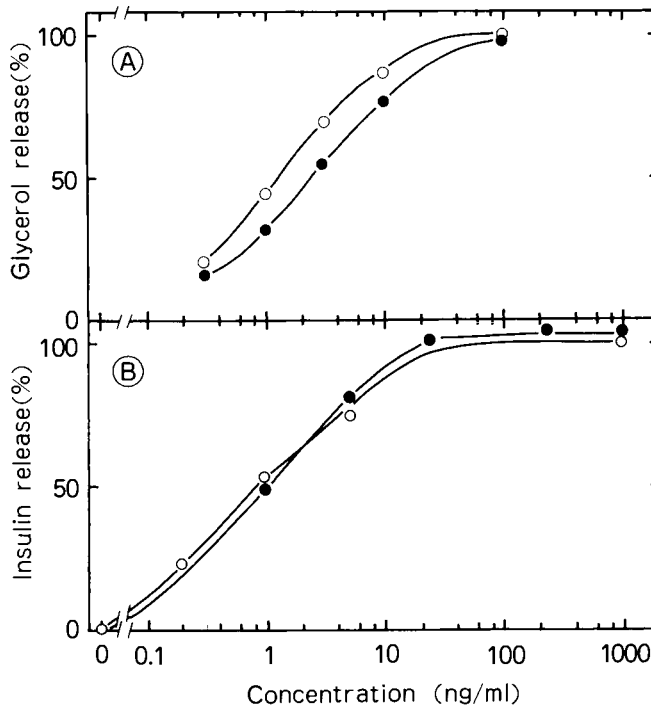

Fig. 8. Release of glycerol from rat adipocytes (A) and of insulin from rat pancreatic islets (B) as enhanced by IAP (○) and acetamidinated IAP (●). Insulin release was measured in the presence of 2 μg/ml of epinephrine to evaluate reversal of α-adrenergic inhibition by the toxin. From (35).

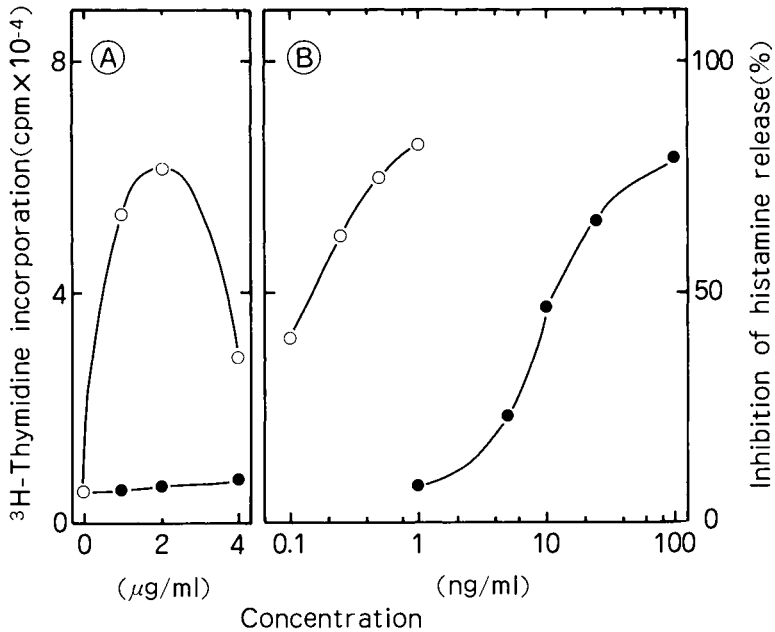

Fig. 9. IAP (○) and acetamidinated IAP (●) stimulate mouse lymphocytes (A) and inhibit compound 48/80-induced histamine release from rat mast cells (B). From (35).

into the cells nor the ability of the A-protomer to mediate its action on the α subunit of Ni. Failure of acetamidination to prevent these actions of IAP is consistent with the conclusion drawn above from the results in Fig. 7 that the free amino groups in the IAP molecule are not essential for the biological activities of the toxin dependent on the internalization of the A-protomer and its subsequent interaction with Ni in intact cells.

Hypotension and positive inotropisim (and chronotropism) were observed for 1-2 weeks when IAP was once injected into spontaneously hypertensive rats (35). Acetamidinated IAP was as effective as was the native IAP in this regard. Enhanced β-adrenergic responses to endogenous catecholamines must be involved in these cardiovascular effects of the toxin, since the effects were antagonized by β-adrenergic antagonists. Probably, the function of Ni negatively coupled to cardiovascular β-adrenergic receptors (18,31) was abolished by IAP-catalyzed ADP-ribosylation. Thus, these actions of IAP *in vivo*, together with the epinephrine-hyperglycemica inhibitory and hyperinsulinemia-inducing actions, came into the same

category of "Class-1" in Table I.

B. Biological Activities of Islet-activating Protein that Inhibited by Acetamidination of the Lysine Residues: "Class-2" Activities

Mitogenic action of IAP disappeared upon acetamidination; acetamidinated IAP did not stimulate the incorporation of thymidine into splenic cells (Fig. 9A). Conceivably, the divalent binding of IAP via the two dimers causing crosslinking of membrane proteins was interfered with by acetamidination of the lysine residues. This idea was confirmed by marked attenuation of the insulin-like action of IAP on adipocytes which also depends on crosslinking of membrane proteins (Fig. 10). The same actions of the B-oligomer of IAP on lymphocytes and adipocytes were also suppressed by acetamidination. Thus, we would like to propose that the biological activities of IAP that were severely impaired by acetamidination of the toxin molecule results from stimulation of cells due to divalent binding of the B-oligomer to membrane proteins.

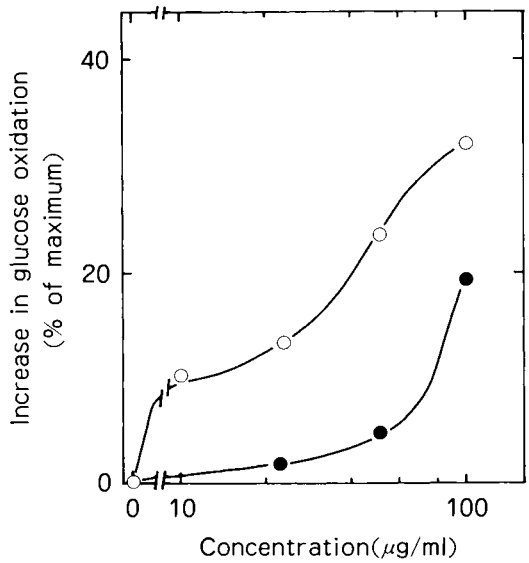

Fig. 10. Stimulation by IAP (○) and acetamidinated IAP (●) of glucose oxidation in rat adipocytes. The increase in glucose oxidation is expressed as a percentage of the maximal increment caused by 5 ng/ml of insulin. From (35).

These activities, including lymphocytosis-promoting (Fig. 11), histamine-sensitizing (Fig. 12) and adjuvant (Fig. 13) activities, of IAP are listed under "Class-2" in Table I.

The "Class-2" activities were distinctly different from the "Class-1" activities in that much higher concentrations of IAP were required. Large amounts of the B-oligomer is probably needed for crosslinking of membrane proteins, whereas ADP-ribosylation proceeds catalytically dependent on a very few molecules of the A-protomer that have been internalized by the aid of the B-oligomer associated at the 1:1 molar ratio.

The widely known action of pertussis toxin to promote lymphocytosis depends on its direct action on lymphocytes (36), in which the same mechanisms are involved as in the toxin's mitogenic action. Stimulation of lymphocytes would trigger certain immune reactions leading to facilitated antibody formation (the adjuvant activity). Moreover, the endothelial cells of pulmonary vessels would contract and shrink, upon stimulation by IAP, permitting outward passage of plasma protein and fluid into the extracellular spaces (the increase in vascular permeability). This would be

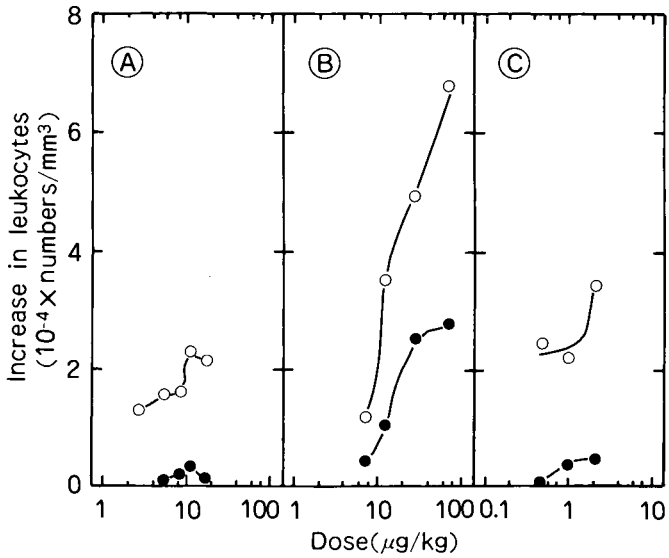

Fig. 11. Lymphocytosis-promoting activities of IAP (○) and acetamidinated IAP (●). The number of leukocytes was measured in rats (A), mice (B) and rabbits (C) 3 days after the i.v. injection of the toxin. From (34,35).

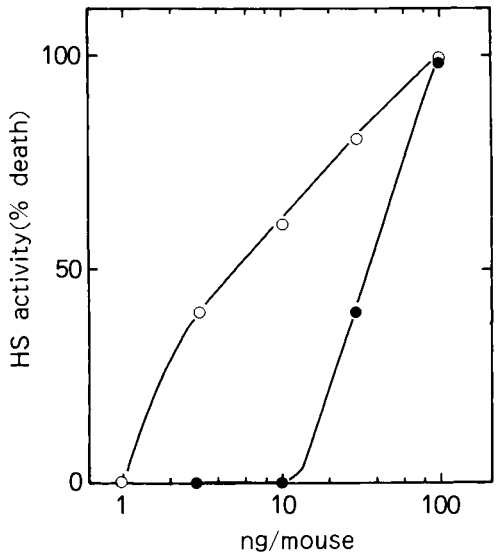

Fig. 12. Histamine-sensitizing activities of IAP (○) and acetamidinated IAP (●). The number of mice killed by 1 mg of histamine is plotted as a percentage of the total number of mice in the same group. From (35).

responsible for IAP-induced increase in histamine death (the histamine-sensitizing activity). The injection of histamine into IAP-treated rodents results in hyperinsulinemia and hence hypoglycemia by the same mechanism as for "Class-1" activities (37). Although this hypoglycemia is partly responsible for IAP-induced enhancement of histamine death (37), the increased vascular permeability is more important, since histamine-sensitizing activity of the toxin was still observed, even when hypoglycemia was prevented by glucose infusion. Since cAMP is inhibitory, rather than stimulatory, to these cellular responses that play essential roles in development of immune or inflammatory reactions, it is unlikely that the A-protomer which acts to increase the cellular cAMP content is involved in these "Class-2" activities of IAP.

C. A Possible Involvement of Other Mechanisms in Certain Biological Activities of Islet-activating Protein

Compound 48/80-induced histamine secretion from rat mast cells was strongly inhibited by prior exposure of the cells to IAP (33). The potency of IAP to inhibit the histamine

secretion was markedly diminished by acetamidination of the lysine residues (Fig. 9B), indicating that the activity should fall into the category of "Class-2" in Table I. Our recent study has shown, however, that the IAP-induced inhibition of histamine secretion, just like the "Class-1" activities of the toxin, arose from ADP-ribosylation of the Mr=41,000 protein by the A-protomer of IAP (38). This action of IAP was not reproduced by its B-oligomer. Moreover, the concentrations of IAP required were much lower than those required for other "Class-2" activities and comparable to those for the "Class-1" activities. Thus, the mechanism for this IAP activity may belong to "Class-1" while susceptibility to acetamidination makes it reasonable to bring this activity under the category of "Class-2". It is therefore classified as "Class-3" in Table I.

The mast cells, or basophils, are the blood cells that originate from the stem cells and are involved in inflammatory or immune responses. The B-oligomer of IAP would bind to the

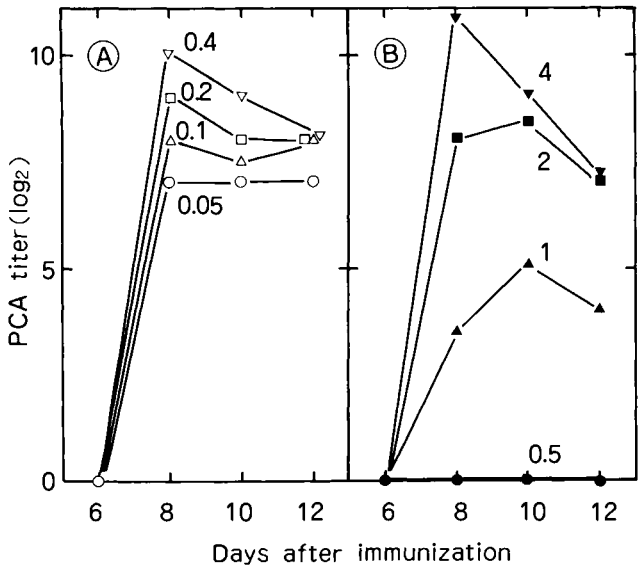

Fig. 13. Adjuvant activity of IAP (A) and acetamidinated IAP (B). The amount of IgE in the circulating blood of rats immunized by DNP-ascaris was assessed at 2 day intervals by a 48-h PCA (passive cutaneous anaphylaxis) test and expressed as the PCA titer. The dose (μg/animal) of the toxin, which was i.v. injected simultaneously with the antigen, is shown on each plot. From (35).

surface of these blood cells in a manner somewhat different from its binding to non-blood cells such as pancreatic islets. This type of binding might be unique in that it depends on the lysine residues which are acetamidinated under the conditions employed in the present study.

Neither a monomer nor a dimer that constitutes the B-oligomer moiety of IAP exhibited, by itself, any of the biological activities discussed above. The hemagglutinin activity was an exception; D1 was as effective as the native IAP in this regard (not shown). Thus, the mechanism for the hemagglutinin activity of IAP must be distinct from the mechanism for either the "Class-1" or "Class-2" activities. It is therefore placed under "Class-4" in Table I. This activity was not affected by acetamidination of the lysine residues in the peptides.

VII. REDUCTIVE METHYLATION OF ISLET-ACTIVATING PROTEIN AND ITS SUBUNITS: PROPERTIES AND BIOLOGICAL ACTIVITIES OF THE HYBRID TOXIN

Reductive methylation is superior to acetamidination as chemical modification of lysine amino groups in protein, since methylation proceeds under more mild conditions (39). The reductive methylation of IAP was undertaken by 2-h exposure of IAP to 10 mM formaldehyde in the presence of 15 mM pyridine borane, a mild reducing agent, at pH 7.0 in an atmosphere of nitrogen. Over 90% of the amino groups of the lysine residues in IAP was dimethylated under these conditions.

TABLE II. Preparation of hybrid IAP

Hybrid IAP (Abbreviation*)		Methylation of components				Yield (%)
		A-protomer	D1	D2	S5	
1	(H-AC)	Yes	No	No	Yes	45.8
2	(H-D1)	No	Yes	No	No	42.9
3	(H-D2)	No	No	Yes	No	57.6
4	(H-DD)	No	Yes	Yes	No	30.1

* Abbreviations: H stands for hybrid, A for the A-protomer, C for the connecting subunit (S5), D1 for dimer-1, D2 for dimer-2, DD for both D1 and D2. H is followed by abbreviations of components which are methylated.

The resultant methylated IAP exhibited the same biological activities as did the acetamidinated IAP; it was as effective as the native IAP in inducing the "Class-1 and 4" activities but did not exhibit the Class-2 and 3" activities (see Table I).

The methylated IAP was dissociated into the A-protomer, D1, D2 and S5 in 8 M urea under similar conditions as employed previously for the dissociation of the native IAP into these components. These methylated monomers and dimers were isolated individually by application to a column of CM-Sepharose. Each of them gave a sharp single band on disc electrophoresis. Combination of the methylated subunits with non-methylated subunits derived from the native IAP afforded four kinds of hybrid IAP with compositions and yields shown in Table II. These hybrid IAPs were then analyzed for representative biological activities under the category of "Class-2 and 3" to study the relative roles of two dimers in the firm binding of the toxin responsible for stimulation of the cells.

Fig. 14 shows mitogenic actions of the native, methylated and hybrid IAP. H-AC and H-D1 were as effective as the native

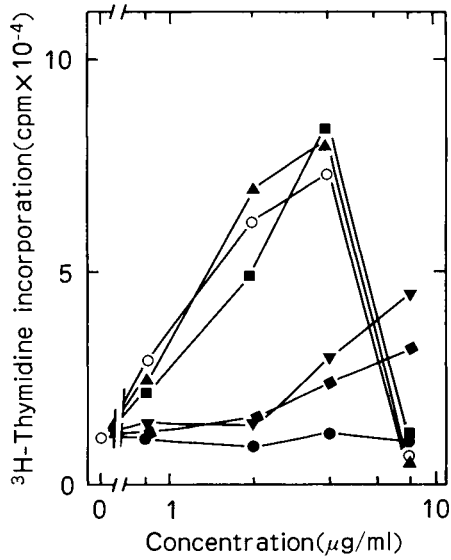

Fig. 14. Mitogenic action of hybrid IAP. Mouse splenic cells were cultured for 48 h with native IAP (○), methylated IAP (●), H-AC (▲), H-D1 (■), H-D2 (▼) or H-DD (◆). See Table II for the subunit compositions of hybrid toxins.

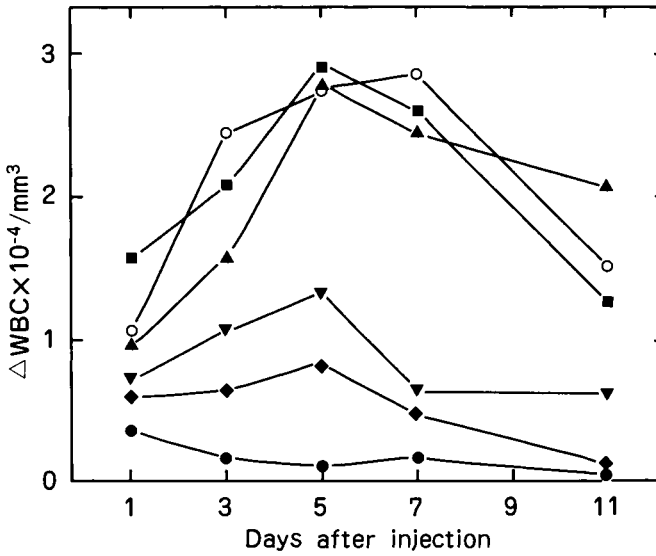

Fig. 15. Lymphocytosis-promoting action of hybrid IAP. Plots are the increments in lymphocytes in rats i.v. injècted with 4 μg of native IAP (○), methylated IAP (●), H-AC (▲), H-D1 (■), H-D2 (▼) or H-DD (◆).

IAP, whereas H-D2 and H-DD resembled methylated IAP in that they were scarcely mitogenic. Thus, there was no interference with mitogenicity when the A-protomer, the connecting subunit (S5) or D1 was methylated (i.e., H-AC and H-D1). Mitogenic action of IAP was lost by the chemical modification of D2 (i.e., H-D2 and H-DD).

Similar effects of methylation of IAP dimers were observed for lymphocytosis-promoting activity of IAP (Fig. 15). The hybrid toxins in which D2 was not modified, H-AC and H-D1, were as potent lymphocytosis-promoting factors as the native IAP. In contrast, only slight increases in lymphocytes were caused by H-D2 and H-DD in which D2 was methylated. It is very likely, therefore, that intact D2 was essential for the "Class-2" activities of IAP. Concomitantly, these results lend a strong support to our idea that the well-known lymphocytosis-promoting action of pertussis toxin arises from its mitogenic action.

Inhibition of histamine release from mast cells by IAP was likewise differentially affected by methylation of its constituent two dimers. H-D1 with intact D2 was as potent as the native IAP in inhibiting histamine release, whereas H-D2 with methylated D2 was, like methylated IAP, much less potent

in this regard (Fig. 16). Thus, the free amino groups in the dimer-2 of IAP play an important role in binding of the toxin molecule to blood cells.

The pivotal role of intact D2 in binding of IAP to blood cells was further demonstrated by competitive inhibition by D2 of the mitogenic action of IAP (Fig. 17B). D2 was 5-times more potent than D1 in inhibiting the mitogenic action of IAP on lymphocytes. The D2-induced inhibition was markedly attenuated, but the D1-induced inhibition was not affected, by methylation of lysine residues. Thus, D2 was more important than D1 in binding of IAP to lymphocytes, and the free amino groups in D2 were, but those in D1 were not, essential for the binding. In sharp contrast, D1 antagonized, more strongly than did D2, the action of IAP on adipocytes to stimulate glycerol release (Fig. 17A). Methylated D1 was as effective as non-methylated D1 in this regard. Methylation of D2, however, abolished the antagonistic effect completely. Thus, D1 was more important than D2 in binding of IAP to non-blood cells and insertion of the A-protomer into the membranes, which was not impaired by methylation as well as acetamidination of lysine residues. It is concluded that the free amino groups in D1 are not, but those in D2 are, involved in its binding to the cell surface.

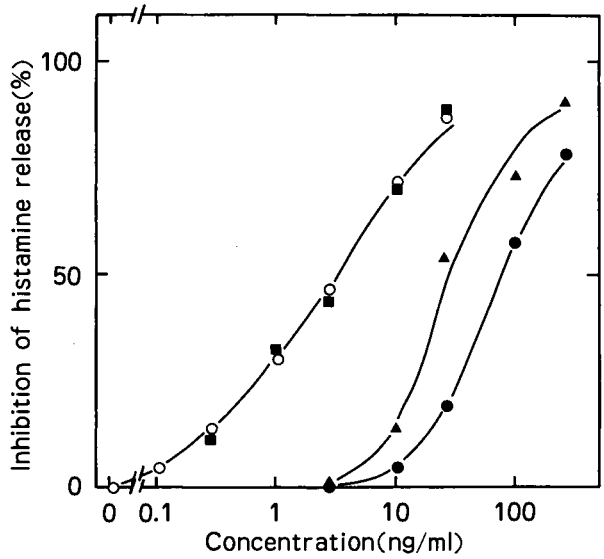

Fig. 16. Inhibition of histamine release from mast cells by native IAP (○), methylated IAP (●), H-D1 (■) or H-D2 (▲). Time of exposure to the toxin was 2 h.

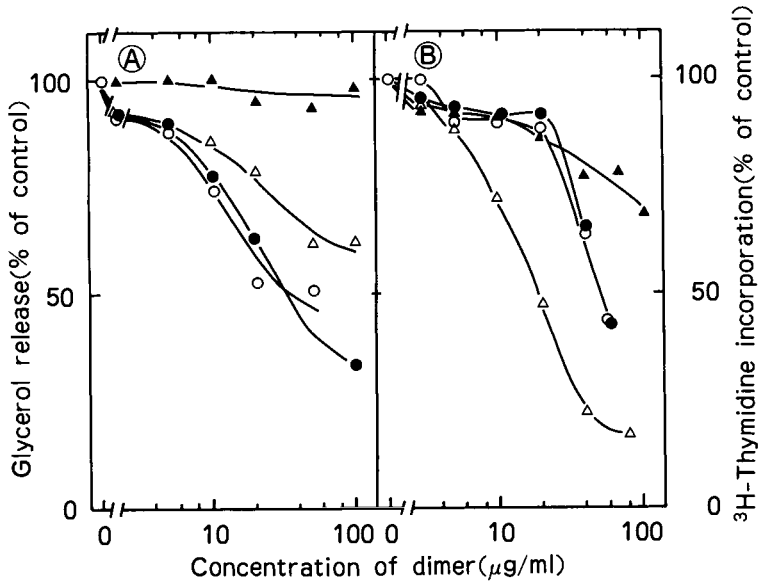

Fig. 17. Inhibition of the actions of IAP on glycerol release from adipocytes (A) and on splenic cells (B) by dimers or methylated dimers. The concentration of IAP was 30 ng (A) or 4 μg (B) per ml. D1 ○, methylated D1 ●, D2 △, methylated D2 ▲.

VIII. CONCLUDING REMARKS

Pertussis toxin, an islet-activating protein (IAP), has been characterized by its peculiarly multiple biological activities including lymphocytosis-promoting (LP), histamine-sensitizing (HS), adjuvant and mitogenic activities in addition to IAP activities. Our recent experimental findings, simply summarized in this article, clearly showed that distinctly different dual mechanisms are involved in these diverse biological activities of the toxin.

One is stimulation of cells such as lymphocytes due to firm (and probably divalent) binding of the B-oligomer to the cell surface. This plays an important role in development *in vivo* of LP, HS and adjuvant activities as well as of mitogenic activity *in vitro*. The free amino groups in the lysine residues in D2, one of the dimers constituting the B-oligomer, may be involved in this type of binding.

The other is the ADP-ribosylation of the α-subunit of Ni by the A-protomer that is transported across the plasma membrane to its target sites within the cells as a result of

binding of IAP to the cell surface via the B-oligomer moiety. D1 appears to play a more important role than D2 in the binding of the B-oligomer to non-blood cells for this purpose. This binding, in contrast to the firm binding to blood cells, was not affected by chemical modification of the lysine amino groups in the IAP molecule. Ni is involved directly in receptor-mediated inhibition, and rather indirectly in activation (18,20,22), of adenylate cyclase. The function of Ni is lost by IAP-catalyzed ADP-ribosylation of its α-subunit (17-19,22). Since cAMP, the product of adenylate cyclase, is a second messenger of a variety of cell stimuli, it is not suprising to find that the interaction of the A-protomer of IAP with Ni leads to development of diverse biological activities of IAP including insulin secretion, inhibition of epinephrine hyperglycemia, hypotension and positive inotropism. Moreover, our recent studies have suggested that the IAP substrate, probably the α-subunit of Ni, is also involved in other receptor-mediated signal transduction leading to phospholipid turnover and Ca mobilization (33,38,40,41). This is the mechanism whereby receptor-mediated histamine release from mast cels was strongly inhibited by IAP (33,38, 41). Possible occurrence of an endogenous ADP-ribosyltransferase sharing the same substrate with IAP has recently been suggested in mammalian hepatic tissues (42,43).

An additional well-known action of pertussis toxin as hemagglutinin seemed not to be explained by either of these two mechanisms; hemagglutinin activity of IAP was reproduced not only by the B-oligomer but also by D1. This effect of D1 was again less sensitive to chemical modification. Identification of the membrane proteins responsible for the seemingly different types of binding of IAP to the cell surface is now under study. Several review articles dealing with IAP are published in 1984 to 1985 (22,31,43-47).

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PERTUSSIS TOXIN: STRUCTURAL ELEMENTS INVOLVED IN THE INTERACTION WITH CELLS

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I. INTRODUCTION

The intoxication of mammalian cells by bacterial toxins results from a sequential series of individual events (1). The course of intoxication requires first binding of toxin to the cell surface, entry of the bound toxin into the appropriate cell compartment, and in some instances chemical modification of a specific cell target by a toxin-catalyzed reaction. Overt signs of intoxication, such as cell death or changes in responsiveness to hormones, become apparent only after these events have taken place. Studies with pertussis toxin (PT) have demonstrated that the toxin catalyzes ADP-ribosylation of a specific cell membrane associated protein (N_1). Chemical modification of N_1 blocks responsiveness of cells to ligands which normally inhibit adenylate cyclase (2-6). However, there is at present only limited information regarding the binding of PT to cells, the nature of the cell surface receptor, or the mechanism by which the toxin enters the cell.

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With a number of bacterial toxins, including diphtheria toxin, cholera toxin and tetanus toxin, the initial interaction of toxin with target cells involves binding of toxin to a specific cell surface receptor. If a given cell type lacks the appropriate receptor, the toxin does not bind and the cell remains insensitive to the toxin. Cell surface receptors have been identified in several instances and have been shown to be specific carbohydrate sequences (7, 8). With cholera toxin and tetanus toxin the carbohydrate moiety is part of a ganglioside (9-13). A glycoprotein is involved in binding to diphtheria toxin but the role of the carbohydrate moiety in binding is not clear (14, 15). PT is also known to interact with glycoproteins including haptoglobin and ceruloplasmin, these two glycoproteins have been shown to inhibit the hemagglutination activity of PT (16). Haptoglobin and fetuin have also been used as affinity matrixes for purification of the toxin (16-18). In the following section we describe a series of studies in which the interaction of pertussis toxin with cells and glycoprotein ligands was used to explore a specific receptor and the structural elements of PT involved in the interaction with cells.

II. Pertussis Toxin Structure and Function

Several methods have been described for the purification of PT (16, 19-22). In the current studies, PT was prepared as described from B. pertussis strain 165 (17). PT, prepared by this method, yields a homogeneous preparation which on SDS gel electrophoresis, after treatment with mercaptoethanol, gives four polypeptides of molecular weights of 30,000, 26,000, 25,500 and 14,000. In accord with the nomenclature proposed by Tamura and coworkers (23), these subunits of PT are termed S1, S2, S3 and S4,5. The results reported here differ somewhat from those of Tamura in that an S5 component with molecular weight of 11,700 was not observed in our preparations. In the absence of thiol reducing agent, each of these components migrates faster suggesting the presence of internal disulfide bonds. The individual subunits (see Figure 3) are assembled into a hexameric structure with a molecular weight of about 120,000 in which the S1, S2, S3 and S4,5 components are present in the respective molecular ratio 1:1:1:3.

In order to identify the elements involved in the interaction of PT with cells and in the catalytic activity, a study was conducted where the subunits of pertussis toxin

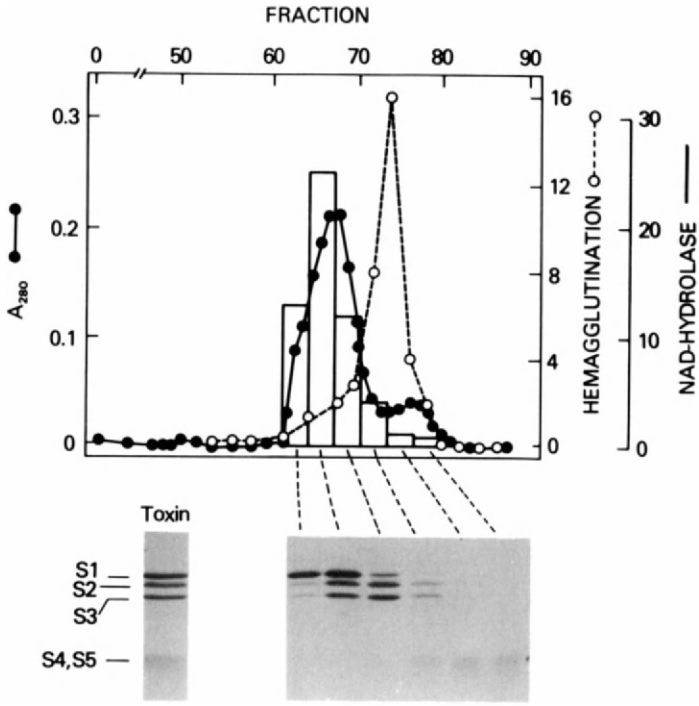

Fig. 1. Partial resolution of pertussis toxin subunits. Pertussis toxin (2.5 mg) was treated with 6 M guanidine hydrochloride for 1 hr and then chromatographed on a Sephacryl S-200 column (1.5 x 80 cm) equilibrated with 6 M guanidine hydrochloride. This treatment results in partial resolution of the pertussis toxin as seen by SDS-gel electrophoresis of pooled fractions shown in the lower portion of the figure. Fractions, pooled as indicated by the open bars, were concentrated 4-fold by ultrafiltration. NAD-hycholase activity (in 10 µl) was assayed by following the release of ^{14}C-carbonyl-nicotinamide from [^{14}C-carbonyl] NAD (24). Activity is expressed as cpm x 10^{-3} released in 100 min at 37°. Hemagglutination activity, with goose RBC's, is expressed as hemagglutination units x 10^{-2} per ml.

were resolved. Treatment of PT with 6M guanidine hydrochloride and subsequent chromatography on a column of Sephacryl S-200 results in partial resolution of the subunits (Figure 1). Two major peaks were observed on the basis of absorbance at 280 nM. Analysis of the fractions by SDS gel electrophoresis revealed the leading edge of the

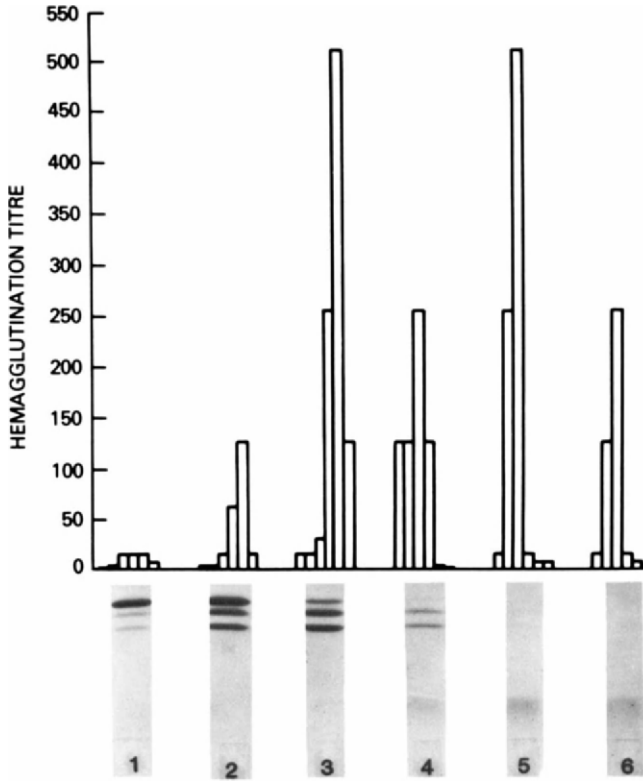

Fig. 2. Reconstitution of hemagglutination activity by mixing partially resolved pertussis toxin subunits. Chromatography of guanidine hydrochloride denatured pertussis toxin on Sephacryl S-200 (see Figure 1) results in resolution of pertussis toxin into six fractions which contain the pertussis toxin subunits as shown by SDS gel electrophoresis in the bottom of the figure. The subunits in lanes 1-6 are from top to bottom S1, S2, S3, and S4,5. Aliquots from the six fractions were mixed with an aliquot of equal volume from each of the other fraction and then assayed for hemagglutination activity with goose RBC's. Histograms above each lane show the hemagglutination resulting from such binary mixtures.

major peak was comprised almost entirely of the S1 subunit, while the trailing end contained the S2 and S3 subunits. The second protein peak contained primarily the S4,5 component. After treatment of pooled fractions with dithiotreitol, NAD-hydrolase activity was assayed. This activity was associated with fractions of the major protein

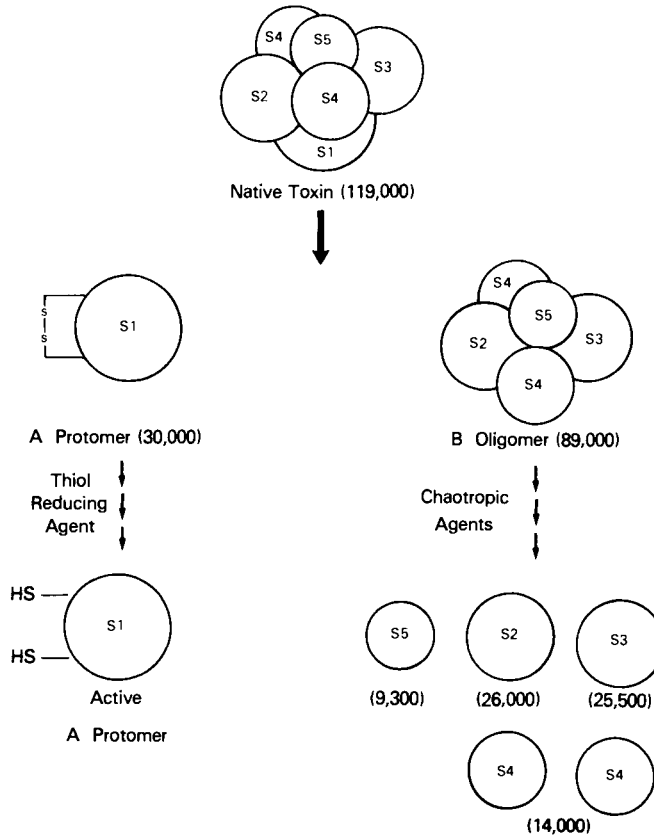

Fig. 3. The subunit structure of pertussis toxin. Native pertussis toxin is a hexamer composed of four or perhaps five nonidentical subuits (17, 23). Treatment of the toxin with mild denaturing agents results in dissociation of the toxin into its A and B components. The A component constitutes the S1 subunit and shows NAD-hydrolase and ADP-ribosyl transferase activity after treatment with thiol reducing agent. The B oligomer blocks binding of toxin to cells and is responsible for the toxins hemagglutinating activity.

peak containing high levels of the S1 component; this result is in accord with the observations made by Ui and coworkers (25). Analysis of the effluent for hemagglutination activity with goose red blood cells showed maximal activity in fractions between the two protein peaks. In an experiment where hemagglutination activity was monitored after fractions obtained from gel filtration were mixed (Figure 2), results were obtained which indicate that the concurrent presence of fractions containing the S2 and S3 components in addition to the S4,5 component is essential for reconstitution of activity. Thus, the current data suggest that interaction of the S4,5 component with the S2 and S3 subunits is necessary for the PT-associated hemagglutination activity. This association of S2, S3 and S4,5 subunits is similar if not identical in structure to the B subunit of PT, described previously, which was shown to block the ability of the holotoxin to interact with adipocytes and C6 glioma cells (26).

We have constructed a model for PT on the basis of these observations (Figure 3). The holotoxin is hexamer composed of one each of the S1, S2 and S3 subunits in addition to the three S4,5 subunits. Mild treatment of PT with a denaturant (23) or presumably interaction of the toxin with the cell leads to a dissociation of the toxin into A protomer and B oligomer. The B oligomer appears to be involved in the initial binding of PT to the cell. This conclusion is reached on the basis of experiments showing that the purified B oligomer blocks intoxication of intact cells with the toxin and from studies demonstrating that reconstitution of hemagglutination activity requires the presence of the toxin subunits comprising the B-oligomer. The catalytic activity of PT is associated with S1 or A subunit. Expression of catalytic activity, either NAD-hydrolysis or ADP-ribosylation, requires treatment with thiol reducing agent indicating that structural rearrangement subsequent to disulfide bond reduction is essential for catalytic activity (17, 26).

III. Temporal Considerations on the Interaction of Pertussis Toxin with Adipocytes

Treatment of isolated rat adipocytes with PT leads to a large increase the rate of lipolysis. This effect proceeds through increased intracellular production of cAMP and has been shown to involve activation of adenylate cyclase by a mechanism where down regulation of adenylate cyclase catalytic activity by inhibitory ligands is blocked (27).

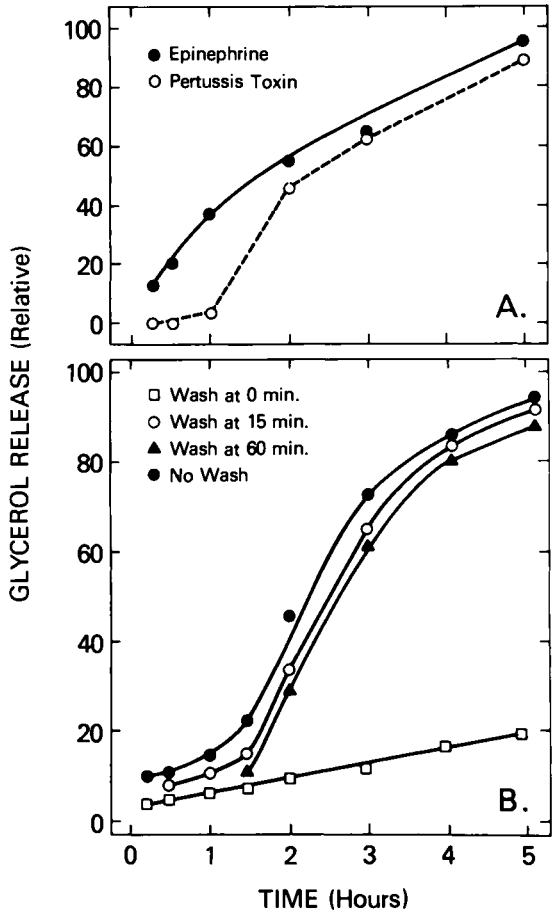

Fig. 4. The effect of pertussis toxin on glycerol release in isolated rat adipocytes. A. Isolated rat adipocytes were incubated with pertussis toxin (500 ng/ml) or epinephrine (0.2 mM). Samples were removed at the indicated times and the amount of glycerol released from triglyceride was determined. Glycerol release is expressed relative to an epinephrine control at 5 hr. B. Adipocytes were treated as in A with pertussis toxin and then washed at the indicated times with pertussis toxin free buffer. Samples were then withdrawn for determination of glycerol.

Differences are observed between the time course for PT-stimulated lipolysis and that obtained with another lipolytic agent such as epinephrine (Figure 4A). Epinephrine treatment leads to an immediate increase in lipolysis. In contrast, the response induced by PT is slow and increased lipolysis is observed only after the cells have been exposed to the toxin for between 60 and 90 minutes.

Since the nature of this lag period between exposure of the cells to PT and lipolysis, is unknown, several experiments were conducted to determine whether it results from a slow initial interaction of toxin with the cells, or from slow entry and processing or from the reaction involving covalent modification of the adenylate cyclase regulatory component N_1. Adipocytes were preincubated with PT for various intervals and attempts were made to remove toxin by washing (Figure 4B). Treatment of cells for as little as 15 min prior to washing, the shortest treatment attempted, had little effect on subsequent activation of lipolysis. This suggests that the initial binding of PT is rapid and that the lag period results from subsequent events.

To further examine the rapidity of binding, the ability of PT neutralizing antibody to block lipolysis was examined (Figure 5). Addition of antibody at 0 time totally blocked PT action, by 30 minutes the addition of antibody was without effect upon PT's action. The half maximal time was about 3 minutes indicating that the interaction of PT with cells is rapid. The data suggests that internalization of the toxin occurs within 30 minutes since neutralizing antibody is without effect at this time. However, it is not known if the specificity of the neutralizing antibody is directed against determinants involved in binding or in catalysis.

PT treatment has been used in a variety of systems to explore the function of the transducing element N_i in regulation of adenylate cyclase. In several of these studies (28, 29) it was necessary to treat cells with PT for extended periods before toxin-mediated effects were observed. These lag periods of 6 hours and longer contrast with the rapid response seen in fat cells and the intermediate response seen in AtT-20 cells (30). The factors responsible for regulating the duration of the lag period remain to be established. It would be of interest to determine if factors such as availability of receptors on the cell surface, rates of internalization or perhaps accessibility of the N_i target affect the duration of the lag period.

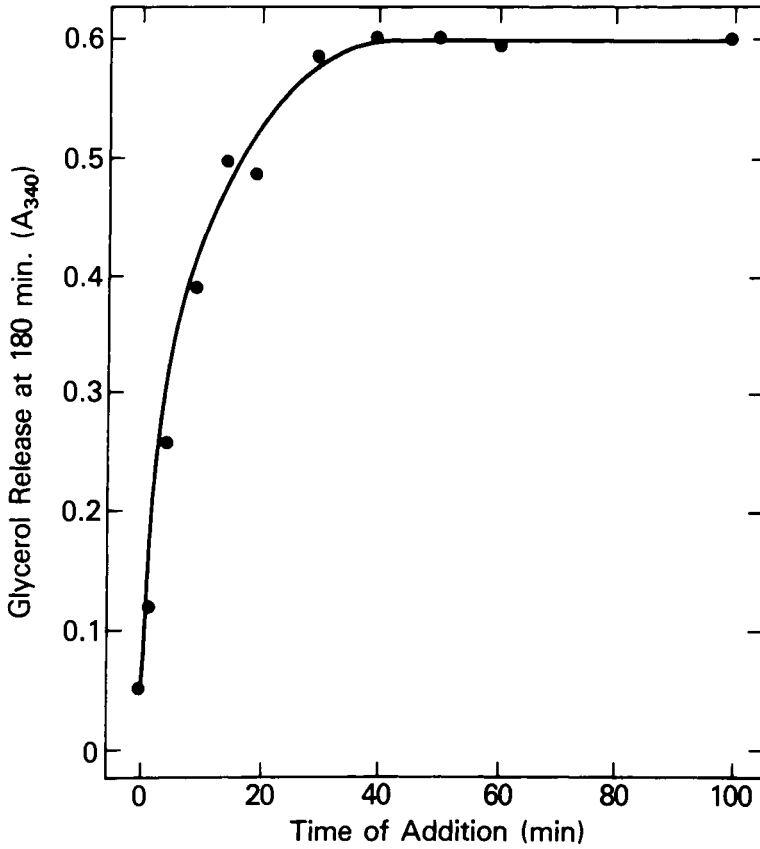

Fig. 5. Neutralization of pertussis toxin induced lipolysis by anti-pertussis toxin antibody. Isolated rat adipocytes were treated with pertussis toxin (500 ng/ml); at the indicated times anti-pertussis toxin antibody was added to the incubation mixtures. The amount of glycerol released (expressed as the absorbance change in the NAD coupled assay (44)) was determined 3 hr after the addition of pertussis toxin.

IV. The Interaction of Pertussis Toxin with Glycoproteins

In addition to the actions of PT *in vivo* and on cell-free preparations, it also functions as a hemagglutinin with avian erythrocytes (31). Irons and MacLennan extended this observation and demonstrated that PT mediated hemagglutination was optimal with goose erythrocytes (16, 32). These workers also showed that serum from a variety of

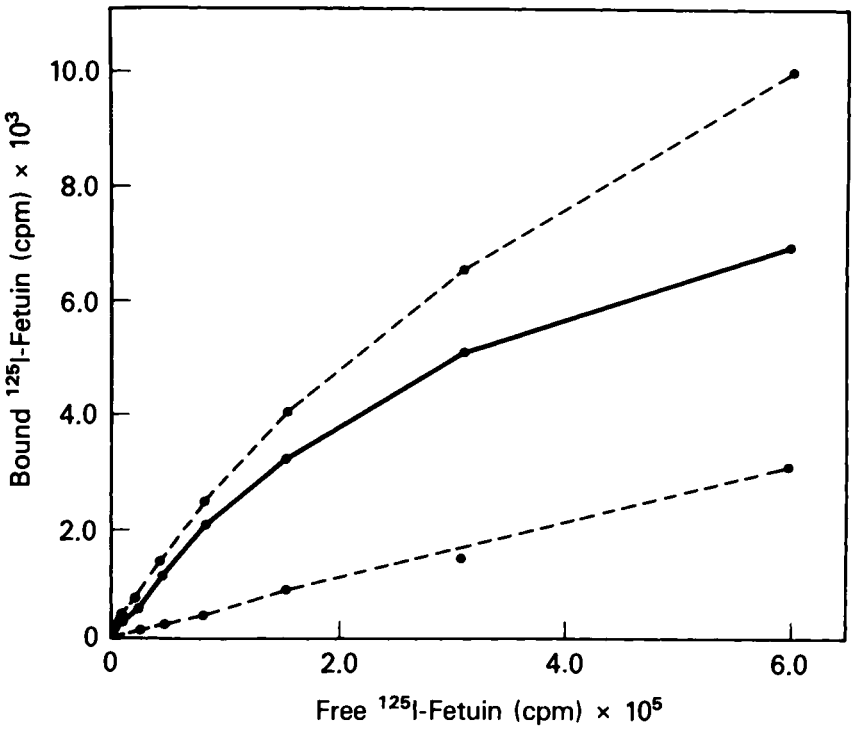

Fig. 6. The dependence of ^{125}I-fetuin binding to immobilized pertussis toxin on fetuin concentration. Microtiter plates were coated with pertussis toxin at 5 μg/ml. After coating, ^{125}I-fetuin (3.9 x 10^4 μCi/μmol) at indicated concentrations, was added and the amount of ^{125}I-fetuin bound was determined after incubation for 75 min at 37°. Total ^{125}I-fetuin bound is shown by the upper broken line. Non-specific binding (lower broken line) was determined by adding a 200-fold excess of unlabeled fetuin. Specific binding of ^{125}I-fetuin is shown by the solid line.

species and certain fractions obtained by Cohn fractionation of human serum (the α-globulin and glycoprotein fractions were most effective) inhibited this hemagglutination. Ultimately, it was established that the inhibitory activity in these preparations was mediated primarily by haptoglobin and ceruloplasmin type III.

The interaction of PT with serum glycoproteins has served as a basis for the affinity purification of PT (16). This approach was first applied by preparing affinity matrix with haptoglobin, however, this glycoprotein is not readily available making its routine use difficult. Independently our laboratory and the laboratory of Per Askelof (17, 18) found that fetuin, a readily available glycoprotein obtained from fetal calf serum, could be used as an affinity matrix.

In order to characterize the interaction of PT with fetuin, a solid phase binding assay was developed. Briefly, microtiter plates are coated with PT and the binding of ^{125}I-fetuin was monitored. Figure 6 shows the results of one such experiment where ^{125}I-fetuin binding was followed as a function of fetuin concentration. After the data is corrected for non-specific binding, the ^{125}I-fetuin binding is concentration dependent and saturable. From this and similar experiments the apparent binding constant fetuin to pertussis toxin was estimated to be between 4 and 7 x 10^{-8} M. The magnitude of this constant is comparable to those observed for the interaction of lectins with their carbohydrate ligands (33, 34). The carbohydrate nature of the receptor for other bacterial toxins and the data presented here provide a basis to suggest that PT binding might involve interaction with the carbohydrate moieties present on fetuin.

V. Carbohydrate Specificity of the Pertussis Toxin Fetuin Interaction

Two distinct classes of oligosaccharide are present on fetuin (35-37). The relatively simple O-linked structure, shown in the lower portion of Figure 7, is attached to the protein at either threonine or serine residues. A more complicated structure, containing mannose branches, is attached through the amide nitrogen of asparagine. A series of experiments were conducted in which deglycosylated derivatives of fetuin and fetuin derived glycosides were tested as inhibitors of ^{125}I-fetuin binding to PT.

The Carbohydrate Moieties of Fetuin

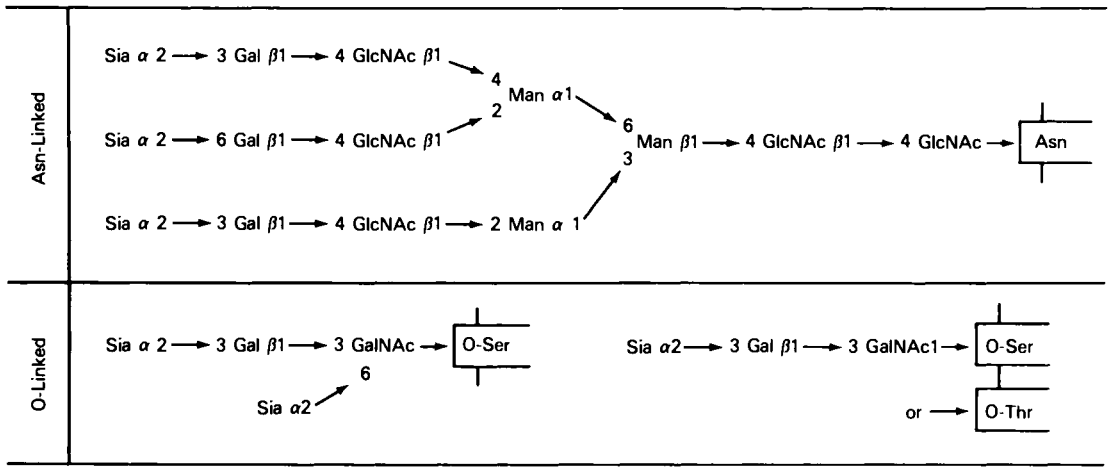

Fig. 7. Carbohydrate structures associated with fetuin. The detailed structures of the asparagine and O-linked carbohydrates present in fetuin are shown above.

Table I shows the results obtained when total removal of carbohydrate by treatment with a crude enzyme preparation from Diplococcus pneumoniae (38) yielded the aglycofetuin derivative. This derivative is ineffective as an inhibitor of ^{125}I-fetuin binding indicating that the carbohydrate moieties are responsible for the pertussis toxin-fetuin interaction. Selective removal of O-linked oligosaccharide, by treatment with alkaline borohydride (39), renders a derivative protein with inhibiting activity indistinguishable from native fetuin. This suggests that asparagine-linked rather than the O-linked oligosaccharide provides the ligand recognized by PT. This conclusion is further supported by the observation that O-linked oligosaccharide is ineffective as an inhibitor. In contrast, the glycopeptide containing the asparagine linked moiety, obtained after proteinase K digestion, is an effective inhibitor of the PT-fetuin interaction.

A series of fetuin derivatives were prepared where terminal sugar residues were sequentially removed by chemical and enzymatic treatment so that the specific carbohydrate residues involved in the PT-fetuin interaction

Table I
Inhibition of ^{125}I-fetuin binding to pertussis toxin by aglyco-fetuin derivatives and fetuin-derived glycosides*

Inhibitor	Protein (μg/ml)	% Inhibition
Fetuin	240	100
Fetuin (O-linked removed)	240	100
Aglycofetuin (O-linked and Asn-linked removed)	2400	2
Neutral sugar (μg/ml)		
O-linked CHO	30	0.5
Asn-linked CHO	1	75
	3	100

*Assays were conducted using the standard microtiter plate binding assay. Inhibitors, at the indicated concentrations, were added to the microtiter wells together with 1 μg/ml ^{125}I-fetuin (3.9 x 10^4 μCu/μmol). After 40 min at 37° the plates were washed and the amount of ^{125}I-fetuin bound was determined.

could be identified (Table II). Fetuin derivatives obtained after removal of sialic acid by mild acid hydrolysis, and subsequent removal of galactose by treatment with the Diplococcus pneumoniae β-galactosidase were nearly as effective as the untreated protein in inhibiting ^{125}I-fetuin binding. When N-acetylglucosamine was subsequently removed by treatment with Jack Bean hexoseamidase, the resultant derivative showed a reduced inhibitory activity. The failure to observe a greater reduction in the potency of the β-galactosidase treated material as an inhibitor results in part from incomplete removal of individual sugar residues at each stage of treatment. Mild acid treatment removed more than 90% of the sialic acid, β-galactosidase in contrast removed only 50% of the available residues and hexosamidase treatment removed only 20% of the N-acetyl glucosamine residues. Thus a substantial number of functional carbohydrate moieties were still present in the hexosaminidase treated derivative. The reduction in inhibition seen with this derivative suggests that the carbohydrate specificity for the PT-fetuin interaction is directed towards N-acetylglucosamine linked to mannose. Since the Jack Bean hexosaminidase does not act on the β-1,4 linkage the data suggest that N-acetylglucosamine linked β-1,2 to mannose is the critical structural element for interaction with PT.

When the same derivatives obtained by sequential removal of terminal sugar residues were tested as inhibitors of PT mediated hemagglutination results were obtained

Table II
Inhibition of ^{125}I-fetuin binding to pertussis toxin by deglycosylated derivatives of fetuin*

Inhibitor	% Inhibition
Fetuin	100
Asialo-fetuin	93
Asialo-galacto-fetuin	101
Asialo-gal-NAcGlu-fetuin	73
Aglyco-fetuin	2.1

*Fetuin binding assays were conducted as in Table I. Unlabeled fetuin and its deglycosylated derivatives were present at 140 μg/ml, ^{125}I-fetuin (2.4 x 10^4 μCi/μmol) was included at 1.8 μg/ml.

Table III
Inhibition of goose-RBC agglutination by deglycosylated derivatives of fetuin*

Inhibitor	Hemagglutination titer
Control	256
Fetuin	4
Asialo-fetuin	64
Asialo-gal-fetuin	32
Asialo-gal-NAcGlu-fetuin	256
Agylco-fetuin	256

*Hemagglutination with pertussis toxin and goose-RBC's was performed after pre-incubation of the toxin (0.3 μg/ml) with deglycosylated fetuin derivatives (0.3 μg/ml) for 15 min at 25°C. Serial dilutions of the reaction mixture with same concentration of inhibitor were then assayed, hemagglutination titer is expressed as the reciprocal of dilution.

consistent with the ^{125}I-fetuin binding data (Table III). Removal of sialic acid and then galactose exerted little effect on hemagglutination, whereas subsequent removal of N-acetyl glucosamine resulted in a fetuin derivative with drastically diminished potency as an inhibitor. These observations suggest that the cell receptors involved in hemagglutination are structurally similar to the asparagine linked N-acetyl glucosamine-mannose bridge structure of fetuin, and further suggest that similar receptors on other cell types may be involved in intoxication by pertussis toxin.

VI. Interaction of Pertussis Toxin with Immunoglobulins

The branched mannose structures linked to N-acaetyl-glucosamine in a β-1,2 orientation is a relatively common structure known to be present in a variety of glycoproteins including cell surface components and the various classes of immunoglobulins (40, 41). However, the structures seen in the immunoglobulins are different from fetuin (Figure 7) in that the mannose branching is biantennary, the degree of substitution with sialic acid and galactose can vary, and the internal N-acetyl glucosamine residues can be linked to

Table IV
Inhibition of I^{125} binding to pertussis toxin by different classes of human immunoglobulins*

Inhibitor	cpm Bound	% Inhibition
Control	3510.8 ± 113.3	-
Fetuin	1107.5 ± 105.8	68.5
IgG	2191.3 ± 238.4	37.6
IgA	1867.8 ± 229.4	46.8
IgM	1580.5 ± 279.3	54.9
IgE	130.0 ± 38.8	96.3

*Assays were conducted as described in Table I. ^{125}I-Fetuin was present at a concentration of 3.6 μg/ml while unlabeled fetuin and the various immunoglobulin were present at 36 μg/ml.

mannose or fucose. To examine if molecules containing a biantennary structure can interact with pertussis toxin, several human immunoglobulins (i.e., IgA, IgE, IgG and IgM) were exmained for their ability to inhibit the binding of ^{125}I-fetuin to pertussis toxin (Table IV). Each of the immunoglobulins tested inhibited binding, thus indicating that a biantennary mannose branch substituted β-1,2 to N-acetylglucosamine and other sugars suffices for the interaction with pertussis toxin.

The inhibition by IgG, IgM and IgA was weak relative to unlabeled fetuin, IgE, in contrast, proved to be more effective. Although we do not know specifically what factors contribute to the differential potency (the carbohydrate structures in each of the immunoglobulin classes are reported as being quite similar (42)) it is possible that factors such as the relative carbohydrate content or surface orientation of the carbohydrate contribute to potency in inhibition of ^{125}I-fetuin binding. The apparent strong interaction of IgE with pertussis toxin is interesting. PT is known to be an effective adjuvant in eliciting an IgE response (see Ishizaka this volume). Whether the interaction of PT with IgE contributes to this phenomena is an intriguing possibility.

VII. Conclusion

The intoxication of mammalian cells by PT leads to the ADP-ribosylation of the adenylate cyclase regulatory compoent N_i with resultant loss in responsiveness to effectors which act in negative modulation of adenylate cyclase. The events leading to intoxication are complex and involve first a binding of PT to a specific cell surface receptor. In the current studies the subunits of PT and a specific carbohydrate structure involved in this interaction were identified. The interaction of PT with goose erythrocytes, as monitored by hemagglutination, requires the concurrent presence of the S2, S3 and S4,5 subunits which together constitute the B subunit (see Figures 1-3) originally described by Tamura and coworkers (23). In addition to its hemagglutination activity, the B subunit has been shown to inhibit intoxication by the holotoxin and function as mitogen stimulating DNA synthesis in mouse lymphocytes (see Ui this volume). Together these data indicate that the B subunit provides the recognition specificity for binding of pertussis toxin to cells. However, the role of the individual toxin protein subunits (i.e., S2, S3 and S4,5) in mediating binding remains to be elucidated.

The interaction of PT with the model ligand fetuin has permitted elucidation of the chemical nature of the receptor. The toxin recognizes structural elements present in the asparagine-linked carbohydrate moieties of fetuin. Analysis of this interaction by sequential removal of terminal sugar residues has established that the minimal carbohydrate structure required contains the branched mannose core with N-acetyl glucosamine attached. Once β-1,2 linked N-acetyl glucosamine is removed using Jack Bean hexosaminidase, a derivative is obtained which no longer interacts with PT. The asparagine linked carbohydrate of fetuin is triantennary containing a β-1,4 linked N-acetyl glucosamine in addition ot the two β-1,2-linked residues. However, the triantennary structure and the β-1,4 linked are not essential for PT binding. Not only does the toxin interact poorly with fetuin derivatives containing only the β-1,4 linkage, but PT can bind effectively to immunoglobulins where the structure is biantennary and the β-1,4 linkage absent (Figure 7). On the basis of these considerations we conclude that minimal structure for PT binding is the biantennary mannose bridge substituted with N-acetyl glucosamine in β-1,2-linkage. From hemagglutination inhibition studies it was shown that these same carbohydrate

structural elements contribute to the interaction of PT with goose erythrocytes. Although it is possible that pertussis toxin can interact with cells by mechanisms distinct from the hemagglutination type activity (see Ui this volume and 43), the current data suggest that recognition of carbohydrate structures such as those found in fetuin and the immunoglobulins is involved in PT binding and leads to subsequent intoxication of cells.

ACKNOWLEDGEMENTS

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GTP-REGULATORY PROTEINS ARE INTRACELLULAR MESSENGERS: A MODEL FOR HORMONE ACTION

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I.INTRODUCTION

It is now firmly established on physiological,molecular and genetic grounds that receptors for a large number of hormones and neurotransmitters, as well as for light in the case of rhodopsin,are linked in the plasma membrane to a family of proteins that have three properties in common: they bind GTP selectively and with high affinity, they degrade the nucleotide to GDP,and they are composed of at least three distinct subunits, one of which is common to the three that have thus far been most extensively characterized (1). Although not yet on firm ground, evidence has accumulated, in part based on the effects of pertussis toxin, that the family of GTP-proteins may have to be increased to include receptors that regulate the metabolism of phosphatidylinositol and those processes related to the release of intracellular calcium (2). Recently, it has been reported that a ras oncogene binds and degrades GTP, thus raising the distinct possibility that hormones involved in cellular growth similarly may be linked through GTP-binding proteins in the transduction process (3).

A few years ago it was proposed that the complexes between receptors and the GTP binding proteins that govern stimulation and inhibition of adenylate cyclase, termed N_s and N_i, respectively, may exist in the form of oligomers (4). Binding of hormones and GTP to these complexes results in disaggregation to smaller, monomeric units which represent the primary "signal" or messenger produced by hormones. Rather than being the first messenger, cyclic AMP produced as a result of monomer action reflects the relative concentrations of monomer N_s and Ni available for interaction with adenylate cyclase. The most important aspect of this hypothesis was the suggestion that a macromolecule rather than a simple organic molecule such as cyclic AMP may be the regulatory signal

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that sets in motion the cascade of events leading to the multiple responses of cells to hormones. At that time it was not known that N_s and N_i contain three distinct proteins, only one of which binds GTP. Nor was it known that an analogue of GTP, such as Gpp(NH)p,and fluoride ion, in the presence of high concentrations of Mg ions, cause dissociation of the GTP-binding subunit(1).Nonetheless, the general concept of a macromolecule being the primary signal of hormone action remains viable.

Here this general concept is extended to suggest that the pleiotropic effects of hormones on their target cells are not due uniquely to the production of a particular signal (such as cyclic AMP or calcium), as in the original second-messenger concept. Rather, as depicted in Fig 1, the alpha subunits released in response to hormones and GTP are capable of interacting with a variety of effector (E) systems within the target cells, each effector producing or transmitting specific "secondary signals" that are in turn, responsible for the pleiotropic effects.

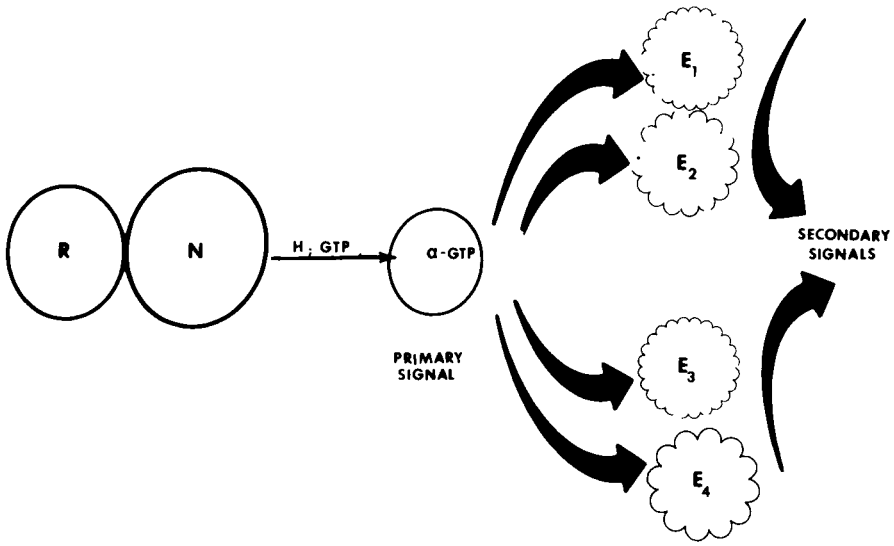

Fig .1. A model depicting the actions of hormones (H) and GTP. Binding of these agents to receptors (R) and N units stimulate release of alpha subunits and subsequent interaction with various effector (E) units that produce secondary signals.

This postulate will be discussed, using a few examples, as a means of explaining certain phenomenon that hitherto were not readily understood on the basis of previous models of hormone action.

II.Multiple Roles of N_s

Perhaps the best documented case for N_s serving multiple roles as a transducing agent is the finding in mouse S49 lymphoma cells that N_s is required for the expression of catecholamine action on both cyclic AMP production and inhibition of Mg transport (5). In the variant S49 cell termed cyc^- which does not contain N_s , neither response is obtained despite the presence of beta adrenergic receptors and adenylate cyclase. Nor does cyclic AMP or activation of the cyclic AMP-dependent protein kinase substitute for catecholamine action, as would be expected if Mg transport were regulated by the kinase. The conclusion from such findings is that N_s serves both as regulator of adenylate cyclase and of Mg transport in these cells.

A multiple role of N_s also seems likely in rat adipocytes. For example, the so-called lipolytic hormones that promote lipolysis in adipocytes by stimulating cyclic AMP production also inhibit glucose transport. In carefully controlled studies comparing levels of cyclic AMP-dependent protein kinase and glucose transport in response to a variety of stimuli, Londos,Cushman, and colleagues concluded that inhibition of glucose transport does not correlate with activation of the kinase and, hence suggested that the stimulants may be acting through a process independent of adenylate cyclase activation (6).These findings, though not proof of N_s acting directly on glucose transport, suggest that the lipolytic hormones may be acting through N_s to mediate both alterations in glucose transport as well as adenylate cyclase activity.

Another possible example is the recent report that beta adrenergic agonists stimulate calcium transport in heart muscle via a process that does not correlate with activation of cyclic AMP-dependent protein kinase since the isolated calcium transporter seems not to be phosphorylated by this kinase(7).

In addition to the above findings, there are numerous reports in the literature showing a lack of correlation between levels of cyclic AMP production in response to hormones and the effects of the hormones on various metabolic processes. Although explanations such as sensitivity of cyclic AMP detection and compartmentation have been offered to explain such discrepancies, it seems equally plausible that, in addition to cyclic AMP production, hormones affect other processes through their ability to activate N_s ; i.e., at subthreshold hormonal concentrations with respect to protein kinase activation, the combined actions of N_s on various effector systems, even though perhaps minimally activated, yield large effects on the metabolism of the cell. Admittedly, this idea is difficult to test. It should be noted ,however, that the premise that cyclic AMP, acting through protein kinase, is responsible for all actions of cyclase -stimulating hormones has not been proven despite years of intensive research.

III. The multiple Roles of N_i

The GTP-binding protein designated N_i mediates the effects of a variety of hormones acting through receptors that affect inhibition of adenylate cyclase.Among an increasing number, such receptors include those for somatostatin,opiates,acetyl choline (muscarinic), and several catecholamine receptors including those for epinephrine and dopamine. One characteristic shared by these receptors is that pertussis toxin, as discussed in several papers at this meeting, blocks the actions of these hormones or neurotransmitters through modification of the alpha subunit of N_i.

An interesting feature of N_i , in contrast to N_s , is that it shares structural,functional, and immunological homology with not only the beta subunit but also with the GTP-binding alpha subunit of transducin, the GTP-binding protein that mediates light activation of a cyclic GMP-phosphodiesterase (8). Such findings raise the possibility that $alpha_i$ is sufficiently malleable in structure to interact with several effectors.

One of the first indications that N_i may have pluralistic functions stems from the report that pertussis toxin-treatment of rats results in marked changes in the effects of catecholamines on adipocytes subsequently isolated from the animals. Among the effects noted were inhibition of ^{32}P incorporation into phosphatidylinositol when stimulated by epinephrine, and enhancement of adenylate cyclase activity

and lipolysis in response to the hormone (2). These effects of catecholamines are typical of alpha-1 and alpha-2 adrenergic receptors, respectively. Hence, these findings suggest that both types of receptors are linked to N_i, if one assumes that pertussis toxin exerts all of its actions uniquely through modification of N_i.

It should be noted that a number of hormones or neurotransmitters that inhibit adenylate cyclase activity through N_i are also capable of stimulating turnover of phosphatidylinositol, a process that is linked to release of calcium from internal cellular stores(9).The designation of receptors as having one or the other function is often made with the use of specific antagonists that block one or the other effect of the agonist. Hence, the designation of alpha adrenergic receptors as alpha-1 and alpha-2. Not excluded,however, is the possibility that a common"signal" emanates from both receptor types, the degree of expression of the signal being determined by the threshold levels required for the signal to promote inhibition of adenylate cyclase versus the processes involved in turnover of phosphatidylinositol. If the alpha subunit of N_i should be the common signal for both, problems of interpretation of receptor types may be encountered.

An example of this type of problem is the recent report showing that epinephrine exerts multiple effects on the islet cell of rat pancreas (10). Epinephrine acts on the insulin-releasing beta cells by blocking insulin release induced by a variety of agents that stimulate cyclic AMP production. This process is sensitive to pertussis toxin,suggesting an involvement of N_i and to the typical apha-2 blocker yohimbine; hence the receptor has features of alpha-2 and therefore should be involved in attenuating adenylate cyclase activity (11). However, it was found that epinephrine did not alter stimulated cyclic AMP production and, in fact, even inhibited insulin release that was stimulated by 8-bromocyclic AMP. It was concluded that epinephrine acts independently of or beyond the step of cyclic AMP production.Although the mechanism is not known,it appears likely that the inhibitory effect is related to alterations in calcium-release since calcium is known to be involved in stimulus-secretion coupling in the pancreas.

Despite the failure to observe effects of epinephrine on cyclic AMP production in intact pancreatic islets, it was shown in the same study that epinephrine inhibited forskolin-stimulated adenylate cyclase in broken cell preparations.It would appear,therefore, that the beta cell contains alpha receptors that mediate two distinct actions of epinephrine, both of which may be mediated by N_i. It is still

not clear, however, whether the penultimate effect -inhibition of insulin release- is exerted through both inhibition of cyclase and a calcium-dependent process.

IV. The Role of N Units in Insulin Action

Insulin exerts a variety of effects on cells, including cell growth, the synthesis of proteins, fats, and carbohydrates, transport of ions, sugars, and amino acids, and the control of levels of cyclic AMP. The insulin receptor has been purified and shown to contain at least two subunits of which one exhibits a tyrosine kinase activity stimulatable by the hormone (12). Despite intensive research,however, there is still no definitive evidence that would explain the pleiotropic effects of insulin. Substances reported to have insulin-mimetic effects are abundant, as are claims for isolation of insulin's "second messengers".

In the past few years, there have been several reports suggesting that GTP may be involved in the expression of insulin's actions at the membrane level. One report suggests that GTP is required for insulin to stimulate a protein kinase in the sarcolemma of muscle (13). Others have reported that GTP is involved in the stimulatory effects of insulin on a phosphodiesterase and on inhibition of adenylate cyclase in rat liver plasma membranes (14).

In adipocytes, insulin stimulates glucose transport by mechanisms that are independent of cyclic AMP production. Insulin reduces cyclic AMP levels in adipocytes possibly due to activation of a low K_m cyclic AMP phosphodiesterase present in intracellular membranes (15). The hormone may also act by inhibiting adenylate cyclase although evidence for direct effects in isolated membranes remains controversial. The bulk of the evidence suggests that insulin acts on the phosphodiesterase and adenylate cyclase via indirect processes.

As stated previously, lipolytic and anti-lipolytic agents such as isoproterenol and adenosine, respectively, exert opposing effects on both adenylate cyclase activity and glucose transport. An intriguing aspect of insulin's action on these processes is the diminished responsiveness of adipocytes to the hormone when the adenosine concentration in the incubation medium is reduced (16,17). Recently, Londos and colleagues have found that insulin at physiological concentrations (10 to 100 microunits per ml) failed to either lower cAMP or inhibit lipolysis in media containing neither stimulants or inhibitors of adenylate cyclase(18).Insulin activity was restored in the presence of agonists, such as

adenosine plus isoproterenol, which act through adenylate cyclase-associated receptors. Interestingly, these ligands restored not only insulin's ability to lower cAMP but also the antilipolytic actions of insulin that are clearly independent of cellular cAMP concentrations. These data indicate that the cyclase-associated receptors regulate events other than cyclase activity, a conclusion reinforced by the finding that adenosine reverses the catecholamine inhibition of insulin-stimulated glucose transport in a manner independent of adenosine effects on cAMP (19). How, one might ask, can these cyclase-associated components (R's and N's) regulate both cellular cAMP concentrations and events apparently independent of cAMP?

As a working hypothesis, Figure 2 illustrates activation and release of the alpha subunits by hormones and GTP operating on both stimulatory and inhibitory receptor/N complexes. It is postulated that the released alpha units can take inactivated states; insulin affects the conversion of both the $alpha_s$ and $alpha_i$ regulatory units to putative inactivated states. As for the nature of these states, one possibility for consideration is that the alpha

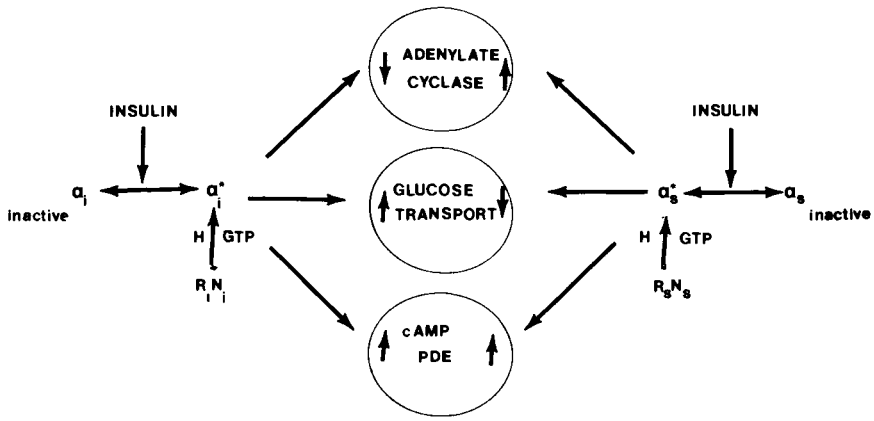

Fig 2. A model for insulin action via modification of the alpha subunits of N_s and N_i. See text for details.

units are phosphorylated by specific protein kinases; the phosphorylated $alpha_s$ is rendered inactive whereas $alpha_i$ is put into an active form. Insulin, in this example, could affect both alpha units by a common process if it regulated a phosphatase that controls the dephosphorylation of the phosphorylated states of the two alpha units. In this manner, it can be readily understood how insulin controls in opposing manner those effectors that mediate secondary signal production. By the same reasoning, the effectiveness of insulin as an antilipolytic agent would be determined by the concentration of the primary signals, $alpha_s$ and $alpha_i$. Hence, when adenosine is absent and therefore $alpha_i$ is not produced in sufficient quantity, insulin's action would be minimal as an antilipolytic effector (and possibly also as a stimulator of glucose transport) since there would not be a counter-regulatory action against the effects of the $alpha_s$ unit.

V. Release of Alpha Units from Membranes

One of the compelling reasons for the general acceptance of cyclic nucleotides, calcium, and other small molecules as second messengers of hormone actdion is that these molecules can readily traverse the various compartments of the cell and therefore impinge upon the various effectors or regulatory systems throughout the cell. Their effective levels in the cell can be regulated by destruction or sequestration, or by export from the cells. On the other hand, there is nothing inherently difficult in viewing relatively large macromolecules, such as the alpha units (41k to 48k) , as messengers in the sense of traveling about the cytosol of cells and reacting with a variety of sensory systems. An important difference from the small organic molecules is that such proteins are malleable in their structure and subject to covalent and non-covalent modification. Thus, one form of the signal arising from the membrane can take numerous structural forms, each of which may have differing affinities for the variety of effector systems to which they are exposed. In fact, calmodulin and other calcium binding proteins are good examples of how macromolecules modulate a variety of cellular processes. The question to be asked at this juncture, therefore, is whether the alpha units are released in soluble form from the plasma membrane in a form accessible for interacting with effector systems in other parts of the cell or on other

segments of the plasma membrane than their origin in the form of N units. There are two studies which strongly suggest that this is the case.

One study showed that supernatant fractions from homogenates of rat liver, heart, skeletal muscle, dog liver, and rabbit liver contained an activity that restored Mg-dependent fluoride- and guanine nucleotide-stimulated adenylate cyclase activity in cyc^- S49 murine lymphoma cell membranes (20). The properties of the soluble material resembled those of $alpha_s$. Moreover, the material was subject to activation by cholera toxin. Approximately 25% of this material was found in the supernatant fraction (300,000 x g). Hence, from these findings it would appear that $alpha_s$ is present in soluble form within cells.

A second study, which is perhaps more relevant to the hypothesis presented here, was also carried out with cyc^- membranes from S49 lymphoma cells which lack the $alpha_s$ unit and is a useful membrane for monitoring the presence of N_s by virtue of the acquired activation of the catalytic unit by the activated $alpha_s$ unit (21). In this study, purified plasma membranes from human erythrocytes, rat liver, and turkey erythrocytes were incubated with the cyc^-membranes without detergents or any known factors that would cause either extraction of membrane components or fusion of the donor and acceptor membranes. When the donor membranes were incubated under conditions that caused activation of N_s , such as with fluoride ion or guanine nucleotides in the presence of high Mg concentrations, or treatment with cholera toxin and NAD followed by GTP and low concentrations of Mg ions, the acceptor cyc^- membrane acquired adenylate cyclase activity that was not detected in control cyc^- membranes. Moreover, such treatment of the donor membranes was required to observe "complementation". One explanation for these findings is that $alpha_s$ is liberated from its N_s complex by the various activators and is subsequently transferred to the cyc^- membranes where it can associate with adenylate cyclase.

A prerequisite for testing this hypothesis is the ability to separate cyc- from the donor membranes subsequent to co-incubation of the membranes under "complementation" conditions. We have succeeded recently in separating human erythrocyte membranes from cyc^- membranes after centrifugation on sucrose gradients that allow the donor membranes (erythrocytes) to sediment whereas all of the cyc^-membranes remain at the surface of the gradient. After co-incubation under complementation conditions, erythrocyte membrane markers (such as acetylcholinesterase) were not detectable in the cyc^-fraction. More critically, when the $alpha_s$ subunit of N_s in the erythrocyte membrane was labeled by treatment with choleragen and ^{32}P-NAD, labeled $alpha_s$ was not detected in the sucrose

boundary layer in the absence of complementation with cyc^-membranes following centrifugation. By these criteria, therefore, it was possible to test whether the subsequently isolated cyc^-membranes would acquire both MgATP-dependent adenylate cyclase (as one indication of complementation by a transferred $alpha_s$ unit) and ^{32}P-labeled $alpha_s$ subsequent to treatment of erythroyte N_s with choleragen and ^{32}P-NAD.

We have completed the initial phase of such studies by demonstrating transfer of $alpha_s$ according to the above criteria. Judged from the amount of ^{32}P-labeled material transfered as $alpha_s$, no more than 10 percent of $alpha_s$ is transferred from the donor membranes to cyc^- when the membranes were co-incubated at a ratio of 10:1. Doubling the amount of $alpha_s$ at a fixed concentration of donor membranes increased the amount of transferred $alpha_s$, suggesting that the "acceptor" sites for $alpha_s$ on cyc^- membranes are the limiting factor in the degree of transfer.

Currently, we are investigating the various conditions required for maximal transfer of $alpha_s$ to human erythrocytes. In order to assess whether hormones promote transfer of $alpha_s$, we plan to employ rat liver and turkey erythrocyte membranes for complementation assays since these membranes contain receptors for glucagon and catecholamines that promote activation of N_s in the presence of guanine nucleotides.

Although incomplete, our findings to date indicate that $alpha_s$ is liberated from human erythrocyte membranes under activating conditions and forms complexes with adenylate cyclase and possibly other effector processes (such as that responsible for inhibiting Mg-transport) in cyc^- membranes. It remains to be determined whether alpha subunits from the other types of GTP-binding proteins involved in membrane transduction processes will behave similarly. In any event, the hypothesis remains tenable that the primary "messenger" of hormones acting through complexes of receptors and GTP-binding proteins are the subunits of N released in response to the concerted actions of hormones and GTP.

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STRUCTURAL AND FUNCTIONAL PROPERTIES OF N_s AND N_i, THE REGULATORY COMPONENTS OF ADENYLYL CYCLASES*

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I. INTRODUCTION

The regulation of adenylyl cyclase by hormones and neurotransmitters requires the simultaneous action of a guanine nucleotide. This was first shown for stimulatory hormones,

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such as glucagon (1), and later for inhibitory hormones as well (2, 3). Two key findings established, at least for stimulatory effects, that guanine nucleotide regulation of adenylyl cyclase is mediated by a protein (N_s) distinct from receptors or from the catalytic component of the system. First, the resolution of GTP-binding proteins from the catalytic activity of adenylyl cyclase (4); and second, the characterization of a genetic variant of the S49 mouse lymphoma cell line (cyc$^-$) deficient in both guanine nucleotide and stimulatory hormone regulation of adenylyl cyclase, yet containing both receptors and a catalytically active adenylyl cyclase enzyme (5, 6). Kinetic experiments (7, 8) suggested that there might also be an inhibitory regulatory protein (N_i) distinct from N_s. It was not possible to prove this to be the case, however, until the demonstration by Katada and Ui (9, 10) that a toxin produced by Bordetella pertussis simulataneously blocks receptor-mediated inhibition of adenylyl cyclase and ADPribosylates a protein of M_r 40,000 that is different from the subunit of N_s that is ADP-ribosylated by cholera toxin. The separate nature of N_i from N_s was subsequently shown by the purification of guanine nucleotide-sensitive, pertussis toxin substrates separate from N_s (11, 12) and by the demonstration that the pertussis toxin substrate of cyc$^-$ S49 cell membranes is coupled to inhibitory regulation of adenylyl cyclase in the absence of a functional N_s (13, 14). Both of these proteins, N_s and N_i, have been purified to homogeneity from a number of different sources (12, 15, 16, 17, 18, 19). The characterization of the structure and function of these two proteins as purified from human erythrocytes is the primary topic to be discussed here.

II. PURIFICATION OF N_s AND N_i

N_s was first purified by Gilman and collaborators from rabbit liver by stabilizing the protein with the presence of NaF and Mg (18). The purification described here (see Table I) is similar to these earlier ones, but was developed with the express intent of avoiding the use of ligands which activate the protein (15). In this case, stabilization of N_s activity is accomplished by using high concentrations of β-mercaptoethanol (20 mM), by avoiding the presence of Mg after the initial solubilization and by including in all of the buffers 30% ethyleneglycol (15). The N_s activity that is thus isolated is stable in these buffers for weeks at 4°C, and indefinitely at -70°C.

Nearly coincident with N_s throughout much of the purification is a protein which is a substrate for ADPribosylation by pertussis toxin toxin (eg. Figs 1 and 2). As also

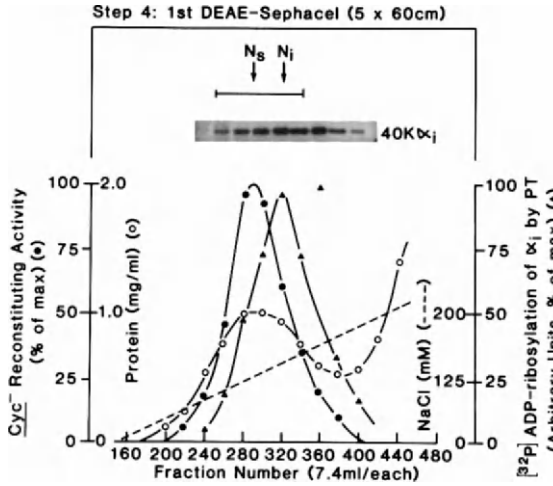


Figure 1: Elution profile of the first chromatographic step of the purification of human erythrocyte N_S and N_i: ion exchange chromotography on DEAE-Sephacel. N_S was localized in the column effluent by its ability to reconstitute GMP-P(NH)P stimulation of the cyc⁻ S49 cell membrane adenylyl cyclase. N_i was localized by its ability to be ADP-ribosylated by pertussis toxin. Inset: autoradiogram of the M_r 40,000 region (α_i region) of a polyacrylamide gel run after labeling N_i with [^{32}P]NAD in the presence of pertussis toxin.

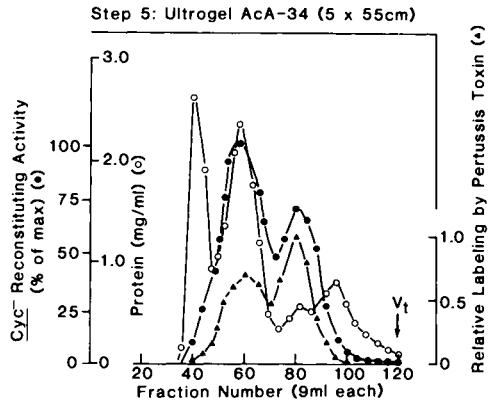
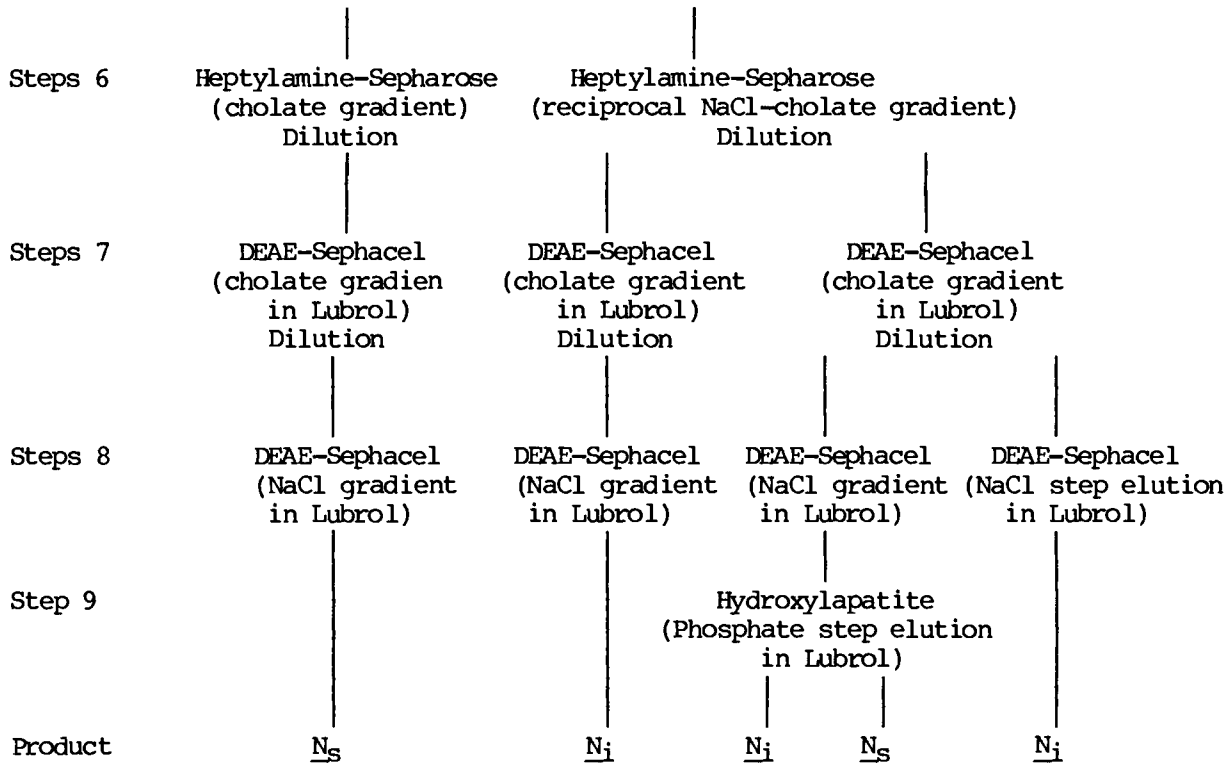


Figure 2: Elution profile of the second chromatographic step in the purification of human erythrocyte N_S and N_i: gel filtration on AcA 34 Ultrogel. For other details see the legend of Fig 1.

Table I

Summary of Purification Scheme Used

	Human Erythrocytes
Step 1	Lysis and Preparation of Washed Membranes
Step 2	Pre-extraction of Membranes
Step 3	Preparation of Cholate Extract
Step 4	DEAE-Sephacel (NaCl gradient in cholate) Dialysis plus Concentration (Amicon PM-10)
Step 5	AcA-34 Ultrogel
	Peak A Peak B


Steps 6
Heptylamine-Sepharose (cholate gradient) Dilution
Heptylamine-Sepharose (reciprocal NaCl-cholate gradient) Dilution
Steps 7
DEAE-Sephacel (cholate gradien in Lubrol) Dilution
DEAE-Sephacel (cholate gradient in Lubrol) Dilution
DEAE-Sephacel (cholate gradient in Lubrol) Dilution
Steps 8
DEAE-Sephacel (NaCl gradient in Lubrol)
DEAE-Sephacel (NaCl gradient in Lubrol)
DEAE-Sephacel (NaCl gradient in Lubrol)
DEAE-Sephacel (NaCl step elution in Lubrol)
Step 9
Hydroxylapatite (Phosphate step elution in Lubrol)
Product
Ns
Ni
Ni
Ns
Ni

shown by Bokoch et al. (11), the final purified protein is structurally related to N_s in that it also contains a peptide of M_r 35,000 (see below) and its hydrodynamic behavior is altered by treatment with guanine nucleotides and Mg (11, 16). This protein, in so far as it can presently be characterized, appears to be the N_i that mediates hormonal inhibition of adenylyl cyclase in intact systems.

One of the differences between the purification of N_s and N_i in the presence or absence of activating ligands such such as NaF is the fact that the proteins not treated by these ligands behave in a heterogeneous manner (Table I). In Fig 2 is shown one of the early steps in the purification, gel filtration on AcA 34 Ultrogel, in which both the N_s and N_i activities split up into two discrete peaks (labeled A and B). The A peak contains a predominance of N_s relative to B, and the B peak contains a predominance of N_i relative to A; there are significant quantities of both proteins in each peak. The functional significance of this is unclear, and may be due to the interaction of the N proteins with contaminating proteins. The presence of NaF (17) appears to convert most of the protein to a single peak, and we have not yet observed any functional difference in the N_s activity obtained in preparations A and B. Neverthelss, the differences evident in this early step carry over to the subsequent steps of the purification. In the finally purified N_s preparations (Table I), that from the A peak contains virtually no contaminating N_i, whereas that from the B peak still contains significant N_i contamination. Thus, although speculative, the N_s and N_i proteins may be functionally heterogeneous with respect to their ability to associate with one another, and this ability may be altered under conditions conducive to activation of the proteins (17).

This purification scheme (15) yields about 1 mg of total N_s protein from 50 g of erythrocyte membranes. This represents about a 5,000-fold purification with 10% recovery. The yield of N_i protein is about twice that of N_s, i.e., about 2 mg, with perhaps 5% recovery since the N_i in the A preparation, representing perhaps one half the total (see Fig 2), has not been purified further. Assuming that the recoveries of the two proteins would otherwise be the same, this represents a minimum of a 4-fold excess of N_i over N_s in the intact erythrocyte membrane.

III. PHYSICAL CHARACTERIZATION OF THE PURIFIED PROTEINS

By hydrodynamic characterization (Table II) N_s and N_i are large proteins, each of 96,000 daltons, with significant detergent binding in Lubrol PX. In the case of N_s, 34% of the calculated molecular weight of the detergent-protein

Table II

Some Molecular Parameters of N_s, N_i and the 40K $\beta\gamma$ Complex

	N_s	N_i	"40K Protein"
$S_{20,w}$ (Corrected for detergent binding)	4.1	4.1	2.0
Stokes' Radius (nm)	5.9	5.6	5.1
Detergent Bound	34%	11%	8%
Molecular Weight (protein)	96,000	96,000	40,000
Subunit Composition	$\alpha\beta\gamma$	$\alpha\beta\gamma$	$\beta\gamma$

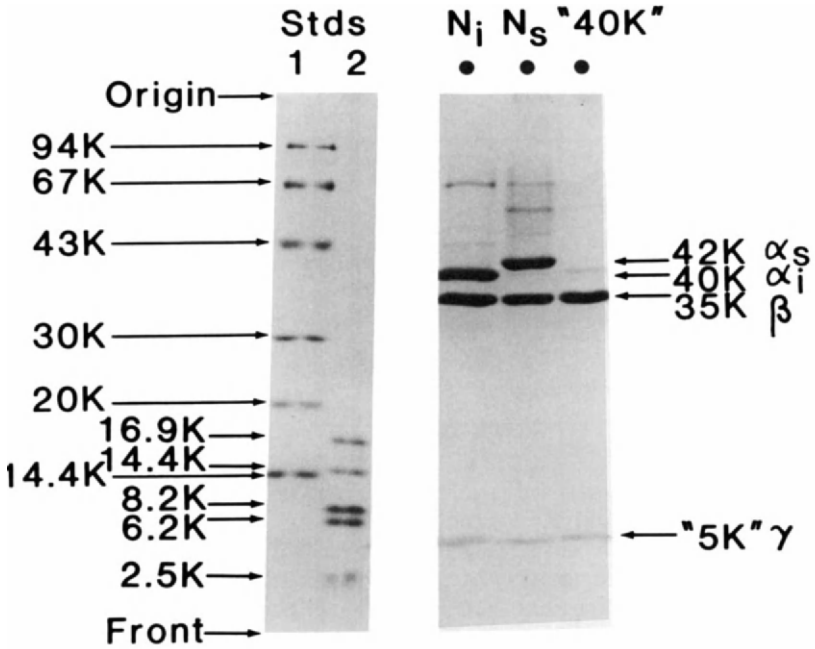


Figure 3: A discontinuous urea and polyacrylamide gradient gel of N_s, N_i and "40K" protein stained with Coomassie blue. The gel shows the standards used to calibrate the gel along with the α, β and γ subunits of N_s and N_i. For further details see Ref. 20.

complex is due to detergent binding. On gradient SDS-PAGE in the presence of urea, both N_s and N_i are $\alpha\beta\gamma$ trimers (Fig 3) with subunits of M_r 42,000 (α_s), 35,000 (β) and approximately 5,000 (γ) for N_s and M_r 40,000 (α_i), 35,000 (β) and approximately 5,000 (γ) for N_i (20). The estimated molecular weights for the α and β subunits of each protein were verified by subjecting them to SDS-PAGE at varying acrylamide concentrations and constructing Ferguson plots (Fig 4). When the α and β subunits of N_s and N_i were compared by two-dimensional peptide map analysis (15), the β subunits were found to be identical, whereas the α subunits, which are the actual substrates for ADP-ribosylation by bacterial toxins, were found to be different (Fig 5). This is in agreement with the findings of Manning and Gilman (21) who compared the same subunits on the basis of amino acid

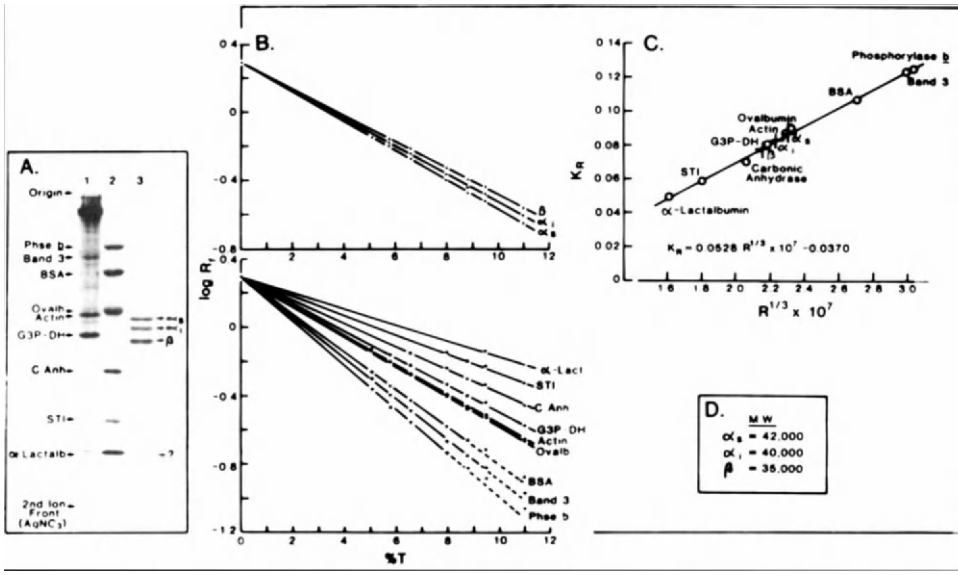


Figure 4. Analysis of the molecular weights of the α and β subunits of N_s and N_i by the method of Ferguson. A mixed preparation of N_i and N_s was electrophoresed at different polyacrylamide concentrations. A) Example of one of the polyacrylamide gels run. B) Plots of the relative mobilities of the subunits of N_s and N_i (top) or standard proteins (bottom) as a function acrylamide concentration. C) Plot of the slope of each line in B versus the cube root of the molecular radius for each standard protein. Also indicated are the positions of the α_s, α_i and β subunits on the standard curve. D) Estimated molecular weights of α_s, α_i and β from the data in panel C. For further details see Ref. 15.

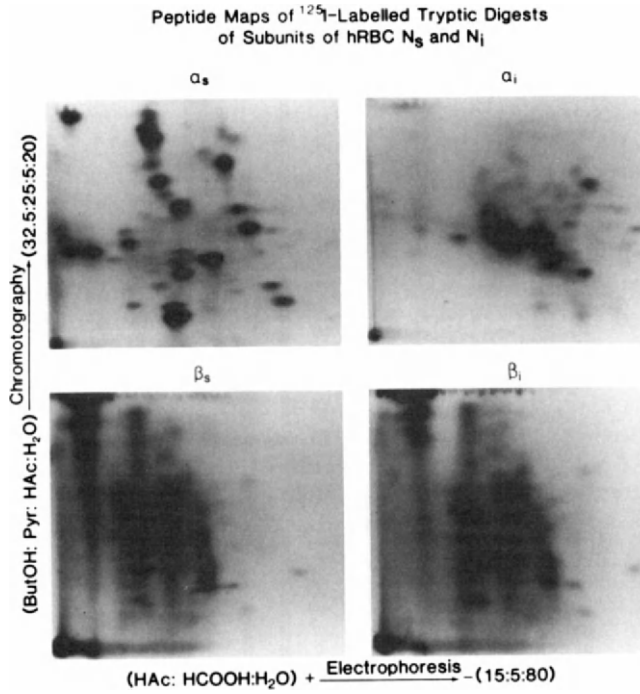


Figure 5: Two dimensional peptide map analysis of the α and β subunits of N_S and N_i. Samples of N_S and N_i were run on a polyacrylamide gel and stained with Coomassie blue. Protein-stained bands corresponding to the α and β subunits of each protein were cut from the gel, iodinated in situ with ^{125}I and chloramine T, and then subjected to tryptic digestion. Peptides freed from the gel matrix were analyzed by thin layer electrophoresis in one direction, followed by thin layer chromatography perpendicular to the first dimension. Shown are autoradiograms made from such maps of α_S, α_i, β_S and β_i. For further details see Ref. 15.

composition and limited proteolysis followed by one-dimensional SDS-PAGE. The β subunits of N_S and N_i are also identical to an M_r 35,000 peptide found in a side fraction of the B preparation from the purification (Table I) which is free of either α_S or α_i. This protein, however, also has associated with it a low molecular weight subunit (γ) of approximately the same size as the γ subunits of N_S and N_i (Fig 3) and behaves hydrodynamically as a 2S complex with M_r 40,000 (Table II). It is referred to by us as "40K protein" or 2S complex.

A. Characterization of γ as Part of N_S and N_i

The presence of a low molecular weight γ subunit asso-

ciated with N_s and N_i was suspected, in part, because of the similarity of these proteins to transducin from retinal rod outer segments. Transducin regulates the activity of a cGMP phosphodiesterase (22, 23) and is itself an $\alpha\beta\gamma$ trimer (24, 25, 26, 27). Its β subunit is structurally related to the β subunits of N_s and N_i (21) and its α subunit is a substrate for both pertussis toxin (28) and cholera toxin (29).

Although a γ-type peptide is present in N_s and N_i preparations (Fig 3), it was necessary to establish some functional relationship of this peptide to the other two subunits especially since the putative γ subunit does not stain well with Coomassie blue and apparently represents very little mass. In order to establish such a relationship, the known effect that Mg and guanine nucleotides have on the size of the N proteins was taken advantage of. Fig 6 shows the results of an experiment with N_i (similar results have been obtained with purified N_s) in which the Stokes' radii of α_i, β and putative γ were assessed by gel filtration

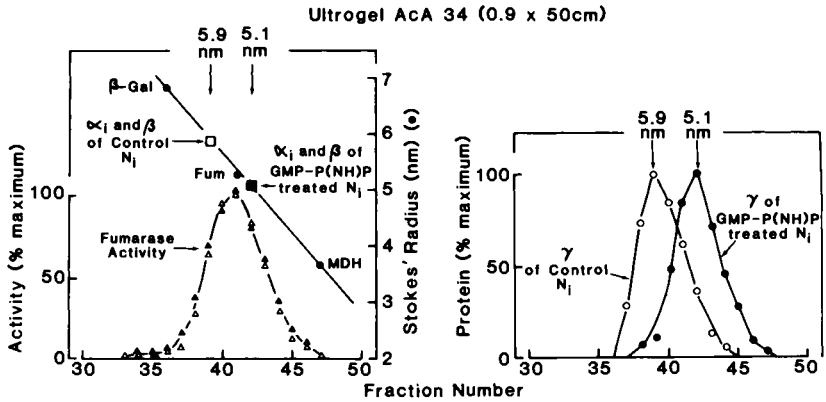


Figure 6: Relationship of the γ subunit to the α and β subunits of N_i. Samples of N_i were incubated at 32°C in the presence either of GDP and EDTA (Control) or GMP-P(NH)P and Mg, and then subjected to chromatography on Ultrogel AcA 34 in the presence of the same ligands plus fumarase as a positional marker on the column. Fractions were collected and analyzed for protein composition on the polyacrylamide gel electrophoresis system shown in Figure 3. The Left panel shows the calibration of the column, the alignment of the fumarase markers in the two column runs, and the position of the α and β subunits of N_i from each column run. The Right panel shows the distribution of silver-stainable material associated with the putative γ subunit during each run, determined by densitometric scanning of silver stained gels.

over AcA 34 Ultrogel, before and after treatment with Mg and GMP-P(NH)P. It can be seen that, whereas the α_i and β subunits of the GMP-P(NH)P- and Mg-treated ("activated") form traveled with a Stoke's radius of 5.1 nm, the subunits of the control GDP- and EDTA-treated form ("unactivated N_i") traveled at 5.9 nm. This is compatible with the idea that the N_i, upon exposure to guanine nucleotides and Mg, undergoes subunit dissociation. In parallel with the α and β subunits of the protein, the low molecular weight peptide also changed its hydrodynamic behavior. This established the functional relationship between this peptide (γ) and the other two subunits: first, because it is unlikely that a small molecular weight peptide could have a Stokes' radius as large as 5.1 or 5.9 nm (equivalent in mass to a protein in excess of 100,000 molecular weight); and second, because the putative γ subunit responds in parallel with the α and β subunits to the treatment with guanine nucleotide and Mg.

At present, our other knowledge of the γ subunits of N_S and N_i is limited. The fact that γ travels at 5.1 nm Stokes' radius after treatment with GMP-P(NH)P and Mg suggests that even after "activation" it remains associated with one of the other two subunits of the protein. Since γ is also found in

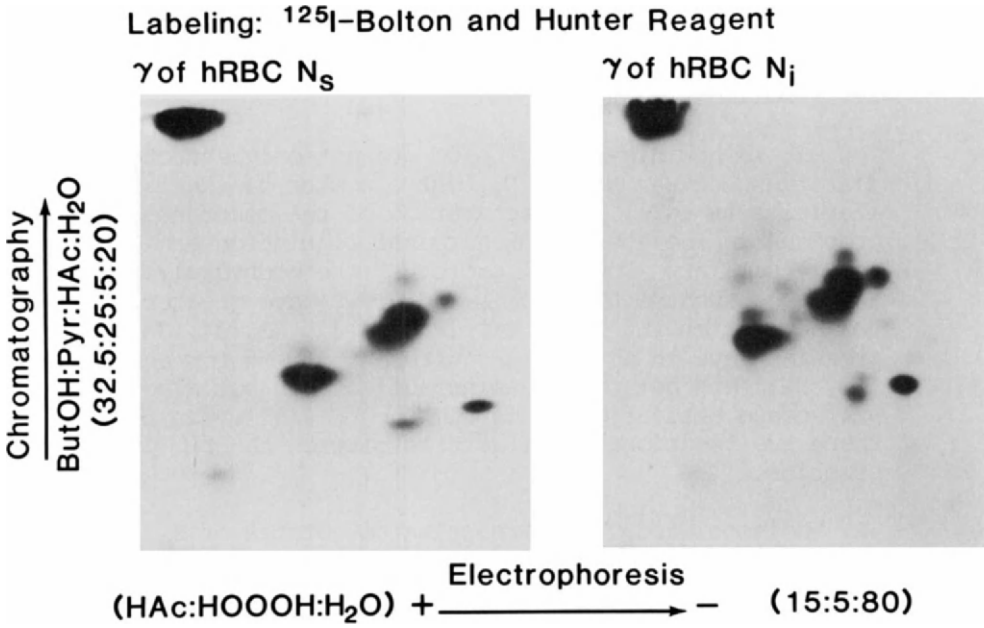


Figure 7: Two dimensional peptide map analysis of the γ subunits of N_S and N_i. The same procedure as described in the the legend to Fig 5 was used except that labeling was done with [^{125}I]Bolton-Hunter Reagent.

association with "free" β subsubunit (Fig 3), it presumably is bound to β in analogy to the $\beta\gamma$ complex of transducin (24). The formation of a $\beta\gamma$ complex is apparently the reason that the "free" β subunit behaves hydrodynamically like an M_r 40,000 protein (Table II). Two-dimensional maps of tryptic peptides of γ subunits separated from N_s and N_i by SDS-PAGE and then labeled in situ with [^{125}I]Bolton-Hunter reagent (30) show them to be very similar if not identical (Fig 7). The stoichiometry of γ to the other subunits is not known. Although there is much less Coomassie blue staining of γ then of β or α, this is actually expected since even at a stoichiometry of 1:1:1 there would be 1/17 to 1/8 as much mass associated with γ than with β or α. It is curious that in comparing N_s to N_i, there often appears to be less γ of N_s than of N_i, when referred to the intensity of α subunit staining. This could be due to differences in stoichiometry for the two proteins. If there is a difference in stoichiometry of γ to α and β of N_s and N_i, this may denote a possible functional difference in the action of β in N_s and N_i./ We have never observed separation of β from γ (31) as is the case for transducin (24). It is therefore possible that γ modulates the activity of the β subunits and that if the stoichiometry of γ to β varies this could lead to functional differences.

IV. FUNCTIONAL CHARACTERIZATION OF N_s AND N_i

It is now clear that there are not only structural similarities between N_s and N_i, but a number of functional similarities as well. The activation of the membrane-bound proteins in the absence of hormonal regulation are qualitatively similar. For both proteins, the nonhydrolyzable GTP analogues such as GMP-P(NH)P and GTPγS have greater efficacy for activation than does GTP itself (32, 33, 34, 35). Both proteins have an absolute requirement for Mg for activation (33, 36), and not only guanine nucleotides but also fluoride will cause activation of both N_s and N_i (32). In addition, there are functional similarities between the two purified proteins.

A. Undissociated, Yet "Preactivated" States of N_s and N_i

In the case of purified N_s and N_i, both have been shown to undergo a reduction in size upon exposure to either guanine nucleotides or NaF in the presence of Mg. This appears to represent subunit dissocation of the proteins (11, 12). As illustrated in Fig 8, and shown recently for both N_s and N_i (37) we have found that there are in fact three discrete states of the proteins regulated by Mg and guanine nucleo-

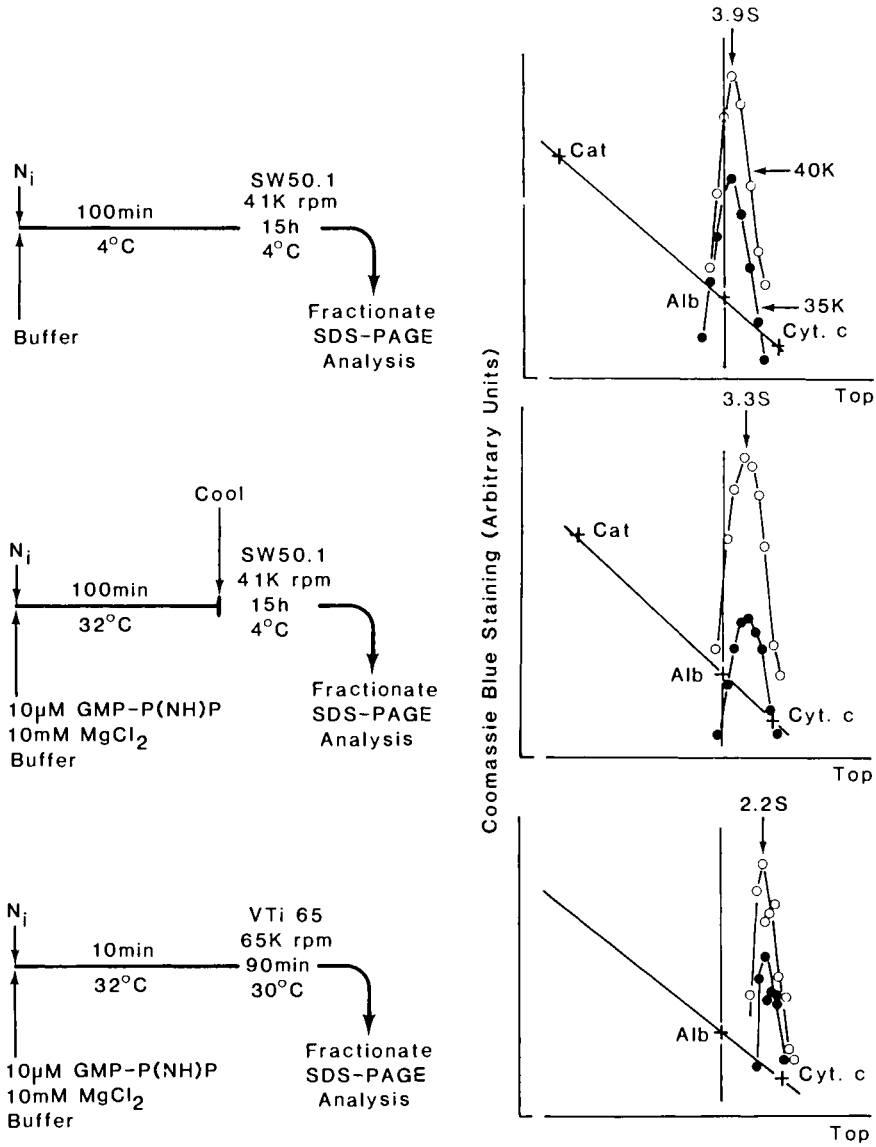


Figure 8: Sucrose density gradient analysis of N_i. Samples of N_i were treated according to the protocols indicated on the Left, and then subjected to analysis on linear 5-20% sucrose gradients along with calibrating standards. The distribution of Coomassie blue-stained α and β subunits of N_i along with the positions of the standard protein markers are shown in the Right panels. Markers are catalase ($S_{20,w}$ = 11.3), bovine serum albumin ($S_{20,w}$ = 4.2) and cytochrome C ($S_{20,w}$ = 1.7).

tides. There is a 4S form of each protein which does not have guanine nucleotide bound and, in the case of N_s, is "unactivated" in the sense that its reconstitution of the adenylyl cyclase of cyc⁻ S49 cell membranes requires the simultaneous addition of nucleotide. This is the form isolated in the above described purification. In addition, there are two forms of each protein which have nucleotide bound and, in the case of N_s, are "preactivated" in that they do not require the simultaneous addition of guanine nucleotide to reconstitute the adenylyl cyclase activity of cyc⁻ S49 cell membranes. These are 2.0S dissociated forms of the the proteins and what we call "3S" forms of N_s and N_i (even

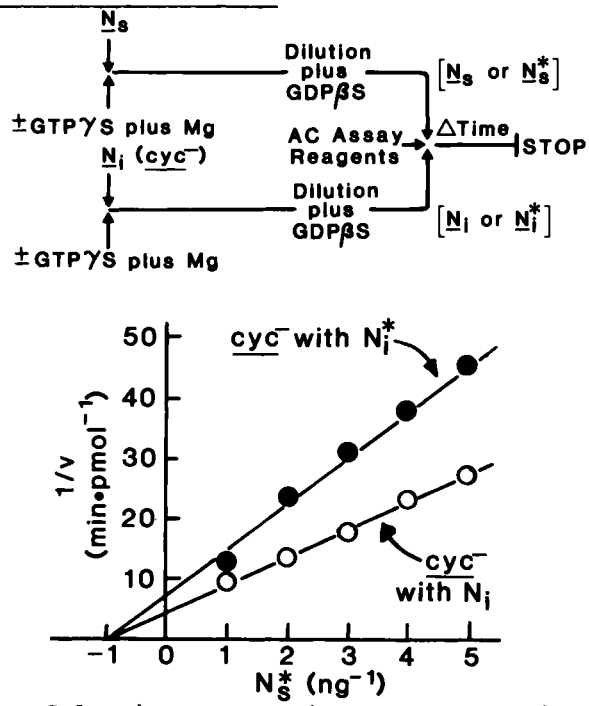


Figure 9: Selective and agonist dependent stimulation of GTPase of N_s by pure beta-adrenergic receptor. N_s and N_i were reconstituted in phospholipid vesicles with pure beta-adrenergic receptor as described in refs. 14 and 51. These vesicles were then assayed for GTP hydrolysis in the absence and the presence of isotoproterenol and isotoproterenol plus excess beta-adrenergic antagonist (alprenolol, Δ). Note that hydrolytic rates obtained with N_i are but 10% of those obtained with N_s. The stimulation of GTP hydrolysis by N_i on addition of isoproterenol, although small, is significant. Inset to right panel: same data as right panel with 20-times expanded y-scale. From 51.

though the actual apparent S value of this form of N_i in 0.5% Lubrol PX is 3.2-3.3S), which are nondissociated. These nondissociated forms, although stable under certain conditions, form in the cold in a Mg- and nucleotide-dependent manner, have nucleotide tightly bound to them and are thought to be precursors of the dissociated forms. The existence of a nondissociated but "preactivated" form, which can also be obtained with NaF (37) may explain the perplexing paradox by which Gilman and collaborators had purified N_S in its undissociated form in the presence of activating ligands (18, 19). It is quite possible that the form, at least of N_S, which is purified in the presence of fluoride is the 3.0S form described here.

B. N_S and N_i as GTPases

In many respects, just as in the case of regulation in the absence of hormone, regulation of N_S and N_i by receptors also resemble each other. The affinity of either stimulatory or inhibitory hormones for their respective receptors is decreased by guanine nucleotides (38, 39, 40) and increased by Mg (41, 42, 43). Both hormonal stimulation and hormonal in-

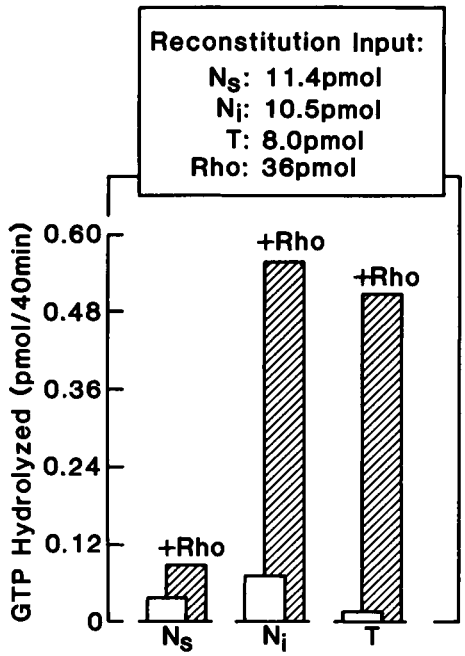


Figure 10: Rhodopsin-mediated stimulation of GTP hydrolysis by N_i. From 51.

hibition of adenylyl cyclase are accompanied by an increased release of guanine nucleotides from membranes (measured by release of prebound radiolabeled nucleotide) which is thought to reflect increased guanine nucleotide exchange (44, 45) and a hormone-dependent increase in GTP hydrolysis (46, 47, 48). In the case of stimulatory hormones, the N_s itself has been shown to have GTPase activity (49, 50), provided it is assayed after reconstitution with stimulatory hormone receptor (50, 51). This is illustrated in Fig 9, where agonist (isoproterenol) stimulation of GTP hydrolysis by a reconstituted β-adrenergic receptor-N_s system is shown. In contrast, purified N_i exhibits GTP hydrolyzing activity (albeit low) even in the absence of a functional hormone receptor (52, 53). Like N_s, GTPase at N_i is stimulated on reconstitution with an appropriate receptor. Interestingly, reconstitution of N_i with rhodopsin (Fig 10) but not a fully purified β-adrenergic receptor also results in stimulation of GTP hydrolysis (54, 51). This indicates that the α subunits of transducin and N_i must share a common domain and that the domain of rhodopsin responsible for interacting with transducin and N_i must resemble the domain of R_i-type receptors that couple to N_i.

C. Kinetic Regulatory Cycles of N_s ands N_i

The data cited above suggest that N_i and N_s are functionally, as well as structurally, very similar proteins and that they each are regulated by an activation/deactivation

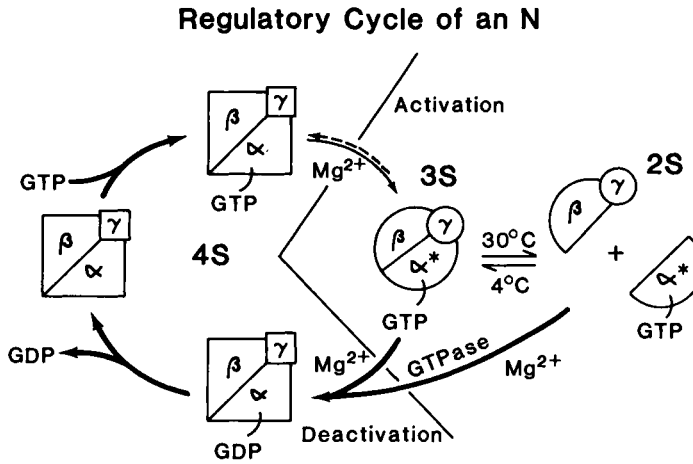


Figure 11: Schematic representation of the kinetic regulatory cycles affecting either N_s or N_i through activation by GTP and Mg and deactivation by hydrolysis of the bound GTP to GDP.

cycle involving GTP hydolysis in the deactivation step. A generalized model for the activation of N proteins by GTP, based in part on the observations summarized here, is shown in Fig 11. As the model is drawn, it is applicable to either N_S or N_i. In both cases there is a guanine nucleotide dependent formation of a high molecular weight $\alpha^{GTP}\beta\gamma$ complex (4S form) which is converted in a Mg-dependent manner to a high molecular weight $\alpha^{*GTP}\beta\gamma$ complex (3S form) capable of dissociating into α^{*GTP} and $\beta\gamma$ components (2S form). The fact that guanine nucleotide is shown to be capable of binding before the Mg-dependent activation step occurs is consistent with the studies of Northup et al. with purified N_S (55) showing that photoaffinity labeling of the protein with a GTP analogue did not require the presence of Mg.

D. Kinetic Differences Between N_S and N_u.

In spite of the overall similarities in the kinetic cycles for the regulation of the activation of N_S and N_i, as indicated in Fig 11, there are differences between these two proteins. Although both proteins require guanine nucleotide and Mg for activation (33, 36), their dependence upon Mg is not identical. Thus, N_S requires rather high (mM) Mg concentrations for activation, whereas N_i requires very low low (on the order of 1 μM) concentrations. Interestingly, hormonal stimulation of N_S in membranes results in a reduction of its Mg requirement for activation to the same low uM concentraions required for the activation of N_i in the absence of an activated hormone receptor (56). This suggests that the shift in affinity for Mg at N_S may not be the only effect of hormonal stimulation and in fact may be but a reflection of another change relating to activation.

Another difference between N_S and N_i is the consequence of cholera or pertussis toxin action on their respective substrates. The ADP-ribosylation of N_S by cholera toxin results in the potentiation of the action of GTP such that it acquires the same efficacy as the nonhydrolyzable GTP analogues (57). The ADP-ribosylation of N_i by pertussis toxin, however, results in a state where GTP is ineffective at activating the protein, even though the action of GMP-P(NH)P is virtually unaltered in comparison (58). Further, while pertussis toxin treatment results in inhibition of hormonal regulation of N_i (e.g., 59, 60, 61, 62, 63, 64), cholera toxin treatment does not interfere with hormonal regulation of N_S (65) and if anything facilitates it (66, 67). These differences occur in spite of the fact that both toxins block the hormonal-stimulation of GTP hydrolysis that is associated with N_S (47) and N_i (59, 68, 64).

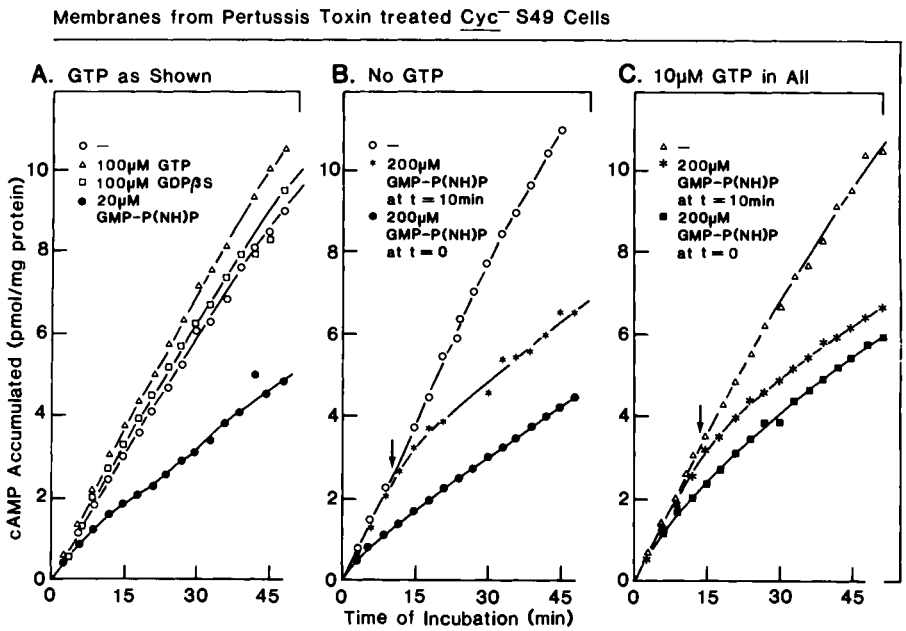


Figure 12: GTP does not inhibit in a persistent manner GMP-P(NH)P-mediated activation of pertussis toxin-treated N_i. Membranes from pertussis toxin-treated cyc⁻ S49 lymphoma cells were incubated (58) for the indicated times. Additions of GTP, GMP-P(NH)P and GDPβS and the times at which they were added are indicated on the figure. Note that GTP is unable to cause inhibition of cyc⁻ adenylyl cyclase (failure to activate N_i) and that GMP-P(NH)P added to an incubation containing GTP results in inhibition of cyclic AMP formation (lack of persistent inhibitory effect of GTP).

E. Rate Limiting Step Affected by Toxins.

The kinetic parameter in the activation process of pure N_S by GTP affected gy ADP-ribosylation with cholera toxin was investigated (70). It was concluded that ADP-ribosylated N_S differs from control N_S in that the interaction strength between α_S and β (presumably $\beta\gamma$) is diminished and that the net effect of cholera toxin treatment is to promote the $\alpha\beta\gamma$ to α^{GTP} plus $\beta\gamma$ subunit dissociation reaction.

It is interesting to note, however, that on treatment

with GTP cholera toxin-treated N_S exhibited a hydrodynamic behavior consistent with formation of a 3S form, rather than a fully dissociated mixture of 2S α_S^G plus 2S $\beta\gamma$ (70).

The kinetic parameter in the turnover cycle of N_i affected by pertussis toxin was also investigated (71). Results with cyc⁻ membranes showing loss of action of GTP and preservation of action of nonhydrolyzable GTP analogues (14, 58) indicate that the actual GTP to GDP reaction was not inhibited. Two approaches were used to test whether subsequent cycling was inhibited because of accumulation of N_i-GDP complexes. The first used cyc⁻ membranes and tested whether pre-exposure of pertussis toxin-treated N_i to GTP would result in inhibition of the effect of GMP-P(NH)P. As illustrated in Fig 12, such persistent inhibition of GMP-P(NH)P action could not be demonstrated, indicating that the inefficiency of GTP to activate N_i was not due to an arrest of the cycle in the N_i-GDP form. The second approach tested the effect of ADP-ribosylation of N_i on its endogenous GTPase activity. As illustrated in Table III, GTP hydrolysis by N_i is unaffected by pertussis toxin treatment. This confirms affect the capacity of N_i to hydrolyze GTP. It would appear that the effect in ADP-ribosylated N_i is its susceptibility to be activated by GTP itself. That is, even though GTP binds it is unable to induce the conformational change that leads to N_S activation.

V. THE MECHANISM OF ACTION OF N_i

From what is now known about the structure and function of N_S and N_i, there are two opposing, although not necessarily mutually exclusive, views about how N_i might bring about its effect on the catalyst. The first is that α_i^{*GTP} (or possibly the $\beta\gamma$ complex from N_i; see below) would induce an inhibited activity state of the catalytic component; and the other, proposed by Katada et al. (64, 72, 73) is that the $\beta\gamma$ complex liberated by the dissociation of N_i upon its activation blocks the subsequent activation of N_S. Certainly the $\beta\gamma$ subunit-mediated deactivation of N_S can occur (74), but the functional significance of this observation for the mechanism of action of N_i is still unclear. Before this mechanism can be fully accepted as the primary action of N_i there are four issues which must yet be settled.

1) The role of subunit dissociation in the activation of N_S has been worked out with the use of nonhydrolyzable guanine nucleotides. It has not yet been shown that the protein dissociates under the influence of GTP, or even under the influence of the nonhydrolyzable analogues in a membrane environment. Nor has it been demonstrated that the $\alpha_S^{*GTP}\beta\gamma$ is not active with respect to the catalyst. Germane to this

Table III

Lack of effect of Pertussis Toxin-mediated ADP-ribosylation on GTPase Activity of N_i

N_i (6 pmol) was incubated for 45 min at 32°C with 2 mM NAD^+, 0.1 mM ATP, 1 µM GTP, 0.1% BSA, 1 mM EDTA and 10 mM Tris-HCl, pH 7.6, in the absence and the presence of pertussis toxin (PT) in a final volume of 25 ul. Ten-ul aliquots were then assayed for GTPase activity in a final volume of 100 ul containing 100,000 cpm [γ-^{32}P]GTP (sp. act. 10^6 cpm/pmol), 1 mM EDTA, 5 mM $MgCl_2$ and 100 nM GTP carried over from the treatment step. Incubations were for 10 min at 32°C and the $^{32}P_i$ liberated was assayed as desribed in (53). One-µl aliquots of the treated N_i were also subjected in parallel to a second ADP-ribosylation treatment in a final volume of 100 µl in the presence of 10^6 cpm of [^{32}P]NAD^+ (sp. act. 10^4 cpm/pmol), 20 µM NAD^+ (carried over from the first treatment step), 1 mM ATP, 1 uM GTP, 0.1% BSA, 10 mM thymidine, 1 mM EDTA and 10 mM Tris-HCl, pH 7.6. The reactions were stopped and incorporation of [^{32}P]ADP-ribose into N_i was determined by autoradiography after separation of N_i by SDS-PAGE.

ADP-Ribosylation Treatment (45 min)		GTPase Activity of N_i*	Incorporation of [^{32}P]ADP-ribose into Treated N_i
Additions	Temperature	(fmol P_i formed)	(intensity of spot on autoradiogram)
--	32°C	157 ± 13	++++
PT	32°C	181 ± 15	-
--	4°C	176 ± 6	++++

*Means of duplicates ± 1/2 the range.

issue is the finding of Northup et al. (75, 76) that the associated 4.0S $\alpha\beta\gamma$ complex when added to cyc$^-$ S49 cell membranes in the presence of Mg and a non-hydrolyzable guanine nucleotide has twice the reconstituting activity of the separated and GTP.S-activated α_s subunit.

2) Data from the cyc$^-$ S49 cell, which has no functional N_s activity (see Ref. 58 for review), indicate that adenylyl

cyclase can be inhibited through N_i without the necessity of the enzyme being stimulated by N_S (32, 33, 58, 14, 31, 73). The degree of inhibition seen in cyc⁻ S49 cell membranes by nonhydrolyzable GTP analogues is equivalent to that seen in intact membrane systems by hormone in the presence of GTP (32). In addition, kinetic data (52, 74) suggest that N_i and N_S act in a noncompetitive manner to regulate the catalyst (Fig 13) and that N_i action is not associated with deactivation of the N_S it is inhibiting (Fig 14). This makes it unlikely that the N_i of cyc⁻ is having an aberrant

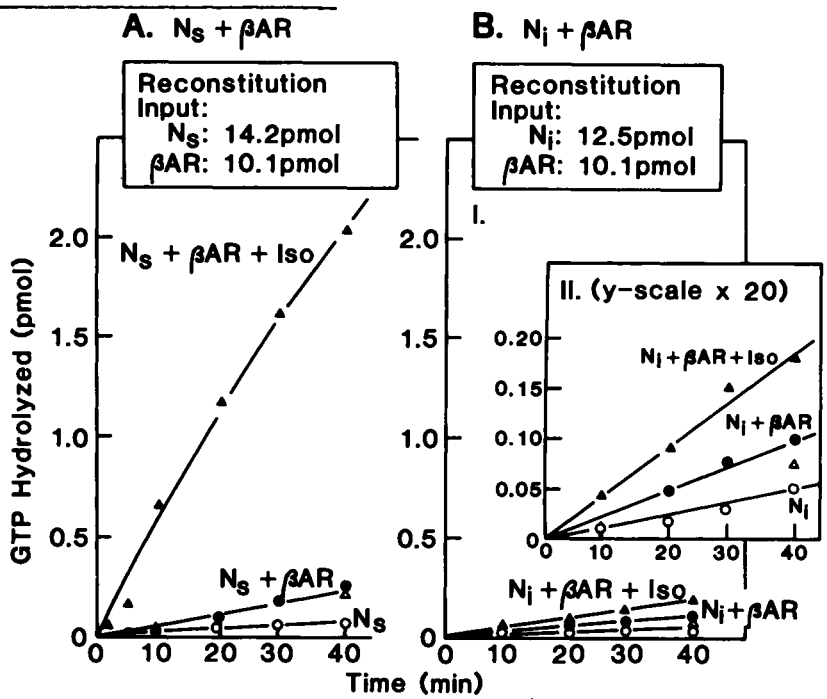


Figure 13: Non-competitive nature of N^i-mediated inhibition of N_S-mediated activation of cyc⁻ membrane adenylyl cyclase. Cyc⁻ membranes (N_i) and N_S were treated or not with GTP.S and magnesium to yield N_S^*, N_i and N_i^*, the activated forms of N_S and the inactive and activated form of N_i. Treated cyc⁻ membranes were then reconstituted with increasing concentrations of N_S^* and the resulting steady state activities (74) were determined and plotted in a double-reciprocal fashion. Note that since straight lines intersecting at a common point of the x axis were obtained, this indicates that the percent inhibition of activity due to N_i activation was constant regardless of the level of N_S^* added. This defines the non-competitive nature of N_i-mediated inhibition of N_S^* action.

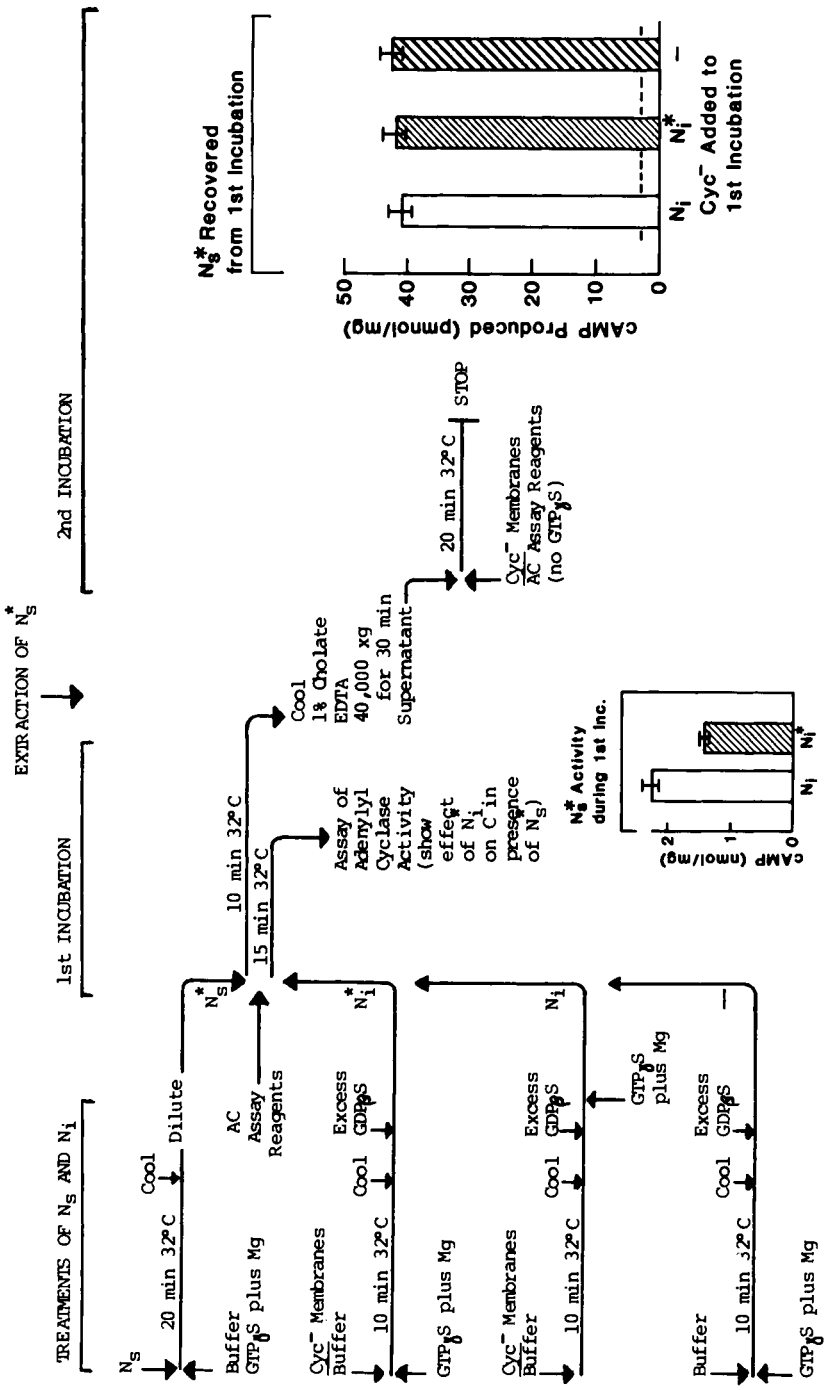

TREATMENTS OF N_s AND N_i
1st INCUBATION
EXTRACTION OF N_s^*
2nd INCUBATION
N_s
20 min 32°C
Cool
Dilute
Buffer
GTPγS plus Mg
N_s^*
10 min 32°C
15 min 32°C
AC Assay Reagents
Cool 1% Cholate EDTA 40,000 xg for 30 min Supernatant
Assay of Adenylyl Cyclase Activity (show effect of N_i^* on C in presence of N_s)
20 min 32°C
STOP
Cyc⁻ Membranes
AC Assay Reagents (no GTPγS)
Cyc⁻ Membranes
Buffer
10 min 32°C
Excess GDPβS
N_i^*
GTPγS plus Mg
Cyc⁻ Membranes
Buffer
10 min 32°C
Excess GDPβS
N_i
GTPγS plus Mg
Buffer
10 min 32°C
Excess GDPβS
—
GTPγS plus Mg
N_s^* Activity during 1st Inc.
cAMP (nmol/mg)
0
1
2
N_i
N_i^*
N_s^* Recovered from 1st Incubation
cAMP Produced (pmol/mg)
0
10
20
30
40
50
N_i
N_i^*
Cyc⁻ Added to 1st Incubation

Figure 14

Figure 14: Lack of deactivation of N_S^* by N_i^*. N_S and cyc$^-$ membranes (N_i) were activated or not by treatment with GTP.S as indicated. The resulting treated forms of N_S and N_i were then mixed to effect reconstitution of the cyc$^-$ adenylyl cyclase system. The effectiveness the N_i^* in cyc$^-$ membranes to inhibit N_S^* was assayed. N_S was then extracted with cholate from parallel mixtures and assayed for its state of activity in a second reconstitution assay. A control N_S^* was carried through the same procedure but omitting cyc$^-$ membranes from the first reconstitution incubation step. Note that even though N_S^* had been exposed to N_i^*, as evidenced by the reduced reconstituted activity obtained during the first reconstition incubation, this N_i^*-mediated inhibition of N_S^* action was not due to deactivation, as evidenced by the recovery of equal N_S^* activity from incubations of N_S^* with cyc$^-$ membranes with unactivated N_i or activated N_i (N_i^*).

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effect on the catalytic component by affecting the site which would normally be occupied by N_S.

3) The kinetic data on the effects of "free 35K" (presumably $\beta\gamma$) to deactivate N_S (74, 75, 76) and to inhibit the adenylyl cyclase activity of membrane preparations (64, 72), are not entirely consistent with a simple model of deactivation of N_S as a mechanism of action of N_i. Although "free 35K", as well as our $\beta\gamma$ 2S complex, readily deactivate a NaF-activated N_S, it deactivates GTPγS or GMP-P(NH)P-activated N_S only very slowly, i.e., with a $t_{1/2}$ on the order of hours (75, 76). Nevertheless, activation of N_i appears to at least partially inhibit a GMP-P(NH)P-activated N_S even in intact membrane systems (75, 77). In addition, although it should be possible to fully inhibit N_S activity by adding a sufficiently high concentration of "free 35K", this apparently does not happen and addition of "free 35K" to membrane preparations leads to inhibition which is no greater than and not additive with an inhibitory hormone and GTPγS (64, 72).

4) Even though cholera toxin treatment of N_S has been reported to result in at least 100-fold reduction in the potency with which $\beta\gamma$ complexes can inhibit N_S activation (70), we have recently found that treatment of the platelet membranes with cholera toxin to the extent that GTP stimulation is comparable to that of GMP-P(NH)P, does not result in a decrease in the efficacy or a shift in the dose-response curves with which epinephrine (acting through N_i) attenuates this adenylyl cyclase.

The four points just summarized make it impossible at present to make a concrete statement about the mechanism of action of N_i. It is quite possible that both mechanisms,

i.e., direct action of N_i on the catalyst and $\beta\gamma$ complex-mediated deactivation of N_s, could each play a role. But, if true, under defined circumstances one might expect one to be more important than the other.

VI. CONCLUSION

The results of the studies summarized here indicate that N_s and N_i are structurally and functionally very similar proteins. The activation of both conform to the generalized scheme shown in Fig 11. There are, however, subtle differences between N_s and N_i indicated by their different depenpencies upon Mg for activation, their GTP hydrolyzing activities in the presence or absence of receptors and their responses to ADP-ribosylation by bacterial toxins. Such differences might be expected, since the two proteins have different functions and dissimilar α subunits, the two proteins may nevertheless have similar rate-limiting steps in traversing the cycle in Fig 11, and toxins may affect the same step but in opposing sense. This rate-limiting step appears to be whatever GTP induces to cause activation of the N protein, and appears also to be the step affected by hormone receptor. One of the key questions yet to be answered about the regulation of adenylyl cyclase is the mechanism of action of N_i. Does it affect the catalytic component directly via its α_i subunit or does it do it via its $\beta\gamma$ complex? What proportion of its effect on intact cells is due to its direct action on the catalytic unit of adenylyl cyclase and what proportion is due to its $\beta\gamma$ complex inhibiting the activity of N_s? Undoubtedly the answer to this question will in part depend on the purification of the missing partners of the system (a true R_i and the elusive catalyst) and on the successful reconstitution of a fully regulated system with structurally-defined proteins.

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PERTUSSIS TOXIN-CATALYZED ADP-RIBOSYLATION: EFFECTS ON COUPLING OF INHIBITORY RECEPTORS TO ADENYLATE CYCLASE

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I. INTRODUCTION: REGULATION OF THE ADENYLATE CYCLASE SYSTEM

The hormone-sensitive adenylate cyclase system is a membrane-associated, multicomponent complex responsible for the synthesis of cAMP from ATP (1-5). The system is dually regulated by different classes of agonists that either stimulate or inhibit catalytic activity (1-5). Agonists exert their effects by initially binding to specific cell surface receptors (1-5). The agonist-receptor complexes are coupled to the catalytic unit through two guanyl nucleotide-binding regulatory proteins termed G_i and G_s, which mediate inhibition and stimulation, respectively (Fig. 1) (4). Both G_i and G_s are heterotrimers composed of α, β, and γ subunits (4,6). The β and γ subunits (35,000 and 10,000 daltons, respectively) appear to be identical in G_i and G_s (4,6). During solubilization and purification, they exist as a stable $G_{\beta\gamma}$ complex (4,6). The α subunits of G_i and G_s, which bind

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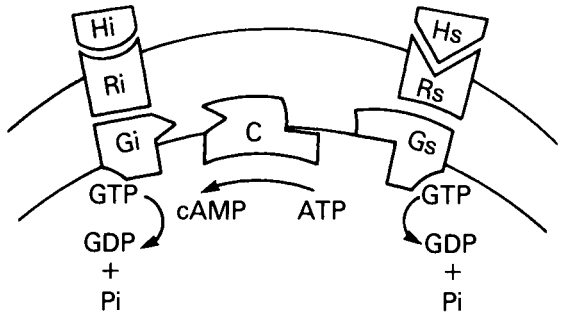

Fig. 1. An adenylate cyclase system. Components of the complex are designated R_s, stimulatory hormone receptor; R_i, inhibitory hormone receptor; G_s, stimulatory guanyl nucleotide-binding protein; G_i, inhibitory guanyl nucleotide-binding protein; and C, catalytic unit; H_i and H_s are inhibitory and stimulatory ligands, respectively. As shown schematically, C is responsible for the conversion of ATP to cAMP. G_s and G_i are in the activated state in the presence of GTP; both proteins are converted to an inactive state by hydrolysis of GTP to GDP and P_i.

guanyl nucleotides, differ in structure (4,7,8); $G_{i\alpha}$ has a molecular weight of 41,000 while molecular weights of 42-45,000 and 47-52,000 have been obtained for two forms of $G_{s\alpha}$ (4,7,9).

Activation of the cyclase catalytic unit by G_s appears to require the dissociation of the inactive complex into $G_{s\alpha}$ and $G_{\beta\gamma}$ (4,8); $G_{s\alpha}$ in the presence of guanosine triphosphate or some stable guanosine triphosphate analogues activates the catalytic unit (4,8). Inactivation of G_s occurs with hydrolysis of GTP and reassociation of $G_{s\alpha}$·GDP with $G_{\beta\gamma}$ to yield an inactive species (4,8,10,11). Stimulatory agonists such as isoproterenol accelerate release of GDP from the inactive species, thereby promoting the binding of GTP (12,13). By enhancing $G_{s\alpha}$·GTP formation, these agents accelerate hydrolysis of GTP by G_s. Activation of cyclase by choleragen-catalyzed ADP-ribosylation of $G_{s\alpha}$ appears to result from two effects. First, the rate of GTP hydrolysis by the ADP-ribosylated $G_{s\alpha}$ is decreased, thus prolonging the life of the active $G_{s\alpha}$·GTP species (14). In addition, the rate of

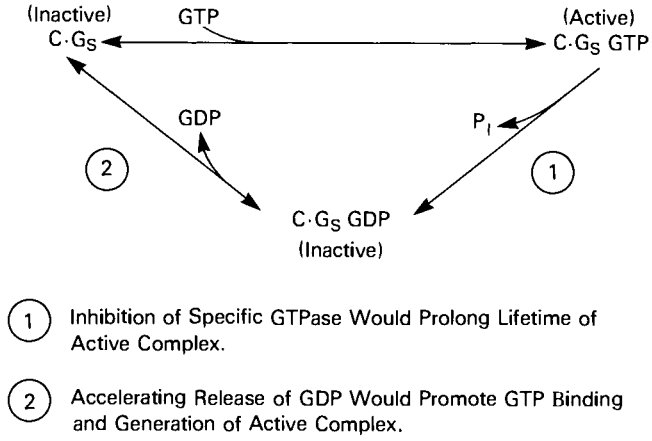

Fig. 2. Activation of adenylate cyclase by choleragen. Definition of G_s and C are given in Fig. 1. Reaction 1: Hydrolysis of GTP bound to $C{\cdot}G_s$ to yield $C{\cdot}G_s{\cdot}GDP$. Reaction 2: Release of GDP from $C{\cdot}G_s{\cdot}GDP$ to give $C{\cdot}G_s$. Choleragen is believed to activate G_s by stabilizing and promoting the formation of the $G_s{\cdot}GTP$ complex.

release of GDP from $G_{s\alpha}{\cdot}GDP$ is accelerated (15,16). Formation of $G_{s\alpha}{\cdot}GDP$ presumably results from a slowed, but significant, hydrolysis of GTP by the ADP-ribosylated $G_{s\alpha}$. Enhanced release of GDP from the $G_{s\alpha}{\cdot}GDP$ complex presumably frees the site so it can once again bind GTP, yielding the active species (Fig. 2). In turkey erythrocyte membranes, the rate of release of GDP by choleragen was approximately an order of magnitude slower than that observed with β-adrenergic agonists, such as isoproterenol (15). The total amount of specifically released GDP was similar with either agent, consistent with the concept that both act through the same guanyl nucleotide-binding protein, G_s (15). Following solubilization in nonionic detergents, the guanyl nucleotide-binding protein and the choleragen substrate coeluted on gel permeation chromatography (16). Further, when the fractions were tested for their ability to restore guanyl nucleotide sensitivity to membranes prepared from S49 cyc^- cells, which are deficient in G_s, it was observed that proteins possessing

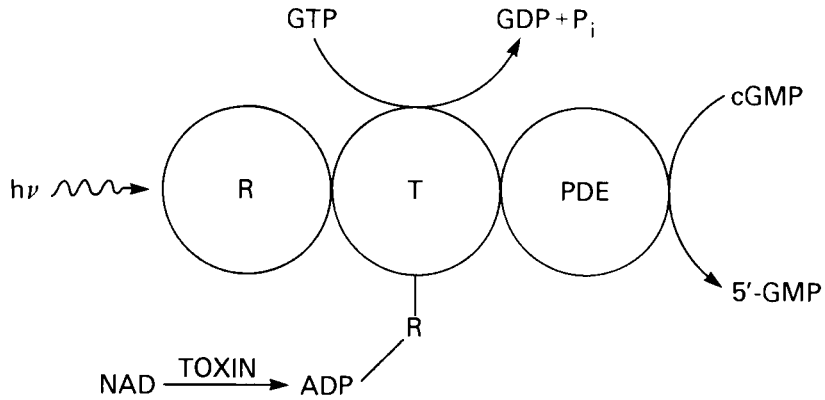

Fig. 3. The rhodopsin-transducin-cyclic GMP phosphodiesterase system of retinal rod outer segments. Components of the complex are represented as R, rhodopsin; T, transducin; and PDE, cyclic GMP phosphodiesterase. Rhodopsin is activated by light (hν). Transducin is active when GTP is bound; hydrolysis of GTP to GDP inactivates it. Choleragen- or pertussis toxin-catalyzed ADP-ribosylation of transducin alters both guanyl nucleotide binding and hydrolysis.

cyc^- recombinant activity cochromatographed with the choleragen substrate and the guanyl nucleotide-binding protein (16). Thus, the target for both the β-adrenergic agonist and toxin was the GTP-binding protein of cyclase.

Interaction of inhibitory agonists with their specific receptors results in the activation of G_i with dissociation into $G_{i\alpha}$ and $G_{\beta\gamma}$, the latter being the complex that G_i and G_s share in common (4,6,17,18). Inhibition of catalytic

activity is believed to result from a combination of two mechanisms (4,18-20). First, dissociation of G_i increases the pool of $G_{\beta\gamma}$, thereby increasing association of $G_{\beta\gamma}$ with the active $G_{s\alpha}$ to form the inactive $G_{s\alpha}G_{\beta\gamma}$ (4,18,19). A second mechanism is based on data demonstrating that hormonal inhibition occurs in membranes prepared from the $G_{s\alpha}$-deficient cyc$^-$ lymphoma cell line (19,20). Since inhibition via the association of $G_{\beta\gamma}$ with $G_{s\alpha}$ is not a possibility, it was proposed that the suppression of catalytic activity resulted from direct effects of $G_{i\alpha}$ on the catalytic unit. As noted previously for the stimulatory arm of adenylate cyclase, an agonist-stimulated GTPase activity is associated with G_i (21-23); with conversion of $G_{i\alpha}$·GTP to $G_{i\alpha}$·GDP, the regulatory protein is converted from an active to an inactive form. Reactivation of $G_{i\alpha}$·GDP then requires release of GDP and rebinding of GTP; indeed, inhibitory agonists stimulate the release of specifically bound guanyl nucleotide and thus make the site available to GTP (24-26).

II. SIMILARITIES BETWEEN THE ADENYLATE CYCLASE COMPLEX AND THE RHODOPSIN-TRANSDUCIN-CYCLIC GMP PHOSPHODIESTERASE SYSTEM

The inhibitory arm of the adenylate cyclase complex shares structural and functional homology with the rhodopsin-transducin-cGMP phosphodiesterase system of retinal rod outer segments (Fig. 3) (4,27-29). Rhodopsin, the photon receptor, is activated by light and corresponds in the photolyzed state to the inhibitory agonist-receptor complex; dark rhodopsin is analogous to the free, inactive receptor (27-31). Activated rhodopsin interacts with the guanyl nucleotide-binding protein, transducin, a heterotrimer of α, β, and γ subunits (39,000, 36,000 and 10,000 Da, respectively); the α subunit binds guanyl nucleotides (29). The protease digestion pattern and the amino acid composition of transducin$_\alpha$ (T_α) and transducin$_\beta$ (T_β) are similar to the corresponding subunits of G_i (32).

Reactions shown to be associated with the inhibitory arm of the cyclase system also are a property of the rhodopsin-transducin complex (28-30,33). In the presence of liposomes containing light-activated rhodopsin, T_α bound guanyl nucleotides such as guanyl-5'-yl imidodiphosphate; binding was facilitated by $T_{\beta\gamma}$ (29). Activation of transducin occurs on dissociation of T_α with bound GTP (or stable analogue) from

TABLE I. Effects of the α and $\beta\gamma$ Subunits of Transducin and G_i of Adenylate Cyclase in the Photolyzed Rhodopsin-stimulated GTPase Reaction

Source of α subunit	GTPase activity · pmol/10 min		
	$No_{\beta\gamma}$	$T_{\beta\gamma}$	$G_{\beta\gamma}$
None	0.03	0.57	0.08
Transducin	4.8	89	53
G_i	1.3	12.9	12.2

The indicated subunits of transducin isolated from bovine retinal rod outer segments or rabbit liver G_i were incubated with photolyzed rhodopsin reconstituted in phosphatidylcholine vesicles, before assay of GTPase activity. Data are from ref. 30.

$T_{\beta\gamma}$ (29); T_α·GTP in turn stimulates the phosphodiesterase and accelerates the degradation of cGMP (29). The precise mechanism of action of T_α·GTP, whether on the cGMP phosphodiesterase directly or on an associated regulatory protein, has not been defined.

Light-activated, but not dark-adapted, rhodopsin stimulated the hydrolysis of GTP by T_α (33); maximal activity was observed in the presence of the transducin$_{\beta\gamma}$ subunits that promoted the replacement of GDP with GTP on T_α (33). In addition to the structural similarities between the inhibitory receptor/G_i and rhodopsin/transducin systems, the G_i and transducin subunits are functionally interchangeable in the rhodopsin-stimulated GTPase reaction (Table I) (30). Rhodopsin enhanced the GTP hydrolysis by $G_{i\alpha}$ (or T_α) and $T_{\beta\gamma}$. Thus, rhodopsin is functionally similar to an inhibitory hormone receptor. In the guanyl nucleotide-binding and GTPase reactions, light-activated, but not dark-adapted, rhodopsin was active (28,29,34). Thus, light-activated rhodopsin would be analogous to the inhibitory agonist-receptor complex while the free receptor would correspond to the dark-adapted form.

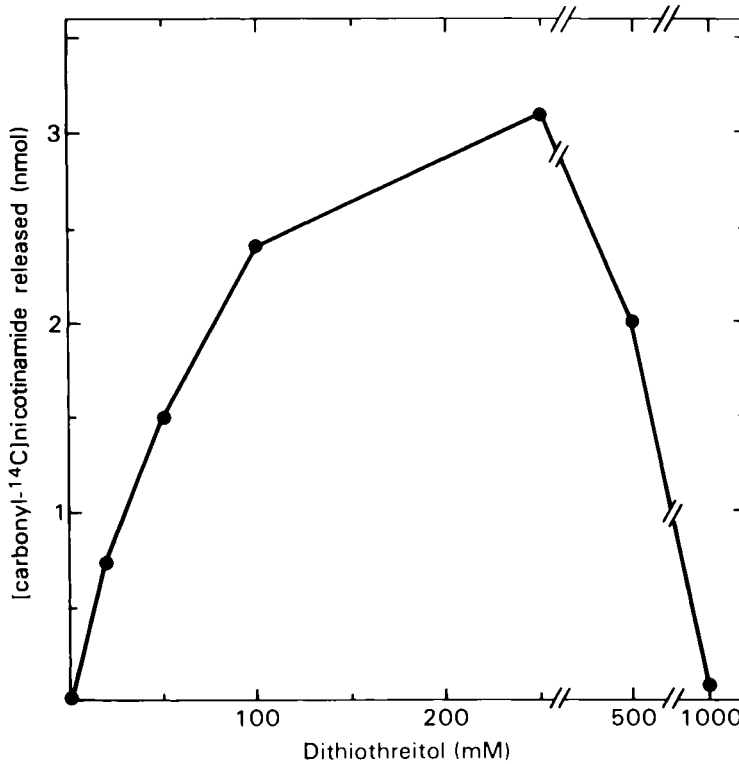

Fig. 4. Effect of dithiothreitol on the NAD glycohydrolase activity of pertussis toxin. Activity was measured by quantifying the release of [carbonyl-^{14}C]nicotinamide from [carbonyl-^{14}C]NAD. Figure is reproduced from ref. 47.

III. ADP-RIBOSYLTRANSFERASE AND NAD GLYCOHYDROLASE ACTIVITIES OF PERTUSSIS TOXIN

Both G_i and transducin are substrates for the ADP-ribosyltransferase activity of pertussis toxin (35-41). Pertussis toxin, a secretory product of Bordetella pertussis, is an oligomeric protein of 117,000 Da composed of five different subunits (S_1,S_2..S_5) (42). The molecular weights of the

component proteins are 28,000, 23,000, 22,000, 11,700, and 9,300 for S_1 through S_5, respectively; there are two S_4 subunits and one each of the others per oligomer (42,43). The binding complex of the holotoxin consists of two heterodimers (S_2S_4) and (S_3S_4) linked by S_5 (42). The enzymatic activity of the toxin resides in the S_1 subunit (42), which catalyzes the transfer of ADP-ribose from NAD to a critical asparagine residue in T_α (and presumably in $G_{i\alpha}$) resulting in its inactivation (Reaction 1) (35,36,39-41,44-46). In the absence of an ADP-ribose acceptor protein, the toxin catalyzes the hydrolysis of NAD to ADP-ribose and nicotinamide (Reaction 2) (47,48).

$$\text{NAD + protein} \longrightarrow \text{ADP-ribose-protein + nicotinamide + } H^{\oplus} \quad (1)$$

$$\text{NAD + HOH} \longrightarrow \text{ADP-ribose + nicotinamide + } H^{\oplus} \quad (2)$$

The holotoxin, although active on cells, appears to be enzymatically inactive (47). Expression of the latent NAD glycohydrolase activity appears to be almost completely dependent on incubation with thiol (Fig. 4) (47); in the absence of a denaturant, such as urea, maximal NAD glycohydrolase activity is observed in ~ 100-250 mM dithiothreitol (47). With the isolated S_1 subunit, significantly lower concentrations of thiol are effective (48).

Reduction of S_1 did not result in significant changes in the mobility of the protein on sodium dodecyl sulfate-polyacrylamide gels; it was therefore proposed that reduction of intrachain disulfide bonds was involved in the activation of the subunit (43,48). When the ability of toxin to ADP-ribosylate $G_{i\alpha}$ in various membrane preparations was examined, it was observed that thiol was almost absolutely required with membranes from human red blood cells but only stimulated the activity with preparations from NG108-15 (Fig. 5) (47). These studies are consistent with the hypothesis that activation of the toxin in cells may involve enzymatic reduction of intrachain disulfide bonds in the S_1 subunit; prior studies have shown that activation of choleragen A protomer in the presence of limiting thiol is enhanced by thiol:protein disulfide oxidoreductase (49).

ADP-ribosylation of the 41 kDa substrate in cell membranes (presumably $G_{i\alpha}$) by pertussis toxin appears to be enhanced by nucleotides, with ATP exhibiting more activity than GTP (Fig. 6) (35). With solubilized and purified $G_{i\alpha}$, no ATP requirement was observed (7). Since ATP also increased the hydrolysis of NAD to ADP-ribose and nicotinamide

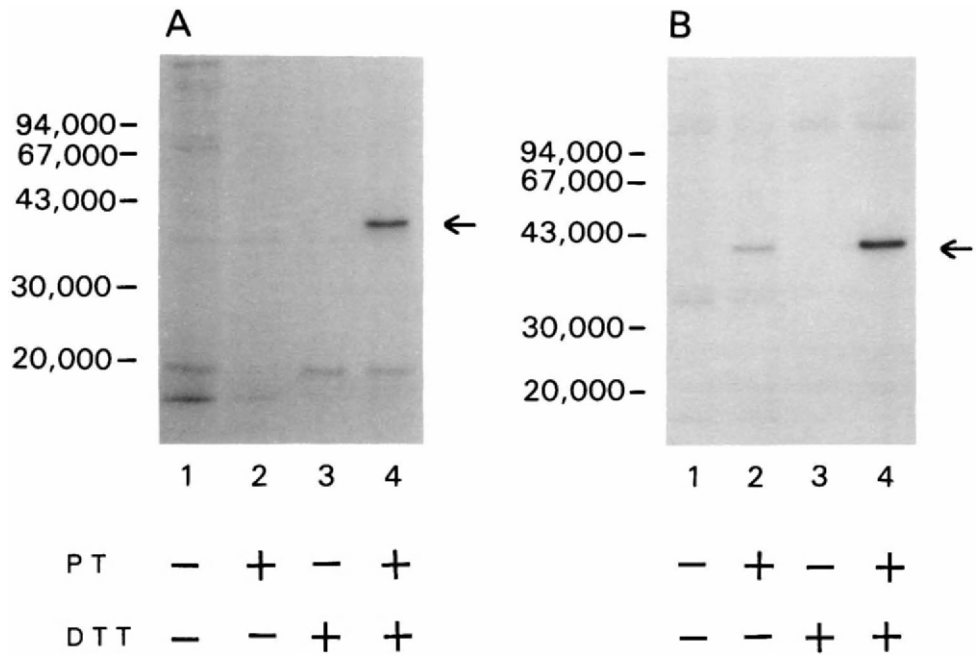

Fig. 5. Effect of dithiothreitol on the ADP-ribosylation of a 41,000 Da protein by pertussis toxin in cell membranes. Membranes prepared from human erythrocytes (A) and NG108-15 cells (B) were incubated with [^{32}P]NAD and, as indicated, PT (pertussis toxin) and DTT (dithiothreitol). The labeled proteins were detected by autoradiography following sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. The mobilities of molecular weight standards are given to the left of both (A) and (B). Figure is reproduced from ref. 47.

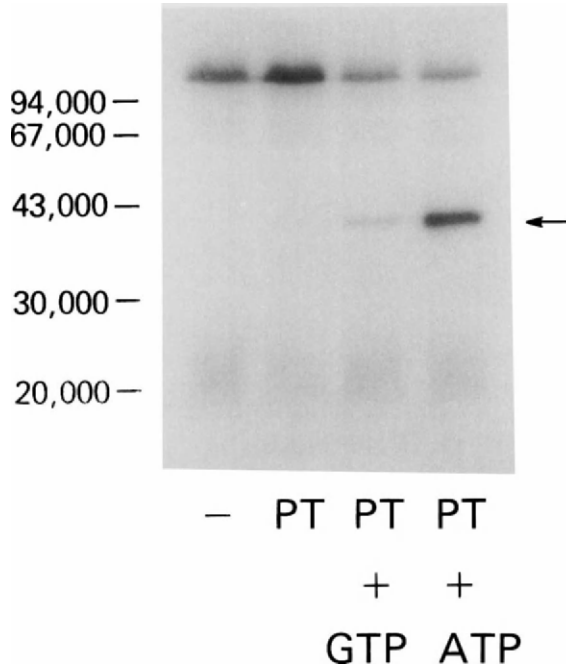

Fig. 6. Effect of ATP and GTP on pertussis toxin-catalyzed ADP-ribosylation of a 41,000 Da protein in membranes from NG108-15 cells. Membranes were incubated with [^{32}P]NAD and, as indicated, PT (pertussis toxin), GTP and ATP. The [^{32}P]ADP-ribosylated proteins were detected by autoradiography following sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. Positions of molecular weight standards are indicated on the left (Hsia, J. A., Moss, J., Hewlett, E. L., Vaughan, M., unpublished data).

(Reaction 2), it would appear that a primary effect of nucleotide may be on the toxin, although a nucleotide site has not been identified. Precedent for the existence of such a site is derived from the studies by Collier and co-workers on diphtheria toxin, where high affinity binding of the dinucleotide ApUp was observed (51-53).

The ability of pertussis toxin to catalyze the ADP-ribosylation of T_α and $G_{i\alpha}$ is clearly influenced by the presence of the $\beta\gamma$ subunits and the status of the receptor. ADP-ribosylation of T_α was enhanced by $T_{\beta\gamma}$ (Fig. 7) (31). Photolyzed rhodopsin, but not the dark-adapted receptor, decreased ADP-ribosylation of T_α and of $T_\alpha \cdot T_{\beta\gamma}$. Since photolyzed

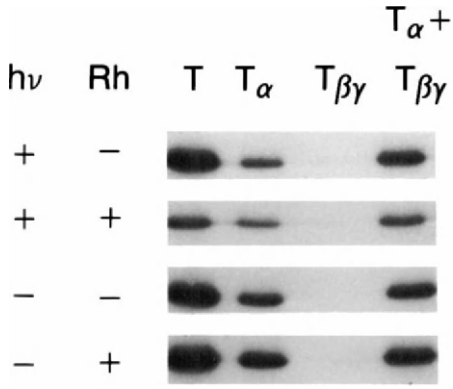

Fig. 7. Effect of rhodopsin and $T_{\beta\gamma}$ on the pertussis toxin-catalyzed ADP-ribosylation of T_{α}. Phosphatidylcholine liposomes with or without rhodopsin (R) were incubated with pertussis toxin, [^{32}P]NAD and, where indicated, in the presence of light (hv) and transducin (T) or transducin subunits (T_{α}, $T_{\beta\gamma}$). The [^{32}P]ADP-ribosylated proteins were identified by autoradiography following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The area of film containing the radiolabeled 39,000 Da subunit of transducin is shown (Watkins, P. A., Kanaho, Y., Hewlett, E. L., Moss, J., unpublished observations and ref. 50).

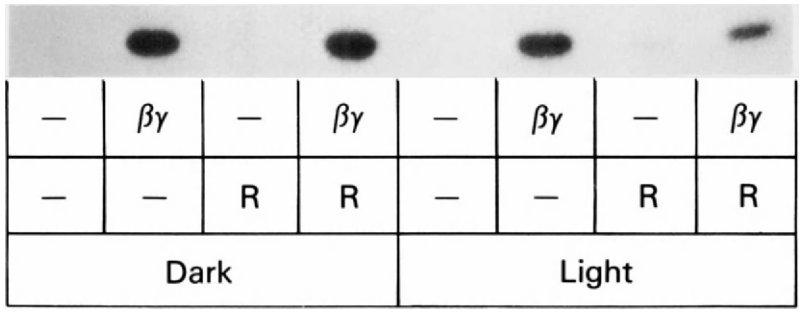

Fig. 8. Effect of dark and photolyzed rhodopsin on pertussis toxin-catalyzed ADP-ribosylation of the 41,000 Da subunit of G_i. Purified $G_{i\alpha}$ was incubated with pertussis toxin, [^{32}P]NAD and, where indicated, $T_{\beta\gamma}$ and photolyzed or dark rhodopsin (R). [^{32}P]ADP-ribosylation was quantified by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis, followed by autoradiography. The area of film containing the [^{32}P]ADP-ribosylated $G_{i\alpha}$, a 41,000 Da protein, is shown. Figure is reproduced from ref. 31.

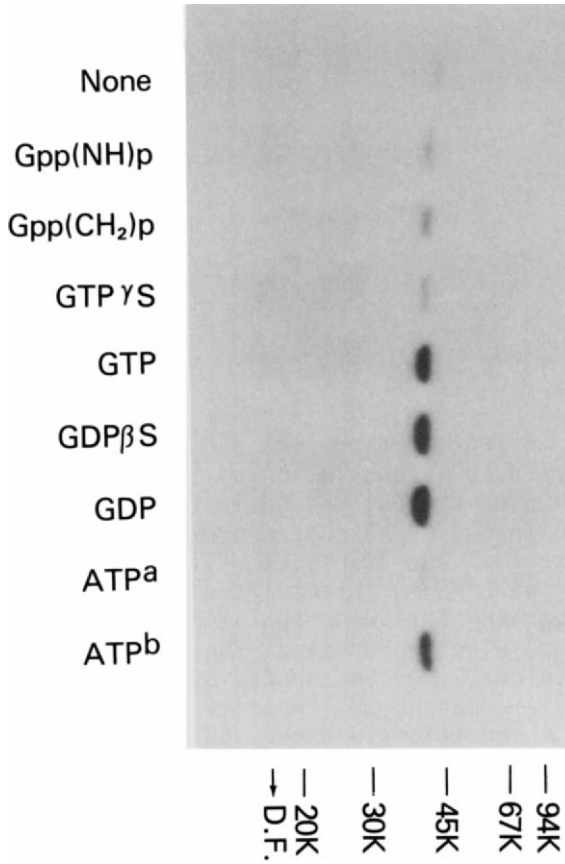

Fig. 9. Effect of nucleotides on pertussis toxin-catalyzed ADP-ribosylation of the 41,000 Da α subunit of G_i. The $G_{i\alpha}$ with $G_{\beta\gamma}$ was incubated with pertussis toxin, [^{32}P]NAD and the indicated guanyl nucleotide (10 μM); concentrations of ATP were (a) 10 μM and (b) 0.5 mM. [^{32}P]ADP-ribosylation of $G_{i\alpha}$ was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by autoradiography. Molecular weight standards are given on the right ordinate. Figure is reproduced from ref. 31.

rhodopsin promotes the active state of transducin, these data support the proposal that pertussis toxin catalyzes the ADP-ribosylation of the inactive form of the guanyl nucleotide-binding protein. To examine further the role of nucleotides and inhibitory receptors in the pertussis toxin-catalyzed ADP-ribosylation, a reconstituted system containing $G_{i\alpha}$

purified from rabbit liver and phosphatidylcholine vesicles containing rhodopsin was used (31). Photolyzed rhodopsin is believed to mimic the active inhibitory hormone receptor-agonist complex while dark rhodopsin is analogous to the inactive ligand-free receptor. ADP-ribosylation of the 41 kDa $G_{i\alpha}$ was stimulated by the addition of $G_{\beta,\gamma}$ and ATP (31). In the presence of $G_{\beta\gamma}$ subunits, dark rhodopsin had no effect on the ADP-ribosylation of $G_{i\alpha}$, whereas photolyzed rhodopsin significantly decreased $G_{i\alpha}$ modification (Fig. 8) (31). In the presence of photolyzed rhodopsin, addition of the non-hydrolyzable GTP analogues, Gpp(CH_2)p or Gpp(NH)p, resulted in a slight enhancement in the ADP-ribosylation reaction. GDP or GDPβS, however, significantly increased modification, almost to the levels observed in the presence of dark rhodopsin (Fig. 9) (31). GTP behaved similarly to GDP and GDPβS, probably because, under the conditions of the assay, GTP was hydrolyzed by $G_{i\alpha}$ to GDP (30,31). Since the presence of both dark rhodopsin and GDP or its analogue favor the inactive state of G_i, it would appear that the substrate for the pertussis toxin-catalyzed reaction is the associated, inactive $G_{i\alpha}G_{\beta\gamma}$ complex (18,31,39).

IV. EFFECTS OF PERTUSSIS TOXIN ON AGONIST RECEPTOR·G_i INTERACTION

The effects of pertussis toxin on the adenylate cyclase and rhodopsin-transducin-cyclic GMP phosphodiesterase systems can be determined by quantifying the appropriate overall reaction or the partial reactions characteristic of the complex. In the case of the adenylate cyclase system, the two best-studied partial reactions are inhibitory agonist stimulation of a GTPase activity and ligand binding to its receptor. For the rhodopsin-transducin complex, effects of toxin on GTPase activity and rhodopsin-transducin interaction have been analyzed.

Pertussis toxin-treated cells exhibit a decreased sensitivity to inhibitory agonists evidenced by a decrease in their ability to inhibit adenylate cyclase (26,46,54-57) or to reduce intracellular cAMP (45,58,59). In addition, in some cells toxin exposure increases sensitivity to stimulatory agonists (35,36,45,46,54,58-60); it was proposed that interference with the inhibitory pathway leads to unopposed action of the stimulatory arm.

The effects of pertussis toxin on adenylate cyclase are manifested in several ways. First, the ability of an

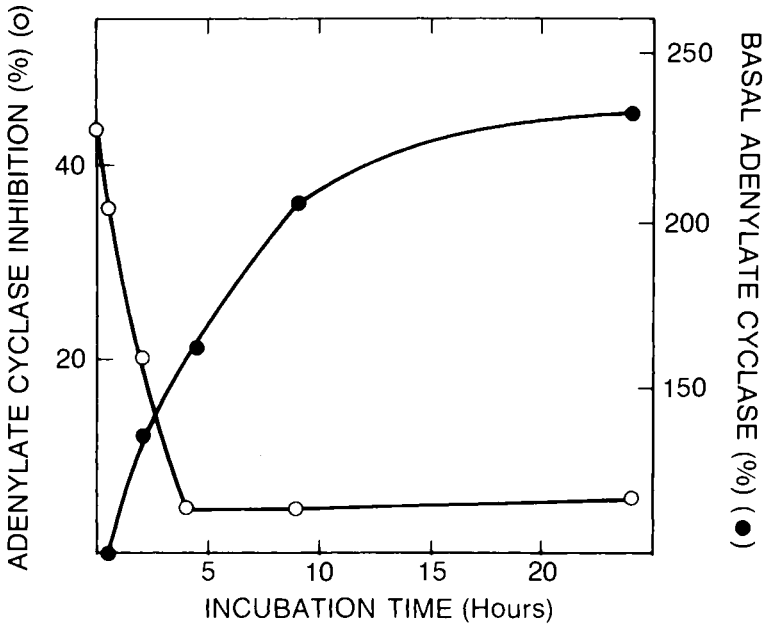

Fig. 10. Effect of pertussis toxin-catalyzed ADP-ribosylation on basal- and opiate-inhibited adenylate cyclase activity in membranes from NG108-15 cells. NG108-15 cells were incubated with pertussis toxin for the times indicated on the abscissa. Membranes were prepared and assayed for adenylate cyclase activity with (O) or without (●) enkephalinamide. Percentage inhibition by enkephalinamide (O) and basal activity as a percentage of that at zero time (●) are on left and right ordinates, respectively. Figure is reproduced from ref. 54.

inhibitory agonist to decrease cyclase activity is abolished (Fig. 10) (54); second, basal adenylate cyclase activity is increased (Fig. 10) (54); and, last, the inhibitory action of GTP on adenylate cyclase is reduced (20,26,55). The increase in basal adenylate cyclase activity is consistent with the hypothesis that the cyclase is subject to tonic inhibition that is relieved following toxin-catalyzed ADP-ribosylation of G_i. These data support the conclusion that the ADP-ribosylation inactivates G_i. A similar conclusion is reached from studies on the rhodopsin-transducin complex where ADP-ribosylation of T_α inhibits its ability to activate the cyclic GMP phosphodiesterase (39).

Both the adenylate cyclase inhibitory and transducin systems exhibit receptor-stimulated GTPase activity (21,30).

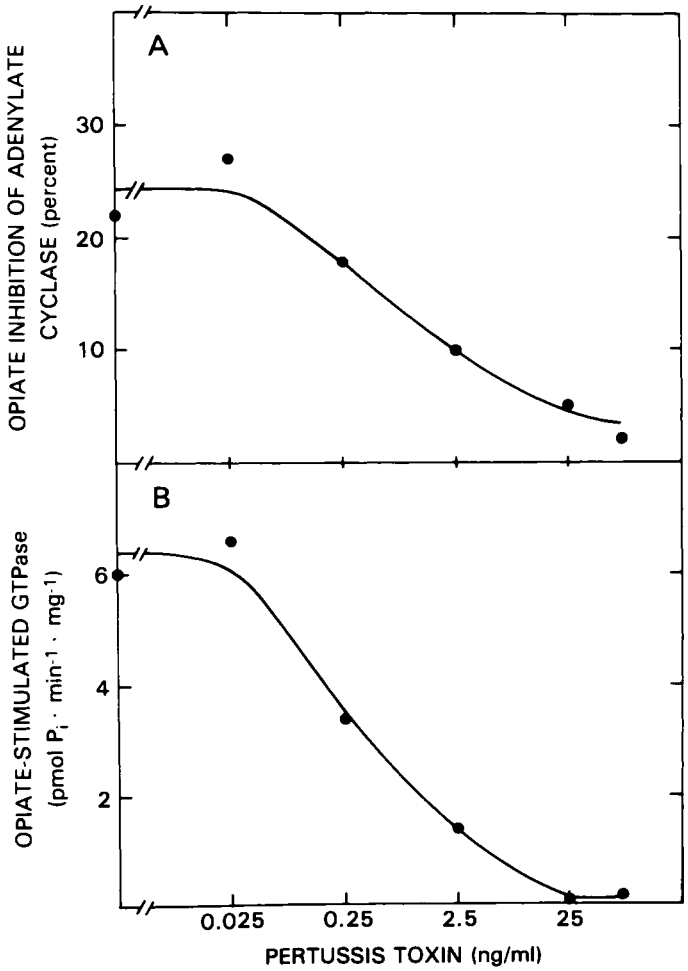

Fig. 11. Effect of pertussis toxin on opiate inhibition of adenylate cyclase and opiate stimulation of GTPase activity. NG108-15 cells were incubated with pertussis toxin for 24 h at the concentration given on the abscissa; membranes were prepared and assayed for adenylate cyclase activity in the presence of PGE_1 with and without enkephalin (A); percentage inhibition of adenylate cyclase activity is given on the ordinate. In (B), GTPase activity was determined in the presence or absence of enkephalin. Opiate stimulation of GTPase is plotted as a function of pertussis toxin concentration. Figure is reproduced from ref. 61.

Table II. Effect of Pertussis Toxin and NAD on Opiate Stimulation of GTPase Activity in Membranes from NG108-15 Cells

Additions	Enkephalin	GTPase activity
		(pmol $P_i \cdot min^{-1} \cdot mg^{-1}$)
None	0	33.4
	+	45.8
NAD	0	35.0
	+	47.0
Pertussis toxin	0	37.9
	+	52.1
NAD plus toxin	0	30.6
	+	35.5

Membranes from NG108-15 cells were incubated with additions as indicated before assay of GTPase activity without or with enkephalin. Data are from ref. 61.

In the case of the cyclase, Koski and Klee (21) demonstrated in membranes from NG108-15 cells that opiate agonists specifically enhanced GTP hydrolysis. Exposure of cells to pertussis toxin reduced opiate-enhanced GTPase activity in parallel with loss of opiate inhibition of adenylate cyclase (Fig. 11) (61). Support for the participation of ADP-ribosylation in toxin action is the observation that in membranes from NG108-15 cells inhibition of opiate-stimulated GTPase activity required incubation with both toxin and NAD (Table II) (61). Toxin treatment blocked agonist-stimulated high affinity GTPase in other systems as well (56). GTPase activity of the rhodopsin-stimulated system was also suppressed by pertussis toxin in an NAD-dependent reaction (Fig. 12) (41; Watkins, P. A., Moss, J., Hewlett, E. L., and Vaughan, M., unpublished observations). The degree of inhibition of GTPase paralleled the extent of ADP-ribosylation of the T_α (Fig. 13) (41).

The effects of pertussis toxin on adenylate cyclase activity and GTPase could be explained by a failure of the ADP-ribosylated G_i to react effectively with the inhibitory receptor-agonist complex. In NG108-15 membranes, the binding of opiate antagonists, diprenorphine (Fig. 14), or naloxone was unaffected by toxin treatment; antagonist interaction with receptor is presumably unaffected by G_i (54). In

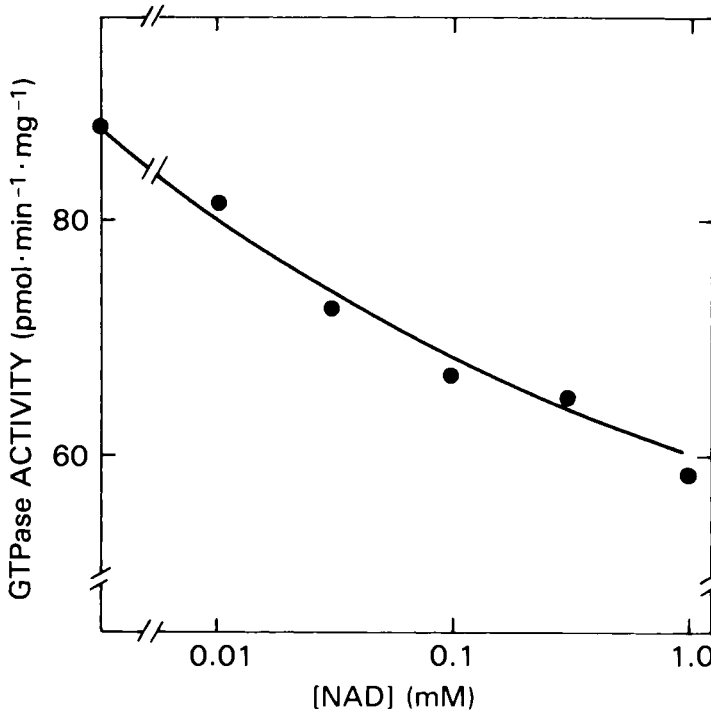

Fig. 12. Effect of pertussis toxin and NAD on GTPase activity in retinal rod outer segments. Rod outer segment membranes were incubated with pertussis toxin and the indicated concentration of NAD before assay of GTPase activity as described in ref. 41 (Watkins, P. A., Moss, J., Hewlett, E. L., Vaughan, M., unpublished observations).

contrast, the ability of the opiate agonist, enkephalinamide, to displace diprenorphine was reduced by toxin treatment, consistent with the hypothesis that agonist affinity, but not receptor number, is reduced in membranes from cells exposed to toxin (Fig. 15) (54). Since the high affinity interaction of agonist with its receptor is presumably dependent on the coupling of receptor to G_i, the loss of the high affinity state is consistent with the proposal that the effect of toxin-catalyzed ADP-ribosylation is to interfere with the coupling of G_i and inhibitory receptor (54,62,63). This conclusion is supported by the observation that the inhibition of enkephalinamide binding by guanyl-5'-yl imidodiphosphate is significantly reduced in membranes from NG108-15

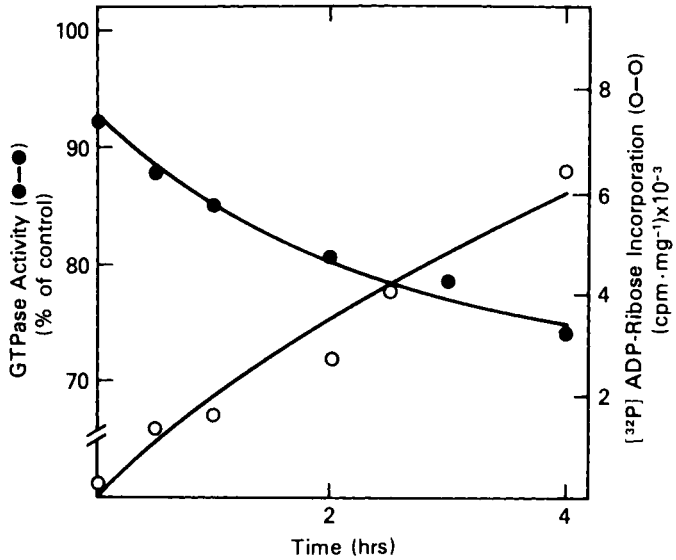

Fig. 13. Correlation between effects of pertussis toxin on rod outer segment GTPase activity and extent of ADP-ribosylation of the transducin α subunit. Photolyzed rod outer segment membranes were incubated with pertussis toxin, guanyl-5'-yl imidodiphosphate, ATP and either NAD (●) or [^{32}P]NAD (O) for the time indicated on the abscissa. Samples with unlabeled NAD (●) were assayed for GTPase activity. Samples from incubations with [^{32}P]NAD were precipitated with trichloroacetic acid, solubilized with sodium dodecyl sulfate containing dithiothreitol, and subjected to polyacrylamide slab gel electrophoresis; radioactivity in gel slices containing the 39,000 Da protein was determined with a liquid scintillation counter and is given as [^{32}P]ADP-ribose incorporated along the right ordinate (O). Figure is reproduced from ref. 41.

cells exposed to toxin (Fig. 16) (54). Evidence that the toxin-catalyzed ADP-ribosylation physically uncouples receptor from guanyl nucleotide-binding protein was obtained in the rhodopsin-transducin system; ADP-ribosylation of transducin enhanced its release from retinal rod outer segments containing the receptor, rhodopsin (39).

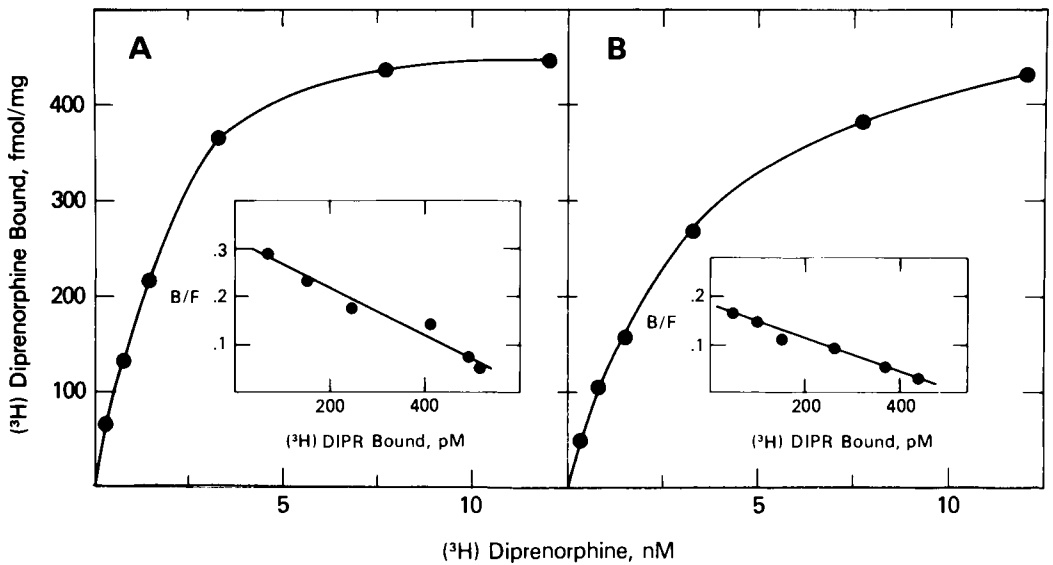

Fig. 14. Effect of pertussis toxin on the binding of [^{3}H]diprenorphine to membranes from NG108-15 cells. Control (A) and pertussis toxin-treated (B) membranes were incubated with the indicated concentrations of [^{3}H]diprenorphine. B_{max} was 550 for (A) and 530 fmol/mg protein for (B); K_d was 2 μM for (A) and 3 μM for (B). These values for control and toxin-treated membranes were not significantly different. Figure is reproduced from ref. 54.

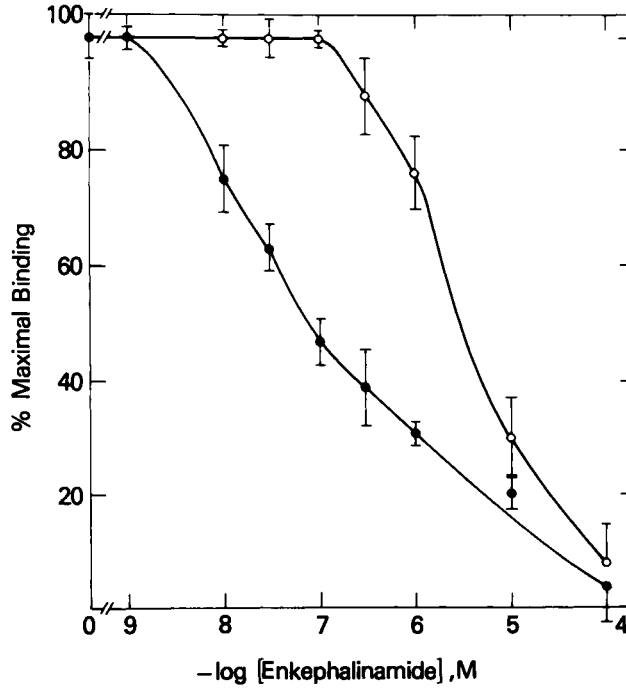

Fig. 15. Effect of pertussis toxin on the interaction of diprenorphine and enkephalinamide with NG108-15 membranes. Specific binding of [^{3}H]diprenorphine by control (●) and pertussis toxin-treated (O) membranes in the presence of the indicated concentration of enkephalinamide is plotted as a percentage of maximal specific binding. Figure is reproduced from ref. 54.

V. CONCLUSIONS

It is evident that studies of the effects of pertussis toxin on adenylate cyclase have significantly enhanced understanding of the interactions involved in the regulation of that complex, multicomponent system. In addition, they have helped to define a family of guanyl nucleotide-binding proteins that play analogous roles in the coupling of receptors to target proteins. The chemical mechanism of toxin action, transfer of the ADP-ribose moiety of NAD to a critical amino acid on G_i and transducin, has furthered investigation into

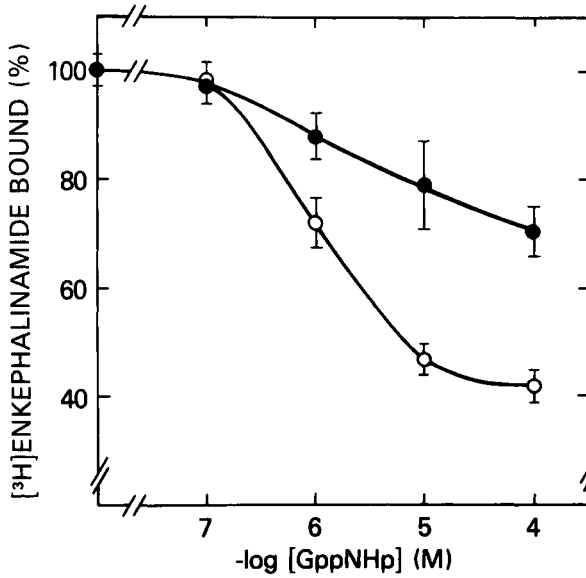

Fig. 16. Effect of guanyl-5'-yl imidodiphosphate on the binding of [^{3}H]enkephalinamide to membranes from control and toxin-treated NG108-15 cells. Membranes from control (O) and pertussis toxin-treated (●) cells were incubated with [^{3}H]-enkephalinamide and the indicated concentration of guanyl-5'-yl imidodiphosphate. Figure is reproduced from ref. 54.

the role of ADP-ribosyltransferases in the regulation of cellular metabolism.

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Resolution of Agonists and Gpp(NH)p Mediated Adenylate Cyclase Inhibition with Pertussis Toxin

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I. INTRODUCTION

Pertussis toxin induces the ADP ribosylation of a 41,000 dalton membrane-bound protein (believed to be the alpha subunit of Ni (1-6)) and attenuates receptor-mediated inhibition of adenylate cyclase activity in all tissues tested to date that contain an inhibitory receptor (7-15). However, how ADP ribosylation of Ni abolishes receptor-mediated inhibition of adenylate cyclase remains unclear. The results presented in this report suggest that the ADP ribosylation of Ni induced by pertussis toxin functionally uncouples Ni from the inhibitory receptor.

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The present study was conducted on tissue from the intermediate lobe of the rat pituitary gland. A D-2 dopamine receptor occurs on the intermediate lobe (Fig. 1). Agonist activation of the D-2 receptor results in inhibition of adenylate cyclase activity (16-20), inhibition of alpha melanocyte stimulating hormone (alpha MSH) release (21-33), and a decrease in the level of mRNA coding for the synthesis of proopiomelanocortin [POMC (24)], the prohormone from which alpha MSH is derived. An inhibitory guanyl nucleotide binding protein [termed Ni (6)] is believed to act as a membrane transducer in converting the binding of an agonist to the D-2 receptor into inhibition of adenylate cyclase activity (16,17). Modulation of adenylate cyclase activity is believed to be a primary mechanism by which the D-2 receptor regulates the functioning of the intermediate lobe cells. A stimulatory beta2-adrenergic receptor associated with a stimulatory guanyl nucleotide binding protein [termed Ns (6)] also occurs in the intermediate lobe. Agonist activation of the beta receptor stimulates both adenylate cyclase activity (25) and alpha MSH release (21-23). Thus, the stimulatory beta adrenergic system and the inhibitory D-2 dopaminergic system work in opposition to each other.

II. BACTERIAL TOXIN-INDUCED [^{32}P]ADP RIBOSYLATION OF Ni AND Ns

Experiments were conducted to determine if the membranes of the intermediate lobe contain a substrate for the pertussis toxin-induced ADP ribosylation (2). Pertussis toxin was found to catalyze the [^{32}P]ADP ribosylation of a 41,000 dalton membrane-bound protein (Fig. 2). Preincubation of the tissue for 16 h with pertussis toxin diminished the subsequent labelling of the 41,000 dalton protein that occurred in the presence of [^{32}P]NAD but did not diminish the ability of cholera toxin to induce the [^{32}P]ADP ribosylation of a 46,000/50,000 protein doublet; this protein doublet is believed to be the two molecular weight forms of the alpha subunit of Ns (26,27). Conversely, preincubation of the tissue with cholera toxin prevented the labelling of the 46,000/50,000 dalton doublet upon a subsequent exposure of the tissue to cholera toxin in the presence of [^{32}P]NAD, but did not diminish the subsequent [^{32}P]ADP ribosylation of the 41,000 dalton protein induced by pertussis toxin. Thus, the 41,000 dalton protein, believed to be a subunit of Ni, is distinct from the 46,000/50,000 protein doublet, believed to be two molecular weight forms of the alpha subunit of Ns (27).

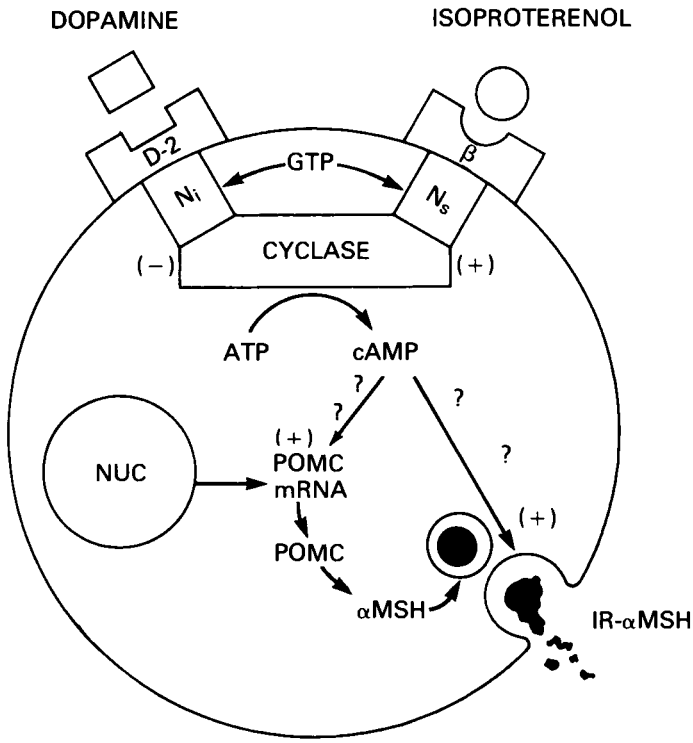

Fig. 1. Diagrammatic representation of receptor regulation of adenylate cyclase activity, peptide hormone release, and POMC synthesis in the melanotroph of the rat intermediate lobe. A β-adrenergic agonist, (isoproterenol) occupies a β-adrenergic receptor interacting with a stimulatory guanyl nucleotide binding component (Ns); GTP interacts with Ns to stimulate adenylate cyclase activity. A dopaminergic agonist (dopamine) occupies a D-2 dopamine receptor (D-2) interacting with a stimulatory guanyl nucletotide binding component (Ns); GTP interacts with Ns to stimulate adenylate cyclase activity. A dopaminergic agonist (dopamine) occupies a D-2 dopamine receptor (D-2) interacting with an inhibitory guanyl nucleotide binding component (Ni); GTP interacts with Ni to inhibit adenylate cyclase activity. GTP activates Ns only in the presence of a β-adrenergic agonist and activates Ni only in the presence of a dopaminergic agonist. Increased cAMP results in the enhanced release of peptide hormones via an unknown mechanism(s). Prolonged activation of the D-2 receptor results in a decrease in the level of mRNA for POMC. Agents such as cholera toxin and forskolin that stimulate adenylate cyclase increase the capacity of

the cell to synthesize POMC and reverse the inhibition of POMC synthesis induced by activation of the D-2 receptor.

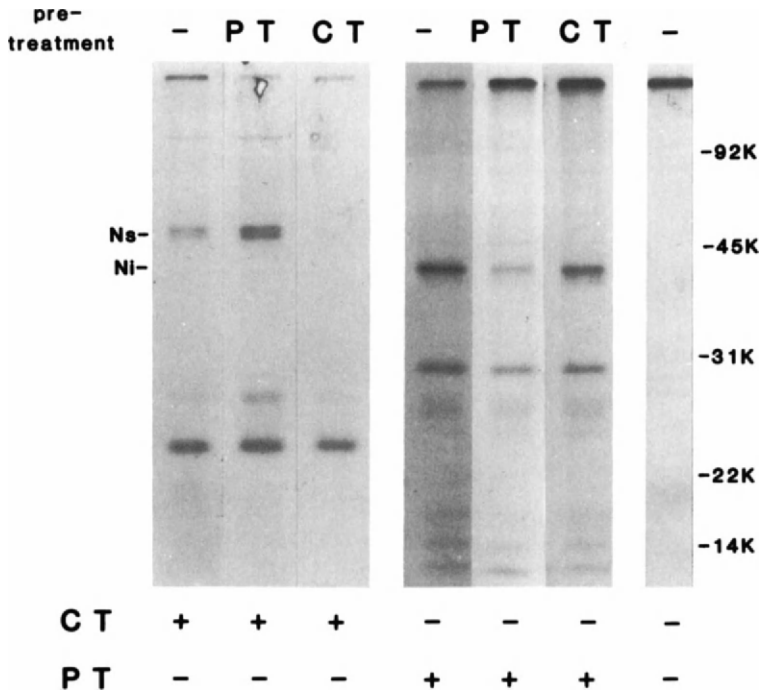

Fig. 2. Cholera toxin induces specifically the ADP-ribosylation of 50,000 and 46,000 dalton membrane components of the rat intermediate lobe; pertussis toxin induces specifically the ADP-ribosylation of a 41,000 dalton membrane component of the rat intermediate lobe. Intermediate lobe tissue was pretreated 18 h with pertussis toxin (PT), cholera toxin (CT) or no toxin (-) as indicated at the top. Membranes were prepared and exposed to [^{32}P]NAD in the absence (-) of toxin or in the presence (+) of either cholera toxin (CT) or pertussis toxin (PT) as indicated at the bottom. The samples were then subjected to SDS gel electrophoresis followed by autoradiography. Migration of molecular weight markers is indicated at the right. The location of the cholera toxin substrate (believed to be subunits of Ns) and the pertussis toxin substrate (believed to be a subunit of Ni) are indicated at the left as Ns and Ni, respectively.

III. EFFECTS OF PERTUSSIS TOXIN ON RECEPTOR- AND GUANYL NUCLEOTIDE-MEDIATED INHIBITION OF ADENYLATE CYCLASE

Intermediate lobe tissue was treated routinely with cholera toxin to activate fully the pathway for stimulation of adenylate cyclase and make more apparent effects on the inhibitory system (17). Thus, in the current study of the inhibitory pathway, intermediate lobe tissue was treated with cholera toxin alone (control) or in combination with pertussis toxin (pertussis toxin). In control tissue, N-n-propylnorapomorphine (NPA), a D-2 dopamine agonist, caused a dose-dependent inhibition of adenylate cyclase activity (Fig. 3). The inhibitory effect of NPA was blocked by fluphenazenine, a dopamine antagonist. Thus, the NPA-induced inhibition of adenylate cyclase activity results from the interaction of NPA with the dopamine receptor. 5'-guanylylimidodiphosphate [Gpp(NH)p], a nonhydrolyzable analog of GTP, also causes a dose-dependent inhibition of adenylate cyclase activity. However, the inhibition induced by Gpp(NH)p was not blocked by fluphenazine. Therefore, Gpp(NH)p acts at a site other than the D-2 receptor. In pertussis toxin treated tissue, NPA failed to inhibit adenylate cyclase activity. In contrast, Gpp(NH)p continued to inhibit cyclase activity in a manner similar to the inhibition seen in control tissue. These findings suggest that although pertussis toxin attenuates the D-2 dopamine receptor-mediated inhibition of enzyme activity, a distinct mechanism, perhaps involving Ni, may exist that permits continued inhibition of adenylate cyclase by the nonhydrolyzable GTP analog.

IV. INTERACTION OF Gpp(NH)p AND GTP AT Ni

As has been reported previously (17), the Gpp(NH)p-induced inhibition of adenylate cyclase can be completely reversed by high concentrations of GTP in control tissue (Fig. 4, left panel). However, if the D-2 dopamine receptor is activated by NPA, GTP can no longer reverse the inhibitory effect of Gpp(NH)p. These data suggested that, in the absence of a D-2 agonist, GTP can compete with Gpp(NH)p for the guanyl nucleotide binding site on Ni but can not activate Ni. When the D-2 receptor is activated by NPA, GTP continues to compete with Gpp(NH)p for occupancy of the guanyl nucleotide binding site on Ni but can now activate Ni to the same extent as Gpp(NH)p. Thus, we propose that stimulation of the D-2 receptor alters the properties of Ni in a manner that allows GTP to activate Ni.

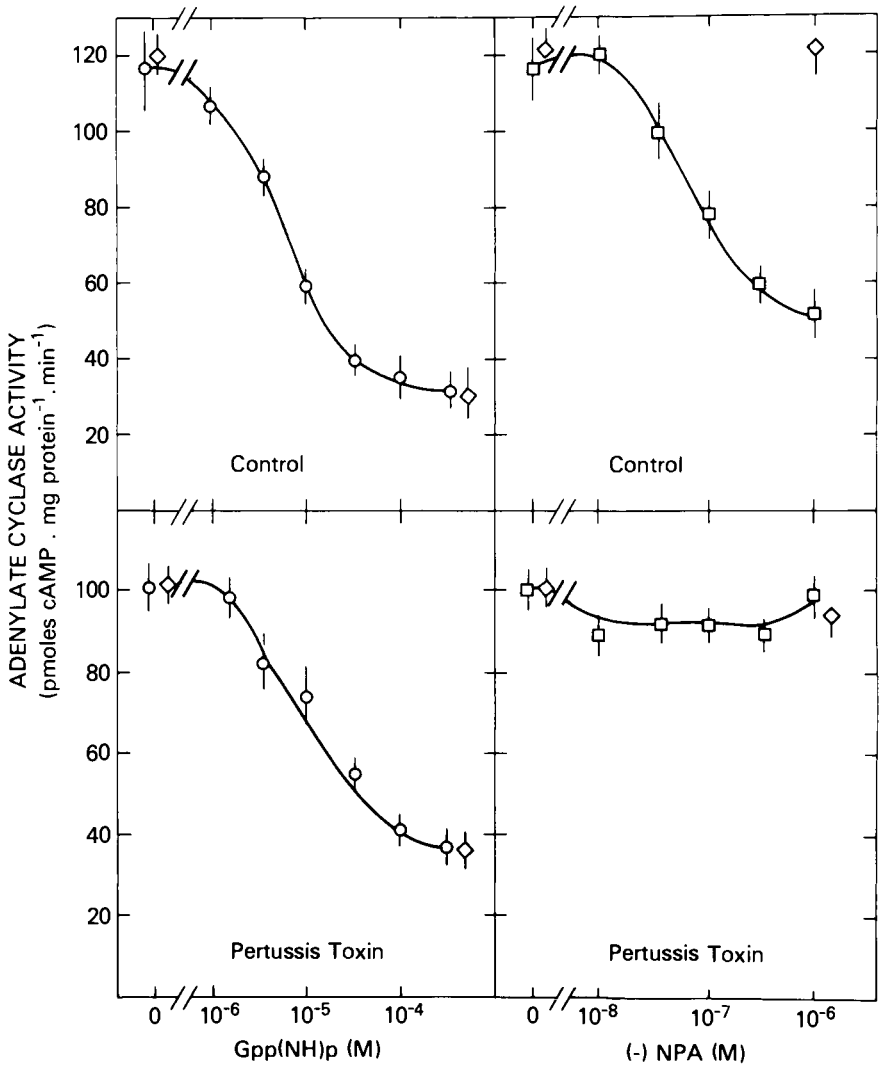

Fig. 3. Pertussis toxin attenuates the (-)NPA-induced, but not the Gpp(NH)p-induced, inhibition of adenylate cyclase activity in cholera toxin-treated intermediate lobe tissue. Intermediate lobe tissue was incubated for 18 h in the absence (upper panels) or in the presence (lower panels) of 1 μg/ml pertussis toxin. During the last 2 h of incubation, cholera toxin (30 nM, final concentration) was added to both experimental groups.

Left panels: at the indicated concentrations, Gpp(NH)p was tested in the absence (open circles) or in the presence (open diamonds) of 10 μM fluphenazine. Right panels: at the indicated concentrations, (-)NPA was tested in the absence (open squares) or in the presence (open diamonds) of 10 μM fluphenazine.

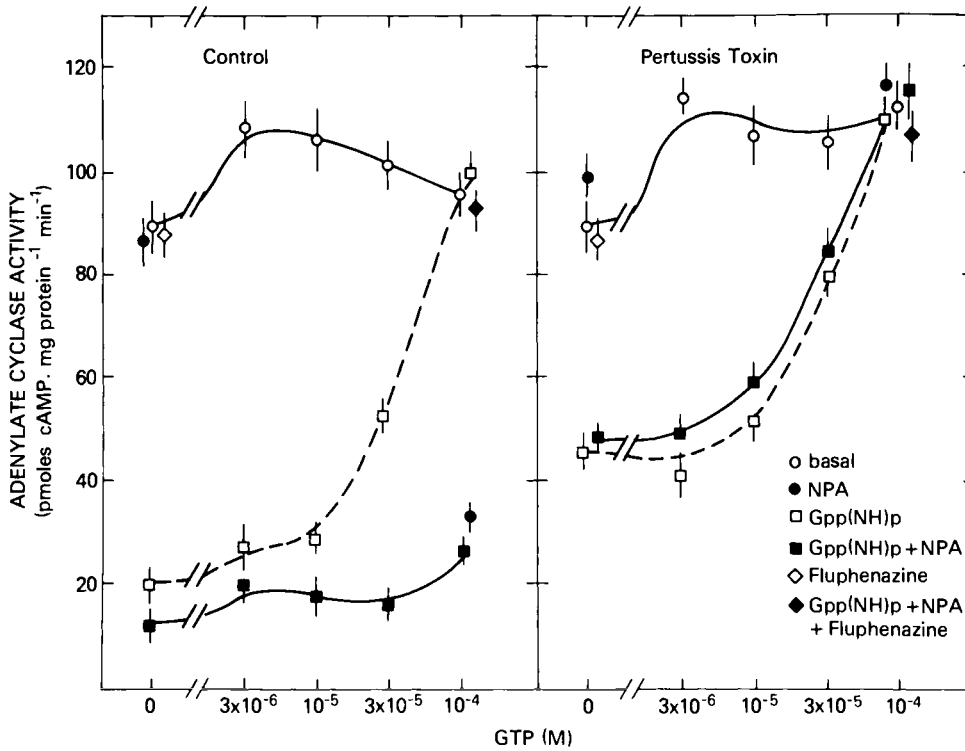

Fig. 4. The effect of pertussis toxin on the interactions among GTP, Gpp(NH)p and (-)NPA. Intermediate lobe tissue was incubated for 18 h in the absence (left panel) or in the presence (right panel) of pertussis toxin (1 μg/ml). During the last two hours of incubation, cholera toxin (30 nM, final concentration) was added to both experimental groups. At the indicated concentrations, GTP was tested alone (open circles) or in combination with 1 μM (-)NPA (filled circles), 30 μM Gpp(NH)p (open

squares), 1 μM (-)NPA and 30 μM Gpp(NH)p (filled squares), or 1 μM(-)NPA, 30 μM Gpp(NH)p and 10 μM fluphenazine (filled diamonds.)

In tissue previously treated with pertussis toxin, high concentrations of GTP also reversed the Gpp(NH)p-induced inhibition of cyclase activity (Fig. 4, right panel). Thus, in pertussis toxin-treated tissue, GTP can still interact with the guanyl nucleotide binding site on Ni. However, when NPA interacts with the D-2 receptor, GTP continues to reverse the inhibitory effect of Gpp(NH)p. Therefore, pertussis toxin appears to abolish the ability of the D-2 receptor to alter the properties of Ni in a manner that allows GTP to activate Ni.

V. PERTUSSIS TOXIN DECREASES RECEPTOR AFFINITY FOR AGONIST BINDING

The direct interaction of dopaminergic drugs with the D-2 receptor was studied with [^{3}H]spiroperidol, a D-2 antagonist (19). Such studies indicated that pertussis toxin did not affect either the number of D-2 receptors or the affinity of the receptors for the radiolabelled antagonist (not shown). However, pertussis toxin did decrease the affinity of the D-2 receptor for NPA, a D-2 agonist (Fig. 5) and abolished the ability of GTP to affect agonist binding (an effect seen in untreated tissue). Similar effects of pertussis toxin have been reported by a number of other laboratories (12,28). These findings support the proposal that one of the normal functions of Ni is to modify the inhibitory receptor in a manner that specifically enhances agonist binding (29-31). If the sole effect of pertussis toxin is to catalyze the ADP ribosylation of Ni, the present data suggest that the ADP ribosylation occurs at a site on Ni that is critical for the interaction of Ni and the inhibitory receptor.

VI. HYPOTHETICAL MODEL OF RECEPTOR MODULATION OF Ni AND THE CONSEQUENCE OF PERTUSSIS TOXIN-INDUCED ADP RIBOSYLATION OF Ni

A hypothetical model of the interaction of the D-2 dopamine receptor, Ni and the catalytic unit of adenylate cyclase and the consequences of treatment with pertussis toxin are presented in Figures 6 and 7, respectively. It is hypothesized that, in the absence of a D-2 agonist, GTP interacts with Ni and is hydrolyzed by the Ni-associated GTPase to GDP which continues to occupy the guanyl nucleotide binding site (Fig. 6). Consequently, no inhibitory effect

on cyclase is observed. If a dopamine agonist activates the D-2 receptor, the activated D-2 receptor interacts with Ni and causes an enhancement in the exchange of GDP for GTP. The rapid replacement of GDP by GTP at the guanyl nucleotide binding site on Ni results in a persistent activation of Ni and inhibition of cyclase activity. If Gpp(NH)p is added to the system in the absence of an agonist, Gpp(NH)p interacts with Ni and causes a persistent activation of Ni since GTPases can not hydrolzye this GTP analog. If excess GTP is added to the system in the absence of a dopaminergic agonist, GTP will compete with Gpp(NH)p for the guanyl nucleotide binding site on Ni. However, if a dopamine agonist is included with GTP, then GTP itself will activate Ni and will not reverse the inhibitory effect of Gpp(NH)p.

The pertussis toxin-induced ADP ribosylation occurs at a site on Ni that is critical for the interaction of Ni and the inhibitory receptor (Fig. 7). Thus, following treatment of the tissue with pertussis toxin, GTP can still interact with the guanyl nucleotide binding site where it is hydrolyzed to GDP. Dopaminergic agonists can still activate the receptor, but the activated receptor can no longer interact with Ni to enhance the rate of exchange of GDP for GTP and therefore can no longer mediate inhibition of adenylate cyclase activity. Gpp(NH)p can still interact with the guanyl nucleotide binding site on Ni and activate Ni. GTP can still compete with Gpp(NH)p for the guanyl nucleotide binding site on Ni and block the inhibitory effect of Gpp(NH)p; however, the inclusion of a dopamine agonist can no longer alter the properties of Ni.

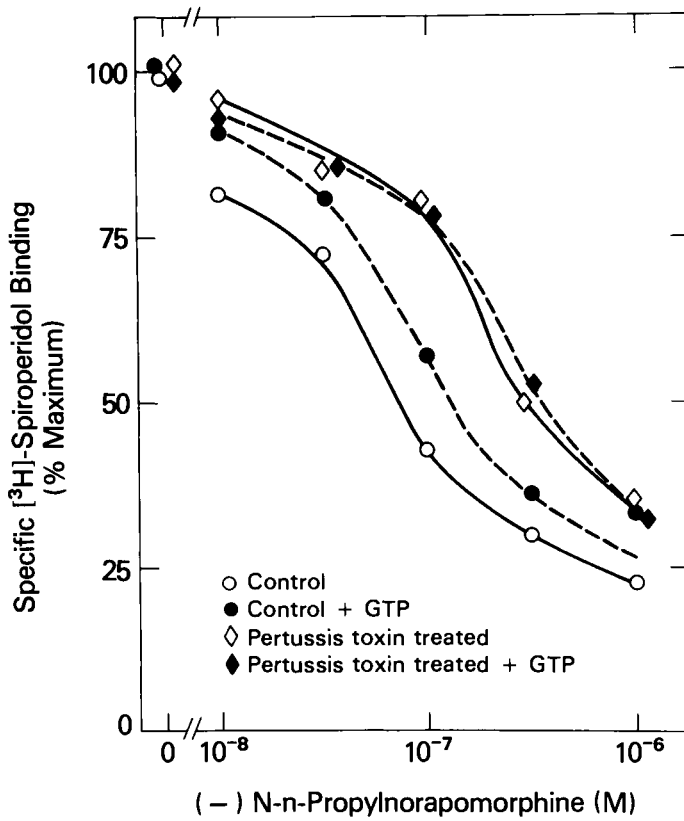

Fig. 5. Effect of pertussis toxin on the competition between [^{3}H]-spiroperidol and (-)NPA for occupancy of specific [^{3}H]-spiroperidol binding sites in the intermediate lobe. Intermediate lobe tissue was incubated for 18 h in the absence (circles) or in the presence (diamonds) of 1 μg/ml pertussis toxin. Specific binding of [^{3}H]-spiroperidol was determined in the absence (open symbols) or the presence (filled symbols) of 100 μM GTP. The amount of [^{3}H]-spiroperidol specifically bound in the presence of (-)NPA is expressed as a percentage of the amount of [^{3}H]-spiroperidol bound in the absence of (-)NPA. [^{3}H]-spiroperidol was present at a final concentration of 2 nM.

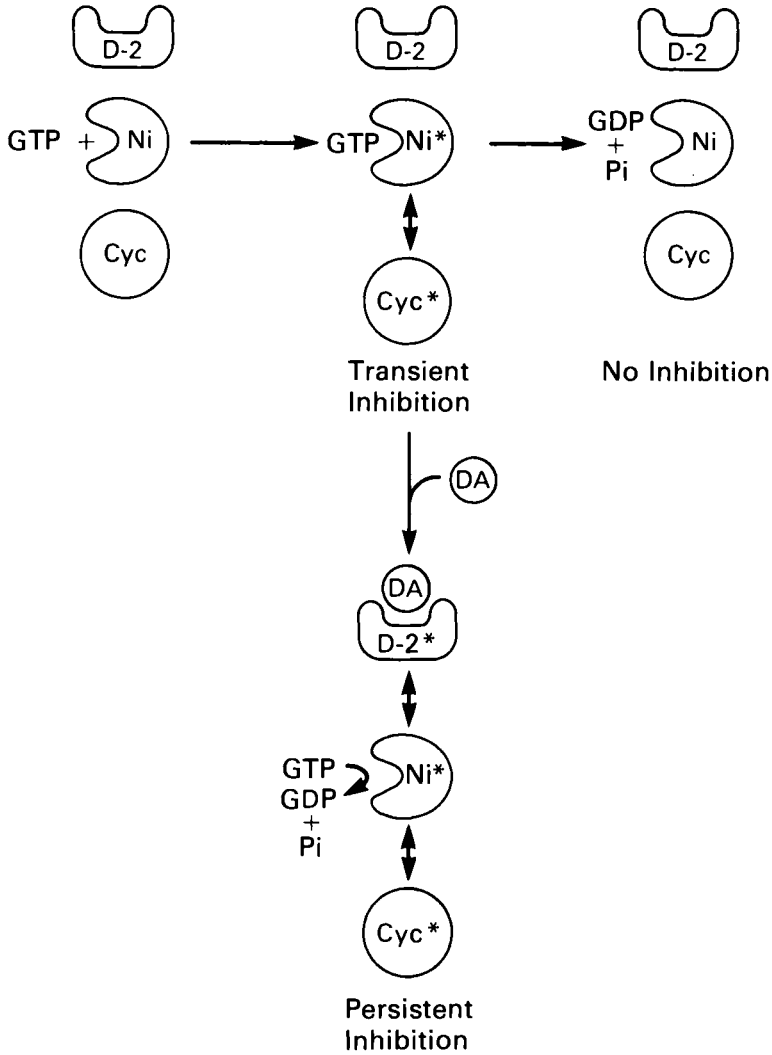

Fig. 6. Hypothetical model the D-2 receptor modulation of Ni. In the absence of a D-2 agonist, GTP interacts with the guanyl nucleotide binding site on Ni, transiently activates Ni, and is hydrolyzed to GDP by a GTPase on Ni. When the D-2 receptor (D-2) is activated by dopamine (DA), the D-2 receptor interacts with Ni and enhances the exchange of GDP for GTP at the guanyl nucleotide binding site on Ni. The persistent activation of Ni causes a persistent inhibition of adenylate cyclase (cyc).

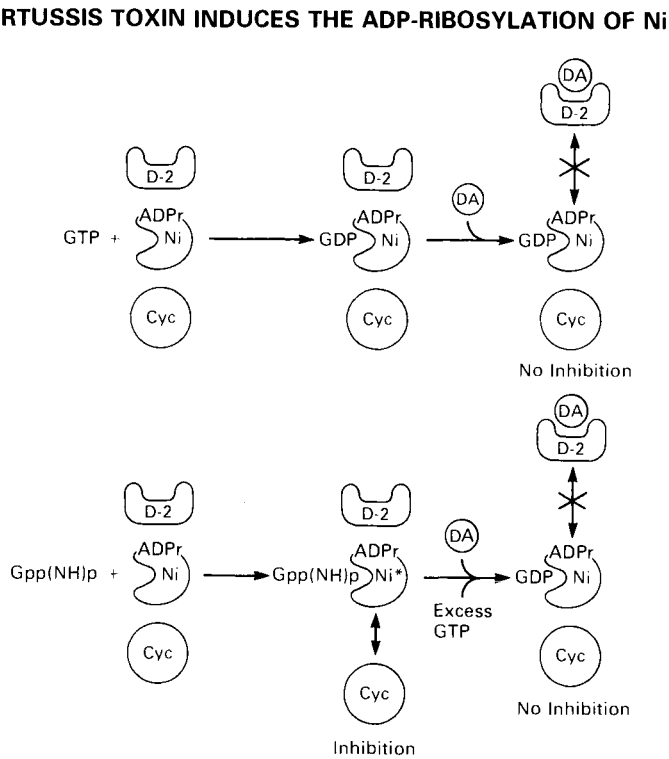

Fig. 7. Hypothetical model of the effect of pertussis toxin on the interaction of the D-2 receptor and Ni. Pertussis toxin catalyzes the ADP ribosylation of Ni at a site on Ni that is critical for the interaction of Ni and the D-2 receptor. In intermediate lobe tissue treated with pertussis toxin, GTP can interact with the guanyl nucleotide binding site on Ni where it is hydrolyzed to GDP. The D-2 receptor binds dopamine with a lower affinity than seen in control tissue because the receptor can not interact with Ni. Furthermore, the binding of dopamine to the D-2 receptor can no longer enhance the exchange of GDP for GTP at Ni and therefore the binding of dopamine to the D-2 receptor can no longer enhance the exchange of GDP for GTP at Ni and therefore can no longer initiate the inhibition of adenylate cyclase (cyc). Gpp(NH)p can still interact with Ni and activate Ni resulting in the inhibition of cyclase activity. GTP can block the inhibitory effect of Gpp(NH)p either in the absence or in the presence of dopamine.

VII. USE OF PERTUSSIS TOXIN AS A PROBE TO DETERMINE THE ROLE OF Ni IN MEDIATING RECEPTOR-INITIATED EFFECTS ON CELL FUNCTION

Activation of the D-2 dopamine receptor in the intermediate lobe results in inhibition of MSH release and a decrease in the capacity of the tissue to synthesize proopiomelanocortin (POMC) (24). In the present study, pertussis toxin was used as a probe to determine the involvement of Ni in mediating these responses of the tissue to activation of the D-2 receptor.

Acute activation of the D-2 receptor by dopamine inhibits cholera toxin-stimulated release of alpha MSH from intermediate lobe cells (Fig. 8). Pretreatment of the cells with pertussis toxin totally abolishes the ability of the D-2 receptor to mediate inhibition of hormone release (Fig. 8, right panel). Prolonged stimulation of the D-2 receptor with bromocriptine, a long acting D-2 agonist, causes a 40% reduction in the capacity of intermediate lobe tissue to synthesize POMC (Fig. 9). Pretreatment of intermediate lobe tissue with pertussis toxin causes a dose-dependent abolition of D-2 receptor-mediated decrease in POMC synthesis. The potency of pertussis toxin in abolishing the dopaminergic inhibition of POMC synthesis is similar to the potency of pertussis toxin in abolishing the dopaminergic inhibition of adenylate cyclase activity (Fig. 9 insert). These results suggest that Ni plays a critical role in the D-2 dopamine receptor-mediated regulation of MSH release and POMC synthesis. However, it is possible that pertussis toxin alters other membrane components in addition to Ni, and it is also possible that Ni can alter the functioning of other membrane components in addition to adenylate cyclase. Therefore, at the present time, one must be cautious in interpreting the results of experiments in which pertussis toxin is used as an experimental tool in studying inhibitory systems.

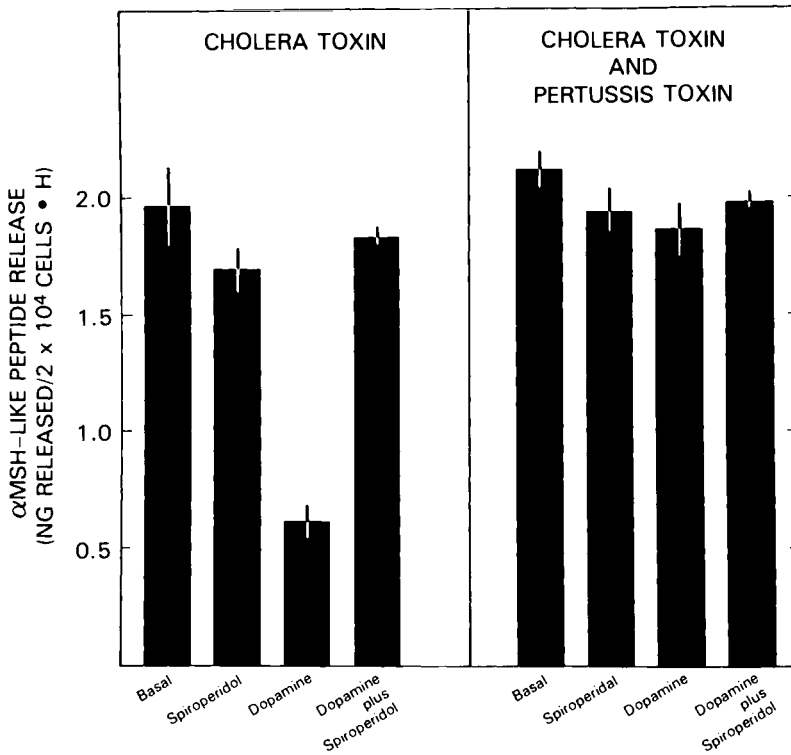

Fig. 8. Pertussis toxin abolishes the dopaminergic inhibition of cholera toxin-induced MSH release from intermediate lobe cells. Intermediate lobe cells were incubated 16 h in the absence (cholera toxin treated) or in the presence (cholera toxin plus pertussis toxin treated) of pertussis toxin. Both groups of cells were then treated for 2 h with 30 nM cholera toxin. The rate of MSH release was then determined in the absence of drugs, in the presence of dopamine, spiroperidol or a combination of dopamine and spiroperidol as indicated.

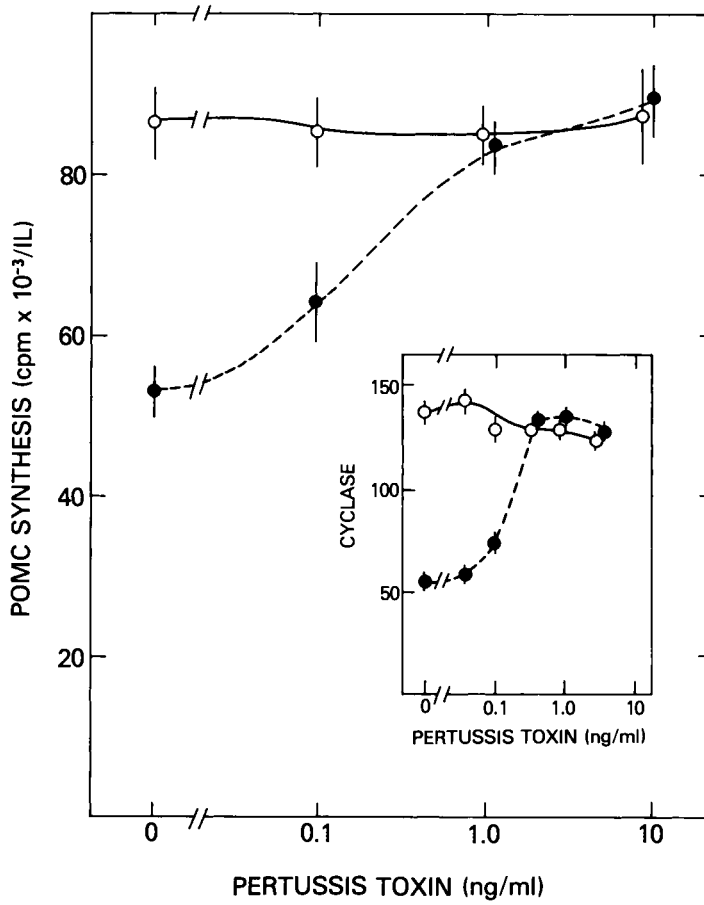

Fig. 9. Pertussis toxin abolishes the capacity of the dopamine receptor to mediate inhibition of proopiomelanocortin synthesis in the intermediate lobe. Intermediate lobe tissue was treated with the indicated concentrations of pertussis toxin for 16 h. The tissue was then tested in the absence (open circles) or presence (filled circles) of dopamine in the adenylate cyclase assay (insert) or incubated an additional 24 h in the absence or the presence of bromocriptine. The tissue treated in the absence (open circles) or presence (filled circles) of bromocriptine was then tested for its ability to synthesize proopiomelanocortin (POMC). (From Cote, T.E., Felder, R., and Sekura, R.D., in preparation).

VIII. CONCLUSION

Ni acts as a membrane transducer by converting the binding of an agonist to its receptor into an inhibition of the enzyme adenylate cyclase. Pertussis toxin has proven to be a useful tool in studies of the interactions among the receptor, Ni and the catalytic unit. The data presented here suggest that Ni has two distinct actions. The first action of Ni is to interact with the receptor (when the receptor is activated by an agonist) in a manner that causes the receptor to bind the agonist in a high affinity state. This action of Ni is abolished by the pertussis toxin-induced ADP ribosylation of the alpha subunit of Ni. Thus, pertussis toxin has no effect on the number of receptors or the affinity of the receptors for antagonist binding. However, the pertussis toxin-induced ADP ribosylation of Ni prevents the interaction between Ni and the receptor and thereby abolishes the induction of high affinity agonist binding and the influence of GTP on agonist binding (see Figs. 5 and 7).

The second action of Ni is to exert an inhibitory influence on the catalytic unit of adenylate cyclase either when a nonhydrolyzable GTP analog activates Ni or when there is a concurrent interaction of an agonist with the receptor and GTP with Ni. The pertussis toxin-induced ADP ribosylation of Ni prevents the activated receptor (the receptor occupied by an agonist) from altering the properties of Ni in a manner that would allow GTP to activate Ni and inhibit the catalytic unit. However, the ability of the nonhydrolyzable GTP analog to inhibit adenylate cyclase is insensitive to pertussis toxin. Thus, pertussis toxin has allowed us to demonstrate that Gpp(NH)p can activate Ni and inhibit adenylate cyclase in the absence of any interaction between Ni and the receptor. The finding that GTP can reverse the inhibitory effect of Gpp(NH)p suggests that hydrolysis of GTP to GDP may occur at Ni and thus prevent a sustained activation of Ni by GTP. It is proposed that the activated receptor in some way enhances the exchange of GTP for GDP at Ni. Since GTP, but not GDP, is believed to activate Ni, the continued replenishment of GTP at Ni would result in a persistent activation of Ni and a persistent inhibition of adenylate cyclase (Fig. 6). The pertussis toxin-induced ADP ribosylation of Ni prevents the activated receptor from enhancing the exchange of GTP for GDP and therefore abolishes the receptor-mediated inhibition of adenylate cyclase (Fig. 7). Indeed, it has been demonstrated that stimulation of the inhibitory opiate receptor enhances the rate at which

GDP is released from cell membranes.
This finding is consistent with the proposal that an inhibitory receptor enhances the exchange of GTP for GDP at Ni. Furthermore, the enhanced release of GDP from these membranes is abolished by pertussis toxin (Moss, in this volume).

Although an understanding of the mechanisms involved in receptor-mediated inhibition of adenylate cyclase is beginning to emerge, important questions remain regarding the events that occur at the molecular level. Ni is composed of three subunits designated alpha, beta, and gamma (4,5,32). The purified trimer has been shown to dissociate when a non-hydrolyzable GTP analog binds to the alpha subunit. It has been proposed that the three subunits may also dissociate in the membrane when there is a concurrent interaction of an agonist with the receptor and GTP with Ni (33). One or more of the dissociated subunits has been proposed to bring about an inhibition of adenylate cyclase. The purified beta subunit of Ni or the purified alpha subunit activated by GTPγS have been shown to inhibit adenylate cyclase activity under certain experimental conditions (33). However, it remains to be determined if the concurrent interaction of an agonist with the receptor and GTP at Ni can actually cause a dissociation of the subunits of Ni.

Finally, data have been presented that suggest that Ni is involved in the dopaminergic inhibition of both alpha MSH release and POMC synthesis (Figs. 8 and 9). It seems unlikely that all the physiological changes that are mediated by Ni are brought about by an inhibition of adenylate cyclase. Thus it remains to be determined if Ni can affect other "seccond messenger systems" in the cell, in addition to adenylate cyclase, in bringing about the numerous physiological responses of the cell initiated by the activation of the inhibitory receptor.

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PERTUSSIS TOXIN DISCRIMINATES SOMATOSTATIN'S REGULATION OF ACTH RELEASE THROUGH ADENYLATE CYCLASE AND NON-ADENYLATE CYCLASE MECHANISMS

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I. INTRODUCTION

The mechanisms by which hormones, acting upon membrane bound receptors, produce their physiologic effect have been studied using a variety of experimental approaches. For example, since cyclic AMP has been proposed as a second messenger for many receptor mediated responses (1-3), the ability of hormones to affect adenylate cyclase activity and cyclic AMP production has served as a measure of hormone action. Since these studies can be performed in cell free preparations they are particularly advantageous for the biochemical identification of hormone receptors and the manner by which the receptors are coupled to effector systems since the studies can be performed in cell free preparations. However, examination of functional responses distal to adenylate cyclase activation, or in fact not involving cyclic AMP, requires intact cells since those responses are not measurable when the cell's integrity disappears. By nature, studies in whole cells are more difficult for biochemical analysis of the coupling of receptors to second messenger systems. However, by measuring a biologic response, such as hormone secretion and changes in second messenger concentration, one can both ascertain the amount of second messenger needed to produce a given biologic response and determine if other intermediate cellular events are initiated to produce this response. Thus, in whole cells the entire

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cascade of cellular events needed to produce a physiologic effect can be evaluated rather than just one step which is the restriction of broken cell preparations.

The anterior pituitary has served as a useful organ to study the coupling of hormone receptors to their physiologic response. The tissue contains a number of biochemically identified receptors and easily measurable biologic responses. To further simplify the system, tumor cell lines of the anterior pituitary have been developed consisting of homogenous populations of cells responsive to various hypothalamic factors and secreting one or more hormones. The homogeneity of the tumors is a characteristic useful in correlating the action of a hormone on its receptor with the functional response that that hormone elicits. These cell lines can therefore be used as model systems to elucidate the precise mechanisms involved in regulating hormone secretion in vivo where heterogenous cell populations are present.

One such cell line is AtT-20/D16-16 which is derived from the mouse anterior pituitary and secretes adrenocorticotropin (ACTH). The release of this hormone can be stimulated by both corticotropin releasing factor (CRF) (4) and by catecholamines acting through β-adrenergic receptors (5). Both CRF and β-adrenergic receptors are coupled to adenylate cyclase, in addition non-receptor coupled activators of adenylate cyclase such as forskolin and cholera toxin are ACTH secretagogues (6,7). Furthermore, forskolin and effector hormones stimulate cyclic AMP-dependent protein kinase activity in these cells, an event that may be closely linked to the secretion of ACTH (8,9). Similar characteristics have been reported for normal corticotrophs in primary cultures of the anterior pituitary (10,11). These data imply that cyclic AMP is a second messenger in the receptor-mediated release of ACTH and that it may initiate a cascade involving protein phosphorylation.

Interestingly, β-adrenergic agonists also depolarize AtT-20 cells and enhance the frequency of appearance of action potentials (12). Similar studies on normal corticotrophs have not been reported and may prove difficult to perform since less than 3% of cells of the anterior pituitary contain ACTH. The electrical activity stimulated by β-adrenergic agonists is associated with an inward Ca^{++} current which may be important for the release of ACTH (12). In fact, high K^{+} concentrations depolarize AtT-20 cells, increase Ca^{++} influx and mobilization and cause ACTH secretion (13,14). Since K^{+} does not affect cyclic AMP formation (15), it is possible that the receptor-mediated release of ACTH involves both adenylate cyclase activation as well as stimulation of

effector systems not utilizing cyclic AMP. This raises the possibility that β-adrenergic and CRF receptors may be coupled to multiple second messenger systems in AtT-20 cells.

In the AtT-20 cell line it was found that ACTH release is also under an important inhibitory influence by the peptide somatostatin (SRIF) (6). Since SRIF can block the release of ACTH induced by secretagogues acting through distinct mechanisms (membrane potential, calcium dependent protein kinase as well as through cyclic AMP), it is possible that SRIF interacts with different effector systems to inhibit ACTH secretion (6,13,14,16).

The use of pertussis toxin has greatly facilitated the understanding of how inhibitory hormones regulate secretion. By catalyzing the ADP-ribosylation of N_i, pertussis toxin effectively removed the inhibitory effect of hormones on adenylate cyclase activity (17,18). This has allowed a closer examination of the other mechanisms by which hormones attenuate stimulus-secretion coupling. The following is a description of some of the work done in the anterior pituitary with pertussis toxin which has helped to elucidate the mechanisms involved in the negative control of hormone secretion.

II. INHIBITORY RECEPTORS, N_i, ADENYLATE CYCLASE AND STIMULUS-SECRETION COUPLING

SRIF is thought to block hormone secretion from the anterior pituitary by inhibiting adenylate cyclase activity (6,7). N_i is believed to couple SRIF receptors to adenylate cyclase in the anterior pituitary since GTP and its analogues reduce SRIF binding to pituitary homogenates (19). That N_i plays a vital role in mediating the actions of SRIF on the secretion of hormones from the anterior pituitary has been substantiated by studies employing pertussis toxin (15,20,21). Previously, it was suggested that pertussis toxin could be used to both biochemically identify N_i as well as inactivate it, thus providing information as to its functional significance (17,18,22). Pertussis toxin catalyses the ADP-ribosylation of a protein in AtT-20 cell membranes with a similar molecular weight as that suggested for N_i in other tissues (15). These data indicate the presence of the inhibitory hormone coupling protein, N_i, in the pituitary. Pretreatment of AtT-20 cells or primary cultures of the anterior pituitary with pertussis toxin blocks the capacity of SRIF to inhibit the action of cholera toxin and forskolin in stimulating release. The effect of SRIF in inhibiting growth hormone releasing factor stimulated growth

hormone release (15,21) is also blocked by pertussis toxin. Inhibition of the cyclic AMP response by SRIF was also prevented in these tissues by treatment with pertussis toxin (15,20,21). Proof that the pertussis toxin neutralization of SRIF's inhibitory actions on hormone release was due to modification of N_i comes from studies in AtT-20 cells (15) where diminished responsiveness to SRIF was shown to be related to ADP-ribosylation of a 41,000 dalton component assumed to be N_i. Furthermore, this effect was dependent on the time of pertussis toxin pretreatment and corresponded to the period required to reduce SRIF's inhibition of forskolin stimulated cyclic AMP formation and ACTH release (Fig. 1). A

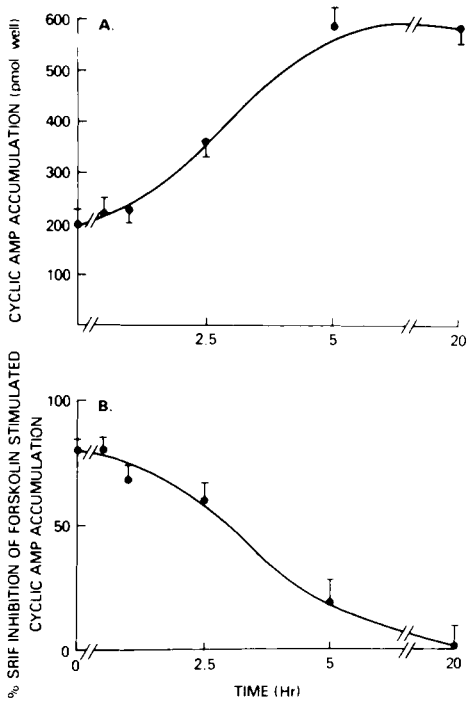

Fig. 1. Time course for pertussis toxin blockade of SRIF's inhibition of stimulated cyclic AMP formation. In A, the effect of forskolin alone on cyclic AMP accumulation is represented after different periods of toxin treatment. In

similar time course was observed by Cronin et al. (21) for the blockade of SRIF's inhibition of growth hormone release from primary cultures of the anterior pituitary. Pertussis toxin's blocking actions in the anterior pituitary are dose-dependent (Fig. 2) and prevented by antibodies raised

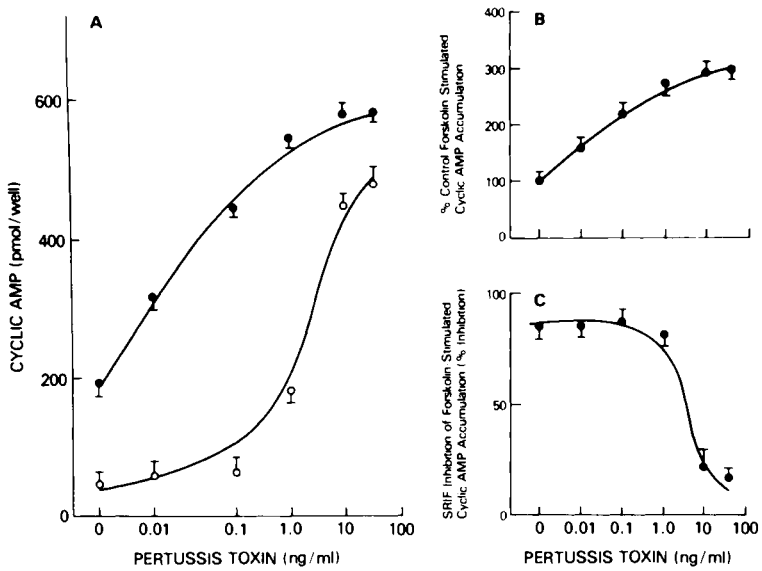

Fig. 2. Dose-dependency of pertussis toxin blockade of SRIF's inhibition of forskolin stimulated cyclic AMP formation. After the treatment, the medium was removed, the cells were washed twice and fresh medium containing forskolin (10^{-4}M) with (O) or without (●) SRIF (10^{-7}M) was added (Inset A). Inset B which is data transformed from Inset A shows the % of control increase of forskolin stimulated cyclic AMP accumulation as a function of toxin concentration. Inset C which is data transformed from Inset A shows the

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B, the % inhibition of forskolin stimulated cyclic AMP formation by SRIF after the different times of toxin treatment. Values represent the results ± SEM of 3 experiments done in triplicate cultures.

reduction in capacity of SRIF to inhibit forskolin's effects also as a function of toxin concentration. Values represent the mean ± SEM of 3 experiments done in triplicate cultures.

against the pertussis toxin protein (21). Furthermore, pertussis toxin induces a permanent alteration in the functioning of N_i since recovery from pertussis toxin's blocking action both of SRIF's inhibition of forskolin evoked ACTH release and cyclic AMP accumulation do not occur (15). Similarly, no recovery of the ability of pertussis toxin to catalyze the ADP-ribosylation of the 41,000 MW substrate in AtT-20 cell membranes occurs once intact cells are first pretreated with pertussis toxin (15). These results suggest that one mechanism by which SRIF diminishes hormone release from the anterior pituitary is by first activating receptors coupled to N_i so as to inhibit the stimulation of cyclic AMP synthesis.

III. PERTUSSIS TOXIN ENHANCES HORMONE RELEASE FROM THE ANTERIOR PITUITARY

Besides removing the hormone inhibition of adenylate cyclase, pertussis toxin treatment facilitates stimulation of cyclic AMP accumulation and in some cases, hormone release (15,20,21,23). Katada and Ui (24) first observed in pancreatic islets that pertussis toxin, while not affecting basal cyclic AMP formation or insulin release, potentiated the ability of glucose to stimulate both of these responses. Cronin et al. (21,23) observed that exposure of anterior pituitary monolayers to pertussis toxin enhanced growth hormone releasing factor induced cyclic AMP synthesis and growth hormone release as well as gonadotropin releasing hormone evoked luteinizing hormone secretion. This potentiating effect was also observed in the AtT-20 cells (15,21). Pertussis toxin pretreatment enhanced CRF, forskolin and cholera toxin induced cyclic AMP accumulation and ACTH release. The potentiation was dependent on the time of pretreatment, the concentration of pertussis toxin used (Fig. 1 and Fig. 2), and was not reversible (recovery was studied for 24 hrs). In contrast, 8-bromo-cyclic AMP, which bypasses adenylate cyclase in stimulating ACTH release, caused the same secretion in control and treated cells. These data suggest that pertussis toxin produces some event in pituitary tissue that makes cells much more capable of producing cyclic AMP. This enhancement cannot be explained by an increase in the number or coupling efficiency of

stimulatory receptors (forskolin and cholera toxin do not act through hormone receptors to stimulate cyclic AMP accumulation) and is most likely not the result of an inhibition of phosphodiesterase activity since the potentiation of forskolin induced cyclic AMP production occurs in the presence of phosphodiesterase inhibitors.

The potentiation of the stimulation of cyclic AMP accumulation following pertussis toxin treatment may be explained by several possible mechanisms. N_i may tonically inhibit adenylate cyclase activity. By removing N_i's influence, as occurs following pertussis toxin treatment, this tonic inhibition may be suppressed so that hormones can stimulate adenylate cyclase activity more effectively. In pancreatic islets, it was observed that following pertussis toxin treatment the ability of 8-bromo-cyclic AMP to stimulate insulin release was enhanced, suggesting that pertussis toxin may induce cellular responses distinct from the removal of N_i inhibition of adenylate cyclase activity (24). In the absence of Ca^{++}, pertussis toxin treatment did not potentiate cyclic AMP accumulation nor insulin release. These conditions also prevented pertussis toxin from blocking SRIF or epinephrine inhibition of insulin release (24). It was suggested from these and other data that pertussis toxin can elevate Ca^{++} mobilization in pancreatic cells so as to facilitate hormone release and overcome SRIF's or epinephrine's inhibition of cyclic AMP synthesis and the secretion of insulin (24). However, a detailed mechanism explaining how pertussis toxin could induce this effect has not been put forth.

In AtT-20 cells, pertussis toxin treatment, as mentioned previously, did not enhance 8-bromo-cyclic AMP stimulated ACTH release (15). In addition, the ACTH release response to K^+ or the calcium ionophore, A23817, was not altered by such treatment (15). Thus, the same changes in Ca^{++} availability reported in pancreatic islets (24) following pertussis toxin treatment probably do not occur in the AtT-20 cells. However, the enhanced cyclic AMP response to forskolin in toxin pretreated AtT-20 cells was prevented by cycloheximide (Fig. 3). The protein synthesis inhibitor itself did not change the levels of cyclic AMP formed during forskolin exposure nor did it reverse the blockade of SRIF's inhibition of cyclic AMP accumulation. Recently, Brooker et al. (25) proposed that forskolin stimulated cyclic AMP formation in C6 glioma cells is mediated by a protein that is not involved in hormone stimulated cyclic AMP synthesis. Cycloheximide treatment was suggested to reduce the turnover of this protein thus explaining the diminished ability of forskolin but not hormones to stimulate the accumulation of

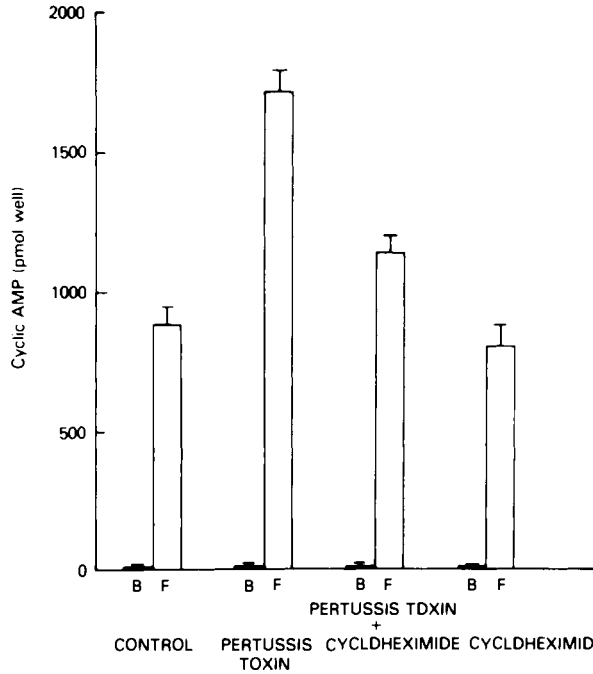

Fig. 3. Cycloheximide blocks the increase of forskolin stimulated cyclic AMP accumulation induced by pertussis toxin. Cells were treated for 6 hrs with pertussis toxin (50 ng/ml), cycloheximide (10μg/ml), their combination or without drug additions (CONTROL). The cells were then washed and treated with (F) or without (B) forskolin (10^{-4}) for 30 mins. Cyclic AMP content was determined as described in the text. Results are the mean ± SEM of 3 separate experiments done in triplicate cultures.

cyclic AMP. The turnover of this putative protein in AtT-20 cells could conceivably be affected by pertussis toxin treatment. Alternatively, pertussis toxin may induce a chronic activation of adenylate cyclase with cycloheximide inducing some non-identified action to neutralize this activation. Whether pertussis toxin's ability to potentiate cyclic AMP stimulation is due to an effect on N_i, Ca^{++} availability or some other intracellular event remains to be determined.

IV. PERTUSSIS TOXIN ALTERS SRIF'S ACTIONS ON NON-ADENYLATE CYCLASE EFFECTOR SYSTEMS

While adenylate cyclase regulation is a primary site by which inhibitory hormones could modify stimulus-secretion coupling, other cellular locations may be equally as important. For instance, membrane potential changes have been closely linked to hormone secretion in pancreatic islets and pituitary cells (18,26-29). Depolarization in some of these cells leads to transient changes in inward Ca^{++} currents, an event associated with if not necessary for secretion. Factors which antagonize this depolarization could prevent the Ca^{++} influx and thereby attenuate the release of hormones. Such mechanisms need not involve cyclic AMP or adenylate cyclase. SRIF has been shown both in the pancreas (26,27) and pituitary (30) to hyperpolarize cells and reduce the frequency of action potentials. In AtT-20 cells, ACTH release has been linked to the increase of frequency of action potentials (12) and membrane depolarizing agents such as K^+ release ACTH (13,14). SRIF and analogues of SRIF inhibit K^+ evoked ACTH release to the same extent and potency as they block forskolin's actions (Fig. 4). In fact,

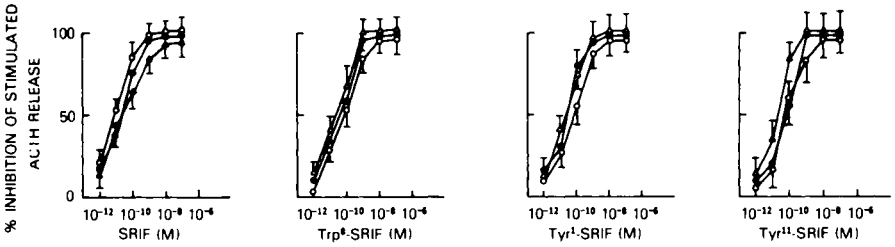

Fig. 4. Inhibition of stimulated ACTH release by SRIF and SRIF analogues. Monolayer cultures of AtT-20 cells were incubated with either forskolin (10^{-5}) (0) 8-bromo-cyclic AMP (10^{-4}M) (●) or K^+ (50mM) (▲) with or without various concentrations of SRIF or SRIF analogues. At the end of this incubation the medium was removed and analyzed for ACTH immunoreactive material. Values are represented as the % inhibition of ACTH release stimulated by each secretagogue.

pharmacologic characterization indicate that there is only one type of functional SRIF receptor on AtT-20 cells. However, K^+ and forskolin release ACTH through distinct intracellular processes. Thus, SRIF receptors must be coupled to different effector systems. This proposal is supported by recent pharmacologic studies. Pretreatment of AtT-20 cells with SRIF reduces the subsequent potency of SRIF to inhibit forskolin and CRF stimulated ACTH release (31). This desensitization is time and dose-dependent, reversible and apparent for the cyclic AMP accumulation as well as the ACTH release response (31). Similar treatment, however, does not alter SRIF inhibition of K^+ evoked ACTH release (Fig. 5)

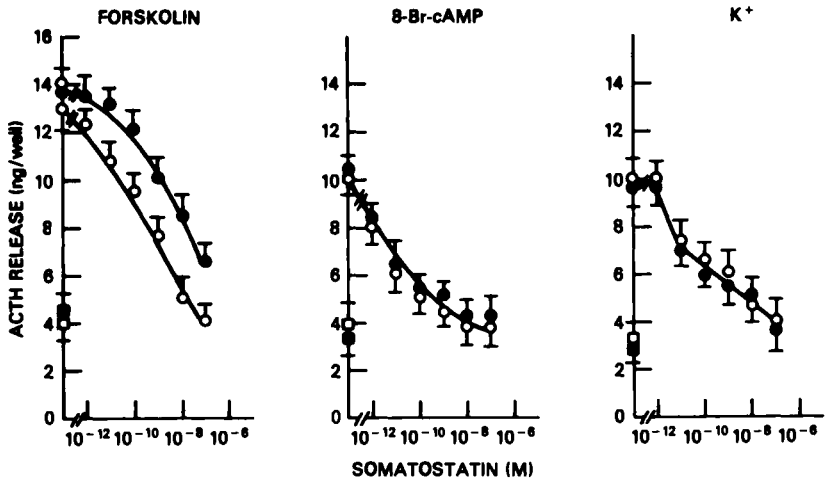

Fig. 5. SRIF desensitization. Monolayer cultures of AtT-20 cells were exposed to DMEM containing 10% fetal calf serum with (●) or without (O) SRIF (10^{-7}M) for 6 hr. At the end of this time the cells were washed twice and then incubated for 30-60 min with forskolin (10^{-5}M), 8-bromo-cyclic AMP (8 Br-cAMP) (10^{-4}M) or K^+ (50mM) with or without different concentrations of SRIF. Basal release in control (□) and treated (■) cells is also represented. The values are the results ± SEM of seven different experiments done in triplicate wells.

suggesting that the coupling of SRIF receptors to adenylate cyclase is regulated differently than the coupling to the mediator of K^+'s effects. Furthermore, elevation of

extracellular Ca^{++} concentration reduces SRIF's inhibition of K^+ but not forskolin evoked ACTH release (Fig. 6). Increased

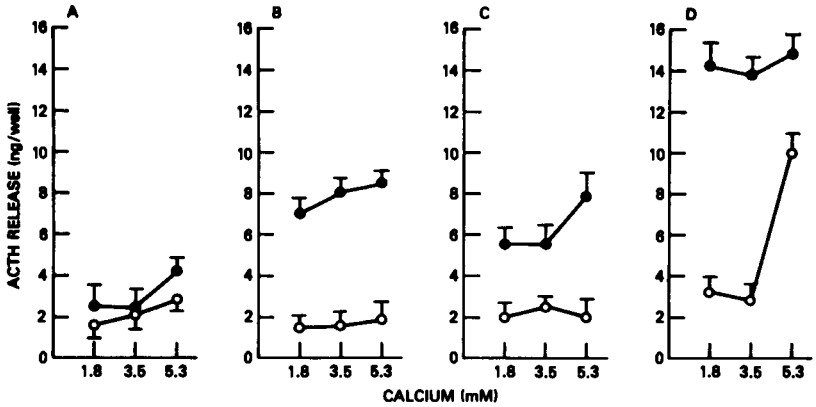

Fig. 6. Effect of $CaCl_2$ on SRIF's inhibition of ACTH release. Monolayer cultures of AtT-20 cells were incubated at 37°C with medium without stimulant (basal release) (A) or with forskolin (10^{-5}M) (B), 8-bromo-cyclic AMP (10^{-4}M) (C) or K^+ (50mM) (D) with (O) or without (●) SRIF (10^{-7}M) plus the addition of $CaCl_2$ at the concentration indicated. The normal concentration of $CaCl_2$ in the medium is 1.8 mM. The values represent the mean ± SEM ACTH release of 3 experiments done in triplicate wells.

Ca^{++} concentrations do not change the potency of SRIF to inhibit the ACTH release response to forskolin (Fig. 7). In fact, removal or addition of Ca^{++} from the medium does not alter SRIF's blockade of forskolin stimulated cyclic AMP formation (Fig. 8) nor does the addition of the calcium ionophore A23817 (Fig. 9). Therefore, SRIF's inhibition of K^+ mediated ACTH release has a Ca^{++} dependency not observed for forskolin. These studies further reveal the different natureof the coupling of SRIF receptors to the various intracellular transducing systems involved in stimulus-secreting coupling in AtT-20 cells.

If pertussis toxin's actions on AtT-20 cells could solelybe accounted for by a disinhibition of adenylate cyclase activity, then it might be expected that SRIF's

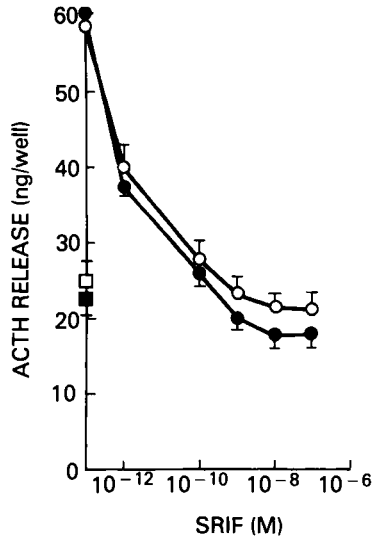

Fig. 7. AtT-20 cells were incubated with low (○) or high (●) $CaCl_2$ and forskolin (10^{-5}M) with SRIF and ACTH release was measured.

regulation of K^+ evoked ACTH release would not be altered by pertussis toxin pretreatment. In fact, after pertussis toxin treatment, SRIF is still capable of inhibiting the ACTH release response to K^+. However, both the potency and magnitude of inhibition by the peptide is significantly diminished. This alteration may be explained in light of recent studies by Cote et al. (32) on dopamine receptors which are negatively coupled to adenylate cyclase in melanotrophs of the intermediate lobe of the pituitary. They observed that pertussis toxin treatment resulted in a reduced ability of dopamine agonists to inhibit ^{3}H-spiroperidol (a dopamine receptor antagonist) binding to homogenates of these cells. Also, the ability of GTP to reduce the potency of dopamine agonists to inhibit ^{3}H-spiroperidol binding was absent in toxin treated cells. From these data, the authors suggested that pertussis toxin uncouples N_i from the dopamine receptor, thus reducing the affinity of these receptors for agonists. The total number

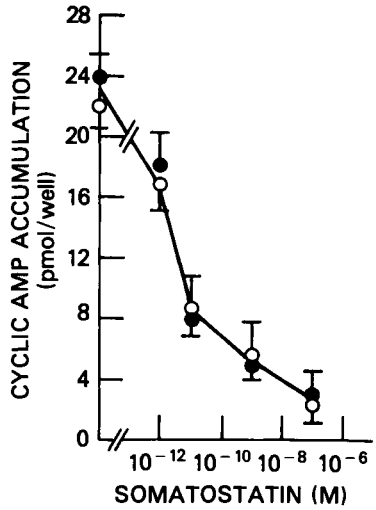

Fig. 8. Lack of effect of Ca^{++} on SRIF'S inhibition of forskolin stimulated cyclic AMP accumulation. AtT-20 cells were incubated for 30 min with DMEM containing forskolin (10^{-5}M), different concentrations of SRIF with (●) or without (0) $CaCl_2$ (1.8 mM). Values represent the mean ± SEM of 3 experiments done in duplicate wells.

of receptors were not changed after this treatment. If these results can be extrapolated to the AtT-20 cells, it may imply that pertussis toxin treatment reduces SRIF receptor affinity for SRIF. This effect could explain the reduction of SRIF's potency and efficiency to block K^+ evoked ACTH release, a response not linked to adenylate cyclase. Therefore, these studies imply a role for N_i in mediating both the inhibition of adenylate cyclase activity as well as to modify inhibitory hormone receptor sensitivity. N_i is thus a crucial protein in the regulation of inhibitory receptors. A similar importance of N_s has been proposed to occur for stimulatory receptors (33-35). Furthermore, recent ligand binding studies have suggested an ability of N_i to regulate stimulatory receptors, since beta-adrenergic agonist binding (^{3}H-HBI) to membranes of cyc$^-$ variants of S49 lymphoma cells,

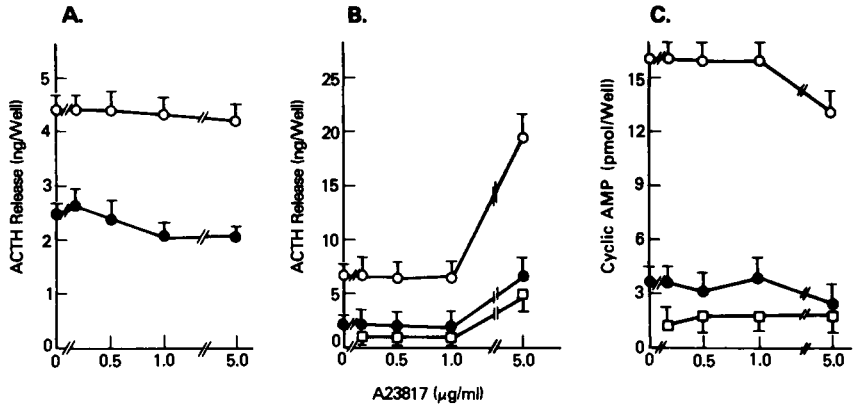

Fig. 9. Effect of the calcium ionophore A23187 on SRIF inhibition of forskolin and 8-bromo-cyclic AMP stimulated ACTH release and forskolin stimulated cyclic AMP accumulation. AtT-20 cells were incubated at 37°C for 60 min with forskolin (10^{-5}M) with (●) or without (O) SRIF (10^{-7}M) and different concentrations of A23187 and ACTH release from (A) and cyclic AMP accumulation in (C) the cells was determined. In the same subcultures, 8-bromo-cyclic AMP (10^{-4}M) with or without SRIF (10^{-7}M) was incubated for 60 min with different concentrations of A23187 and ACTH release or cyclic AMP accumulation of 3 experiments done in triplicate wells. □ A23817 alone.

deficient in functional N_s but containing N_i, is inhibited by GTP (36). An interaction of N_i and stimulatory receptors is further supported by the reconstitution studies of Asano et al. (37). Thus, by controlling receptor sensitivity, guanine nucleotide binding proteins appear to have an important potential role in the regulation of stimulus-secretion coupling.

V. CONCLUSION

ACTH release is stimulated by several different hormones acting through a variety of apparently independent intracellular mechanisms. CRF and catecholamines activate adenylate cyclase and cyclic AMP dependent protein kinase in corticotrophs (8,9,11). Phosphorylation of several substrates by cyclic AMP dependent protein kinase may be an important event in the ACTH release response to these secretagogues, however, this has not been demonstrated (8). Vasopressin also causes ACTH secretion but this peptide does not increase cyclic AMP production in normal corticotrophs (38). This hormone may alter phosphatidyl inositol turnover as suggested in other tissues to trigger ACTH release. In line with this proposal, phorbol esters activate calcium dependent protein kinase activity and ACTH release from AtT-20 cells (16,39,40). Since diacylglycerol is a breakdown product of inositide turnover and also activates calcium dependent protein kinase, it is possible that some hormones may stimulate this pathway to cause ACTH release. Furthermore, catecholamines depolarize AtT-20 cells, and increase transient Ca^{++} influx (12). Since Ca^{++} is necessary for stimulus-secretion coupling, this mechanism may be yet another alternative mechanism involved in the secretion of ACTH. The requirement for multiple ACTH secretagogues as well as intracellular transducing system may be explained by a need to maintain continuous sensitivity of the corticotroph to stimulation. Thus, while each secretagogue can desensitize independently (for review, 41) complete refractoriness of the corticotroph has not yet been observed. This factor may insure that the organism can always respond to some form of stress.

Opposing the stimulatory influence of hypothalamic CRF-like substance is the peptide SRIF. Also of hypothalamic origin, SRIF blocks ACTH release as well as the secretion of other pituitary hormones (6,41). Such an inhibitory influence may allow the brain to fine-tune the output of the anterior pituitary. Consistent with this major inhibitory role of SRIF is the ability of this peptide to stop all secretagogues from releasing ACTH from AtT-20 cells. This action does not appear to be non-selective since the effect occurs at minute concentrations of SRIF and since a number of structurally related analogues produce the same effect. Furthermore, high affinity SRIF binding sites have been identified on AtT-20 cells and have been proposed to mediate SRIF's inhibitory action (42). If SRIF's actions are specific it suggests that either there are multiple types of SRIF receptors coupled to different effector systems, only one

receptor linked to multiple transducers, or SRIF acts upon a crucial event needed for all secretagogues to cause ACTH secretion. While the pharmacology of SRIF is insufficient to properly characterize receptors (due to the lack of antagonists) use of the available analogues suggests that only a single SRIF receptor regulates ACTH release. How a single type of SRIF receptor could couple to second messenger systems of diverse molecular makeup is not known. One possibility is that the SRIF receptor may interact with a family of membrane components each able to couple with the SRIF receptor but also possessing different moieties so as to link with various second messenger systems. Such membrane components may be the multiple guanine nucleotide binding proteins suggested to exist by Rodbell (43). In fact, SRIF binding in the pituitary is regulated by guanine nucleotides (19). Alternatively, SRIF may regulate ACTH release through mechanisms which have not as yet been described. Since stimulus-secretion coupling may be a very rapid event, studies examining the rapid effects of secretagogues and SRIF on membrane potential and changes in intracellular transducing systems as well as ACTH release will be required to fully explain the mechanisms by which SRIF ultimately regulates the ACTH release process.

The development of agents such as pertussis toxin has advanced our knowledge of the molecular mechanisms controlling stimulus-secretion coupling. Availability of other toxins or agents capable of identifying other types of guanine nucleotide binding proteins may help to explain the coupling mechanism of inhibitory hormones with non-adenylate cyclase transducing systems. While helping to clarify the role of N_i in physiologic systems, pertussis toxin has also revealed further complexities. For instance, how does pertussis toxin enhance the cyclic AMP and hormone release responses. In addition, it will be important to determine the mechanism by which pertussis toxin treatment alters biologic responses not involving adenylate cyclase such as SRIF's inhibition of K^+ evoked ACTH release. The manner by which pertussis toxin promotes such multiple intracellular events may aid in the understanding of how this agent itself produces its biologic action. Identification of these adaptive responses may also further elucidate the feedback systems and the various intricate control mechanisms in the cells that presumably regulate in some manner the stimulus-secretion process.

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GENETIC AND FUNCTIONAL STUDIES OF PERTUSSIS TOXIN SUBSTRATES[1]

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I. INTRODUCTION

Current research in many laboratories focuses on the structure and functions of a family of GTP-binding regulatory proteins, each of which carries information across a membrane from a detector/ receptor protein to an effector enzyme. Three members of the family have been identified: G_s, G_i, and retinal transducin. The first two proteins stimulate (G_s) or inhibit (G_i) adenylate cyclase activity when they are activated by specific receptors for hormones or neurotransmitters. Transducin, a protein found in high abundance in the rod outer segments (ROS) of vertebrate retina, couples photoexcitation of rhodopsin to stimulation of a specific cGMP phosphodiesterase (PDE). The membrane-bound p21-ras protein, an oncogene product that binds and hydrolyzes GTP (1,2), is probably a fourth member of this protein family, although the detector and effector elements that are coupled by p21-ras have not yet been identified.

Evidence for homology among the G proteins and transducin (3-5) includes their similar subunit structures (each is a heterotrimer of α, β, and γ subunits), similar amino acid

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compositions of the respective subunits, and the occurrence of a guanine nucleotide binding site in each protein's α subunit. For each of these proteins, the excited signal detector (hormone receptor or rhodopsin) activates the coupling protein by catalyzing exchange of GTP for GDP at the guanine nucleotide binding site; this reaction is followed by separation of the coupling protein's α and β γ subunits. In addition, each of the three proteins has a GTPase activity, which serves to terminate the active state. Light or specific hormones maintain activity of the cognate coupling protein by promoting repeated replacement of GDP by GTP, a process that results in light- or hormone-stimulated GTP hydrolysis (3,4,6). For transducin and G_s, the active (GTP-bound) α subunit stimulates the effector enzyme, adenylate cyclase or PDE; the mechanisms by which G_i inhibits adenylate cyclase are under investigation (7).

The two G proteins and transducin exhibit another striking homology: The α subunit of each protein serves as a substrate for ADP-ribosylation by one or both of the exotoxins of V. cholerae or B. pertussis. The toxin-catalyzed covalent modifications have proved useful for identifying the coupling proteins and as probes for molecular interactions between subunits and with other proteins. Indeed, the action of pertussis toxin (hereafter termed PT) led to discovery of G_i, as described by Ui and co-workers in this symposium. More recent exploration of PT's effects strongly suggests that G_i mediates effects of certain signals that do not depend on alterations of cAMP synthesis, as described below.

This review will summarize studies from our laboratory in three areas: (a) Studies of retinal transducin as a target for toxin-catalyzed ADP-ribosylation; (b) use of PT for assessing the role of G_i in chemotactic activation of human neutrophils; (c) initial efforts aimed at molecular cloning of the genes that encode transducin and the G proteins.

II. BACTERIAL TOXINS AND RETINAL TRANSDUCIN

A. Transducin's Role in ROS — a Molecular Model for Subunit Interactions

Recent investigations of the biochemistry of signal transduction in ROS (3-5,8) outlined a system in which information flows from the exciting photon through rhodopsin and transducin to activation of cGMP hydrolysis. The

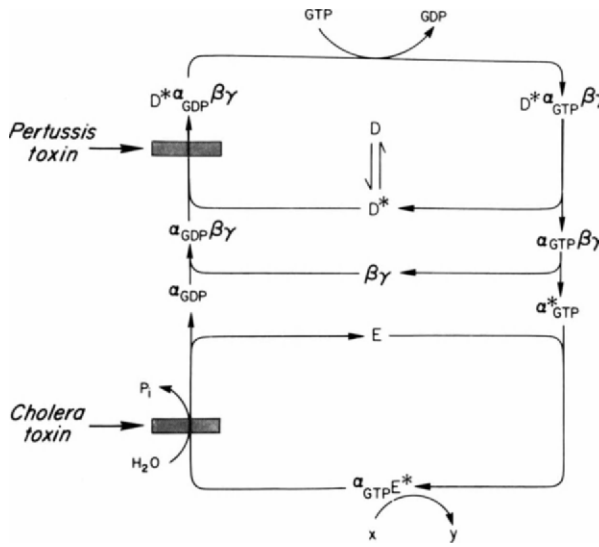

Fig. 1. Scheme depicting interactions between subunits of G proteins (α, β, and γ) and detector (D) and effector (E) components of GTP-dependent transmembrane signaling. The bars indicate positions at which the process is interrupted by toxin-catalyzed ADP-ribosylation. See text for details.

retinal system provides enormous amplification of the exciting signal: A single photon may activate hundreds of transducin and PDE molecules and stimulate hydrolysis of millions of cGMP molecules (3).

Fig. 1 depicts a generalized model of the molecular interactions involved in transduction of signals between detector (D) and effector (E) molecules by GTP-binding proteins. Subunits of the coupling protein are denoted α, β, or γ. The model is based upon that proposed for transducin by Fung's laboratory (9,10) and studies of the G proteins by Gilman and his colleagues (11). α^*-GTP, the "active" conformer of the α subunit, can activate E; this active conformation decays, however, concomitant with hydrolysis of the

γ-phosporyl bond of GTP. Free βγ then binds to α-GDP, and the αβγ complex has a high affinity for the activated detector component (D^* = hormone-receptor complex or photoexcited rhodopsin). Guanine nucleotides and D^* heterotropically displace one another from binding to αβγ. As a result, GTP can replace GDP on the guanine nucleotide binding site of α, with concomitant dissociation of αβγ from D^*. (This reaction, resulting in release of transducin from photoactivated ROS, forms the basis of the purification of transducin.) A conformational change in α (to α^*), stabilized by GTP, reduces its affinity for βγ, and free α^*-GTP is released to activate E once again.

Compared to the amounts of receptor and G proteins obtainable from plasma membranes, ROS contain vast amounts of rhodopsin, transducin, and the effector enzyme, cGMP PDE. The relative abundance of the retinal proteins has allowed biochemical experiments that have been difficult or impossible to perform with components of hormone-sensitive adenylate cyclase. For example, Fung's laboratory was able to radiolabel functionally active α and βγ subunits of transducin and study their interaction with rhodopsin. They showed that neither the α nor the βγ subunit of transducin, alone, can bind to photorhodopsin, while the two together do bind to the photoreceptor in the absence of guanine nucleotides (9,10).

A series of studies from Gilman's laboratory have demonstrated similar subunit interactions of the G proteins. Using resolved subunits of the G proteins, these workers showed that G protein activation (by guanine nucleotides or fluoride ion) is accompanied by separation of α from βγ and that addition of excess βγ (derived from G_i) can reverse activation of the α subunit of G_s (summarized in (11)).

A recent elegant study (12) demonstrated functional interchange of subunits among the G proteins and transducin: Photorhodopsin stimulated GTP hydrolysis by purified G_i, by purified transducin, and by heterologous mixtures in which the α and βγ subunits were derived from different proteins. Thus, the α subunit of G_i hydrolyzed GTP in a light-dependent fashion when mixed with βγ derived from transducin, and transducin's α subunit hydrolyzed GTP when mixed with βγ derived from G_i. These results imply that βγ of either protein can bind to α of the other, and that a binding site on photorhodopsin is recognized by heterologous αβγ heterotrimers.

B. The Toxins

Cholera toxin (hereafter termed CT) played a useful part in the early characterization of G_s, because toxin-catalyzed ADP-ribosylation allowed the protein's α subunit to be radiolabeled with (^{32}P) ADP-ribose (13-15). ADP-ribosylation by CT stabilizes the GTP-bound active conformation of the α subunit of G_s, and promotes dissociation of α from βγ (16), thereby activating cAMP synthesis and reducing hormone-stimulated GTPase activity (see Fig. 1).

Purification of PT by Ui and his colleagues allowed them to show that treatment with the toxin caused attenuation of the inhibition of adenylate cyclase produced by inhibitory ligands, including α-adrenergic, muscarinic, and opiate agonists. They also showed that the toxin has an A-B structure similar to that of CT, and that it acts by catalyzing ADP-ribosylation of a 41-kDa polypeptide that is bound to the plasma membrane (17-20). Later work showed that the 41-kDa polypeptide is in fact the α subunit of G_i (21,22). ADP-ribosylation by PT stabilizes transducin and G_i in the GDP-bound inactive form, as described below.

C. CT and transducin

We reported (8) that CT catalyzes ADP-ribosylation of the α-subunit of transducin in ROS, and thereby inhibits its light-stimulated GTPase activity. Parallels between CT-catalyzed ADP-ribosylation of G_s and transducin are striking: (a) Both proteins serve as substrates for the toxin only in the membrane-bound form; (b) activation of the detector molecule by hormone (23) or light (8) enhances toxin-catalyzed ADP-ribosylation (indeed, in our studies (8) CT did not ADP-ribosylate transducin in dark-adapted ROS); (c) binding of a stable GTP analog, guanylyl-5'-imidodiphosphate, enhances ADP-ribosylation, both in the adenylate cyclase system (23) and in ROS (8). In summary, in both systems the preferred substrate for CT is the "active," GTP-bound conformation of the coupling protein, and the covalent attachment of ADP-ribose stabilizes the coupling protein in the active conformation.

Because CT requires such similar conditions for ADP-ribosylating both G_s and transducin, and because the functional effects of ADP-ribosylation are also similar, it is likely that CT ADP-ribosylates similar sites in the two proteins. Accordingly, we sought to identify the site at which CT attaches ADP-ribose to transducin. ADP-ribosylated transducin was partially purified and then subjected to

tryptic hydrolysis. The ADP-ribosylated fragment, purified on a boronate column (24) followed by high performance liquid chromatography, was a tetrapeptide with the sequence SER-ARG-VAL-LYS. Arginine was the ADP-ribosylated amino acid (25).

This is the first identification of an ADP-ribosylated arginine from a protein that is specifically ADP-ribosylated by CT. Incubations of CT with NAD^+ and various amino acids had previously indicated that the guanidinium group of arginine can serve as an acceptor for ADP-ribose (26). Like CT, two other bacterial toxins affect cellular metabolism in eukaryotes by mono-ADP-ribosylating specific cellular proteins (19,20,27). Diphtheria toxin ADP-ribosylates a modified histidine residue, termed diphthamide, on Elongation Factor 2 (28) and PT ADP-ribosylates an asparagine near the carboxyl terminus of retinal transducin (29). In addition, ADP-ribosylation of an arginine on RNA polymerase has been reported during T4 phage infection of E. coli (30). Thus, each of the toxins ADP-ribosylates a unique amino acid on its specific protein substrate.

Because of the close structural and functional similarities between G_s and transducin, the amino acid sequence around the CT substrate site on G_s will probably resemble that found in transducin; we predict that the ADP-ribosylated amino acid will be arginine.

D. PT and Transducin

The striking parallels between hormone-sensitive adenylate cyclase and the rod photoreceptor suggested that ROS might contain a protein analogous to G_i. Preliminary results of exposure of photoactivated ROS to toxin and (^{32}P) NAD^+ were quite disappointing: The toxin appeared to catalyze very little labelling of any protein. Eventually we discovered that this disappointing outcome resulted from performing the experiments with photoexcited ROS. We subsequently found that pertussis toxin very rapidly ADP-ribosylates one polypeptide in dark-adapted ROS, and that this reaction is markedly inhibited by photoexcitation of rhodopsin (31).

A second surprise was that the polypeptide ADP-ribosylated by pertussis toxin in ROS is the α subunit of transducin itself. We had initially expected to find a substrate distinct from transducin, just as G_i is distinct from G_s. The polypeptide ADP-ribosylated by pertussis toxin, however, was clearly transducin's α subunit.

As a substrate, PT prefers the inactive GDP-bound form

of transducin -- a preference directly opposite to that of CT for the same protein. Thus, light and exposure to GTP analogs inhibit ADP-ribosylation by PT but increase ADP-ribosylation by CT. Conversely, dark adaptation and GDP enhance ADP-ribosylation by PT but markedly attenuate the reaction catalyzed by CT. The two toxins ADP-ribosylate quite distinct sites on the α subunit: The amino acid sequence at the substrate site for PT is the carboxy-terminal octapeptide GLU-ASN-LEU-LYS-ASN(ADP-ribose)-GLY-LEU-PHE (29), which bears no resemblance to the sequence we found for CT.

ADP-ribosylation by PT dramatically alters the function of transducin (31). The PT-catalyzed covalent modification produces inhibition of transducin's light-activated GTPase activity in ROS, and blocks the signal-coupling capacity of transducin, preventing photoactivation of phosphodiesterase.

Although PT, like CT, inhibits the GTPase activity of transducin, it does so by a different mechanism. Covalent modification by PT appears to interfere with exchange of GTP for GDP, by stabilizing transducin in the inactive GDP-bound state (Fig. 1). Just as with CT, the conformational state stabilized by PT-catalyzed ADP-ribosylation is similar or identical to the preferred substrate conformation for the toxin. The increased stability of the GDP-bound state is accompanied by diminished ability of the modified protein to interact with photoexcited rhodopsin (D^* in Fig. 1), the catalyst for GTP-GDP exchange, as indicated by its decreased affinity for bleached ROS membranes (31).

This interpretation suggests that PT treatment may decrease the affinity of the cognate coupling protein, G_i, for hormone receptors that inhibit adenylate cyclase. Indeed, the toxin abolishes high affinity binding of α-adrenergic and muscarinic agonists to their respective receptors in a cultured cell line (32). In adenylate cyclase systems, this high affinity binding of agonists (which is decreased or absent in the presence of GTP analogs) is considered an index of receptor coupling to guanine nucleotide-binding regulatory proteins. As a second corollary of the present findings with transducin, we predict that pertussis toxin will ADP-ribosylate G_i better in the presence of stable GDP analogs than in the presence of GTP analogs or of agonists that inhibit adenylate cyclase. Initial experiments in our laboratory are consistent with this prediction.

What is the biological significance of ADP-ribosylation sites on transducin for both PT and CT? Why do toxin-catalyzed modifications at these sites cause parallel functional consequences in transducin and the two G proteins?

We imagine that the genes encoding transducin and a primordial G protein diverged in the course of evolution from a common ancestral gene. This divergence presumably occurred before the subsequent separation of genes for the G_s and G_i proteins, each of which presumably retained the ADP-ribosylation site for only one of the toxins. It is difficult, however, to imagine why any of these proteins serves as a substrate for ADP-ribosylation by a bacterial toxin, or why the ADP-ribosylation sites appear to have been conserved. Preservation of the two substrate sites in transducin suggests that the sites are functionally important. Perhaps they serve as recognition sites for other polypeptides -- e.g., $\beta\gamma$ subunits, effector enzymes, signal detectors, or even endogenous ADP-ribosylating enzymes.

III. CHEMOTACTIC ACTIVATION OF NEUTROPHILS

Investigations in many laboratories have begun to unravel the complex series of biochemical events that comprise the response of neutrophilic leukocytes to chemotactic stimuli, a response that is essential for survival of the organism. In model systems in vitro (reviewed in (33,34)), neutrophils are activated by several chemical stimuli, including the tripeptide formyl-met-leu-phe (FMLP), leukotriene-B_4 (LTB_4), and components of complement. One response is chemotaxis itself: Neutrophils migrate toward these ligands in vitro as well as in vivo. Some of the biochemical events triggered by chemotactic ligands include, in approximate temporal order: An increase in turnover of phosphatidylinositol and in the intracellular concentration of calcium ion; an increase in phospholipid methylation; a small and transient increase in cellular cAMP; release of arachidonic acid and its metabolism to products dependent upon cyclo-oxygenase and lipoxygenase; secretion (under appropriate circumstances) of lysosomal hydrolases, such as β-glucuronidase; and generation of superoxide anion (34-37).

Considerable evidence (35) indicates that the elevation of intracellular calcium somehow mediates the subsequent secretory and superoxide anion responses; indeed, entry of extracellular calcium, facilitated by treatment with a calcium ionophore, will trigger both of these responses. The mechanism by which binding of ligands such as LTB_4 and FMLP to cell surface receptors causes the increase in intracellular calcium has not been well defined; the elevated cytosolic calcium derives in part from intracellular stores, and

in part from elevated permeability of the plasma membrane to calcium ion. Phorbol esters, presumably acting by stimulation of protein kinase C, can activate the secretory and superoxide anion responses without elevating intracellular calcium (34-37).

An increase in intracellular calcium also precedes release of histamine from mast cells stimulated by ligands that cross-link cell-surface IgE or by compound 48/80. Experiments with calcium ionophores and depletion of cellular calcium indicate that the increase in intracellular calcium in mast cells is both necessary and sufficient for histamine release triggered by these stimuli. Ui's laboratory recently reported (38) that treatment of mast cells with PT caused ADP-ribosylation of a 41-kDa polypeptide, presumably identical to the α subunit of G_i, and concomitant inhibition of histamine release mediated by IgE or by compound 48/80. PT treatment did not prevent histamine release in response to extracellular calcium plus a calcium ionophore. These results implied that G_i (or a similar protein) mediates ligand-stimulated histamine release from mast cells, probably at a step proximal to the increase in intracellular calcium.

The similarity between the mast cell and neutrophil responses led us to ask whether PT treatment might also inhibit activation of neutrophils by chemotactic factors. Indeed, the ability of guanine nucleotides to reduce binding affinity of FMLP for its receptors on neutrophil membranes (39) already suggested that the FMLP receptor might be functionally linked to a GTP-binding protein. Initial experiments performed in collaboration with Daniel Goldman and Edward Goetzl (D. Goldman _et al._, unpublished) strongly suggest that G_i (or a G_i-like protein) plays an important role in neutrophil activation.

Treatment of human neutrophils with PT (100 ng/ml for 2 hr) markedly inhibits the following responses of human neutrophils to LTB_4 and FMLP _in vitro_: Chemotaxis; increased intracellular calcium, measured by fluorescence of the calcium-sensitive dye quin2; and secretion of β-glucuronidase. PT does not block the secretion of β-glucuronidase stimulated by calcium ionophore or phorbol ester. As in mast cells and other cell types, PT catalyzes ADP-ribosylation of a 41-kDa polypeptide in neutrophil membranes. PT-catalyzed incorporation of radiolabel from (^{32}P) NAD^+ into the 41-kDa polypeptide is much reduced in membranes prepared from intact neutrophils exposed to 100 ng/ml PT for 2 hr, indicating that PT can ADP-ribosylate the polypeptide in intact neutrophils.

The simplest interpretation of these results is that

activation of G_i by receptors for FMLP or LTB_4 mediates the increase in intracellular calcium, which in turn triggers chemotaxis and other biochemical events involved in neutrophil activation. An alternative explanation is that PT treatment blocks inhibition of adenylate cyclase, and that a resulting (higher than normal) increase in neutrophil cAMP attenuates the chemotactic response. This interpretation is in accord with observations that exogenous cAMP analogs can partially inhibit certain components of the neutrophil activation response, particularly the generation of superoxide anion. This alternative explanation is unlikely to be correct, however, because PT treatment blocks the increase in intracellular calcium triggered by chemotactic stimuli, while cAMP analogs do not.[3]

The exciting implication of the neutrophil and mast cell experiments is that in both cases a protein substrate of pertussis toxin, presumably G_i of a closely related protein, transduces a signal that is not primarily mediated by altered concentration of a cyclic nucleotide. If the simple explanation of the experiments is correct, PT's inhibitory actions indicate that the α (or $\beta\gamma$) subunit of a GTP-binding regulatory protein interacts with an effector distinct from adenylate cyclase or PDE. Although the identity of this effector (or effectors) is unknown, it presumably regulates mobilization of calcium. The most obvious candidates for such an effector role are the enzymes involved in turnover of phosphatidylinositol (PI), in view of recent observations (e.g., (40)) indicating that inositol triphosphate, a PI metabolite, can mobilize calcium from intracellular stores into the cytoplasmic compartment.

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[3]Experiments initiated independently in Ui's laboratory (M. Ui, personal communication) showed that PT treatment blocks several components of the neutrophil chemotactic response without increasing or prolonging the transient elevation in cellular cAMP that occurs early in the response. Similarly, these workers reported that PT's inhibition of histamine release from mast cells was not accompanied by increased elevation of mast cell cAMP (38).

IV. MOLECULAR CLONING OF GENES ENCODING TRANSDUCIN SUBUNITS

A. Rationale

A number of laboratories have initiated attempts to isolate the genes that encode subunits of transducin and other GTP-binding regulatory proteins. Achievement of this goal will be rewarding, for the following reasons:

1. Detailed understanding of the relation between structure and function of the GTP-binding coupling proteins will constitute a significant advance. Homologies among these proteins make it likely that new information gained in one system will be applicable to the others. The relatively low abundance of the proteins in cells, however, limits the rate of acquiring new information by classical biochemical techniques. Characterization of the corresponding genes should provide a more convenient method for determining primary structure of the proteins. Recent advances in techniques for altering protein structure by *in vitro* mutagenesis and for expressing polypeptide products of mutant genes in intact cells open attractive avenues for correlating structure and function of the GTP-binding protein molecules involved in signal transduction.

2. Mutations affecting one of the regulatory proteins, G_s, produce instructive phenotypes in tissue culture cells and cause an inherited human disease. Different mutations in a single gene, the structural gene for the α subunit of G_s, produce three quite distinct mutant phenotypes in S49 mouse lymphoma cells: Uncoupling of G_s from receptors, uncoupling of G_s from catalytic cyclase, and apparent total loss of G_s activity (41). Pseudohypoparathyroidism, a human disorder of resistance to hormones that utilize cAMP as a second messenger, results from partial deficiency of G_s activity in endocrine target cells (42). This dominantly inherited disorder probably results from mutation of a gene encoding one of the subunits of G_s. Molecular cloning and sequencing of the G_s genes will make it possible to define the molecular basis of these mutations.

3. The responsiveness of adenylate cyclase to stimulatory ligands is altered by glucocorticoid and thyroid hormones (43-45) and during differentiation of cells and tissues (46-48). Some of these changes may be caused by altered expression of genes encoding subunits of G_s or G_i. Isolation of the relevant genes will provide probes for assessing expression of the corresponding mRNAs and allow us to study cellular mechanisms that regulate their expression.

4. The family of GTP-binding coupling proteins may

include additional proteins besides G_s, G_i, transducin, and p21-ras.[4] For example, Sternweis has observed a 39-kDa pertussis toxin substrate in brain that may prove to be encoded by a gene distinct from the gene for the α subunit of G_i (P. Sternweis, personal communication). Homologies in structure and function of known members of this family of proteins suggest that the corresponding genes -- and those of additional GTP-binding proteins -- may share common or similar base sequences. If so, molecular cloning of a few such genes may provide hybridization probes for identifying genes that encode additional members of the family. In any case, comparisons of the genes and the deduced amino acid sequences of the corresponding polypeptides will help to identify functionally critical structural features of the proteins and will set the stage for exploring the evolution of the entire family.

B. Experimental Strategy and Initial Results

We have begun by attempting to clone cDNAs for the subunits of transducin, because this protein's higher abundance provided us with two essential tools -- antisera of high titer directed against resolved subunits and sufficient protein to allow determination of amino acid sequence.

Adopting the vector and techniques developed by Young and Davis (50,51), we have used antibody probes to identify recombinants of interest in a lambdagt11 expression library containing bovine retinal cDNAs. Jeremy Nathans of Stanford University kindly provided a bovine retinal lambdagt10 cDNA library, which he had used to isolate the cDNA encoding rhodopsin (52). Retinal cDNA inserts were excised from lambdagt10, using the restriction nuclease Eco RI, and transferred into the unique Eco RI site of lambdagt11, situated in the coding region of the latter vector's inducible β-galactosidase gene (50,51). If the cDNA inserts are inserted in the proper orientation and reading frame, they will be expressed under appropriate conditions as part of a hybrid phage protein, which should contain epitopes of the retinal protein encoded by the cDNA. cDNAs of interest can therefore be detected by antibody screening of phage

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[4] Finkel and Cooper recently reported that p21-ras binds to the transferrin receptor, and that transferrin attenuates this binding (49); this is reminiscent of the binding of transducin and the G proteins to their respective detector elements.

plaques. Phage identified with the antibody probe are then purified and their cDNA inserts characterized.

Using this procedure, we have isolated cDNAs that encode peptide sequences recognized by either anti-βγ or anti-α antisera. When a recombinant phage expresses a protein that produces a strong signal in the antibody screen, its insert must still be validated as a cDNA specific for the polypeptide of interest. As described below, we have validated one cDNA fragment corresponding to the γ subunit of transducin (53); we are presently characterizing cDNAs that may correspond to the β and α subunits.

1. Partial cDNA encoding the γ subunit

Using the retinal lambdagt11 expression library, we identified five bacteriophage that reproducibly express antigen detected with anti-βγ antisera from each of two rabbits. To date we have characterized the cDNA inserts of three of these: All three contained identical 153-bp inserts that hybridized to a a single mRNA species (650 nucleotides long) in extracts of bovine retina but not in similar extracts of bovine heart, liver, and brain. Because such an mRNA would be too short to encode the 35-kDa β subunit of transducin, we compared the amino acid sequence deduced from the cDNA sequence to the amino acid sequence of transducin's γ subunit reported by McConnell et al. (54): The sequence of 117 bases at the 3' end of the insert predicted an amino acid sequence identical to the N-terminal 39 amino acids of the 69-residue polypeptide sequenced by McConnell et al. Thus the cDNA inserts of these three phage represent identical fragments of the cDNA corresponding to the γ subunit. The restriction enzyme we used to transfer inserts from lambdagt10 to lambdagt11 presumably cut the cDNA at an internal Eco RI site, causing loss of the sequence corresponding to the remaining 30 amino acid residues of the polypeptide plus a stretch of bases corresponding to the 3'-untranslated region of the cDNA.

The McConnell sequence begins with an unusual N-terminal amino acid, proline. Our cDNA sequence predicts a methionine just preceding this proline residue, plus 11 additional amino acids. The cDNA fragment is terminated at the 5' end by an Eco RI site which, like the 3' site, was presumably cleaved when the inserts were transferred between phage libraries. The significance of these additional residues cannot be determined until the entire cDNA has been isolated and its DNA sequenced. All 12 amino acids may form part of an N-terminal peptide that is removed from the preprotein during post-translational processing of the

polypeptide in vivo. Alternatively, the codon preceding the N-terminal proline of the McConnell sequence is in fact the initiation codon, and the N-terminal methionine is removed during processing of the mature polypeptide. In that case, the bases upstream to the methionine codon would correspond to part of the 5'-untranslated region of the γ mRNA.

Our success in isolating the partial γ cDNA fragment is encouraging, in that it demonstrates effectiveness of the antibody screening technique for isolating transducin cDNAs. In addition, the observation that mRNA hybridizing with γ cDNA is found only in retinal extracts implies that the γ subunit of transducin differs from the corresponding subunits of G_s and G_i. This implication is presently only tentative, however: The relatively low abundance of G proteins in other tissues, as compared to transducin in retina, may result in a correspondingly low abundance of γ subunit mRNA, at a level below too low to detect with our current assays.

The amino acid compositions and proteolytic peptide maps of β subunits derived from the G proteins and transducin are indistinguishable (29). This finding suggests that the β polypeptides of all three are products of the same gene. If so, differences among their associated γ subunits must be responsible for an important difference in behavior of the respective βγ complexes: The βγ complex of transducin is an extrinsic membrane protein easily dissociated from ROS membranes in the presence of light and GTP, while the βγ complexes of G_s and G_i can only be solubilized from membranes in the presence of detergent. This line of reasoning leads us to predict that the γ polypeptides of G_s and G_i will contain stretches of hydrophobic amino acid residues that are absent from the corresponding subunit of transducin, and perhaps that γ subunits of the G proteins promote adherence of their βγ complexes to membranes.

2. Other cDNAs identified with anti-βγ antisera

We have not yet characterized cDNA inserts of the two remaining phage in the retinal lambdagt11 library that were detected with anti-βγ antisera. The possibility that β subunits of transducin and the regulatory cyclase proteins are identical led us to screen with the same antisera a lambdagt11 expression library containing cDNA from rat brain. The 803-bp insert of one recombinant phage detected in this way hybridizes to a 3.2-kb mRNA in extracts of bovine retina, heart, and liver. This result indicates that the cDNA insert does not correspond to transducin's γ subunit, which is encoded on a much smaller mRNA; rather, it represents a gene expressed in several tissues. Further

experiments will be required to determine whether this insert contains part of a cDNA that encodes β subunit polypeptides of the signal-transducing proteins.

3. A retinal cDNA fragment identified with anti-α antisera

Screening of the retinal lambdagt11 library with anti-α antisera detected eight positive plaques. Of these, we are currently characterizing the largest cDNA insert, which is 2.4 kb long. This cDNA insert hybridizes to an mRNA (approximately 2.4 kb) that is present in extracts of bovine retina but that was not detected in extracts of heart, liver, and brain.

To determine whether this cDNA fragment represents the gene for the α subunit of transducin, we are taking two approaches: 1. We will test the lambdagt11 phage for reactivity with antisera raised against the synthetic peptides corresponding to tryptic fragments of α that contain its ADP-ribosylation sites for CT and PT.[5] Binding of either or both of these antisera to the hybrid protein encoded by this cDNA will make it highly likely that the cDNA does encode the α polypeptide. 2. For more definitive validation of the cDNA, we are determining the base sequence of the insert. The cDNA will be validated if the amino acid sequence deduced from it contains an internal tetrapeptide and a carboxy-terminal octapeptide that correspond, respectively, to established sequences of the CT and PT ADP-ribosylation sites of transducin's α subunit.

V. SUMMARY

Recent findings in many laboratories indicate that pertussis toxin substrates -- including G_i, transducin, and perhaps other membrane proteins -- play essential roles in transducing signals across membranes. Further elucidation of the precise molecular basis of these transduction processes will come from continuing biochemical investigation, combined with isolation, analysis, and experimental manipulation of the genes that encode the proteins involved.

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[5]The synthetic peptides and antisera were prepared in collaboration with M. Tsubokawa and J. Ramachandran of Genentech, Inc.

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BIOCHEMICAL BASIS OF THE ADJUVANT EFFECTS OF PERTUSSIS TOXIN FOR THE IgE RESPONSE [1]

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I. INTRODUCTION

My major interest in the past 10 years was to find some immunological maneuvers to regulate the IgE antibody formation. Some years ago, we thought about the possibility that the IgE antibody response may be regulated not only by antigen-specific helper and suppressor T cells but also through isotype-specific mechanisms. We initiated our studies on this line using nematode infection which selectively enhances the IgE synthesis, and found that T cells of rats infected with *Nippostrongylus brasiliensis* (Nb) form soluble factors which have affinity for IgE and selectively enhance or suppress the IgE response (1,2). Subsequent studies revealed that the formation of IgE-potentiating factor or IgE-suppressor factor is not unique for the nematode infection. It was found that IgE-potentiating factor is formed by T cells under various conditions in which the IgE synthesis is being enhanced. One of the examples is treatment of rats with *Bordetella pertussis* vaccine (BP), which is the best adjuvant for the IgE antibody response. The factor was detected in the serum of animals which received a single injection of the vaccine, and lymphocytes in their peripheral blood released IgE-potentiating factor (3). In order to analyze the mechanisms involved, I asked Dr. Munoz for his collaboration. In this presentation, I would like to summarize our experiments in which pertussigen

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was employed, and discuss the nature of T cell factors induced by the toxin.

II. RELATIONSHIP BETWEEN THE IgE-POTENTIATING FACTOR AND IgE-SUPPRESSIVE FACTOR

Before discussing cellular mechanisms for the formation of IgE-potentiating factor by BP-treatment, I would like to spend some time to explain the relationship between the IgE-potentiating factor and IgE-suppressive factor (which would be effector molecules for the regulation of IgE response). These factors are comparable in molecular weights (ca 15,000), and have affinity for IgE (1-3) (Table I). The two factors are antigenically related; antibodies against IgE-potentiating factor react to IgE-suppressive factor and Fc_ε receptors ($Fc_\varepsilon R$) on B and T cells (4). The differences between the two factors are carbohydrate moieties in the molecules. IgE-potentiating factor has affinity for lentil lectin and Concanavalin A (Con A). In contrast, IgE-suppressive factor does not bind to these lectins but has affinity for peanut agglutinin (PNA). Further analysis of the two factors suggested that IgE-potentiating factor contains both N-linked oligosaccharide and O-linked oligosaccharide, and their terminal sugar residues are sialic acid (5) (Fig. 1). The IgE suppressive factor does not contain N-linked oligosaccharide but has O-linked oligosaccharide of which terminal residues are N-acetylgalactosamine-galactose. The differences in carbohydrate have biologic significance. Evidence was obtained that the N-linked oligosaccharide in IgE-potentiating factor is essential for its activity to potentiate the IgE response (5).

TABLE I. Comparisons Between IgE-Potentiating Factor and IgE-Suppressive Factor

Properties	IgE-Potentiating Factor	IgE-Suppressive Factor
Source	$Fc_\varepsilon R^+$ W $3/25^+$ T cells	W $3/25^+$ T cells
Molecular weight	13,000 - 15,000 daltons	13,000 - 15,000 daltons
Affinity for IgE	+	+
Affinity for:		
Lentil Lectin	+	-
Concanavalin A	+	-
Peanut Agglutinin	-	+

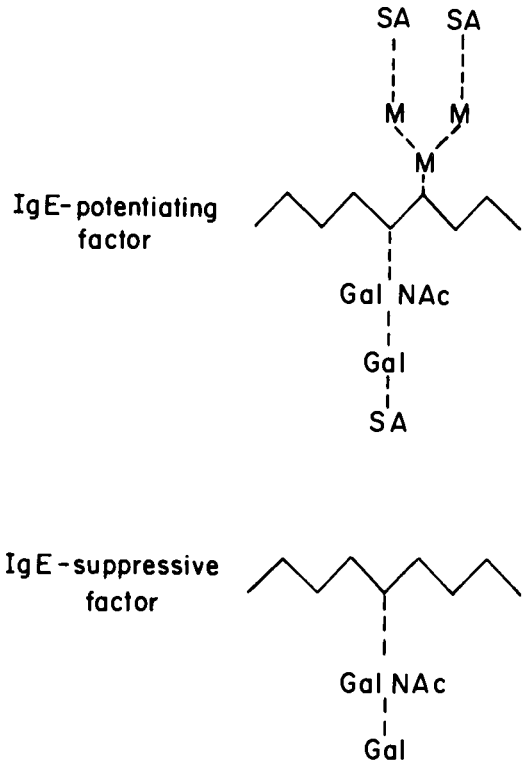

Fig. 1. Oligosaccharides associated with IgE-potentiating factor and IgE-suppressive factor. IgE-potentiating factor contains both N-linked oligosaccharide and O-linked oligosaccharide, while IgE-suppressive factor has only O-linked oligosaccharide.

Another important finding obtained in this series of work was that the same T cells have capacities to produce both IgE-potentiating factor and IgE-suppressive factor, and that the nature of IgE-binding factors formed by the cells is decided by their environment. For example, rat T cells activated by Con A (10 μg/ml) produced IgE-potentiating factor upon incubation with IgE (6). However, incubation of the Con A-activated cells with IgE together with tunicamycin resulted in the formation of IgE-suppressive factor (6). Switching of the Con A-activated cells from the formation of IgE-potentiating factor

to the formation of IgE-suppressive factor was also achieved by pretreatment of the cells with glucocorticoids, or incubation of the cells with IgE in the presence of lipomodulin, a phospholipase inhibitory protein (7). On the other hand, T cells activated by Con A (1 μg/ml) produced IgE-suppressive factor upon incubation with IgE (6). However, the same cells produced IgE-potentiating factor if an activator of phospholipase, such as melittin or monoclonal anti-lipomodulin, was added to the cells together with IgE (8). A more critical evidence was obtained using a T cell hybridoma, which produce IgE-suppressive factor upon incubation with IgE (9). The same hybridoma produced IgE-potentiating factor in the presence of anti-lipomodulin (10). More recently, Drs. K. Moore and C. Martons succeeded cloning the gene for IgE-binding factors. Their work in collaboration with us showed that IgE-potentiating factor and IgE-suppressive factor share common structural gene, and the nature of the factors is decided during post-translational glycosylation process.

III. CHARACTERIZATION OF A T CELL FACTOR INDUCED BY PERTUSSIS TOXIN

Cellular mechanisms for the selective formation of IgE-potentiating factor by BP-treatment were analyzed by fractionating the peripheral blood mononuclear cells of BP-treated rats (Fig. 2). The results showed that monocytes of BP-treated animals released an interferon-like substance which in turn stimulated normal T cells to form IgE-binding factors. However, normal lymphocytes stimulated by the interferon-like substance formed a mixture of IgE-potentiating factor and IgE-suppressive factor. It was found that T cells of BP-treated rats released another soluble factor, which enhanced the N-glycosylation of IgE-binding factors during their biosynthesis. Thus, a mixture of interferon-like substances from monocytes and glycosylation enhancing factor (GEF) from T cells of BP-treated rats induced normal T cells to form IgE-potentiating factor (11). Another biologic activity of GEF is to enhance the expression of $Fc_{\varepsilon}R$ on B cells. The proportion of $Fc_{\varepsilon}R^{+}$ cells in normal rat mesenteric lymph node (MLN) cells increases upon incubation overnight with IgE. If the cells were treated with IgE together with GEF, the increase of $Fc_{\varepsilon}R^{+}$ cells was enhanced. It appears that assembly of N-linked oligosaccharide is essential for the expression of $Fc_{\varepsilon}R$ on the cell surface, and therefore that assembly of the oligosaccharides to precursor molecules enhanced $Fc_{\varepsilon}R$ expression.

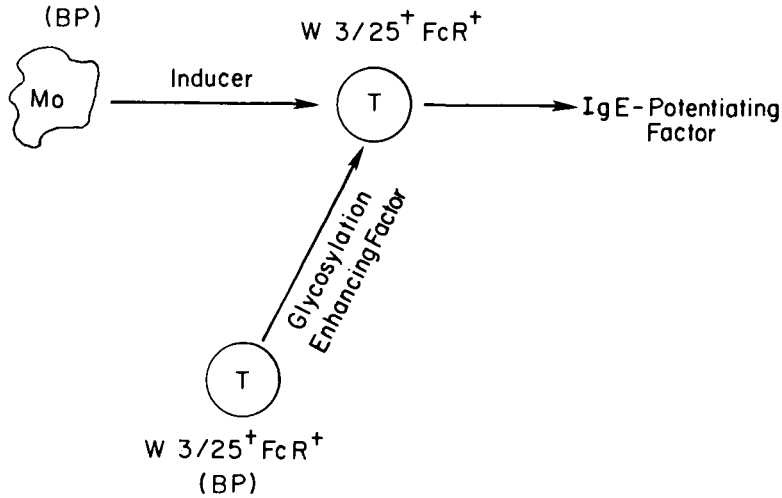

Fig. 2. Schematic model for the selective formation of IgE-potentiating factor by BP-treatment. BP activates monocytes (MO) for the formation of inducer (interferon-like) substance which in turn stimulates normal W $3/25^+$ (Lyt 1^+) T cells to form IgE-binding factors. A subset of T cells in BP-treated rats release glycosylation enhancing factor which enhances the assembly of N-linked oligosaccharides to IgE-binding factors during their biosynthesis.

Since pertussigen and BP have similar adjuvant effects with respect to the IgE response (12), we expected that pertussigen may induce the formation of IgE-potentiating factor. However, incubation of normal rat lymphocytes with 0.2 μg/ml pertussigen resulted in the formation of GEF rather than IgE-potentiating factor. In our previous experiments, we found that incubation of normal lymphocytes of Lewis strain rats with rat IgE resulted in the formation of IgE-binding factors. About 1/2 of the factors formed by IgE-stimulation had affinity for lentil lectin, while the remaining 1/2 did not have the affinity. Therefore, the factors formed by normal lymphocytes did not show biologic activities. However, if one incubates normal lymphocytes with IgE in the presence of culture filtrates of pertussigen-stimulated lymphocytes, all of the IgE-binding factors had affinity for lentil lectin and the factors potentiated the IgE response (13). The results indicate that pertussigen stimulates normal T cells to form GEF. Fractio-

nation of culture filtrates revealed that GEF has the molecular weight of approximately 25,000, and isoelectric point of pH 6.6 or higher. The lymphokine is clearly separated from IL-2. A unique property of GEF is that the lymphokine has a lectin-like property. The factor binds to acid-treated Sepharose and could be recovered from the beads by elution with lactose or galactose (13). This property is important for the activity of this factor. GEF does not enhance the glycosylation of IgE binding factors if galactose or lactose is present in culture medium (14). Another unique property of GEF is that this lymphokine appears to have a kallikrein-like protease activity. GEF is inactivated by irreversible inhibitors of serine esterase, such as phenylmethyl sulfonyl fluoride (PMSF) and diisopropylfluorophosphate (DFP). The lymphokine can not exert its function in the presence of benzamidine or synthetic substrate of trypsin. In contrast, synthetic substrates of chymotrypsin did not prevent the action of GEF. Furthermore, GEF can be purified by using p-aminobenzamidine agarose. This lymphokine bound to the beads and was recovered by elution with benzamidine. Our hypothesis that GEF is a serine protease is supported by the fact that trypsin, kallikrein and plasmin could enhance the glycosylation of IgE-binding factors. Among the proteolytic enzymes, GEF is most likely to be kallikrein-like enzyme, because GEF and kallikrein were not inactivated by N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK) which inactivated both trypsin and plasmin. Furthermore, bradykinin, a cleavage product of kininogen by kallikrein, enhances the glycosylation of IgE-binding factors at concentrations of 10 to 100 ng/ml (14).

It is well known that pertussis toxin is a T cell mitogen. However, cell proliferation is not required for the formation of GEF. A 1 hr incubation of normal lymphocytes with 0.2 μg/ml pertussigen is sufficient for the release of GEF. Other mitogens, such as Con A, could induce the release of GEF from normal lymphocytes at the concentration of 5 to 10 μg/ml. GEF is spontaneously released from MLN cells of rats infected with the nematode, Nippostrongylus brasiliensis (15). Our experiments also demonstrated that spleen cells of rats immunized with alum-absorbed keyhole limpet hemocyanin (KLH), which induces the IgE antibody response, released GEF upon incubation with homologous antigen (15). Thus, GEF satisfies all requirements of a lymphokine, but this factor is a kallikrein-like enzyme. These findings suggest that some other lymphokines may also have enzymatic activity.

IV. BIOCHEMICAL EFFECTS OF GEF ON T LYMPHOCYTES

In order to study biochemical mechanisms through which GEF enhances the glycosylation of IgE-binding factors, we employed T cell hybridoma 23A4 cells, which produce unglycosylated IgE-binding factors upon incubation with IgE (16). Separate studies on the hybridomas showed that the cells produce another soluble factor, which inhibits the assembly of N-linked oligosaccharides to IgE-binding factors during their biosynthesis. As described, incubation of normal MLN cells with IgE results in the formation of IgE-binding factors, one half of which had affinity for lentil lectin and the remaining one-half does not. However, if one incubates normal MLN cells with IgE in the presence of culture supernatant of 23A4 cells, essentially all IgE- binding factors formed by normal MLN cells failed to bind to lentil lectin (Table II). The hybridoma derived soluble factor, which were called glycosylation inhibiting factor (GIF), has the molecular weight of approximately 15,000 daltons, and could be absorbed with monoclonal anti-lipomodulin-coupled Sepharose (15,16). Separate experiments indicated that GIF is a fragment of phosphorylated lipomodulin (17). It appears that the selective formation of unglycosylated IgE-binding factors by the hybridomas may be due to the presence of phospholipase inhibitory protein in the cells (15).

It is obvious that GEF and GIF have opposite functions with respect to the glycosylation of IgE-binding factors. Indeed, GEF and GIF compete with each other (c.f. Table II). If one incubates normal MLN cells with a mixture of the two factors together with IgE, and IgE-binding factors formed by the cells were fractionated on lentil lectin Sepharose, the factors distributed approximately equally between the effluent and eluate fractions. We wondered if GEF might inactivate or digest GIF. Thus, we incubated GIF with GEF for 24 hr and then removed GEF from the mixture by absorption with acid-treated Sepharose. The results showed that GIF was recovered from the mixture, indicating that GEF did not inactivate GIF. It was also found that GEF did not inhibit the formation of GIF by the hybridoma cells.

Unexpected findings in the series of experiments were that GEF enhanced the release of GIF from the hybridoma cells. If one incubates the hybridoma cells in usual culture medium for 1 hr, GIF was not detected in the culture supernatant. However, 1 hr culture supernatant of the same cells with purified GEF contained a detectable amount of GIF. The same principle applies to normal cells. Incubation of normal spleen cells with GEF results in the release of GIF within 1 hr. If

TABLE II. Antagonistic Effect Between Glycosylation Enhancing Factor and Glycosylation Inhibiting Factor on Normal MLN Cells [a]

Normal MLN cells cultured with	IgE-binding factors in culture filtrate[b]	
	unfractionated	effluent/eluate
IgE	44	21/23
GIF + IgE	45	45/0
GEF + IgE	40	2/37
GIF + GEF + IgE	43	24/19

a Normal MLN cells were incubated overnight with 10 μg/ml IgE in the presence of GIF, GEF or a mixture of the two, and IgE-binding factors in culture filtrates were determined by rosette inhibition.

b Culture filtrates were absorbed with lentil lectin Sepharose and the beads were eluted with α methyl mannoside. Numbers in the column represent the percentage of rosette inhibition between $Fc_{\varepsilon}R^{+}$ lymphocytes and IgE-coated erythrocytes.

no GEF was added, even 24 hr culture supernatant did not contain a detectable amount of GIF (Table III). The release of GIF from the hybridoma cells and normal lymphocytes was observed even when the cells had been pretreated with cyclohexamide. Hirata (18) has reported that lipomodulin lost phospholipase inhibiting activity upon phosphorylation, but the phosphorylated lipomodulin could be reactivated by dephosphorylation. Hirata et al (19) also indicated that most of "lipomodulin" in culture supernatant is phosphorylated. Since GIF released from T cell hybridomas is a fragment of phosphorylated lipomodulin (17), we anticipated that GEF might have enhanced the phosphorylation of intracellular lipomodulin, and thereby enhanced the release of phosphorylated lipomodulin (GIF). If this is the case, one can expect that phosphorylation (or release) of lipomodulin results in the activation of phospholipase in the cells. Thus, we determined whether GEF enhances the release of arachidonic acid or its derivatives. In the experiments shown in Table IV, 23A4 cells were preincubated with ^{14}C-arachidonic acid to label their phospholipids. The cells were then incubated either with affinity-purified GEF or bradykinin, and the release of ^{14}C-arachidonate was determined. The results show that both GEF and bradykinin induced the release of ^{14}C-arachidonate from the cells, indicating that phospholipase in the hybridoma was activated by GEF.

TABLE III. Kinetics of the Release of Glycosylation Inhibiting Factor From Normal MLN cells After Exposure to Glycosylation Enhancing Factor [a]

Supernatant of MLN Cells		Nature of IgE-binding Factors[b]	
cultured with	incubation period	unfractionated	effluent/ eluate
	hr		%
GEF	0	55	30/28
	1	47	40/7
	2	45	43/2
	4	45	44/1
	10	44	42/4
None	0	52	25/29
	2	53	29/28
	10	55	29/30
Medium		54	27/29

a Normal MLN cells (10^7/ml) were incubated with or without purified GEF for various period of time. Culture supernatants were treated with 1 mM PMSF and alkaline phosphatase, and assessed for the presence of GIF.

b Normal MLN cells were incubated with IgE in the presence of a culture supernatant, and IgE-binding factors formed in the cultures were fractionated on lentil lectin Sepharose. Numbers represent percent rosette inhibition.

Recently, we explored biochemical mechanisms through which GEF induces the activation of phospholipase. As will be described in the next section, we found that GEF induced phospholipid methylation in mouse mast cells. Thus, we determined whether GEF may induce phospholipid methylation in 23A4 hybridoma cells. Measurements of the incorporation of ^{3}H-methyl groups into phospholipids showed a transient increase in phospholipid methylation at 30 sec after the addition of GEF. It was also found that GEF enhances the formation of diacylglycerol (DAG). In the experiment shown in Fig. 3, phospholipids in 23A4 cells were labeled by preincubation with ^{14}C-arachidonic acid. Aliquots of the cells were incubated either with purified GEF or a control preparation, and lipids in the cells were extracted. Determination of ^{14}C-arachidonic acid- labeled DAG by thin layer chromatography showed that GEF induced a transient increase in DAG in 23A4 cells. In order to explore the biologic significance of the

TABLE IV. Induction of ^{14}C-Arachidonate Release from 23A4 Cells by GEF or Bradykinin

Ligand added	cpm in supernatant[a]	cpm Δ released[b]	% Released[c]	Δ % Released[d]
None	12094	0	14.5 (7.8)[e]	0 (0)
GEF, 0.1 ml	29922	17828	32.9 (16.6)	18.4 (8.8)
GEF control, 0.1 ml	12114	20	15.1 (7.5)	0.6(-0.3)
Bradykinin, 0.1 μg	14154	2060	17.3 (8.2)	2.8 (0.4)
1 μg	19968	7874	23.9 (13.0)	9.4 (5.2)

a Average of duplicate samples. Difference between duplicate samples was less than 5% of the average.

b Spontaneous release of ^{14}C-arachidonate was subtracted.

c cpm in supernatant/(cpm in supernatant + cpm in pellet).

d The percentage of spontaneous release was subtracted.

e Numbers in parentheses are results of a separate experiment of the same design.

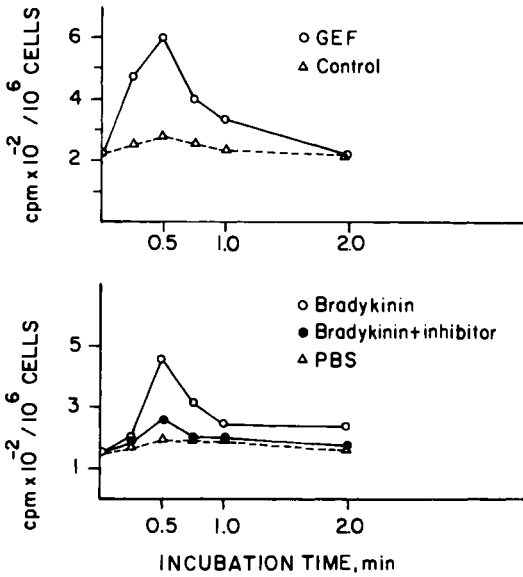

Fig. 3. Diacylglycerol (DAG) formation in 23A4 hybridoma by GEF (top) or bradykinin (bottom). Hybridoma cells were precultured with ^{14}C- arachidonic acid. Aliquots of the cells were challenged with purified GEF or bradykinin (1 μg/ml), and ^{14}C-labeled DAG in the cells was measured by thin layer chromatography. Aliquots of the cells were incubated with bradykinin in the presence of 0.5 mM 3 deaza-adenosine and 0.1 mM L homocysteine-thiolactone, which inhibit the activation of methyltransferases (•). The inhibitors of methyltransferases inhibited DAG formation.

formation of DAG, we determined the effect of permeable DAG, i.e., oleoyl acetyl glycerol (OAG) (20), on 23A4 cells. The cells were incubated with either OAG or diolein for 1 hr and culture filtrates were assessed for the presence of GIF. The results clearly showed that OAG, 30 μg/ml, induced the release of GIF, while the same concentration of diolein failed to do so. Another biochemical effect of GEF is to increase ^{45}Ca uptake into 23A4 cells. Kinetics of ^{45}Ca uptake by 23A4 cells are shown in Fig. 4. It is clear that GEF enhances ^{45} Ca uptake.

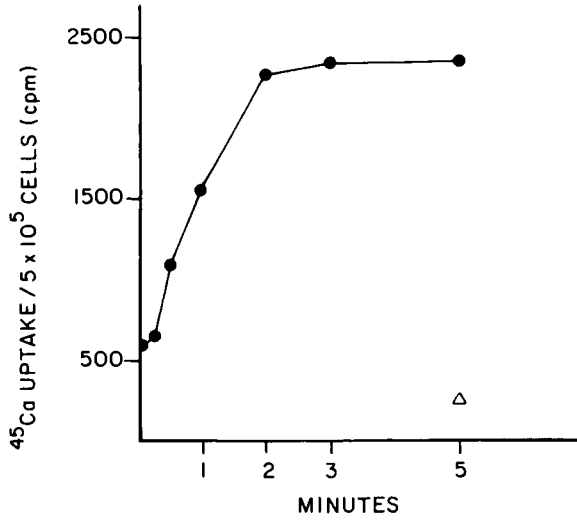

Fig. 4. $^{45}Ca^{2+}$ uptake by 23A4 cells upon stimulation with GEF. Aliquots of the cells were incubated with $^{45}Ca^{2+}$ and challenged with purified GEF (●) or a control preparation (▲), and uptake of $^{45}Ca^{2+}$ was followed.

A transient formation of DAG and an increase in Ca^{2+} uptake suggest that GEF may induce the activation of protein kinase C (21). If the protein kinase were involved, one can expect that phorbol myristic ester, which directly activates protein kinase C, (22,23), may enhance the release of GIF, and induce the glycosylation of IgE-binding factors. Thus, we incubated 23A4 cells or normal MLN cells for 1 hr with IgE together with various concentrations of tetradecanol-phorbol-13-acetate (TPA). Cells were washed, resuspended in fresh medium and then cultured overnight to obtain IgE-binding factors. Table V shows the results of fractionation of culture filtrates on lentil lectin Sepharose. It is clear that TPA switched 23A4 cells from the formation of unglycosylated IgE-binding factor to the

TABLE V. Enhancement of N-Glycosylation of IgE-Binding Factors by 12-O-tetradecanol-phorbol-13-acetate (TPA)[a]

Concentration of TPA added to cells	IgE	IgE-binding factors formed by 23A4 cells effluent/eluate[b]	IgE-binding factors formed by Normal MLN Cells effluent/eluate[b]
nM		%	%
0	+	40/1	14/17
2	+	25/13	7/26
20	+	2/33	0/33
200	+	0/34	0/25
2000	+	0/38	ND
2000	-	0/0	0/0
GEF[c]	+	0/38	9/36

a 23A4 cells or normal MLN cells were cultured for 1 hr with IgE in the presence of various concentrations of TPA or GEF. Cells were washed, resuspended in fresh culture medium and cultured overnight. Culture supernatants were filtered through CF 50A membranes, extensively dialyzed and then fractionated on lentil lectin Sepharose.

b Distribution of IgE-binding factors between the effluent and eluate fraction from lentil lectin Sepharose.

c The final dilution of GEF in 23A4 cell or normal MLN cell culture was 1:10.

formation of glycosylated form. The phorbol ester also changed the nature of IgE-binding factors formed by normal MLN cells.

Finally, we tried to confirm that GIF released from 23A4 cells is indeed phosphorylated lipomodulin. The hybridoma was preincubated for 1 hr with ^{32}P-phosphoric acid and aliquots of the cell suspension were incubated with either purified GEF, bradykinin or a control preparation. Culture supernatant was then absorbed with anti-lipomodulin coupled Sepharose, and the beads were eluted with glycine HCl buffer, pH 3.0. The eluates were dialyzed, lyophilized and analyzed by SDS-PAGE. A radiolabeled band of 13K daltons was demonstrated in the supernatants of GEF-stimulated cells and bradykinin stimulated cells. The results confirmed that GEF induced the release of phosphorylated lipomodulin from the hybridoma cells.

Summarizing the biochemical analysis described above, one may speculate the following pathway. It appears that GEF activates methyltransferases and phospholipase C in 23A4 cells.

The activation of these enzymes leads to the formation of diacylglycerol and Ca^{2+} uptake. One may speculate that DAG and an increase in Ca^{2+} induces the activation of protein kinase C. It is not known whether protein kinase C phosphorylates lipomodulin, or this enzyme activates other protein kinase for the phosphorylation of lipomodulin. In any event, lipomodulin is inactivated by phosphorylation, and causes the activation of phospholipase in the cells. It is not known why the activation of phospholipase enhances the assembly of N-linked oligosaccharides to IgE-binding factors. However, an enhancement of N-glycosylation of IgE-binding factors by an activator of phospholipase support this hypothesis.

V. ACTIVATION of MAST CELLS BY GEF

Biochemical activities of GEF suggested that the target of this factor may not be restricted to a subset of T cells. Since BP and pertussis toxin have various biological effects, we wondered if some of the effects might be explained by GEF. Thus, we determined whether the lymphokine might activate mouse mast cells for mediator release, or change the susceptibility of mast cells to IgE-dependent mediator release. GEF was prepared by incubation of normal spleen cells with pertussigen, and purified by p-aminobenzamidine agarose. The preparation was treated with TLCK to inactivate trypsin-like enzymes other than kallikrein. Culture supernatants of the same spleen cells incubated without pertussigen were similarly processed and used as a control. In the experiments shown in Fig. 5, purified mouse mast cells were labeled with ^{14}C-arachidonic acid and then incubated with purified GEF for 10 min. The results clearly showed that GEF induced the release of both histamine and arachidonate from mast cells. The minimum concentration of purified GEF for histamine release from mouse mast cells was comparable to that required for switching rat T cells from the formation of IgE-suppressive factor to the formation of IgE-potentiating factor. Similar experiments were carried out with bradykinin. The results showed that 1 ng/ml to 1 μg/ml of bradykinin induced the release of both histamine and arachidonate from mast cells. An important finding is that galactose inhibited histamine release by GEF, but not the histamine release by bradykinin. The results indicate that the effector substance in the purified GEF preparation exerts its function through the binding to galactose residues on the cell surface. Since GEF has affinity for galactose, this finding supports the concept that GEF is indeed responsible for mediator release from mast cells.

We wondered if a suboptimal dose of GEF for histamine release might increase the sensitivity of mast cells for IgE-

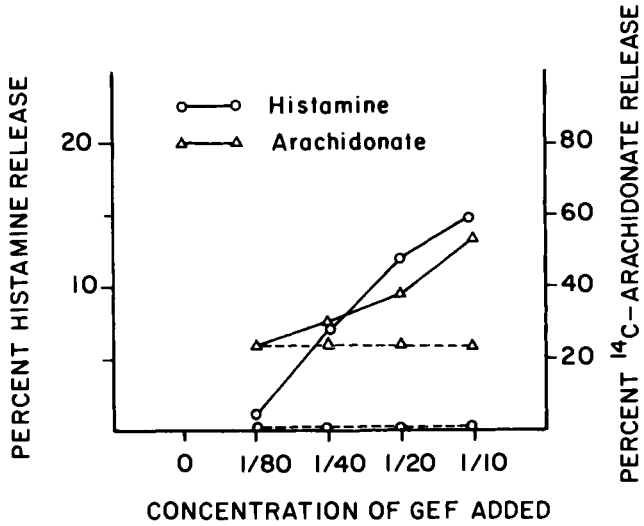

Fig. 5. Release of histamine and ^{14}C-arachidonate from mouse mast cells. Cells were preincubated with ^{14}C-arachidonic acid and aliquots of the cells were incubated with serial dilutions of purified GEF for 20 min. Dotted lines represent histamine (o----o) and arachidonate (Δ----Δ) release by a control preparation.

mediated histamine release. To test this possibility, mouse mast cells were sensitized with monoclonal mouse IgE anti-DNP antibody, and an aliquot of the cell suspension was incubated for 1 hr with a suboptimal concentration of GEF in the absence of Ca^{2+}. The cells were washed to remove free GEF, and then challenged with various concentrations of antigen in the presence of Ca^{2+}. It is apparent in Fig. 6 that GEF-treatment enhanced the antigen-induced histamine release.

Previous studies on IgE-mediated histamine release have shown that the bridging of IgE-receptors on mast cells induced

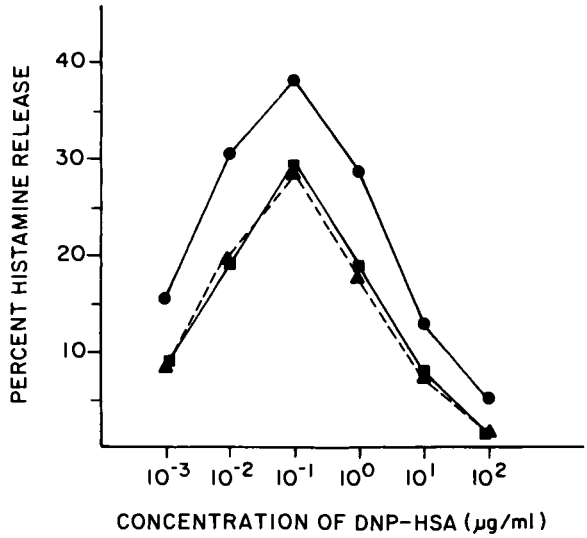

Fig. 6. Enhancement of antigen-induced histamine release by pretreatment of mast cells with GEF. Mast cells sensitized with anti-DNP IgE antibody were pretreated with GEF (●) or a control preparation (■) in the absence of Ca^{2+}. The third set of the sensitized cells were untreated (▲). The three sets of sensitized cells were incubated with various concentrations of DNP-HSA and histamine released from the cells were measured.

phospholipid methylation, and a transient increase in intracellular cAMP levels, and these processes are followed by Ca^{2+} influx and histamine release. Incorporation of methyl groups into phospholipids and intracellular cAMP levels reached maximum 15 sec after the bridging and then gradually declined. ^{45}Ca-uptake reached maximum at 2 min and histamine release reached maximum at 3 to 5 min (24,25). Kinetics of the sequential biochemical events are somehow specific for the bridging of IgE-receptors. Other histamine releasing agents, such as compound 48/80 and Ca^{2+}-ionophore, do not induce phospholipid

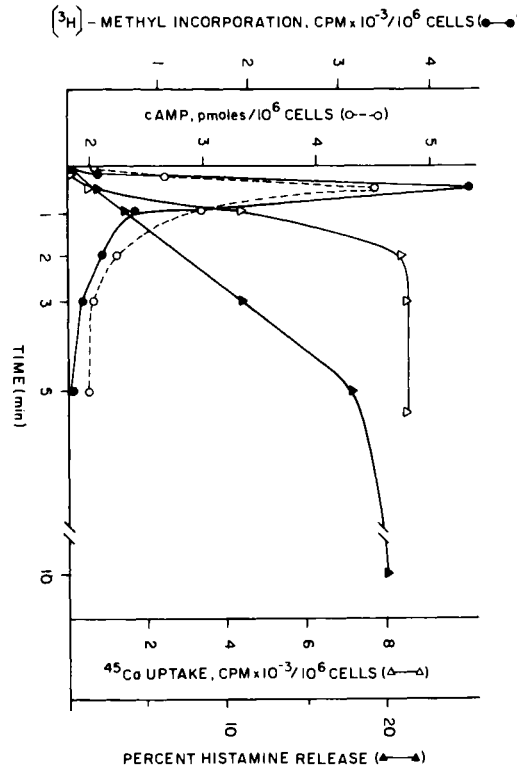

Fig. 7. Kinetics of ^{3}H-methyl incorporation into phospholipids (●), cAMP rise (o), $^{45}Ca^{2+}$ uptake (Δ) and histamine release (▲) induced by GEF. The same mast cell preparation was employed for all measurements.

methylation. Active fragments of complement components, such as C5a and C3a, also induced histamine release from mast cells (26). Although C3a and C5a induce phospholipid methylation, cAMP rise is slower than that induced by the bridging of IgE receptors, and was inhibited by indomethacin (27). Thus, we determined whether GEF and bradykinin might induce such biochemical events. The results of the experiments with GEF is shown in Fig. 7. It is apparent that the lymphokine induces phospholipid methylation, cAMP rise and $^{45}Ca^{2+}$ uptake in the same sequences as those observed in IgE-dependent histamine

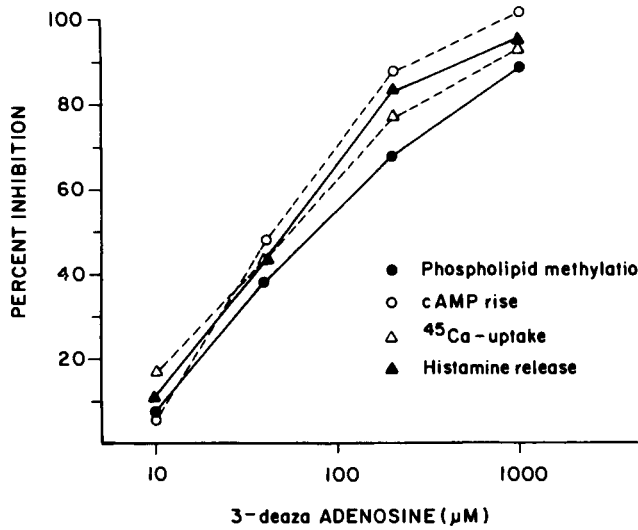

Fig. 8. Inhibition of GEF-induced ^{3}H-methyl incorporation into phospholipids (●), cyclic AMP rise (o), $^{45}Ca^{2+}$-uptake (Δ) and histamine release (▲) by 3-deaza-adenosine and L-homocysteine thiolactone. Mast cells were preincubated for 30 min with varying concentrations of 3 deaza adenosine and 100 μM L-homocysteine thiolactone, and the cells were challenged with GEF. ^{3}H-incorporation into phospholipids, cAMP level, $^{45}Ca^{2+}$-uptake and histamine release were measured at 30 sec, 30 sec, 2 min, and 5 min, respectively (c.f. Fig. 7).

release. The initial rise in cAMP induced by GEF was not inhibited by indomethacin, indicating that the activation of adenylate cyclase is not the result of prostaglandin synthesis. In the IgE-mediated histamine release, inhibitors of methyltransferases inhibit Ca^{2+}-uptake and histamine release (24). Similar results were obtained in GEF-induced histamine release. In the experiment shown in Fig. 8, mast cells were suspended in Tyrode solution containing 10 μM to 1 mM 3 deaza-adenosine

together with 100 μM L-homocysteine thiolactone. After 60 min at 37°C, the treated cells and untreated cells were challenged with GEF, and incorporation of ^{3}H-methyl groups into phospholipids, the cAMP levels, ^{45}Ca uptake and histamine release were determined at optimal times when these values reached maximum. It is apparent that inhibitors of methyltransferases inhibited not only phospholipid methylation but also cAMP rise, ^{45}Ca uptake and histamine release in a similar dose-dependent fashion. The results indicate that phospholipid methylation is essential for the cAMP rise, Ca^{2+} uptake and histamine release by GEF.

VI. SUMMARY

Pertussis toxin stimulates normal T cells to release a soluble factor (GEF), which enhances the assembly of N-linked oligosaccharides to IgE-binding factors. The oligosaccharide provides the IgE-binding factors with the biologic activity: enhancement of the IgE synthesis. GEF is a kallikrein-like enzyme and has a molecular weight of approximately 25,000. The factor has lectin-like properties and exerts its function through the binding to galactose residues on the surface of target cells. It was also found that GEF triggers normal mouse mast cells for histamine and arachidonate release. Pretreatment of mast cells with a suboptimal concentration of GEF enhanced the IgE-dependent histamine release from the cells. GEF induces phospholipid methylation in both T cell hybridomas and mouse mast cells, and enhances ^{45}Ca uptake. In the lymphocyte system, GEF appears to induce phosphorylation of lipomodulin, a phospholipase inhibitory protein, and the release of phosphorylated lipomodulin. As a consequence, GEF activates phospholipase and induces the release of arachidonate. The results indicated that GEF enhances the glycosylation of IgE-binding factors through the activation of phospholipase. The release of GEF from T cells by pertussis toxin appears to explain some biologic activities of the toxin.

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EFFECT OF PERTUSSIS TOXIN ON THE HORMONAL RESPONSIVENESS OF DIFFERENT TISSUES[1]

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I. INTRODUCTION

It has been known for a long time that the administration of pertussis vaccine to rodents induces changes in the sensitivity of many cell types to hormones and mediators, such as histamine (1) and epinephrine (2,3). In 1975 Sumi and Ui reported that the increase in insulin level induced by the administration of beta-adrenergic agonists was strongly exaggerated in pertussis-sensitized rats as compared to controls and that the decrease in blood insulin caused by alpha adrenergic agonists was effectively reversed by the vaccine. Later, Ui and his co-workers purified and characterized the toxin responsible for these effects (5,6): pertussis toxin (named by them Islet-Activating-Protein for its effects on the pancreatic islets). Katada and Ui in 1979 reported that pertussis toxin enhanced insulin secretion and cyclic AMP accumulation in islets by activating native calcium ionophores (7). In 1980, the group of Kato reported that the adrenoceptor involved in inhibiting insulin secretion was of the $alpha_2$ subtype (8).

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While I was in the laboratory of Dr. John Fain, we developed a model for the action of adrenergic amines which suggested that $alpha_1$-adrenoceptors mediate those effects of catecholamines involving phosphatidylinositol turnover and calcium signalling whereas $alpha_2$-adrenergic effects were due to inhibition of adenylate cyclase (9-11). Beta adrenoceptors were known to activate adenylate cyclase. Thus, although it was clear that $alpha_2$-adrenergic-blockade would result in an enhanced beta-adrenergic response, the relationship between this and the putative calcium ionophores proposed by Katada and Ui was obscure.

In order to more carefully define the effect of pertussis toxin studies were initiated using hamster adipocytes as the model system. This model system has the beauties of being easily obtained, extremely sensitive to adrenergic amines and having $alpha_1$, $alpha_2$ and beta adrenergic receptors (9,10). It was observed that the administration of pertussis vaccine to hamsters produced a marked decrease in the sensitivity of their adipocytes to a variety of agents that inhibit adenylate cyclase in the hamster adipocyte (through receptor-mediated GTP-dependent processes) such as $alpha_2$-adrenergic amines, phenyl-isopropyl-adenosine and prostaglandin E_2 (12). This blockade of the receptor-mediated inhibition of adenylate cyclase was evidenced by both cyclic AMP levels and lipolysis (12). It was suggested that pertussis toxin present in the vaccine might block the transfer of inhibitory information from the receptors to adenylate cyclase probably at the level of the coupling between the receptor and the cyclase (12). Independently, Hazeki and Ui reported that pertussis toxin blocks the inhibitory action of muscarinic cholinergic agents and adenosine on cyclic AMP accumulation in isolated rat heart cells (13).

So that we could pursue our studies on the effect of pertussis toxin in this system it was necessary for us to initiate the purification of the toxin in our laboratory. In the present paper I will review our findings using purified pertussis toxin as a tool to better understand the mechanisms of signal transduction involved in the actions of hormones.

II. RESULTS

A. Adipocytes

The purification of pertussis toxin was much more complicated than expected; most of the procedures resulted in inactive preparations or in extremely low yields of active toxin.

Finally, a purification procedure was implemented similar to those of Arai and Sato (14) and Yajima and co-workers (5). By this method the toxin was purified approximately 1800-fold from vaccine concentrates (generously provided by the authorities of the National Institute of Hygiene of Mexico). Administration of as little as 1 μg of toxin/ 100 g of body weight to hamsters resulted in blockade of the inhibition of adenylate cyclase in their adipocytes, by agents that act through receptor-mediated GTP-dependent processes, such as $alpha_2$-adrenergic amines, prostaglandins, phenyl-isopropyl-adenosine and nicotinic acid (15), hence, reproducing my findings with the whole vaccine (12) (see Table I). These results have also been reproduced adding the toxin *in vitro* (16).

We made some additional observations as well. Firstly, the inhibitory action of 2', 5'-dideoxyadenosine on cyclic AMP accumulation was not affected by the toxin (15); this is consistent with the idea that the actions of adenosine on fat cell adenylate cyclase are mediated through two types of sites (17) and further suggests that dideoxyadenosine does not work through the inhibitory coupling mechanism (Ni) but rather on the cyclase itself or on a neighboring structure (see Fig.1). Adenosine is especially important in fat cells because it is an endogenous regulator of lipolysis (18,19). We observed using either the whole vaccine (12) or the purified toxin (unpublished) that the basal rate of lipolysis was significantly higher in adipocytes from treated animals as compared to controls. This action of the toxin is at least partially due to

TABLE I. Effect of Pertussis toxin on the hormonal regulation of cyclic AMP levels in hamster adipocytes. Pertussis toxin (10 μg/100 g) was administered i.p. three days before the experiment was performed. Adipocytes were isolated and incubated as described (15).

	Cyclic AMP (pmol/10^6 cells)	
Addition	Control	Pertussis
Basal	75 ± 5	80 ± 10
Isoproterenol 10^{-6}M	5840 ± 410	9050 ± 895
Isoproterenol + clonidine 10^{-5}M	675 ± 205	8410 ± 500
Isoproterenol + PIA 10^{-6}M	120 ± 30	6814 ± 750
Isoproterenol + PGE_2 10^{-7}M	180 ± 30	7600 ± 850
Isoproterenol + Nicotinic Acid 10^{-5}M	140 ± 30	7000 ± 900

reversal of the endogenous inhibition of lipolysis produced by adenosine (12). Endoh *et al.* (20), Sekura and Manclark (21) and Olansky *et al* (12) have shown that pertussis induced lipolysis in adipocytes and that this effect can be used as an *in vitro* assay for the toxin. This enhanced lipolysis has significant physiological repercussions *in vivo* since it provokes disturbances in the lipid metabolism of the animals. We observed that administration of pertussis vaccine to hamsters produces a severe fatty liver, hyperlipidemia and ketosis (23). All

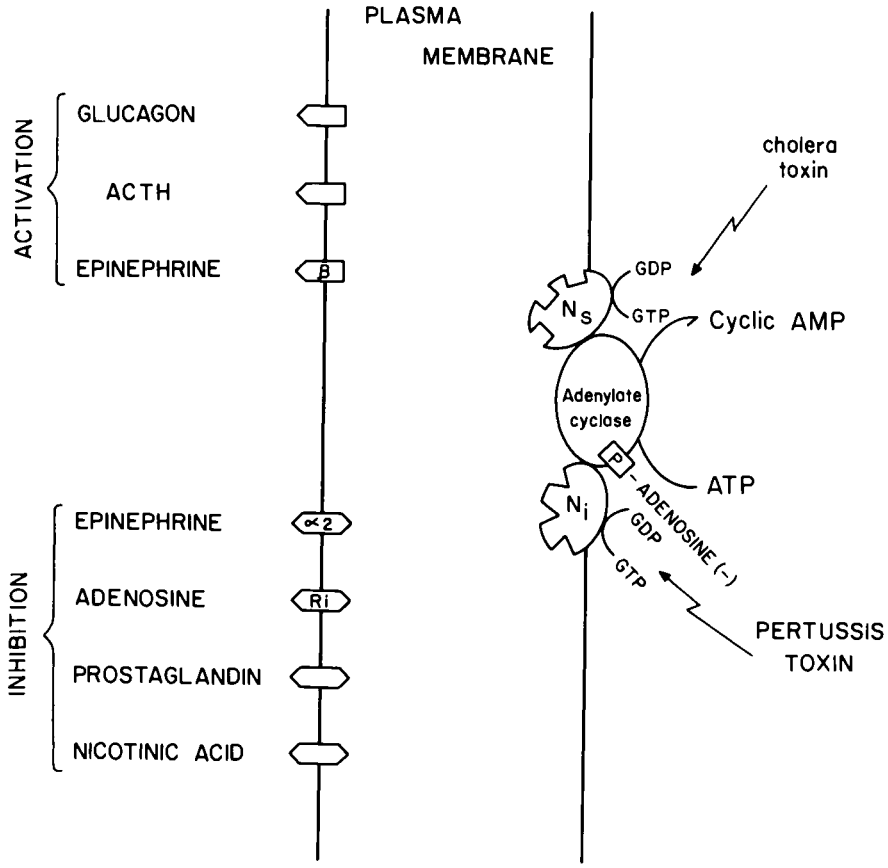


Fig. 1. Model for the action of pertussis toxin on adipocytes.

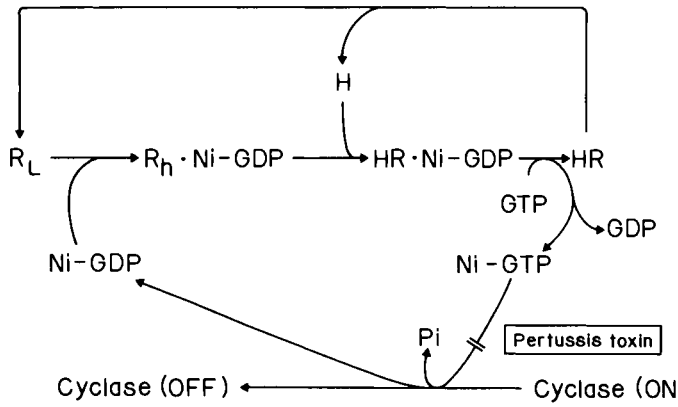
these data can be explained as the result of the altered regulation of lipolysis in adipose tissue, i.e. enhanced lipolysis generates a significant increase in the level of free fatty acids in plasma (23), which in turn intensifies the uptake of these metabolites by the liver (it is known that the uptake of fatty acids by the liver is directly proportional to the concentration to which it is exposed). Fatty acids can be either oxidized or esterified in the liver; both pathways are increased in toxin-treated animals. The enhanced oxidation results in marked production of ketone bodies (ketosis) and the intensified esterification results in a large output of triacylglycerols (hypertriacylglycerolemia) and accumulation of these lipids in the liver (fatty liver). It should be mentioned however, that the severity of the changes varied significantly with the different lots of vaccine concentrates employed and that their magnitude is smaller when the purified toxin is employed (unpublished). This raises the possibility that other component(s) of the vaccine might also play a role in these effects.

Secondly, the action of agents that activate adenylate cyclase such as beta adrenergic amines, ACTH or forskolin was significantly enhanced in adipocytes from animals treated with pertussis toxin (10 μg/100 g 3 days before the experiment was performed) as compared to controls (15). These data were explained as the result of lack of restraint of adenylate cyclase activity resulting from the ADP-ribosylation of Ni (15). We took advantage of this effect of pertussis toxin and showed that in adipocytes from rabbits, which are relatively insensitive to beta adrenergic amines, the administration of pertussis toxin markedly magnified their sensitivity to these compounds (24). The data suggest that in rabbit adipocytes, beta adrenergic amines are ineffective as stimulators of adenylate cyclase due to an excess of Ni (24). Another interesting observation in this regard was made by the group of Brian Hoffman (25) who observed that adipocytes obtained from old rats have a markedly decreased sensitivity to lipolytic agents which was significantly improved by the administration of pertussis vaccine to these animals (25). It has been observed that adipocytes from hypothyroid animals have a decreased responsiveness to agents that activate adenylate cyclase (26). In spite of a great deal of effort by several groups, to determine the site of the defect in the cell response, it has remained unclear. A recent observation, which may, in part, explain the decreased responsiveness of the adipocytes from hypothyroid animals, is that the amount of Ni (but not Ns), as reflected by toxin-catalyzed ADP-ribosylation, seems to be significantly increased in these cells as compared to those from euthyroid animals (27). These data point to a

possible regulatory role of Ni, suggesting that the total amount of Ni or the balance Ni/Ns might regulate the responsiveness of cells to hormones and that these total amounts or balances of coupling factors vary widely among different species (phylogeny), in the course of development (ontogeny), according to cell type (differentiation) or even in different physiological or pathological conditions.

The elegant work of Katada and Ui (28) clearly showed that the target of pertussis toxin is a subunit of Ni with a Mr of 41 kDa. This has also been documented by the same group in fat cells (29). However, recent findings by Malbon and co-workers (30) indicate that in fat cells, the toxin targets are two proteins (Mr 40 kDa and 41 kDa) which seem to be homologous but not identical.

Two major functions are known for the guanine nucleotide regulatory proteins: a) transfering the information from the receptors to adenylate cyclase and b) regulating the state of affinity for agonists of these receptors (see Fig. 2). It is generally accepted that the activation of the guanine nucle-



H-HORMONE

R-RECEPTOR

Fig. 2. Model for the action of Pertussis toxin on adenylate cyclase.

otide regulatory proteins is produced by the release of GDP from the binding sites in exchange for GTP. Hydrolysis of GTP to GDP on the nucleotide regulatory proteins terminates the activation of the cyclase as the turnoff reaction. It has been shown that the ability of GTP to activate Ni, and in this way inhibit adenylate cyclase activity, is blocked by pertussis toxin in fat cells (31,32). Interestingly, the inhibitory action of hydrolysis-resistant analogues of GTP is not completely blocked by the toxin (31,32). We interpret this data as suggesting that the hydrolysis-resistant GTP analogues might act on Ni and other sites as well to inhibit adenylate cyclase. Only their effect on Ni is blocked by pertussis toxin. Jakobs and his group (32) have shown that the ADP-ribosylation of Ni by pertussis toxin leads to blockade of the GTPase activity associated to inhibition of adenylate cyclase. Recently, Murayama and Ui (33) reported that pertussis toxin completely abolished the stimulation of GDP release (from membranes prelabeled with GTP) by agents that inhibit adenylate cyclase. On the other hand GTP and the hydrolysis-resistant analogues of this guanine nucleotide induce the formation of the low affinity state for agonists of receptors coupled to adenylate cyclase. We recently reported that pertussis toxin administration decreased the formation of the high affinity state for agonists of the adipocyte $alpha_2$-adrenergic receptors (34). It is possible that the blockade of the GTPase activity of Ni by pertussis toxin (32) could explain: a) the action of the toxin on GDP release (33), i.e., the membranes prelabeled with GTP cannot hydrolyze it to GDP and b) the effect on the state of affinity of the receptors (34), i.e., allowing GTP to remain permanently bound to Ni and therefore decreasing the formation of the high affinity state for agonists. Nevertheless, this would result in constitution of the active form of the guanine nucleotide regulatory protein (Ni-GTP) which would produce a tonic inhibition of adenylate cyclase. Since this does not happen the data suggest that the ADP-ribosylation catalyzed by pertussis toxin results, (in addition to the above mentioned changes) in a form of Ni uncapable of effective interaction with the catalytic subunit of adenylate cyclase (see Fig. 2).

Activation of $alpha_1$-adrenergic receptors in fat cells produces a marked increase in the labeling of phosphatidylinositol and phosphatidic acid (9, 10, 35, 36). Interestingly, in adipocytes from pertussis toxin-treated rats the $alpha_1$-adrenergic-mediated labeling of phosphatidylinositol was severely diminished (37). These results are intriguing, because $alpha_1$-adrenoceptors are not linked to adenylate cyclase. We studied the $alpha_1$-adrenergic-mediated stimulation of phospholipid metabolism in other systems and concluded that this effect of pertussis toxin is unique for adipose tissue and not a general phenomenon (38). The molecular basis of this action

of the toxin remains to be established.

Finally, an interesting observation regarding the actions of insulin was made. Administration of pertussis toxin did not block the antilipolysis produced by insulin, in contrast to the blockade by the toxin of the antilipolytic effect of other agents (12, 37-40). The study of other actions of insulin, such as glucose oxidation, glucose conversion into lipids and phosphodiesterase activation indicated that the toxin does not block insulin action. This is of significance since there is some evidence suggesting a role of GTP (and therefore possibly a guanine nucleotide regulatory protein) mediating the action of insulin (41).

B. Hepatocytes

Epinephrine induces hyperglycemia and this effect is severely impaired in rodents and humans after the administration of pertussis vaccine (42-44) and also in patients infected with *Bordetella pertussis* (45). This effect could be due to several causes: hyperinsulinemia, a defect in hepatic glycogenolysis or a combination of both. Epinephrine-induced hyperinsulinemia in rodents treated with pertussis vaccine was already demonstrated (3,4). However, the effect of pertussis toxin on liver metabolism had not been evaluated. Kreutner *et al.*(3) reported that the activation of phosphorylase induced by epinephrine was dminished in rats treated with pertussis vaccine. However their experiments did not allow to differentiate between a defect in the hormonal responsiveness of the hepatocyte and a partial blockade of the epinephrine-induced activation of phosphorylase by the associate hyperinsulinism (3). $Alpha_1$-adrenoceptors are the main type of adrenoceptor controlling hepatic metabolism in the normal rat hepatocytes (46-48). Our studies with rat adipocytes indicated that the $alpha_1$-adrenergic responsiveness of this type of cell was significantly decreased by the toxin (37). Based on these data we decided to study the effect of pertussis toxin administration on the hormonal responsiveness of hepatocytes.

The ureogenic action of epinephrine in hepatocytes from normal adult rats is mediated through the activation of $alpha_1$-adrenoceptors. In hepatocytes from rats treated with pertussis toxin, beta-adrenoceptors in addition to $alpha_1$-adrenergic receptors became involved in mediating this effect of the amine (38). The accumulation of cyclic AMP in response to epinephrine or isoproterenol was markedly increased in hepatocytes from toxin-treated rats as compared to controls. The accumulation of cyclic AMP due to glucagon was also significantly increased with respect to the controls. Interestingly, the labeling of phosphatidylinositol was stimulated to a similar extent by

epinephrine in hepatocytes from control or pertussis toxin-treated rats (38). This data indicate that a) the $alpha_1$-adrenergic responsiveness of hepatocytes was not modified by pertussis toxin and b) that the action of agents that stimulate adenylate cyclase, such as glucagon and beta-adrenergic agonists, is significantly magnified. The former data suggest that the action of pertussis toxin on $alpha_1$-adrenergic effects observed in adipose tissue (37) is a unique action rather than a general effect of the toxin. The later data are in agreement with our findings in other systems (15,16,24,25) and indicate that in hepatocytes pertussis toxin releases a constraint on adenylate cyclase activity by blocking the inhibitory coupling mechanism (Ni) (38).

$Alpha_2$-adrenoceptors are present in hepatocytes and their action inhibiting adenylate cyclase has already been shown in isolated membranes (49,50). However attempts to identify $alpha_2$-adrenergic effects in isolated hepatocytes have been unsuccessful in my laboratory. Experiments are in progress to study the action of pertussis toxin on the $alpha_2$-adrenergic modulation of adenylate cyclase in liver membranes.

Hepatocytes in culture rapidly develop beta-adrenergic responsiveness (51,52). This seems to be partially due to an increase in the synthesis of beta-adrenoceptors (52). However, other factors may be possibly involved. In a recent meeting (53) Ui reported that the amount of Ni as reflected by pertussis toxin-catalyzed ADP-ribosylation of the 41 kDa subunit is significantly decreased in liver cells in culture (53). Interestingly, nicotinamide partially blocked this effect which raises the possibility of endogenous ADP-ribosylation (53) as a mechanism of controlling the amount of active guanine nucleotide regulatory proteins.

C. Ileum

The isolated guinea pig ileum is one of the most widely used pharmacological models for the study of the actions of opiates and $alpha_2$-adrenergic amines. These agents modulate the release of acetylcholine (which is the neurotransmitter that triggers contraction), by the motoneurons of the myenteric plexus of the ileum. These opiate and $alpha_2$-adrenergic receptors seem to be coupled to adenylate cyclase in an inhibitory fashion (54) but no direct proof of this is available. We used pertussis toxin as a tool to study the putative involvement of Ni in the action of opiates and adrenergic agents in the ileum. What we did was to study the effect of the administration of the toxin on the inhibition of contraction by morphine and adrenergic agents in the electrically stimulated ileum.

Pretreatment of guinea pig with pertussis toxin did not modify the magnitude of the response of the ileum to electrical stimulation or to exogenously administered acetylcholine (55). The initial experiments trying to demonstrate any effect of the toxin were very disappointing. No clear effect was evidenced in animals treated with the toxin 2 or 3 days before the experiment was performed. However, six days after treatment neither morphine nor the adrenergic agents used were able to inhibit electrically stimulated ileum contraction (55). It was exciting to observe that the response to morphine was partially restored 10 days after treatment and fully restored at 18 days (55) (see Fig. 3).

The data indicate that pertussis toxin is able to block these actions and suggest a possible involvement of Ni in the effects of $alpha_2$-adrenergic agents and opiates in the ileum. Nevertheless, what was most interesting is the very slow onset of the action of the toxin and the gradual recovery (55). The time-course of the action of the toxin suggests that a very small amount of toxin reaches the myenteric plexus *in vivo* but that this small amount remains active for many days. The slow recovery of the response to the agents indicates that either the toxin remains active inside the cells for a long time or the turnover of Ni is very slow in this type of cells or both.

We have now been able to reproduce this effect of the toxin *in vitro* (in a few hours) but it does require a large amount of toxin (manuscript in preparation).

D. Cardiovascular system.

$Alpha_1$ and $alpha_2$-adrenoceptors are present in the vascular system of the rat; upon stimulation, these adrenoceptor subtypes mediate a pressor response (56). Therefore, the pithed rats was considered as a suitable model to study the effect of pertussis toxin on the mechanism of action of alpha-adrenergic amines. In addition, it had been observed that the $alpha_2$-adrenergic-mediated pressor response was significantly affected by calcium antagonists (57), and it was of interest to study the possible involvement of Ni in the mechanism of signal transduction.

We carried out some experiments using the pithed rat as a model evaluating the increase in diastolic pressure produced by the administration of adrenergic agonists. The findings were essentially identical using whole vaccine (58) or pertussis toxin (59). It was observed that the administration of these preparations did not affect the pressor response due to $alpha_1$-adrenergic-selective agents (such as methoxamine) but markedly diminished the effect of selective $alpha_2$-adrenergic drugs (such as clonidine or azepexole (BHT933). The effect of

the toxin was dose-dependent (59). These data indicated several facts: a) that the signalling process of $alpha_1$-adrenergic amines is not affected by the toxin or at least it is not affected to a similar extent than that of $alpha_2$ at the doses employed; this is consistent with our findings using hepatocytes and further stresses the difference with adipocytes (see previous sections); and b) that the mechanism of signal transduction is different for $alpha_1$ as compared to $alpha_2$-adrenoceptor-mediated actions.

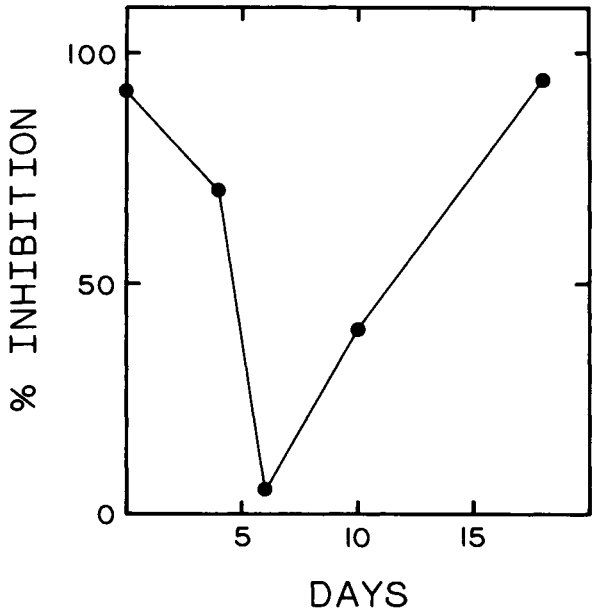


Fig. 3. Effect of pertussis toxin on the inhibition of contraction produced by 10^{-6} morphine. Pertussis toxin (60 μg/kg i.p.) was administered to guinea-pigs. Intestinal segments were electrically stimulated via two stainless steel electrodes and the contraction recorded isometrically (55).

Considering the previous findings and the action of calcium antagonists (57) we proposed the following hypothesis: activation of $alpha_2$-adrenoceptors inhibits, through the action of Ni, adenylate cyclase activity; the subsequent decrease in cyclic AMP alters the membrane permeability to calcium which in turn may trigger contraction. We have tried to test this hypothesis. Unfortunately, our efforts have been handicapped by the absence of a simple model to study $alpha_2$-adrenergic-mediated contraction at a cellular level.

Another observation using this model was the induction of severe tachycardia in the rats injected with the vaccine or the pertussis toxin preparation (58,59). We suggested that this effect of the toxin was probably related to its ability to block receptor-mediated inhibition of adenylate cyclase in heart cells in culture (13).

Therefore, we decided to study this effect more closely. In the conscious rat the heart rate is modulated through the balance between the sympathetic tone (beta-adrenergic, activatory of adenylate cyclase) and the parasympathetic tone (muscarinic cholinergic, inhibitory of adenylate cyclase). In the pithed rat, where no sympathetic or parasympathetic tones exist, the effect of the toxin on heart rate was no longer evident, which suggested that the action of the toxin was due to lack of constraint of the sympathetic tone (59).

This action of pertussis toxin on heart rate probably has clinical significance. It has been reported that immunization with pertussis vaccine precipitates paroxysmal supraventricular tachycardia in patients with predisposition (60). Interestingly, and consistent with our findings, the paroxysmal tachycardia was controlled with the administration of propranolol (60).

We took a step further in this problem. The number and affinity of the muscarinic cholinergic receptors of the heart were studied. It was observed that the number of muscarinic receptors in the heart did not change significantly in pertussis toxin-treated rats as compared to controls (manuscript in preparation). Interestingly, and consistent with our previous findings using fat cells (34), it was observed that the affinity of these receptors for their agonists was significantly decreased. No further effect of guanine nucleotides on their affinity was observed (manuscript in preparation).

E. Kidney

Rat kidney membranes contain also $alpha_1$ and $alpha_2$-adrenoceptors (10). These adrenoceptor subtypes seem to be located in different structures and involved in different functions. Thus, $alpha_1$-adrenoceptors seem to be involved in

the control of gluconeogenesis whereas $alpha_2$-adrenoceptors seem to modulate renin release and water flux (62). In addition, both subtypes of adrenoceptor ($alpha_1$ and $alpha_2$) are regulated by guanine nucleotides in their affinity state for agonists (10). Therefore, we selected this system to address several questions: What is the effect of pertussis toxin on the regulation by guanine nucleotides of the affinity state?, Is the affinity of these adrenoceptors modulated through the same regulatory protein? Has the treatment with pertussis toxin any physiological significance?.

Pretreatment of rats with pertussis toxin (50 μg/100 g, 3-5 days before the experiment was performed) did not induce any change in the number of $alpha_1$ or $alpha_2$-adrenergic receptors of renal cortex membranes or their affinity for antagonists. However, the affinity for epinephrine of the $alpha_2$-adrenoceptors was markedly decreased and also the ability of guanine nucleotides to modulate their affinity was diminished in membranes from toxin-treated animals as compared to controls (63). Interestingly, using the same membranes no effect of pertussis toxin treatment was observed neither on $alpha_1$-adrenoceptor affinity nor on its regulation by guanine nucleotides (63). These results suggest that the modulation of $alpha_1$ and $alpha_2$-adrenoceptor affinity by guanine nucleotides is probably exerted through different molecular entities.

What is the physiological repercussion of this effect of pertussis toxin in the kidney? To address this question the activity of renin in plasma was studied. Renin is a protease that catalyzes the release of angiotensin I from angiotensinogen; it is synthesized, stored and secreted by the juxtaglomerular apparatus of the kidney. Beta-adrenergic activation stimulates renin release whereas $alpha_2$-adrenergic activation inhibits the secretion of this protease (62). It was observed that the modulation of renin release, as reflected by the plasma renin activity, was markedly affected by the administration of the toxin (64). Administration of epinephrine did not modify plasma renin activity in control rats but enormously increased it in rats injected with the toxin (64). This effect of the toxin was reproduced in control rats if yohimbine was administered before the catecholamine (64) (see Fig. 4). On the other hand, the ability of clonidine to decrease plasma renin activity was significantly decreased in toxin treated animals as compared to controls whereas the increase in plasma renin activity produced by isoproterenol was magnified (see Fig. 4). These data indicate that pertussis toxin blocks the $alpha_2$-adrenergic modulation of renin release and magnifies the ability of beta-adrenergic amines to stimulate its release (64).

Recently in collaboration with the group of Dr. Anderson (U. of Colorado) we observed that the ability of prostaglandin

E_2 to inhibit adenylate cyclase and osmotic water permeability in rabbit cortical collecting tubular epithelium is blocked by pertussis toxin (submitted for publication). These two set of data clearly indicate that the action of the toxin has physiological repercussion.

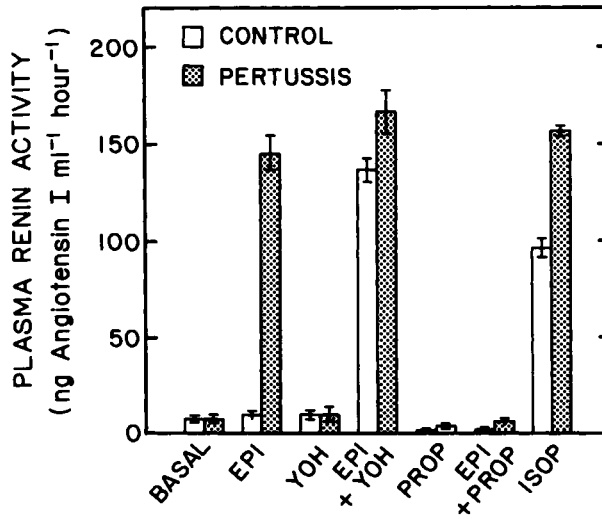


Fig. 4. Effect of pertussis toxin on the adrenergic regulation of plasma renin activity. Pertussis toxin (50 μg/100 g i.p., 3 days before the experiment was performed) or vehicle was administered to the rats. Animals were injected subcutaneously with the agents indicated: EPI, epinephrine (10 μg/100 g); YOH, yohimbine (100 μg/100 g); PRO, propranolol (100 μg/100 g) ISO, isoproterenol (10 μg/100 g) Plasma renin activity was quantified as described (64).

III. CONCLUSION

Undoubtedly pertussis toxin has been a powerful tool to study the mechanisms of hormone action. Our knowledge of the entities that participate in the transduction through adenylate cyclase has increased spectacularly in the last few years. We are now thinking in terms of well characterized subunits of the guanine nucleotide regulatory complexes, in terms of the interplay that such subunits may have in the plasma membrane to modulate cell responsiveness, in terms of balance between these complexes and even considering that other guanine nucleotide regulatory complexes may participate in the process of signal transduction for the action of hormones that are not linked to adenylate cyclase. Obviously, all this has to be contemplated in the context of the present development of the biological sciences; however, I do think that without pertussis toxin all this would have taken much more time.

Fortunately, science is an endless route and there are at least as many things to do as already done. At the present we know nothing about the chemical nature of the putative receptor for pertussis toxin or its relative abundance in the different cells. The toxin has a lag period of days *in vivo* (whole animal) to hours *in vitro* (cell culture). We know nothing about the absorption, distribution and inactivation of pertussis toxin once is injected into an animal. It is postulated that the B component of the toxin binds to the surface receptors to enable the A component to enter the cell where it acts (6). However, we do not know anything about how this takes place. In addition, it has been postulated that the active promoter of the toxin may require some "processing" to be fully active (65). The nature of this "processing" remains to be elucidated.

We have used the administration of pertussis toxin to experimental animals as one possible approach. Obviously this approach is not without problems; once the toxin is administered it acts on many cells, and changes secondary to the action of the toxin in a specific cell may alter the responsiveness of other cells. This is specially important if we consider that the toxin alters the secretion of a variety of hormones and mediators. However, our results always have internal consistency and when possible we have reproduced *in vitro* our findings *in vivo*. In addition it is important to know what happens to animals under the action of pertussis toxin for several reasons. Firstly, pertussis toxin is present in the pertussis vaccine currently used throughout the world and is very probably involved in the pathogenesis of whooping cough.

Therefore, in one way (prophylaxis) or another (infection), our children, which are the population at the highest risk, come in contact with it. It is clear that the vaccine is safe and has been very effective in controlling the disease; but in some cases there are problems which can be directly associated to the administration of the vaccine. These cases are important and the only way to give these patients a reasonable scientific treatment is to know what are the major disturbances produced by the toxin. From a medical point of view, to establish the role of pertussis toxin in the pathogenesis of the disease is very important.

Pertussis toxin seems to be one of the most important antigens in the pertussis vaccine (66-69). The selection of the significant antigens will probably result in the replacement of the whole cell vaccine currently employed by an acellular preparation which ideally would result in better vaccine without side effects. Actually in Japan an acellular preparation is being employed and the Japanese health authorities seem to favor this acellular preparation rather than the traditional vaccine, since it is reported to have fewer local and systemic side of effects. We are currently attempting to develop an acellular vaccine (70).

The use of pertussis toxin to explore the role of Ni in the action of a hormone or neurotransmitter in a specific cell or tissue seems to be a valid approach. Ni seems to be the substrate of pertussis toxin in most cells (as mentioned before, in fat cells two homologous proteins are ADP-ribosylated by pertussis toxin (30) but the physiological meaning of this is far from clear); this does not necessarily mean that it is the only substrate. Therefore, care should be exercised when this approach is employed, to be as sure as possible of the conclusions by using the appropriate controls. It is clear that pertussis toxin is being one of the most powerful tools to elucidate the mechanisms of action of hormones. I am sure that the commercial availability of this toxin will speed up even more the progress in this field.

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EXTRACELLULAR ADENYLATE CYCLASE OF BORDETELLA PERTUSSIS

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Among the many biologically active factors produced by Bordetella pertussis, four may have major effects on the production of cAMP (1). In addition to pertussis toxin, there are effects on adenylate cyclase by lipopolysaccharides and by a second, heatstable factor not yet thoroughly identified, and there is one (or more) adenylate cyclase. The cyclase was first detected in commercial pertussis vaccines where it was found to be remarkably stable (2).

High adenylate cyclase activities are present in virulent strains of Bordetella pertussis. Table I lists a number of common laboratory strains but dozens of other Phase I strains are rich in this activity (3-8). Fresh isolates from active cases of whooping cough also show brisk adenylate cyclase activity and this appears to be a useful diagnostic tool (Table I). When strains become degraded and lose their virulence (Phase IV), such organisms no longer produce adenylate cyclase as well as a number of other activities considered to be virulence factors. Commercial vaccines, in which Bordetella adenylate cyclase was first discovered (1, 9), contain highly significant amounts of the enzyme in some preparations but little in others. Whether this results from the method of inactivation (formaldehyde, merthiolate, etc.) or from other causes is not clear at present.

There are two other members of the genus Bordetella, namely B. parapertussis and B. bronchoseptica. The former causes infections in man (10) and the latter infects rabbits, causes kennel cough in dogs, and atrophic rhinitis in swine (11, 12). Both of these species produce abundant

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TABLE I. Adenylate Cyclase Activity in Bordetella Species

Bordetella strain	Source[a]	Basal	Plus[b] calmodulin	Comment	Reference
		nmole/mg protein/min			
B. Pertussis					
BB 114	org.	0.4	265		3
BB 27	org.	0.5	274		3
Tohama I	media	20	250		6
Clinical Isolates (Mexico)	org.	0.13-0.73	0.78-8.7	Diagnostic	(unpubl.)
Commercial Vaccines	org.	0-0.9	-	Per Single Human Dose	9
L 51 (Phase IV) avirulent	org.	0.001-0.008	0.001-0.009	Degraded[c] strains	3
BB 182-IV	org.	~ 0	~ 0	"	5
B. Parapertussis					
2305-4	media	0.056	1.7[d]	Higher activity than in B. pertussis	4
BB 21449				Equals B. pertussis activity	5

TABLE I (continued)

Bordetella strain	Source[a]	Basal	Plus[b] calmodulin	Comment	Reference
		nmole/mg protein/min			
B. Bronchoseptica					
L_3 72-1					4
	media	0.042	1.2[d]		
D-2	org.			~ 1/2 B. pertussis activity	5

[a]Intact organisms or culture media.

[b]The concentration of calmodulin is variable but near maximal.

[c]Degraded strains have lost various virulence factors but can be identified by DNA hybridization.

[d]Activity per 10^9 cells; stimulation by calmodulin was from contamination in creatin kinase (19) and was partial; basal activity calculated from average stimulation in B. pertussis (4).

adenylate cyclase. Endoh et al. (4) found higher adenylate cyclase activity in these two species than in B. pertussis, suggesting (6) that adenylate cyclase cannot be the only virulence factor since the B. parapertussis is less virulent. On the other hand, the enzyme must certainly contribute since these two species do not produce pertussis toxin, believed to be a major factor in B. pertussis. This subject will be discussed in the next section (see Hewlett this volume).

Although when judged to be present B. pertussis cyclase shows very high activity as compared to most eukaryotic systems, the absolute values of enzyme levels are quite variable and depend not only on the strain but also on

1) The length and temperature of incubation;

2) The composition of the medium, for example, Mg^{2+} (13). In another case, McPheat et al. (14) have shown that the presence of nicotinic acid reduces adenylate cyclase activity in some strains of Bordetella but not others;

3) Residual calmodulin from growth medium or other additions may raise basal values and/or lower the apparent stimulation by added calmodulin (see below).

4) Storage tends to increase basal adenylate cyclase activity but decrease stimulability. This has also been observed by others (6).

It will be seen in Table I that enzyme activity is associated with both intact organisms and the culture medium. We showed in 1976 that the bulk of adenylate activity in strain 114 was extracytoplasmic, being readily assayed with exogenous ATP and exquisitely sensitive to trypsin, in contrast to an intracytoplasmic enzyme such as malic dehydrogenase (15). The extracytoplasmic location of the cyclase has been repeatedly confirmed, but quantitative data on the partition among the various cell compartments are sparse and seem to vary with different strains and growth conditions (6, 14, 15). About 90% of the adenylate cyclase of strain 114 is extracytoplasmic whereas the remainder appears to be within the cell or otherwise unavailable. The fraction secreted into the culture medium has decreased in strain 114 to $<$ 10% but may be greater in other hands (6, 14). It is also not established that the enzyme is of the same form in the different compartments, although the different molecular weights found for cell-associated and medium adenylate cyclases suggest that differences are real (see below).

The properties of the adenylate cyclase of strain 114 in intact organisms and culture medium are summarized in Table II (16). Although stimulated activity is generally much higher than in crude eukaryotic systems, regulation of

TABLE II. Modulators of B. Pertussis Adenylate Cyclase

Agent	Comment
General	
Guanine Nucleotides	No effect
$Mg^{2+} > Mn^{2+}$	Curves cross at low concentrations
dATP > ATP > APPNHP	
Forskolin	No effect
Inhibitors	
Fluoride	$IC_{50} \sim 4$ mM
Phenothiazines	via Calmodulin (and other?)
Troponin I	1:1 Complex with Calmodulin
High Ca^{2+}	Not via Calmodulin
Gangliosides	$GD_1a > GM_1 > GM_3$
Activators	
Calmodulin	Up to 1000 Fold; Ca^{2+} not required; protein from brain, peanut, T. Pyriformis, and Octopus
Troponin C	Potency $<10^{-3}$ of Calmodulin; contamination?
Parvalbumin	Potency $<10^{-4}$ of Calmodulin; contamination?
1,10-Phenanthroline (not 4,7-Phenanthroline)	2-3 Fold; via divalent metal
Phospholipids	3-10 Fold; at concentrations below critical micelle concentration

catalytic activity differs markedly. Thus, guanine nucleotides have no effect until concentrations are large enough to compete for the available Mg^{2+} ion. The preferred divalent cation is Mg^{2+} and maximal activity exceeds that attainable with Mn^{2+}, although at very low levels the concentration curves cross. Substrates are similar to those effective in eukaryotic systems although the relative efficiencies may differ. GTP is not a substrate. At high ATP concentrations substrate inhibition may be observed as shown in Figure 1. This occurs at both low levels of stimulation (or basal activity) and with the maximally activated enzyme. Forskolin in concentrations up to $1 \cdot 10^{-4}$ M is ineffective as it is in the soluble testicular adenylate cyclase. Unlike eukaryotic cyclases, fluoride is inhibitory with an IC_{50} of ~ 4 mM (Table II). Other halides inhibit at substantially higher concentrations (16). Like other cyclases, high Ca^{2+} concentrations ($\geq 1 \cdot 10^{-4}$ M) are inhibitory.

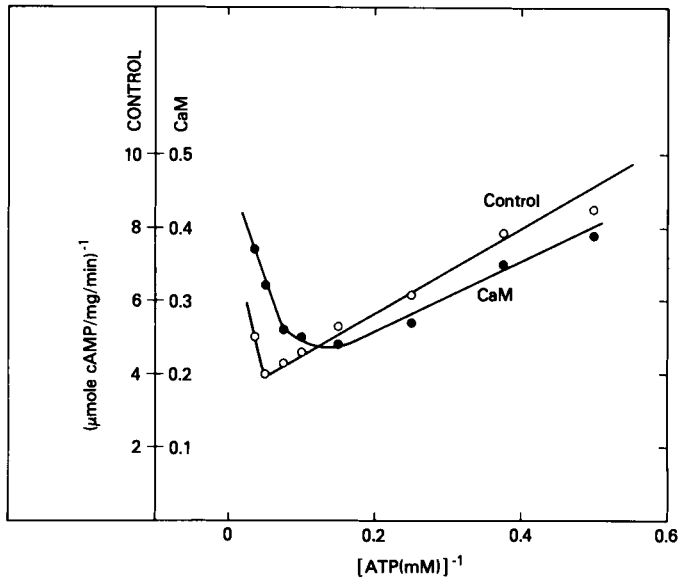

Fig. 1. Substrate inhibition in Bordetella adenylate cyclase. The medium contained 50 mM Tris HCl buffer pH 7.9, 10 µM added $CaCl_2$, 30 mM $MgCl_2$, 1 µM calmodulin. Incubation was for 8 min at 30° and was started with 1.37 µg of DEAE purified adenylate cyclase. Maximum specific activity was 4.5 µmoles/mg/min.

The B. pertussis cyclase is activated up to 1000-fold by bovine brain calmodulin. The first clue that this might be the case comes from the growth conditions preceding the assay: if the organisms had seen blood agar (B-G medium) in a recent passage, then "basal" activity was found to be very high (17). Addition of red cell lysates or hence proteins to organisms with low basal cyclase activity caused brisk activation as did other proteins including antipertussis immune sera. The widespread distribution of the activator in tissue extracts eventually led to the identification of calmodulin as the active material (18).

Activation of B. pertussis adenylate cyclase by calmodulin resembles the activation of other enzymes such as phosphodiesterase or myosin light chain kinase in many, but not all, respects. Half maximal activation in crude preparation occurs at 10-40 nM - somewhat less sensitive than bovine brain phosphodiesterase (15). These values can be reduced by 1-2 orders of magnitude in the presence of phospholipids (see below). The degree of stimulation is determined to a large extent by the basal level of activity which in turn depends on residual calmodulin present from previous exposures or the contamination by carrier proteins or regenerating systems added (19, 20).

As expected for calmodulin-mediated reactions, trifluoperazine and other phenothiazines are inhibitory (Table II) (18). However, such agents are relatively nonspecific and hence poor diagnostic tools for the identification of calmodulin participation in crude systems. Troponin I, which forms a 1:1 complex with calmodulin competes effectively with the cyclase for the available calmodulin (Table II, 6).

The specificity for calmodulin is fairly strict as far has been investigated. Other calcium binding proteins such as troponin C or parvalbumin have very low stimulatory activity; at these levels contamination by calmodulin that is not visible in gels, etc., is impossible to rule out (Table II, 18, 21). On the other hand, all calmodulins so far tried, including octopus, Tetrahymena pyriformis, peanut, testes and brain, have been active with the B. pertussis cyclase, although there appear to be some differences in the extent of stimulation attainable at least in spheroplast membranes (Fig. 2). It is also important to know which of the protein domains is involved in the activation and studies on calmodulin fragments are in progress. Finally, we have found that calmodulin attached to Affigel 15 is still able to stimulate.

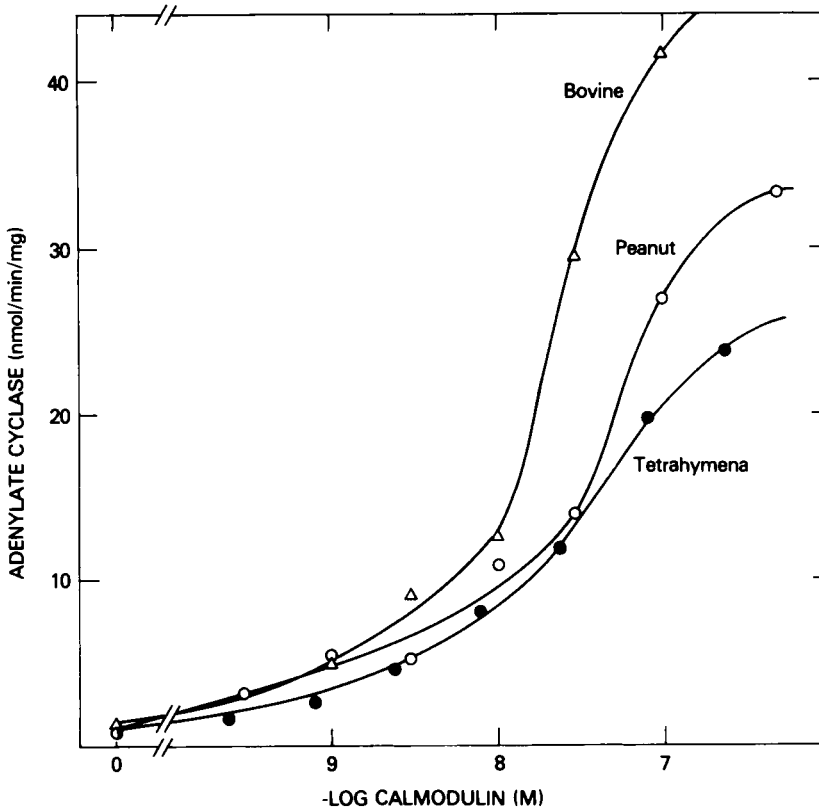

Fig. 2. Adenylate cyclase activity in B. Pertussis spheroplasts as stimulated by calmodulins of various origins. The Tetrahymena and peanut proteins were generously supplied by Dr. Tom Vanaman. Assay conditions as described in ref. 20.

The high sensitivity of the B. pertussis adenylate cyclase stems in part from the high degree of stimulation attainable. That is, even when half-maximal activation occurs at 10 nM, substantial stimulation can be detected by 1/100th that concentration or less. For this reason we have devised an assay procedure for calmodulin applicable to both purified proteins and crude tissue preparations (20). For this purpose a stable and reproducible enzyme preparation was needed and spheroplast membranes proved to be convenient. Sensitivities down to 1 pg were readily attained and could be improved upon if necessary. Crude tis-

sue fractions could be prepared by boiling, taking great care to minimize coprecipitation with the boiled protein. It became readily apparent that a great many purified, pure or even crystalline protein preparations available commercially contained enough calmodulin to yield spurious results if added to cyclase assays (19, 20). The probable identity of the cyclase activator so detected with calmodulin was shown by the fact that the activity: 1) resists boiling; 2) cochromatographs with authentic calmodulin; 3) is inhibited by trifluoperazine; and 4) stimulates Ca^{2+}dependent cyclic nucleotide phosphodiesterase. It is also easy to follow changes in tissue concentrations of calmodulin with various manipulations such as hormone stimulation, dedifferentiation, etc. Tracheal washes (rat) contain abundant calmodulin as do antisera, especially if there has been some hemolysis (unpublished observations). One important consequence of these findings is that regenerating systems frequently used with cyclase assays markedly raise basal enzyme activities and obscure results relating to the effects of calmodulin (see e.g. ref. 4). In our hands bacterial pyruvate kinase was acceptable for regeneration of ATP and was virtually free of calmodulin. The second problem that must be dealt with is the choice of carrier proteins for dilution of enzyme or calmodulin. These are frequently contaminated and must be screened before use. One possible source are prokaryotic proteins lacking untoward activities since prokaryotes are believed not to contain calmodulin. We have found bacterial amylase to be useful. (However, two recent reports on E. Coli and Myxococcus xanthus have claimed the presence of calmodulin-like activity (21, 22)). In any case, contamination with calmodulin is a serious problem in the evaluation of basal adenylate cyclase activity in B. pertussis.

There is one major difference between the calmodulin effect observed in this cyclase and the response of phosphodiesterase - Ca^{2+} is not an absolute requirement for promotion of calmodulin-induced stimulation of the B. pertussis adenylate cyclase. We first noticed this as an inability to arrest catalytic activity of the calmodulin-stimulated enzyme by addition of excess EGTA (18). Since then it has become apparent that in log calmodulin/vs adenylate cyclase plots, discontinuities are frequently observed and in some cases there may be a true plateau (Fig. 3). This occurs with intact organisms, spheroplast membranes and enzyme found in the medium and suggested two separate components to the activation process (23): a) a Ca^{2+}-sensitive portion seen at low calmodulin concentrations and b) a Ca^{2+}insensitive portion that requires about

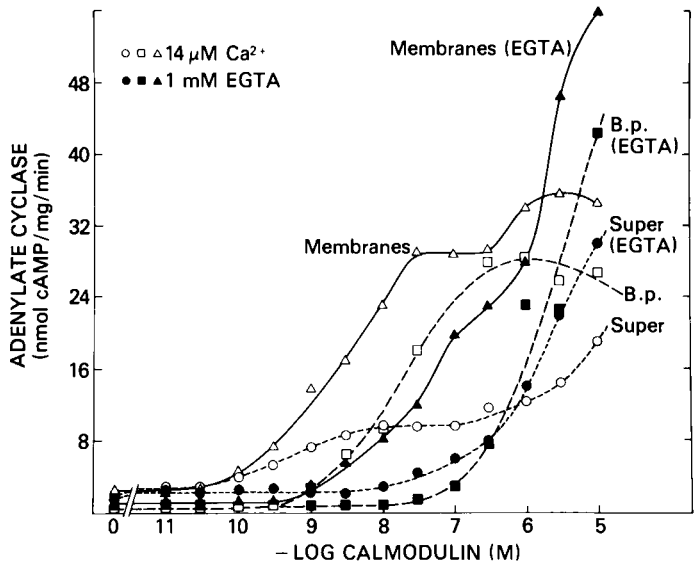

Fig. 3. Activation of different preparations of adenylate cyclase from B. pertussis by bovine brain calmodulin in the presence of 15 μM total Ca^{2+} or 1.0 mM EGTA. Assay conditions as in ref. 23.

two orders of magnitude more activator protein. Using a different strain of B. pertussis, Greenlee et al. (6) have independently found that the point for 50% activation was shifted from ~ 0.1 nM to ~ 50 nM by addition of excess EGTA. We have recently found similar shifts in the pure adenylate cyclase from strain 114. This would tend to rule out the presence of two enzymes with different affinities but cannot distinguish a single enzyme with two sites (one for Ca^{2+}·calmodulin, the other for Ca^{2+}-free calmodulin) from a model with a single site with different affinities for the two forms of calmodulin. Since there are marked changes in the conformation of calmodulin upon occupancy of the Ca^{2+}-binding sites (24), we favor the second model at the present. At 10^{-8} M calmodulin it can be calculated that the concentration of Ca_1^{2+}·calmdoulin was ~ $7 \cdot 10^{-17}$ M (21) hence it appears highly probable that Ca^{2+}-free calmodulin has a stimulatory effect on the B. pertussis cyclase. A few other examples of Ca^{2+}-independent interactions of calmodulin with other proteins have been recorded (see ref. 22) but interactions with the Ca^{2+}-free conformation are certainly the exception.

A property often exhibited by calmodulin-activated enzymes is their ability to interact also with other hydrophobic surfaces, particularly phospholipids and certain other amphiphiles. The adenylate cyclase of B. pertussis responds in a similar manner (Table II). To avoid complications from interactions of these amphiphiles with cell membranes or other particulates, we studied primarily the soluble enzyme of the culture medium or purified preparations (25). The stimulation is less dramatic than obtained with calmdoulin and generally amounted to 3-10 fold. Characteristically, stimulation is not very sensitive to structural changes in the phospholipid such as the polar head group, chain length or degree of unsaturation. However, gangliosides are inhibitory. In fact, various nonionic detergents are effective and in the Triton-X series there is a slight increase in potency with increasing hydrophile-lipophile balance (HLB) number. Ca^{2+} plays an enhancing role also in this form of activation (25). Of considerable interest is the fact that stimulation occurs at concentrations of phospholipid below the critical micelle concentration (provided short chain lecithins are used to attain measurable values of this parameter). The same is true for the Triton X series. This suggests that there are high affinity hydrophobic binding sites on the cyclase. Nevertheless, an important point is that these hydrophobic sites appear to be at loci other than those that interact with calmodulin since the phospholipid effects are additive with maximal calmodulin concentrations. Furthermore, they enhance the sensitivity to calmodulin by an order of magnitude (25).

Attempts to purify the enzyme from the culture medium led initially to a single polypeptide chain of Mr = 70,000 (16). This enzyme was, however, poorly activated by calmodulin and we have since attempted to purify the enzyme from urea extract of intact B. pertussis organism. This led to an activity that yielded a single band in SDS-PAGE with an Mr of 47,000 that could still be activated 50-fold by calmodulin even in the presence of EGTA. The specific activity of a recent preparation was 85 μmoles cAMP/min/mg protein. This value is compared with several other prokaryotic cyclases in Table III. This enzyme can be further stimulated by phospholipids hence the attainable specific activity may be as much as 10-fold greater. The yield of this pure cyclase was very low and we are currently trying to increase the yield for physical studies on the enzyme, antibody production, etc. The relation between the 70 kDa enzyme and the 47 kDa enzyme may be revealed by this approach but is not clear at present. Proteolytic cleavage has not been vigorously ruled out - such reactions generally

TABLE III. Bacterial Adenylate Cyclases

Organism	Degree of Purification	Molecular weight	Specific activity	Ref.
		kDa	μmole/min/mg	
Streptococcus Salivarius	3200 X	-	0.41	26
Brevibacterium Liquefaciens	Pure	46 x 2	30	27
Bacaillus Anthracis	~ 80%	79	20	28
Bordetella Pertussis	Homogeneous	47	85	present study

activate basal activity but abolish the response to specific activators. This is the opposite of what has been observed so far with the two cyclases identified by us. Nor can we, as yet, account for the different sizes reported for the impure enzymes proposed by others: 77 kDa (6), 39 kDa (29) and 50 and 180 kDa (8). However, the possibility that different forms of the adenylate cyclase exist is a real one and is being explored. It should be pointed out that B. anthracis secretes an adenylate cyclase with properties very similar to the one discussed here. Its reported size is 89 kDa (28, 30).

A virulence function of this extracellular adenylate cyclase seems highly probable from its association with virulent strains and absence in degraded strains as well as its presence in the disease-producing but pertussis toxin deficient strains - B. parapertussis and B. bronchoseptica. Early data suggest the existence of extracellular factors that inhibited neutrophils (31). Subsequently, Confer et al. (29, 32) have shown that one of these is likely to be a form of the adenylate cyclase discussed above, which is believed to invade a number of cells and there find both ATP and calmodulin leading to the rapid production of huge and paralyzing levels of cAMP. This, in turn, is felt to protect the Bordetella against phagocytosis. Whether or not a cofactor is required for invasion of the host cell as in the case of B. anthracis (28, 30) is not established at present.

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BORDETELLA ADENYLATE CYCLASE TOXIN: ITS INTOXICATION[1] OF MAMMALIAN CELLS AND EFFECTS ON CELL FUNCTION

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I. INTRODUCTION

The *Bordetella* species produce an adenylate cyclase which has several novel features. As described by Wolff in this volume, the enzyme is predominantly extracytoplasmic in location (1), is activated by the eukaryotic calcium-binding regulatory protein, calmodulin (2,3), and is unaffected by mammalian hormones, guanine nucleotides (4), or forskolin. When this unusual enzyme was first purified from supernatant culture medium of *B. pertussis*, it was tested for its ability to affect cultured mammalian cells which would be expected to respond to an increase in cAMP concentration. In those experiments (Hewlett and Wolff, unpublished results), the purified enzyme had no demonstrable biological effects and its potential as a toxin and virulence factor was cast into doubt. Several years ago, however, there were two major developments which led to a renewed interest in *Bordetella* adenylate cyclase and a recognition of its toxin activity.

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First, Leppla discovered that Bacillus anthracis edema factor (EF) was a calmodulin-dependent, extracytoplasmic adenylate cyclase similar in enzymatic properties to the Bordetella enzyme (5). This factor, which had no toxin activity alone, was able to cause edema when combined with another protein from B. anthracis, called protective antigen (PA), and injected into the skin (6). Leppla demonstrated the molecular basis for this activity in vitro; that is, EF plus PA caused increased cAMP levels and elongation in Chinese hamster ovary cells while neither factor alone had any effect (5). The accumulation of cAMP apparently resulted from PA-facilitated entry of EF into the target cell with subsequent production of cAMP from endogenous ATP. The similarity to Bordetella adenylate cyclase suggested that a separate factor (analogous to PA) might also be required for the Bordetella enzyme to have such an effect. The second important observation was that of Confer and Eaton who found that urea extraction of intact B. pertussis organisms resulted in the solubilization of a form of adenylate cyclase with very high enzymatic activity and the ability to increase cAMP levels in human neutrophils and alveolar macrophages (7). The functional consequence of this intoxication was impairment of the oxidative burst and the ability of these cells to kill ingested bacteria. They also reported that, in contrast to the material extracted from whole cells, supernatant culture medium, which was the source of the nontoxic enzyme previously purified had little activity when added to the target cells.

Together these observations strongly suggested that indeed there was a form of the Bordetella adenylate cyclase which is able to bind to and intoxicate mammalian cells, as well as a nontoxic form. Because crude materials were used in these studies, the data were only indirect that the Bordetella adenylate cyclase, rather than some other bacterial component capable of activating the endogenous adenylate cyclase of the mammalian target cell, was responsible for the observed effects.

II. EVIDENCE FOR TOXIN ACTIVITY OF BORDETELLA ADENYLATE CYCLASE

Two general approaches have been employed to document the role of the Bordetella adenylate cyclase as a toxin capable of elevating cAMP levels in extract-treated target

cells. First, transposon Tn5 - produced mutants of B. pertussis, deficient in specific virulence factors (8,9), have been used to demonstrate that the extracytoplasmic adenylate cyclase is responsible for increases in cAMP and associated changes in target cell function (10,11). For example, extracts from two wild type strains of B. pertussis (BP338 and 165), as well as from mutants deficient in hemolysin production only (BP349) or pertussis toxin only (BP357), were able to elicit massive cAMP accumulation in S49 lymphoma cells (Hewlett, Weiss et. al. submitted for publication, 12) (Table 1). In contrast, extracts from a mutant deficient in hemolysin

Table I. Effect of extracts from wild type and Tn5 mutant B. pertussis organisms on cAMP levels in S49 lymphoma cells

Addition[+]	Phenotype	S49 cAMP (pmol/mg protein)
None	--	12.6
Extract from:		
BP338*	HLY^+ AC^+ PT^+	17,940
165	HLY^+ AC^+ PT^+	23,420
BP347	HLY^- AC^- PT^-	13.5
BP348	HLY^- AC^- PT^+	13.8
BP349	HLY^- AC^+ PT^+	9,181
BP357	HLY^+ AC^+ PT^-	10,561

[+]S49 cells were incubated with additions at 37°C for 1 hour, the medium then removed and intracellular cAMP extracted by 0.1N HCl.

*parental strain for Tn5 mutants
HLY = hemolysin
AC = adenylate cyclase toxin
PT = pertussis toxin

and adenylate cyclase, but able to produce pertussis toxin (BP348) and an avirulent mutant unable to produce any known virulence factors (BP347) were without effect on cAMP levels in S49 cells. Together, these data indicate that Bordetella adenylate cyclase can function as a toxin and that this molecule, and not pertussis toxin, is responsible for the profound increases in cAMP levels seen in cells treated with Bordetella extract.

A second approach has been used to distinguish between an intracellular action of Bordetella adenylate cyclase toxin and an activation of the endogenous adenylate cyclase of the target cell by some other component of the bacterium. When the target cell adenylate cyclase was activated at any of three levels, through the β-adrenergic receptor (by isoproterenol), the guanine nucleotide-binding protein, N_s (by cholera toxin), or the catalytic subunit (by forskolin), it remained susceptible to inhibition by the inhibitory hormone, somatostatin (12,13, Hewlett et al. submitted for publication). Exposure of S49 lymphoma cells to extract from BP338 resulted in cAMP accumulation (Table 1) which was an order of magnitude greater than that achieved by stimulation of the endogenous adenylate cyclase by any agonist (data not shown). Furthermore, the Bordetella extract-elicited cAMP accumulation was unaffected by the inhibitory agonist, somatostatin (data not shown). These data indicate that in cells exposed to Bordetella extract, cAMP is being generated by the autonomous bacterial enzyme functioning independently of cellular regulatory mechanisms and thus support the hypothesis that the Bordetella adenylate cyclase is indeed a toxin capable of entering and intoxicating the target cell.

III. TARGET CELL TYPES AND CONSEQUENCES OF BORDETELLA ADENYLATE CYCLASE INTOXICATION

A number of cell types have been investigated for their susceptibility to intoxication by Bordetella adenylate cyclase toxin (Table 2). From these studies, it has become apparent that in addition to its likely role as a virulence factor for B. pertussis (8), this novel enzyme/toxin is an extremely useful probe for the study of cAMP-regulated processes in diverse cell types. In their initial studies (vide supra), Confer and Eaton showed that neutrophils and alveolar macrophages have impaired

Table II. Target cells intoxicated by *Bordetella* adenylate cyclase toxin and the functional consequences of intoxication

Target Cell	Effect	Reference
Polymorphonuclear leukocyte	Inhibition of chemotaxis, phagocytosis, superoxide generation, and microbial killing	8
	Alteration of PMN-induced inhibition of carbohydrate incorporation by *Coccidioides immitis*	15
Alveolar macrophage	Inhibition of superoxide generation	8
Peripheral blood monocyte	Inhibition of oxidative burst	14
Natural killer (NK) cell	Inhibition of target cell lysis	16
HL-60 promyelocytic leukemia cells	Decreased growth rate and enhanced differentiation	17
S49 lymphoma cells	Inhibition of growth	12, unpublished data
Rat pituitary cells	Enhanced hormone secretion	18

chemotaxis, a reduced oxidative response and decreased killing capacity after treatment with *Bordetella* extract (7). Subsequently, Pearson et al. demonstrated similar effects in human peripheral blood monocytes and determined that at one concentration of adenylate cyclase toxin it is possible to block the oxidative response to soluble and

particulate stimuli without affecting phagocytosis (14). Galgiani et al. found that in addition to blocking the oxidative burst, the toxin could attenuate neutrophil-induced inhibition of N-acetylglucosamine incorporation by arthroconidia of Coccidioides immitis (15). Finally, Slungaard et al. have demonstrated that phenotypic characteristics of several malignant cell lines, including growth rate, can be affected by treatment with this adenylate cyclase toxin (17).

Another immune effector cell function which can be impaired by adenylate cyclase toxin is the cytotoxic activity of human natural killer (NK) cells. Exposure of the human peripheral blood non-adherent mononuclear cell fraction which includes large granular lymphocytes (natural killer cells) to Bordetella pertussis extract containing adenylate cyclase toxin and pertussis toxin resulted in 98% inhibition of cytotoxic activity against ^{51}Cr-labelled target tumor cells (16) (Table 3). In this assay system, however, there appeared to be a contribution of both adenylate cyclase toxin and pertussis toxin, since extract from B. parapertussis (containing adenylate cyclase toxin only) was less potent in inhibiting cytotoxicity (67% inhibition) and purified pertussis toxin alone could cause partial inhibition. These data serve as the first example of a combined effect of two toxins from B. pertussis apparently by different mechanisms.

Table III. Effect of Bordetella adenylate cyclase toxin on NK cell cytotoxicity (16)

Addition to Effector Cells	% Inhibition of Cytotoxicity
B. pertussis extract (adenylate cyclase toxin and pertussis toxin)	98
B. parapertussis extract (adenylate cyclase toxin)	67
Purified pertussis toxin (10 ng/ml final concentration)	62

*Lysis of ^{51}Cr-labelled K562 tumor cells quantitated by release of ^{51}Cr.

The extreme magnitude of cAMP accumulation and the fact that all of the observed effects were inhibitory raised the possibility that the actions of the adenylate cyclase toxin might represent a consequence of ATP depletion, rather than being mediated by the accumulated cAMP. Studies of hormone secretion from rat pituitary *in vitro* have allayed such concerns and further illustrated the utility of this toxin as a research tool (18, Cronin et al. submitted for publication). Exposure of dispersed cultures of rat pituitary to the extract from wild type, but not adenylate cyclase-deficient *B. pertussis*, results in rapid stimulation of growth hormone and prolactin secretion and gradual enhancement of luteinizing hormone secretion. Similar effects are seen with other agents which increase cAMP levels by activation of the endogenous pituitary cell adenylate cyclase, supporting the hypothesis that the observed effects of adenylate cyclase toxin are mediated by cAMP.

IV. PROPOSED STRUCTURE FOR *BORDETELLA* ADENYLATE CYCLASE TOXIN

Several features of the *Bordetella* adenylate cyclase toxin have suggested that the molecule may follow the A/B model proposed for a number of bacterial toxins. In this model the A or active subunit possesses enzymatic activity and the B or binding subunit enables the A subunit to interact with and enter target cells (19). Therefore, the A subunit can have enzymatic activity, but alone cannot intoxicate intact target cells. The analogy with anthrax toxin, in which EF is the putative A subunit and PA the B subunit, provides indirect support for this contention. In this study, fractionation of *Bordetella* extract by ion exchange chromatography (Figure 1) or gel permeation chromatography (20) has revealed multiple peaks

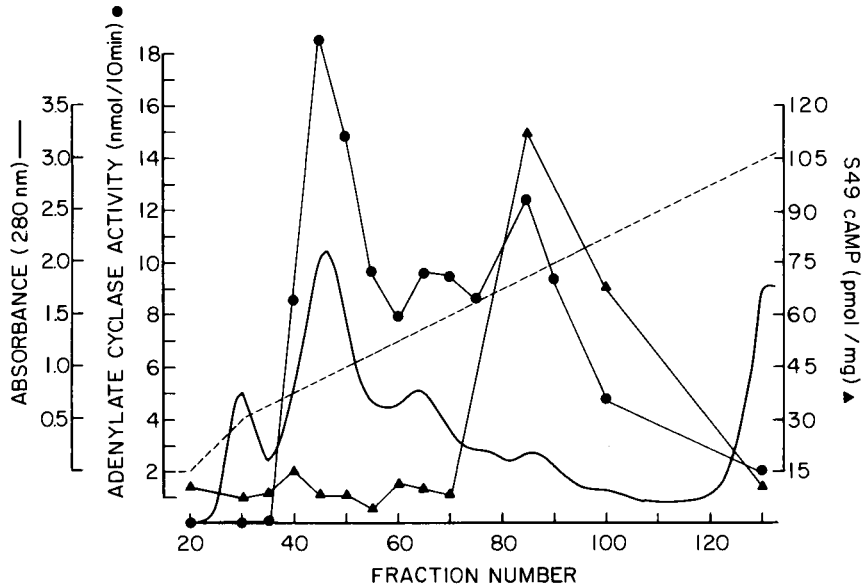

Figure 1. Ion exchange chromatographic fractionation of B. pertussis urea extract. Urea extract dialyzed against ammonium acetate (0.01M) was loaded onto a 40 ml. DEAE-sephadex column. The column was washed and eluted with a NaCl gradient from 0-500 mM in ammonium acetate. Elution profile was determined spectrophotometrically at 280 nm. Adenylate cyclase enzymatic activity is shown in closed circles and adenylate cyclase toxin activity (cAMP levels in S49 cells) shown in closed triangles.

of adenylate cyclase enzymatic activity, only one of which has significant adenylate cyclase toxin activity (as measured by ability to increase S49 cell cAMP levels). The adenylate cyclase toxin activity has an apparent M_r >100,000 and has been postulated to represent the holotoxin molecule (20). An additional peak with lower apparent molecular weight and enzymatic activity only, may be the isolated A subunit equivalent which lacks a B subunit and thus possesses no toxin activity. This is

presumably analogous to the enzyme originally purified from the culture medium of B. pertussis (4). Final resolution of these issues awaits further purification of both forms of the toxin/enzyme.

In conclusion, many details about this unusual toxin remain to be elucidated. In addition to determination of the toxin structure, major areas of investigation are the mechanism of binding and entry of the toxin into host cells and the localization of the enzyme once internalized. Better definition of the intracellular location of the adenylate cyclase and the resultant cAMP accumulation may greatly enhance the understanding of the entry mechanism. It is clear that further studies of this novel enzyme/toxin will be useful not only to Bordetella research, but will also contribute to many disciplines in which cAMP-dependent processes are involved.

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