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# Promotion of lipolysis in rat adipocytes by pertussis toxin: Reversal of endogenous inhibition

(adenosine/cyclic AMP/adenylate cyclase/cholera toxin)

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**ABSTRACT** **Pertussis toxin (PT), a protein produced by *Bordetella pertussis*, was studied for its effect on lipolysis in isolated rat epididymal adipocytes. Exposure of adipocytes to pertussis toxin resulted in a significant increase in cyclic AMP levels and lipolysis after a lag of 1–2 hr. Both the maximal rate of lipolysis and the time lag (beyond 1 hr) were PT concentration-dependent. Heat treatment (95°C, 30 min) or incubation with specific antibody directed against PT eliminated the ability of toxin to increase lipolysis. Cell-free culture medium from *B. pertussis*, but not from nontoxigenic *Bordetella* species, had the same effect on lipolysis as purified toxin. Comparison of the PT effect with the known lipolytic effect of cholera toxin (CT) revealed that the two toxins elicited responses that were indistinguishable in time course and magnitude. In contrast, the adenylate cyclase (EC 4.6.1.1) activities in membranes prepared from PT- or CT-treated adipocytes were different. Adenylate cyclase activity in membranes from control (untreated) adipocytes was inhibited 35–64% by the adenosine analogue $N^6$-(L-2-phenylisopropyl)-adenosine. As expected from previous studies, membranes from CT-treated adipocytes demonstrated an increased basal activity but showed the same proportional inhibition by $N^6$-(L-2-phenylisopropyl)-adenosine as controls. On the other hand, membranes from adipocytes exposed to PT (400 ng/ml for 4 hr) showed no increase in basal adenylate cyclase activity but had reduced sensitivity to $N^6$-(L-2-phenylisopropyl)-adenosine inhibition, with the maximal effect ranging from 11 to 30% at $10^{-6}$ M $N^6$-(L-2-phenylisopropyl)-adenosine. These data support the hypothesis that PT promotes cyclic AMP-dependent lipolysis in a manner quantitatively equivalent to CT, but by a different mechanism involving increased cyclic AMP levels resulting from loss of responsiveness to endogenous inhibitors such as adenosine.**

Pertussis toxin (PT), a protein exotoxin from *Bordetella pertussis*, has been shown to modify the hormone responsiveness of a number of *in vitro* cultured cells (1–4, ¶). In general, PT treatment results in an augmentation of the response to hormones or other agents that cause cyclic AMP accumulation and a reduction or abolition of the response to inhibitory hormones that inhibit cyclic AMP accumulation. In all cases, there has been little or no effect on basal levels of cyclic AMP in the absence of added hormone.

Endoh *et al.* (5) and Sekura and Manclark (6) have shown that PT induces lipolysis in isolated rat adipocytes and that this effect can be used as an *in vitro* assay for toxin. Neither study addressed the mechanism of lipolysis or the apparent paradox of a primary toxin effect in the absence of hormonal stimulation. Recently, Garcia-Sainz studied lipolysis in adipocytes from pertussis vaccine-treated hamsters (7). Although there was no change in the basal rate of lipolysis, isoproterenol-stimulated cyclic AMP accumulation and lipolysis in cells from vaccine-treated animals was less sensitive to inhibition by $\alpha$-adrenergic agonists, prostaglandin $E_2$, and adenosine.

In the present study, we have characterized the effect of purified PT on lipolysis in dispersed rat epididymal adipocytes and on the adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activity in membranes from those cells. The results indicate that the PT effects on adipocytes are consistent with the understanding of its mechanism of action in other cells and illustrate the potential utility of this toxin as a tool for studying the regulation of cellular hormone responsiveness.

## MATERIALS AND METHODS

Bovine serum albumin (fraction V), adenosine, adenosine deaminase, isobutylmethylxanthine, ATP, GTP, isoproterenol, and cholera toxin (CT) were purchased from Sigma. $N^6$-(L-2-Phenylisopropyl)-adenosine was from Boehringer Mannheim. [$\alpha$-$^{32}$P]-ATP was prepared enzymatically from adenosine and $^{32}$P (obtained from New England Nuclear) by the method of Johnson and Walseth (8). Collagenase was purchased from Worthington. Ro 20-1724 was generously supplied by W. E. Scott (Hoffmann–La Roche).

**Preparation of the PT Protein.** PT was prepared from the supernatant culture medium of *B. pertussis*, strain 114, by modification of published methods (9–11). Organisms were grown in modified Stainer–Scholte medium (12) for 44–48 hr and the culture medium was then clarified by centrifugation. Toxin was concentrated on hydroxylapatite (Calbiochem) and eluted with phosphate buffer (100 mM, pH 7.0) containing sodium chloride (0.5 M) at 4°C as described (9). The hydroxylapatite eluate was further purified by affinity chromatography. Cyanogen bromide-activated Sepharose 4B was reacted with sheep hemoglobin. Ten milligrams of hemoglobin was coupled per gram of Sepharose gel. Two hundred milliliters of pooled human plasma (from approximately 10 donors) was passed over the 35 ml of hemoglobin-Sepharose column to allow haptoglobin-hemoglobin binding. The gel was then washed extensively with phosphate buffer (100 mM, pH 7.0) containing NaCl (0.5 M) and finally with potassium thiocyanate (3 M). This procedure created a haptoglobin affinity system functionally equivalent to that described by Irons and MacLennan (10).

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Abbreviations: PT, pertussis toxin; CT, cholera toxin.

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The hydroxylapatite eluate was passed over the affinity column at room temperature and the column was washed sequentially with sodium phosphate (100 mM, pH 7.0) with NaCl (0.5 M) and then with Tris·HCl (100 mM, pH 10) containing NaCl (0.5 M). The adsorbed toxin was eluted with KI (3 M) in Tris·HCl (100 mM, pH 10) with NaCl (0.5 M) and was concentrated and dialyzed against phosphate buffer (100 mM, pH 7.0) with NaCl (0.5 M) by ultrafiltration on an Amicon PM-10 membrane. The resultant preparation of toxin, containing 120–510 μg of protein per ml, either yielded a single Coomassie blue-stained band on polyacrylamide gels in nondissociating buffers or demonstrated a subunit pattern identical to purified toxin on $NaDodSO_4$/polyacrylamide gel electrophoresis. Biological activity was evaluated by production of histamine sensitization in mice (13) and by induction of the characteristic morphologic clustered growth pattern in Chinese hamster ovary cells (14). Antibodies against PT and filamentous hemagglutin were generously provided by James Cowell (Pertussis Branch, Office of Biologics, National Center for Drugs and Biologics, Food and Drug Administration).

**Cell Preparation.** Epididymal fat pad adipocytes were prepared from 150- to 250-g Sprague–Dawley rats. Each fat pad was cut into 1- to 3-mm pieces, placed into 5 ml of Dulbecco modified Eagle medium containing 1% bovine serum albumin and 1 mg of collagenase per ml (pH 7.5), and agitated for 1 hr at 37°C. Cells were washed through 250-μm nylon mesh to obtain a single cell suspension and an aliquot was counted in a hemocytometer.

**Lipolysis.** Cells were suspended in Krebs–Ringer phosphate buffer with 5% bovine serum albumin (pH 7.45) at 1.0–1.5 × $10^5$ cells per ml, with additions as indicated. Cells were maintained at 37°C with gentle agitation and the reaction was terminated at the described times by the addition of trichloroacetic acid to give a final concentration of 5%. Glycerol release was measured in aliquots of those trichloroacetic acid-precipitated samples by an enzymatic fluorometric assay by using the method of Laurell and Tribbling (15). In preliminary experiments, no added adenosine was included, but after the importance of adenosine was appreciated, $10^{-7}$ M adenosine was added to the washing and incubation buffers to approximate usual tissue levels. This produced no significant difference at the 2- to 4-hr time points but resulted in more uniform values of glycerol at the very early time points.

**Adenylate Cyclase and Cyclic AMP.** A single cell suspension of fat cells was prepared as for lipolysis and incubated at 37°C in Krebs–Ringer phosphate buffer with 5% bovine serum albumin (pH 7.45) with additions for the indicated lengths of time prior to membrane preparation. Cells were osmotically lysed by using the method of Birnbaumer *et al.* (16). Adenylate cyclase activity was assayed according to the method of Salomon *et al.* (17), which was modified by the addition of NaCl (80 mM), GTP (1 μM), and adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) (1 unit/ml) and the substitution of Ro 20-1724 (100 μM) for unlabeled cyclic AMP. Adenylate cyclase assay results are expressed as pmol of cyclic AMP formed per min/mg of protein. Cyclic AMP was measured in trichloroacetic acid extracts of intact adipocytes by automated radioimmunoassay (Gammaflow, Squibb) (18). Proteins were assayed by the method of Lowry (19) with bovine serum albumin as the standard.

## RESULTS AND DISCUSSION

Incubation of adipocytes with PT resulted in a dose-dependent increase in the rate of lipolysis. As shown in Fig. 1, increasing toxin concentration both increased the rate of lipolysis and reduced the lag, with minimal lag of 1 hr. At toxin concentrations of 4 ng/ml or less, there was no detectable change in lipolysis at 4 hr.

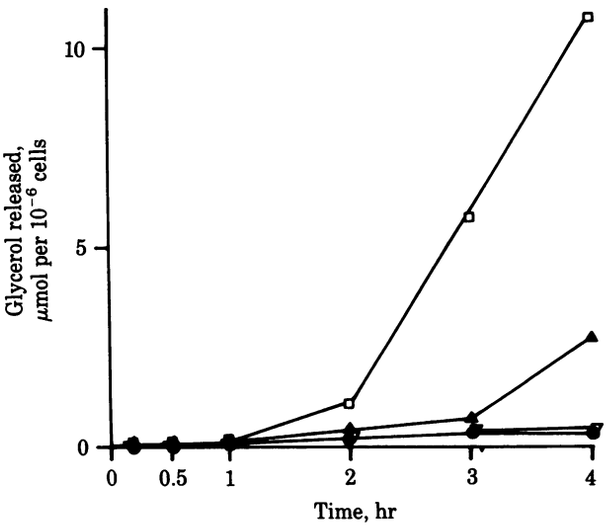


FIG. 1. Time and concentration dependence of PT-induced lipolysis. PT (final concentrations: 400 ng/ml, □; 40 ng/ml, ▲; 4 ng/ml, ▽) or control buffer (●) was added to fat cell suspensions at time zero. Aliquots were removed at the times indicated and assayed for glycerol accumulation.

Several features established the specificity of this effect for PT. First, the lipolytic activity was lost when the toxin preparation was heated at 95°C for 30 min (data not shown), consistent with the recognized heat lability of this toxin (13).

Second, when PT was incubated with anti-PT antibody, the toxin-mediated induction of lipolysis was prevented. In contrast, exposure of toxin to antibody against filamentous hemagglutinin, an outer membrane protein of *B. pertussis*, had no effect on the lipolytic action of the toxin (Table 1). Finally, cell-free supernatant culture media from two toxigenic strains of *B. pertussis*, 114 and UVa-1, were able to elicit lipolysis of the same magnitude. Culture media from nontoxigenic *Bordetella* species, *B. parapertussis* and *B. bronchiseptica*, as well as nontoxigenic *B. pertussis* strain UT 25-80 had no such effect.

Because other bacterial exotoxins are known to induce li-

Table 1. Lipolysis induced by purified PT or crude culture media from *Bordetella* organisms

| Addition | Glycerol released, μmol per $10^{-6}$ cells |
|---|---|
| None | 0.16 ± 0.004 |
| PT (400 ng/ml) | 4.35 ± 0.000 |
| + Anti-PT | 0.16 ± 0.000 |
| + Anti-FHA | 4.83 ± 0.015 |
| Crude culture medium from | |
| *B. pertussis* 114 | 4.54 ± 0.055 |
| *B. pertussis* UVa-1 | 4.35 ± 0.076 |
| *B. bronchiseptica* 469 | 0.15 ± 0.000 |
| *B. bronchiseptica* JOH | 0.15 ± 0.003 |
| *B. parapertussis* 501 | 0.14 ± 0.003 |
| *B. pertussis* UT 25-80 | 0.14 ± 0.023 |

The standard lipolysis assay was used to test for neutralization of PT effect by mixing with antitoxin antibody (anti-PT) or antifilamentous hemagglutin (anti-FHA) for 30 min before addition to the fat cell suspension. Crude culture medium from the indicated *Bordetella* species was added in a final dilution of 1:100. Incubation was for 4 hr. Aliquots of incubation medium were removed and assayed for glycerol. Results are expressed as mean ± SD. Strain UT 25-80 is a degraded nontoxigenic strain of *B. pertussis*.

polysis in rat adipocytes (20), we felt that comparison of the PT effect with that of CT might be useful in elucidating the mechanism involved. As demonstrated in Fig. 2, PT (400 ng/ml) and CT (1 μg/ml) induced lipolysis with indistinguishable time courses and of equivalent magnitudes. Because the activity of triglyceride lipase (EC 3.1.1.3), which catalyzes lipolysis, is regulated through a cyclic AMP-dependent protein kinase (21), an increase in the rate of lipolysis implies either an increase in intracellular cyclic AMP concentration or, less likely, a direct effect on the kinase or the lipase. The possibility that PT might be acting by increasing intracellular cyclic AMP levels was surprising in that other cultured cell systems in which the toxin has been studied respond with markedly altered hormone responsiveness but with no change in basal cyclic AMP levels (1–5). The predominant effect of PT in those systems has been to decrease or abolish the effect of inhibitory hormones that reduce cyclic AMP levels. Because lipolysis in isolated adipocytes is thought to be under tonic inhibition by endogenous cellular products, such as adenosine (22), we considered the possibility that the stimulation of lipolysis by PT might simply reflect relief of such inhibition with resultant increase in cyclic AMP and lipolysis.

To test this hypothesis, we first compared the PT-induced lipolysis with that elicited by several agents known to interfere with adenosine effects. Adenosine deaminase (1 unit/ml) increased lipolysis from the time of addition to the incubation medium, but the magnitude was not as great as that induced by PT (400 ng/ml) (data not shown). On the other hand, isobutylmethylxanthine (1 mM) stimulated lipolysis without a lag and at a rate parallel to that obtained with PT after 2 hr of incubation (Fig. 3). Although methylxanthines are known to inhibit phosphodiesterase, it has been shown by Londos *et al.* (23) that lipolysis is increased at concentrations lower than those required to inhibit phosphodiesterase. This inhibitory effect of methylxanthines is attributed to antagonism of the action of adenosine at its R-site receptor. This concept is further supported by the observation that under the conditions employed here a phosphodiesterase inhibitor without adenosine receptor-blocking activity, Ro 20-1724, had no effect on lipolysis (Fig. 3).

To determine if the induction of lipolysis by isobutylmethylxanthine and PT was, indeed, mediated by cyclic AMP, we measured adipocyte cyclic AMP levels. As shown in Table 2,

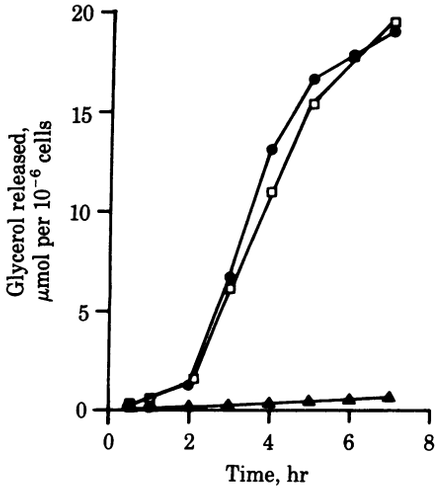


FIG. 2. Time course of lipolysis produced by PT or CT. PT (400 ng/ml, □), CT (1 μg/ml, ●), or control buffer (▲) was added to fat cell suspensions at time zero. Aliquots were removed at times indicated and assayed for glycerol accumulation.

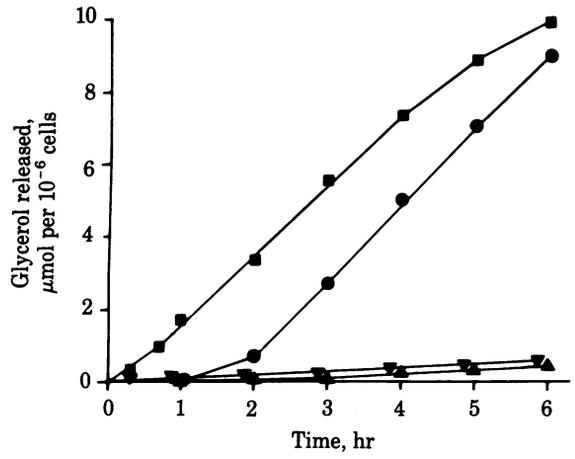


FIG. 3. Time course of isobutylmethylxanthine- or PT-induced lipolysis. Isobutylmethylxanthine (1 mM, ■), Ro 20-1724 (1 mM, ▼), PT (400 ng/ml, ●), or control buffer (▲) was added to fat cell suspensions at time zero. Aliquots were removed at times indicated for glycerol determination. Ethanol, in which the Ro 20-1724 was dissolved, had no effect on lipolysis.

isobutylmethylxanthine elicited a 3-fold increase in the cyclic AMP concentration of cells exposed to the drug for 30 min, whereas PT-treated cells, not yet manifesting lipolysis, had no change in cyclic AMP concentration. On the other hand, after 2 hr of incubation the cyclic AMP level (mean ± SEM) in PT-treated cells had increased to 62.2 ± 0.8 pmol/mg of protein and that in isobutylmethylxanthine-treated cells was persistently elevated at 22.3 ± 0.4 pmol/mg of protein. These data are consistent with the observations of Butcher *et al.* (24), indicating that only a small (2- to 3-fold) elevation of cyclic AMP concentration is sufficient to induce maximal lipolysis.

However, with these data it was not possible to determine whether PT was acting to block adenosine effects, as isobutylmethylxanthine, or activating the adenylate cyclase directly, as CT. In fact, the 1-hr latency exhibited by PT was equivalent to that required by CT and was in contrast to the rapid onset of isobutylmethylxanthine-promoted lipolysis. The latency in CT action has been attributed to time required for toxin to penetrate the cell and initiate its ADP-ribosylation of the guanine nucleotide binding protein G/F, ultimately resulting in acti-

Table 2. Effect of isobutylmethylxanthine and PT on rat adipocyte cyclic AMP

| Addition | Cyclic AMP, pmol per $10^{-6}$ cells | |
|---|---|---|
| | 30 min | 2 hr |
| None | 13.5 ± 0.6 | 12.6 ± 0.2 |
| Isobutylmethylxanthine (1 mM) | 29.8 ± 2* | 22.3 ± 0.4* |
| PT (750 ng/ml) | 13.0 ± 0.5† | 62.2 ± 0.8* |

Rat adipocytes were isolated and prepared as described for lipolysis experiments, except that they were suspended in Krebs–Ringer phosphate buffer with 1% bovine serum albumin. Cells were incubated with control buffer (none), isobutylmethylxanthine, or PT for the times indicated, and the reaction was terminated by the addition of trichloroacetic acid to a concentration of 10%. The trichloroacetic acid was removed by five extractions using 5 vol of water-saturated ether, and the resultant solution was assayed for cyclic AMP by automated radioimmunoassay. The data represent points assayed in quadruplicate from a representative experiment. However, due to changes in absolute values of cyclic AMP with rat size, data from the five experiments demonstrating the same results were not averaged.

* Significantly different from control (no additions) at $P < 0.001$ by unpaired Student's $t$ test.

† Not significantly different from control.

vation of the adenylate cyclase and increase in cyclic AMP levels (25). However, it has been noted that cyclic AMP accumulation and lipolysis in CT-treated cells remain capable of inhibition by adenosine analogues (26) and adenylate cyclase in membranes from CT-treated cells is sensitive to $\alpha$-adrenergic inhibition (27).

These features of the CT effect were used to investigate further the mechanism of PT-induced lipolysis. We compared the adenylate cyclase activity of adipocyte membranes from control, PT-treated (400 ng/ml, 4 hr), and CT-treated (1 $\mu$g/ml, 4 hr) adipocytes for their response to the adenosine deaminase-resistant adenosine analogue $N^6$-(L-2-phenylisopropyl)-adenosine (Fig. 4). In control membranes [in the experiment shown in Fig. 4, basal activity (mean ± SD) = 38 ± 1.5 pmol/min per mg of protein], $N^6$-(L-2-phenylisopropyl)-adenosine inhibition was maximal at $10^{-7}$ M and in four separate experiments ranged from 35 to 64% of basal adenylate cyclase activity. Membranes derived from adipocytes treated with CT manifested the expected 6- to 10-fold increase in activity (232 ± 7.0 pmol/min per mg of protein) but were inhibited equally by $N^6$-(L-2-phenylisopropyl)-adenosine. On the other hand, PT treatment yielded membranes with a small, but reproducible, decrease (mean ± SD) in adenylate cyclase activity relative to control (27 ± 0.8 pmol/min per mg of protein) but a significantly reduced sensitivity to $N^6$-(L-2-phenylisopropyl)-adenosine inhibition (relative to control and CT). The $N^6$-(L-2-phenylisopropyl)-adenosine inhibition ranged from 11 to 30% under the conditions described and could be totally abolished with higher toxin concentration or longer duration of exposure. Thus, it was apparent that although the time course of PT-induced lipolysis is identical to that elicited by CT, there is no permanent activation of the adenylate cyclase. Rather, the lipolytic effect of PT results from the reduced inhibition by adenosine or other inhibitors endogenous to the adipocyte (or both). The consistent decrease in basal adenylate cyclase activity seen in membranes from PT-treated cells was also present in membranes from isobutylmethylxanthine-treated cells (data not shown). This effect may reflect a cyclic AMP-mediated desensitization, as has been demonstrated for a variety of hormone-sensitive adenylate cyclase systems (28, 29), or an inhibitory effect of one or more products of the massive lipolysis induced in these cells (unpublished data).

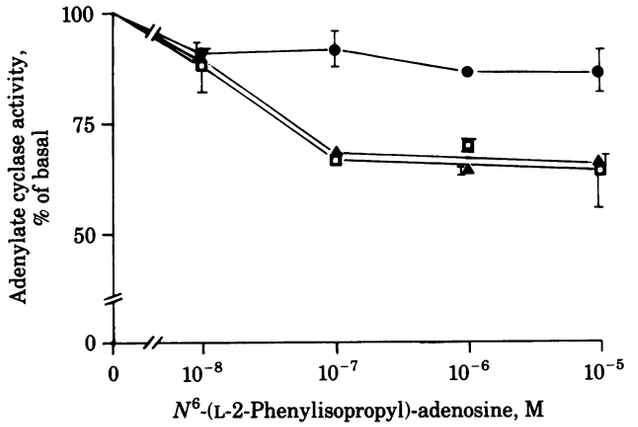


FIG. 4. Effect of $N^6$-(L-2-phenylisopropyl)-adenosine on adipocyte membrane adenylate cyclase. Intact adipocytes were treated with PT (400 ng/ml, 4 hr), CT (1 $\mu$g/ml, 4 hr), or control buffer. Membranes were prepared by the described method (13) and assayed for adenylate cyclase activity without or with $N^6$-(L-2-phenylisopropyl)-adenosine in the concentrations indicated. Results (mean ± SD) shown are from a representative experiment in which control membrane adenylate cyclase activity (75 $\mu$g of protein per assay tube, ▲) in the absence of $N^6$-(L-2-phenylisopropyl)-adenosine was 38 ± 1.5 pmol/min per mg of protein. Membranes from CT-treated cells (65 $\mu$g of protein per assay tube, □) had activity of 232 ± 7.0 pmol/min per mg of protein, whereas those from PT-treated cells (67 $\mu$g of protein per assay tube, ●) had activity of 27 ± 0.8 pmol/min per mg of protein.

Although the mechanism of PT-induced increases in cyclic AMP and lipolysis appears to be different from that of CT, there are important similarities between the two toxins. The PT-mediated reduction in inhibitory hormone responsiveness has been shown to be associated with ADP-ribosylation of a membrane protein, $M_r$ = 41,000, which is clearly distinct from that modified by CT (30, 31). In light of the extensive data by Ui and his co-workers (1–3, 30, 32) and others (refs. 7, 33, and 34; unpublished data) as well as that presented here, it appears likely that the target protein modified by PT is the guanine nucleotide-dependent regulatory protein postulated to couple inhibitory receptors to the adenylate cyclase complex (referred to as $G_i$ or $N_i$) (35). NAD-dependent covalent modification of $N_i$ would alter the molecule in such a way that inhibitory coupling to the adenylate cyclase is impaired or blocked. The wide spectrum of inhibitory agents affected by PT, including $\alpha$-adrenergic (1, 2, 7), muscarinic cholinergic (3, ¶), opiate (3, 31, ¶), and dopaminergic (4) as well as adenosine (2, 7), suggests that all of these compounds may exert their influence through this common pathway, much as the stimulatory agonists for adenylate cyclase require the presence of the guanine nucleotide binding component, G/F (also referred to as $G_s$ or $N_s$) (30–35).

In conclusion, we believe that lipolysis in an isolated adipocyte preparation provides a sensitive assay for the biological activity of PT in the ng/ml range, as proposed by Endoh *et al.* (5). We have provided evidence that the toxin acts by blocking endogenous inhibitory mechanisms. With this understanding it is clear that this microbial product will be a useful tool for further dissection of cellular metabolic regulation and hormone responsiveness in adipocytes and other cells.

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