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Pertussis toxin stimulates delayed-onset, Ca^{2+}-dependent catecholamine release and the ADP-ribosylation of a 40 kDa protein in bovine adrenal chromaffin cells

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Pertussis toxin was found to stimulate catecholamine release from bovine adrenal chromaffin cells in a Ca^{2+}-dependent manner and in the absence of any stimulatory or inhibitory agonists for this cell. The release of catecholamine was associated with the ADP-ribosylation of an approx. 40 kDa protein present in the total membrane fraction. These results are consistent with the existence of an exocytosis-linked G-protein.

Exocytosis; Pertussis toxin; G-protein; ADP-ribosylation; Stimulus-secretion coupling; (Adrenal medulla)

1. INTRODUCTION

The exocytotic release of catecholamines from bovine chromaffin cells can be stimulated by a variety of secretagogues whose common mechanism of action appears to involve elevation of the cytosolic free Ca^{2+} concentration [1]. More recently however, the possibility had been raised that a class of GTP-binding proteins (G-proteins) might also be directly involved in exocytosis [2,3]. Hitherto, G-proteins has been thought to play an essential role only in the coupling of cell surface receptors to certain enzymes and ion channels [4].

One experimental approach used to investigate such an involvement of G-proteins in stimulus-secretion coupling in the bovine chromaffin cell has been to study the effects of GTP analogs on catecholamine release from permeabilized cells. This approach however, has yielded contradictory and inconclusive results. Using chromaffin cells permeabilized by high-voltage discharge, Knight and Baker [5] reported that Ca^{2+}-dependent catecholamine release was inhibited by GTPγS, whereas GMP-PNP had no effect. Bittner et al. [6], however, found that both GTPγS and GMP-PNP induced modest Ca^{2+}-independent catecholamine release from digitonin-permeabilized cells. Pretreatment of cells with pertussis toxin before permeabilization with digitonin had no effect on Ca^{2+}- or GMP-PNP-induced catecholamine release although a substrate for toxin-catalyzed ADP-ribosylation was found to be present.

More recently, using another experimental approach, Tanaka et al. [7] have investigated the effects of pertussis toxin on secretagogue-induced catecholamine release from intact bovine chromaffin cells. It was found that pretreatment of cultured chromaffin cells with pertussis toxin caused the cells to become hyper-responsive to the secretagogues nicotine and KCl. The acquisition of this hyper-responsiveness to secretagogues was coincident with the toxin-catalyzed ADP-ribosylation of a 40 kDa membrane protein. Pertussis toxin has been extensively used as a probe for G-protein function because it catalyzes the ADP-ribosylation of the α-subunit of certain G-

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proteins causing them to become uncoupled from their associated cell surface receptors.

Whilst reinvestigating the effects of pertussis toxin on cultured bovine chromaffin cells, we were able to confirm that pretreatment of cells with pertussis toxin indeed potentiated secretagogue-induced catecholamine release. However, we also found that pertussis toxin itself induced substantial catecholamine release. This phenomenon had not been reported previously and seemed obviously relevant to the fundamental problem of how the toxin, and possibly a G-protein substrate, might be involved in the exocytotic process. Here, we report a preliminary characterization of this phenomenon, and suggest that the secretory potency of pertussis toxin may serve to direct our attention to profoundly important steps in the regulation of exocytosis.

2. EXPERIMENTAL

2.1. *Preparation and culture of chromaffin cells*

Bovine adrenal chromaffin cells were isolated from adrenal medullae by collagenase digestion [8] and purified on a Percoll gradient [9]. The cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DME/F12) with 15 mM Hepes, L-glutamine and phenol red (Sigma, MO), supplemented with 26 mM $NaHCO_3$, 5% (v/v) heat-inactivated fetal calf serum (Gibco, NY) 100 μg streptomycin/ml, 100 U penicillin/ml, 250 ng Fungizone/ml (Gibco) and adjusted to pH 7.3. Cells were plated at a density of 10^6 cells/1.5 ml per 2 cm^2 in 24-well cluster plates (Costar, MA) and maintained at 37°C in a CO_2 incubator for 2–4 days before experiments.

2.2. *Catecholamine release experiments*

Pertussis toxin (List Biological, CA) was suspended in water at a concentration of 100 μg/ml and subsequently diluted as required in DME/F12 culture medium containing 15 mM Hepes and L-glutamine but without phenol red as supplied by Sigma, supplemented with 26 mM $NaHCO_3$ and adjusted to pH 7.3. This medium was used for all experimental cell incubations with the exception of the 1 h incubation described in the legend to fig.3 in which the $CaCl_2$ concentration of the medium needed to be manipulated. In this case, an extracellular release medium of the following composition was used: 118 mM NaCl, 4.7 mM KCl, 10 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 10 mM glucose and 25 mM Hepes adjusted to pH 7.3. All incubation volumes were 0.5 ml, and incubations were performed at 37°C in a CO_2 incubator. Catecholamine release was measured as described [10].

2.3. *ADP-ribosylation experiments*

After incubation of cells with pertussis toxin as described above, the incubation medium was removed and the plate placed on ice. To each well was added 0.4 ml ice-cold homogenization medium consisting of 10 mM Mes/Tris (pH 6.5), 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride and 0.1 mg leupeptin/ml. The cells were vigorously scraped off the bottom of the wells, the medium removed and the wells washed with a further 0.1 ml homogenization medium. Cell homogenates from duplicate wells were pooled and centrifuged at 164 000 × g_{av} for 40 min in a Beckman SW60 rotor. The membrane pellets were resuspended in 40 μl of 75 mM Tris-HCl (pH 7.5) and subjected to pertussis toxin-catalyzed ADP-ribosylation basically as described by Ribeiro-Neto et al. [11]. Thus, 10 μl of membrane suspension was incubated together with 10 μl activated pertussis toxin (prepared by incubating 1 vol. of 100 μg toxin/ml with 0.1 vol. of 0.275 M dithiothreitol at 32°C for 30 min), 10 μl of 60 μM [^{32}P]NAD (final spec. act. approx. 3 Ci/mmol; New England Nuclear, MA) and 30 μl of a mixture containing 10 mM thymidine, 1 mM ATP, 0.1 mM GTP and 1 mM EDTA/Tris (pH 7.5) for 45 min at 32°C. The reaction was terminated by the addition of 0.75 ml ice-cold 25% (w/v) trichloroacetic acid and the samples were stored overnight at 4°C. The samples were then centrifuged for 15 min at 4°C in a RT6000B benchtop centrifuge (Sorvall, DE), the supernatants discarded and any residual trichloroacetic acid removed from the protein pellets by washing twice with diethyl ether. The dry pellets were then subjected to a reduction/alkylation procedure which involved heating for 1 min at 90°C in 40 μl of a solution containing 5% (w/v) SDS and 10 mM dithiothreitol, allowing the samples to return to room temperature and then incubation for a further 15 min at room temperature after adding 10 μl of 0.1 M *N*-ethylmaleimide. Finally, 50 μl SDS-electrophoresis sample buffer was added and the samples were processed and subjected to electrophoresis on 12.5% (w/v) polyacrylamide/0.1% (w/v) bisacrylamide slab gels and autoradiography as described [10].

3. RESULTS

Our initial studies of the effects of pertussis toxin on chromaffin cells confirmed the finding of Tanaka et al. [7] that preincubation of cells with toxin resulted in potentiation of the secretory responses to nicotine and KCl (not shown). However, we also found that incubation of cells with pertussis toxin alone (100 ng/ml) resulted in the induction of catecholamine release after a lag period of approx. 2 h (fig.1). This lag time presumably represents the time required for the toxin to enter the cells and is a well known characteristic of the toxin's action on other cell types [12,13]. Over the course of an 8 h incubation with the toxin there was an approx. 15-fold increase in the amount of catecholamine released over control values, although by the end of this period the rate of release had begun to decline. There was no associated increase in the release of lactate dehydrogenase from toxin-treated cells (not shown), indicating that the cells remained intact during incubation with toxin. In their report of the

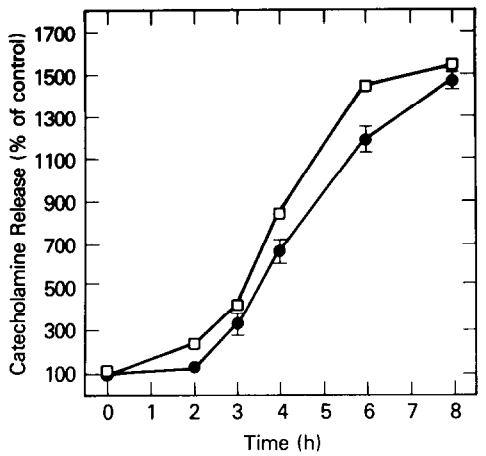

Fig.1. Time course of catecholamine release induced by pertussis toxin. Cells were incubated with pertussis toxin (100 ng/ml) for the indicated time intervals, and catecholamine release measured. Released catecholamine was calculated as a percentage of total cellular catecholamine and then expressed as a percentage of the control release observed after an 8 h incubation in the absence of toxin (control release varied from 0.5 to 0.9% of total cellular catecholamine for this series of experiments). Values shown are means ± SE ($n = 8$) in the case of (●), and means of duplicates for an experiment conducted in parallel to the experiment shown in fig.4 in the case of (□). For further details see section 2.

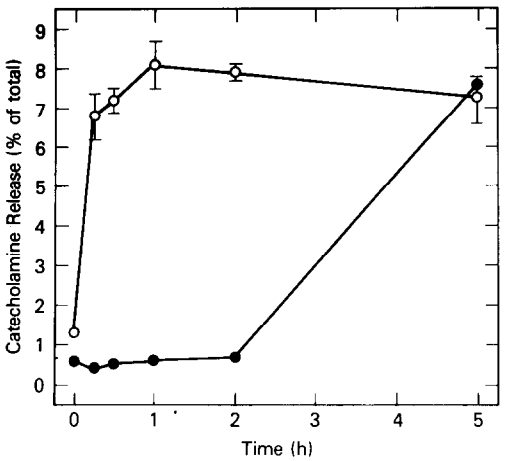

Fig.2. Effect of preincubation of cells with pertussis toxin for various times on subsequent catecholamine release. Cells were preincubated with pertussis toxin (100 ng/ml) for the times shown on the abscissa and the medium removed and assayed for catecholamine (●). To the cells was then added culture medium not containing pertussis toxin and the cells incubated such that the total time elapsed since the start of preincubation with toxin was 5 h. The medium was then again removed and assayed for catecholamine. The curve shown by (○) represents the total catecholamine released during the 5 h period, i.e. the amount shown by (●) plus the amount released subsequently after removal of pertussis toxin. The zero time points represent the catecholamine released in a 15 min preincubation (●) and this amount plus the catecholamine released during a subsequent 4 h 45 min incubation (○) both in the absence of toxin. The 5 h time points represent the catecholamine released during a single 5 h incubation with toxin (●) or the total release during consecutive 2 h and 3 h incubations both with toxin (○). It was necessary to obtain separate 0 h and 5 h points for the two curves due to the small amount of catecholamine release physically induced simply by changing the medium. Results were calculated as a percentage of total cellular catecholamine present at the start of the experiment and are shown as means ± SD ($n = 3$) for a representative experiment. For further details see text.

effect of pertussis toxin pretreatment on secretagogue-induced catecholamine release from bovine chromaffin cells, Tanaka et al. [7] showed that after a 6 h preincubation with toxin the basal rate of catecholamine release measured over a 5 min period was not significantly greater than control. However, unlike the present experiments, the amount of catecholamine released during the 6 h preincubation was not shown.

It has been reported that in certain cells the effects of pertussis toxin remain apparent after the toxin has been removed from the medium and that only short exposure times are required for the toxin's effects to be manifest [12,13]. To determine whether the same was true for chromaffin cells, cells were preincubated with toxin for various times up to 2 h, the medium removed and replaced with medium without toxin. The cells were then incubated so that the total time of the preincubation with toxin and subsequent incubation without toxin was 5 h. The catecholamine released into both the preincubation and incubation media was determined (fig.2). From the data it can be seen that when cells were preincubated for up to 2 h with toxin there was no catecholamine released into the preincubation medium. However, during the subsequent incubation in the absence of toxin the amount of catecholamine released was virtually the same whether the cells had seen the toxin for 15 min or 2 h and was equivalent to the amount released during a straight 5 h incubation with toxin. Experiments in which the cells received a wash with toxin-free medium between the toxin preincubation and subsequent toxin-free incubation showed essentially identical results but with a higher basal release and greater experimental

variability caused by the extra wash step (not shown). The results of this experiment thus imply that tight binding of enough toxin to the cells to induce a maximal response occurs within 15 min, and this cannot be reversed by removing toxin from the medium. The toxin does not begin to induce catecholamine release, however, until after 2 h of the initial exposure of the cells to the toxin.

Classical exocytosis in the bovine chromaffin cell requires the presence of extracellular Ca^{2+} and we therefore tested the pertussis toxin-stimulated secretory process for such dependence. To perform this experiment cells were preincubated for 2 h in culture medium (which has a Ca^{2+} concentration of 1.05 mM) in the absence or presence of toxin. The medium was then replaced by an extracellular salt solution containing various concentrations of $CaCl_2$ in the absence of pertussis toxin, and catecholamine release measured after 1 h incubation. As shown in fig.3, toxin-induced catecholamine release was indeed dependent on extracellular Ca^{2+} concentration in a saturable manner, with half-maximal release being apparent at approx. 0.3 mM Ca^{2+}. We observed that basal catecholamine release was much greater in the extracellular salt solution than in culture medium, and so we found it necessary to incubate the cells for 1 h in order to be able to measure the toxin-induced release over the elevated baseline. From these data however, we conclude that pertussis toxin-activated catecholamine release is Ca^{2+}-dependent over a physiological range of Ca^{2+} concentrations.

The action of pertussis toxin on many cells appears to be mediated by the toxin-catalyzed ADP-ribosylation of G-proteins, and in the chromaffin cell the toxin-induced potentiation of the secretory response to secretagogues has been shown to be coincident with the ADP-ribosylation of a 40 kDa protein [7]. It was therefore important to learn whether catecholamine release stimulated by pertussis toxin itself was also coincident with the ADP-ribosylation of the same or a similar substrate. We approached this question by comparing the ADP-ribosylation state of chromaffin cell membrane proteins with catecholamine release occurring as a function of time and toxin concentration. Thus chromaffin cells were incubated for various times with pertussis toxin after which the cells were homogenized and a total membrane fraction prepared. This fraction was incubated

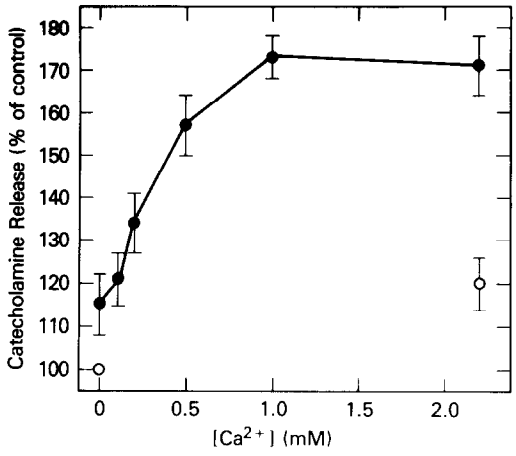

Fig.3. Effect of Ca^{2+} concentration on catecholamine release induced by pertussis toxin. Cells were preincubated for 2 h in the absence (○) or presence (●) of pertussis toxin (100 ng/ml). The medium was then removed and discarded and the cells incubated for a further 1 h in an extracellular release medium containing various concentrations of added $CaCl_2$. This medium was then removed and assayed for catecholamine. Catecholamine release was calculated as a percentage of total cellular catecholamine and then expressed as a percentage of the control release observed from cells preincubated in the absence of toxin and incubated at 0 mM added $CaCl_2$ (control release was variable between experiments and varied from 1.5 to 7.6% of total cellular catecholamine). Values shown are means ± SE ($n = 18$).

with activated pertussis toxin and [^{32}P]NAD and the incorporation of ^{32}P into proteins determined by electrophoresis and autoradiography (fig.4). Cells which were incubated in the absence of pertussis toxin have a major substrate of approx. 40 kDa for ADP-ribosylation catalyzed by the activated toxin. In the case of membranes prepared from cells that had been incubated with pertussis toxin for increasing times, after a 2 h lag there is a progressive decrease in the extent of incorporation of ^{32}P into this protein. Catecholamine release was measured in parallel for this particular experiment and for comparison the results are shown in fig.1 (unfilled squares). The results are qualitatively consistent with the hypothesis that toxin-induced ADP-ribosylation of the 40 kDa protein and catecholamine release are related.

Also compelling were data comparing the toxin dose-response of catecholamine release and ADP-ribosylation of the 40 kDa protein. The dose-response curve for catecholamine release over a 5 h

period at various pertussis toxin concentrations is shown in fig.5. The threshold concentration for activation of catecholamine release was found to be ~1 ng/ml and the dose-response curve was found to reach an apparent plateau by 1000 ng/ml. For the particular experiment depicted by the unfilled squares in fig.5, the extent of ADP-ribosylation of the 40 kDa protein was measured in parallel as shown by the inset to fig.5. It can be seen that for cells that had been exposed to increasing concentrations of pertussis toxin, the extent of ^{32}P incorporation into the 40 kDa protein progressively decreased. Thus, exposure of the cells to pertussis toxin results in the endogenous ADP-ribosylation of this protein. Furthermore, this decline in the extent of ^{32}P incorporation of the 40 kDa protein was coincident with the development of secretory capacity.

4. DISCUSSION

In this paper we show that pertussis toxin stimulates catecholamine release from cultured bovine chromaffin cells in a Ca^{2+}-dependent manner. The Ca^{2+} dependence of this process is quite reminiscent of exocytosis stimulated by the more physiological secretagogues carbachol and KCl in terms of the effective Ca^{2+} concentration range [14]. Although the kinetics of pertussis toxin-stimulated secretion are much slower than those known for carbachol and KCl [14], slow time courses of exocytosis are known for certain secretagogues such as Ba^{2+} [15] and phorbol ester [10]. Furthermore, Ca^{2+}-activated catecholamine release from digitonin-permeabilized cells, a process which may model exocytosis, exhibits slow kinetics [16,17].

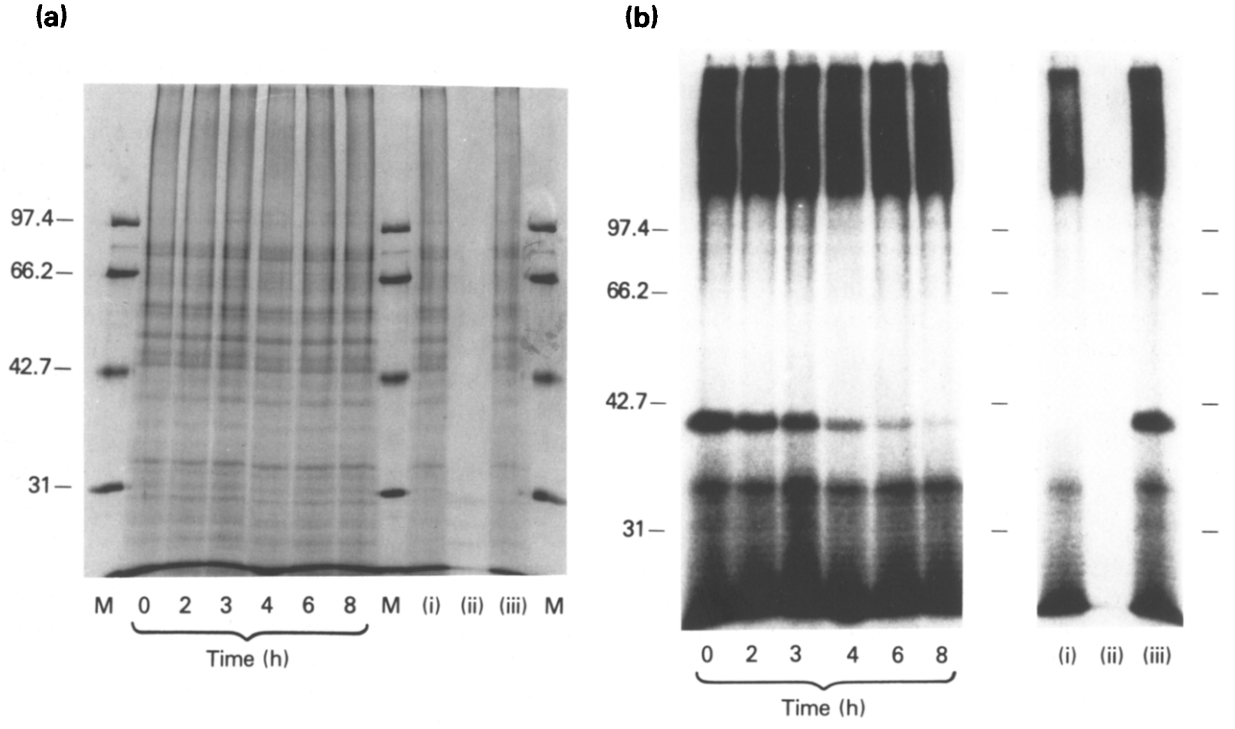

Fig.4. Time course of ADP-ribosylation induced by pertussis toxin. Cells were incubated with pertussis toxin (100 ng/ml) for the indicated time intervals, the zero time point representing cells incubated for 8 h in the absence of toxin. The cells were then scraped into a hypotonic homogenization buffer and a total membrane fraction prepared. This fraction was then exposed to [^{32}P]NAD and activated pertussis toxin, and ^{32}P-labeled proteins were detected by SDS-polyacrylamide gel electrophoresis and autoradiography. The electrophoretogram is shown in (a) and autoradiogram in (b). Tracks labeled M contain molecular mass markers. Tracks labeled (i)-(iii) represent control incubations with [^{32}P]NAD in which (i) membranes prepared from cells which had not been treated with pertussis toxin were incubated in the absence of activated pertussis toxin; (ii) activated pertussis toxin was incubated in the absence of any membranes; and (iii) membranes prepared from cells which had not been treated with pertussis toxin were incubated with activated toxin for 60 min instead of 45 min to show that maximum incorporation of ^{32}P had taken place. For further details see section 2.

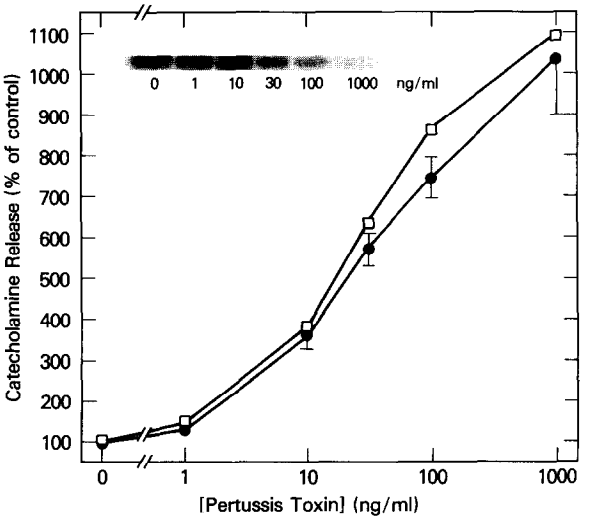

Fig.5. Effect of pertussis toxin concentration on catecholamine release and ADP-ribosylation. Cells were incubated for 5 h with various concentrations of pertussis toxin. The graph shows the amount of catecholamine released under the different conditions calculated as a percentage of total cellular catecholamine and then expressed as a percentage of the control release observed in the absence of pertussis toxin (control release varied from 0.5 to 1.6% of total cellular catecholamine for this series of experiments). Values shown are means ± SE ($n = 10$) in the case of (●), and means of duplicates for an experiment conducted in parallel to the ADP-ribosylation experiment shown in the inset in the case of (□). The inset shows the extent of ^{32}P incorporation into the 40 kDa pertussis toxin substrate after scraping the cells, preparing a total membrane fraction and exposing this fraction to [^{32}P]NAD in the presence of activated pertussis toxin. Only that part of the autoradiogram containing the 40 kDa protein is shown. For further details see text.

Pertussis toxin has been found to have stimulatory effects on secretion in other cells. Pretreatment of rat pancreatic islets with pertussis toxin increases insulin release induced by glucose and other secretagogues, and blocks the α-adrenergic inhibition of glucose-induced insulin release [12,18]. These effects of the toxin were dependent on the presence of extracellular Ca^{2+}. In anterior pituitary cells pretreatment with pertussis toxin increases basal and growth hormone releasing factor-induced growth hormone secretion [19], and basal and gonadotropin releasing hormone-stimulated luteinizing hormone release [20].

Pertussis toxin-activated catecholamine release is likely to involve the ADP-ribosylation of one or more membrane-bound proteins of approx. 40 kDa. The evidence for this is based on the coincidental ADP-ribosylation of such a protein(s) as a function of time and toxin concentration and the development of a secretory response. However, it is difficult to reconcile the finding that pertussis toxin induces catecholamine release in the absence of any exogenous receptor agonist with the current understanding of the mechanism of action of the toxin, which is supposed to involve the uncoupling of a receptor from a G-protein through the ADP-ribosylation of the latter.

It is possible that pertussis toxin-induced catecholamine release is analogous to the pertussis toxin-induced lipolysis in rat adipocytes [21]. Adenosine released from the adipocytes inhibits adenylyl cyclase and lipolysis through G_i, but pertussis toxin blocks this inhibition with the result that the toxin apparently induces spontaneous glycerol release with kinetics very similar to the toxin-induced catecholamine release. Thus, pertussis toxin-induced catecholamine secretion may be due to the blockade of inhibition of secretion mediated by some endogenous agent.

However, it is also possible that an as yet unidentified G-protein is present in chromaffin cells which acts to inhibit the secretory process. ADP-ribosylation of this protein catalyzed by pertussis toxin could block its inhibitory action and thus explain the stimulatory effect of the toxin on basal and secretagogue-induced catecholamine release. Of interest in this respect is that Toutant et al. [22] have recently demonstrated the presence of substrates for pertussis toxin in subcellular fractions of bovine chromaffin cells enriched in secretory granules as well as in plasma membrane fractions. The possibility of a G-protein being directly involved in exocytosis has been invoked by Barrowman et al. [2] and Cockroft et al. [3] as a result of experiments with permeabilized neutrophils and mast cells, respectively. Melancon et al. [23] and Segev et al. [24] have also provided evidence for the involvement of a G-protein in the processing of vesicles for fusion during protein transport through the Golgi stack, thus possibly indicating a role for G-proteins in membrane fusion events in general. Further investigations into the mechanism of the stimulation of catecholamine release by pertussis toxin in bovine chromaffin cells will hopefully help to clarify the role of G-proteins in stimulus-secretion coupling in this cell.

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