The inhibition by pertussis and tetanus toxins of evoked catecholamine release from intact and permeabilized bovine adrenal chromaffin cells

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Pertussis toxin stimulates both basal and nicotine-evoked catecholamine secretion from intact bovine adrenal chromaffin cells, as well as Ca^{2+}-evoked release from permeabilized cells. Tetanus toxin inhibits all these effects; it reduces the secretion of intact cells treated with pertussis toxin to the basal level, and decreases by about 50% Ca^{2+}-evoked release from permeabilized cells whether or not previously stimulated by pertussis toxin.

Tetanus toxin; Pertussis toxin; Adrenal cell; Catecholamine; Exocytosis

1. INTRODUCTION

Tetanus toxin is a potent neurotoxin that inhibits neurotransmitter release at central inhibitory synapses, causing spastic paralysis. It is synthesised by *Clostridium tetani* as a single polypeptide chain (M_r 150000). An active two-chain toxin consisting of heavy (M_r 100000) and light (50000) chains with an internal disulphide bond is generated after subsequent proteolytic processing. The heavy chain binds to the outer plasma membrane of cells, and this leads to the internalization of the light chain [1]. Once internalized, the light chain probably catalyses a reaction which leads to the toxicity; it has been shown to be capable of inhibiting exocytosis of catecholamines when introduced directly into permeabilized adrenal chromaffin cells [2,3]. Tetanus toxin shares this two-component structure with other bacterial toxins such as diphtheria toxin [4] and botulinum toxin [5]. The binding of the heavy chain to gangliosides and other receptors on the cell surface is well established [6], but how the internalized light chain then inhibits neurotransmitter release is not clear.

Pertussis toxin has recently been shown to stimulate both nicotine- and high $[K^+]$-evoked release of catecholamines in chromaffin cells [7–9]. There are three G-proteins (of M_r 41000, 40000 and 39000) that are specifically ADP-ribosylated by pertussis toxin in adrenal chromaffin cells [10], but the acquisition of this hyper-responsiveness to secretagogues was coincident with the toxin-catalysed ADP-ribosylation of only one of them (M_r 40000) [8]. Pertussis toxin affects neither intracellular free $[Ca^{2+}]$, nor $^{45}Ca^{2+}$ uptake [11], but it does markedly increase the affinity of exocytosis for Ca^{2+} [9], suggesting that G-proteins are involved in the direct control of Ca^{2+}-sensitive exocytosis in these cells.

In this paper, we report the effect of tetanus toxin on pertussis-stimulated catecholamine release by adrenal chromaffin cells. Our results indicate that tetanus toxin does not block exocytosis by affecting G-proteins, and that it might have a target at or near the plasma membrane affecting the movement of granules to the adjacent active release sites and/or fusion of granules to the membrane.

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2. MATERIALS AND METHODS

2.1. *Preparation and culture of chromaffin cells*

Bovine adrenal chromaffin cells were isolated from adrenal medullae by protease and collagenase digestion [12]. The cells were plated in 24-well multiwell plates (Gibco) at 0.5×10^6 cells per well, and maintained in Dulbecco's Modified Eagles medium containing 15 mM Hepes, pH 7.4, supplemented with 10% foetal calf serum, 100 units penicillin and streptomycin/ml, 50 μg gentamycin/ml, 0.25 μg fungizone/ml (Gibco) and 50 μg/ml ascorbic acid, at 37°C in a CO_2 incubator for 3 days. The medium was changed on day 3 to Dulbecco's Modified Eagles medium as above but without foetal calf serum, and the cells were maintained in culture for 4–7 days.

2.2. *Catecholamine release experiments*

The cells were incubated with different concentrations of pertussis toxin (from the PHLS Centre for Applied Microbiology and Research, Porton, Wiltshire, UK, and purified by the method of Irons and Gorringe [13]) for different times as described below. The medium was removed and the cells were washed once with Locke's solution (165 mM NaCl, 5.6 mM glucose, 5.0 mM Hepes, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, pH 7.6). The cells were then stimulated with 10 μM nicotine or 55 mM KCl in Locke's solution for 10 min at room temperature. The supernatant was removed and the cells were lysed with 1% Triton X-100. Aliquots of the supernatant and the Triton fraction were assayed fluorimetrically for total catecholamine (adrenaline and noradrenaline) [14].

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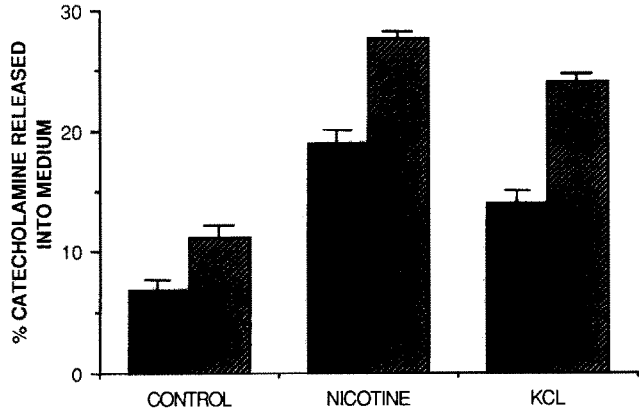

Fig. 1. The effect of pertussis toxin on basal and secretagogue-induced catecholamine release. Cells were incubated for 20 h with 0.9 nM pertussis toxin (hatched bars) or without toxin (solid bars). 10 μM nicotine, 55 mM KCl, or Locke's solution without secretagogue was added to the cells for 10 min, and the amount of catecholamine released into the medium determined as a percentage of total cellular catecholamine. Values shown are means ± SD ($n=3$).

2.3. *Permeabilization of chromaffin cells*

The cells were permeabilized by incubation with 10 μM digitonin (Sigma) in medium containing 139 mM NaCl, 20 mM piperazine-*N,N'*-bis(2-ethanesulphonic acid), 2 mM MgATP, 5 mM EGTA, pH 6.6, with or without 0.7 μM tetanus toxin (a gift from Dr U. Weller of the Justus-Liebig-Universität, Giessen) for 16 min [15]. The solution was then replaced by a similar medium without digitonin or tetanus toxin, and with or without 10 μM free Ca^{2+} ions. The catecholamines released into this medium and the cell fraction were estimated fluorimetrically as described in the previous section.

3. RESULTS

Our initial results on the effect of pertussis toxin on the catecholamine release confirmed previous findings [7–9] that preincubation of intact cells with 0.9 nM pertussis toxin resulted in the stimulation of nicotine-evoked exocytosis by 50% and K^+-evoked exocytosis by 70% (Fig. 1). The effect of the toxin on basal catecholamine release was the same; it was enhanced by 70%.

The dose dependency of stimulation of nicotine-evoked catecholamine release by pertussis toxin is shown in Fig. 2. The half-maximal effective concentration of pertussis toxin (9×10^{-4} nm) is similar to that previously reported by Tanaka et al. [7]. The stimulation of basal release by pertussis toxin is also dose-dependent (Fig. 2), but, although the concentration for half-maximal effect was also about 9×10^{-4} nM, rather higher toxin concentrations were required to reach the maximum effect.

Cells maintained in culture with 0.18 nM pertussis toxin over various lengths of time showed a stimulation of nicotine-evoked catecholamine after a lag period of 2–4 h, and a maximum stimulation of 60% after 6 h exposure (Fig. 3). This lag is presumably the time taken for the toxin to enter the cells and express its effect [16]. The effect of pertussis toxin was not reversed even when the toxin had been removed from the medium for a two-day period (data not shown).

Pertussis toxin also stimulated catecholamine release by 40% from cells that had been permeabilized with digitonin and were then treated with 10 μM Ca^{2+} (Fig. 4). Permeabilization of cells followed by incubation with EGTA led to a variable background release of catecholamine, but even this was stimulated in the presence of the toxin.

Our previous unpublished work and that of others [2,3] have shown that tetanus toxin inhibits Ca^{2+}-sensitive catecholamine release from digitonin-permeabilized cells. We therefore looked at the effect of

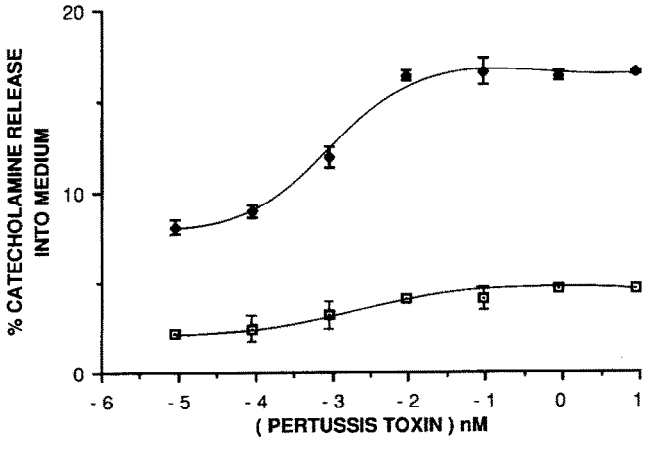

Fig. 2. The effect of pertussis toxin concentration on nicotine-induced catecholamine release. Cells were incubated for 20 h with various concentrations of pertussis toxin (9 to 9×10^{-5} nM). The graph shows the percentage of catecholamine released into the medium with (◆) or without (□) challenge with 10 μM nicotine for 10 min. Results are shown as means ± SD ($n=3$).

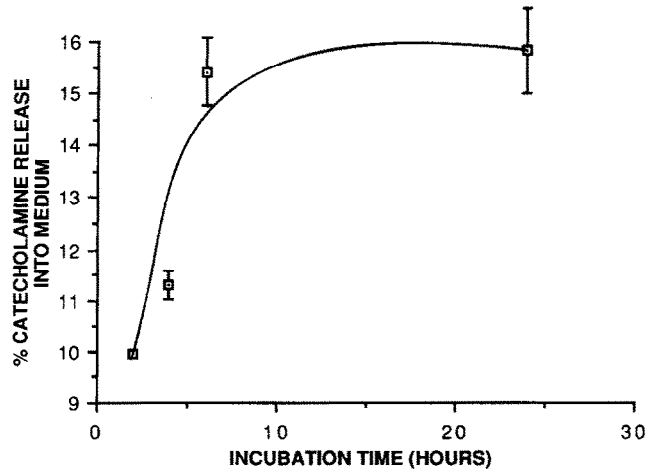

Fig. 3. A time course of catecholamine release induced by pertussis toxin. Cells were incubated with pertussis toxin (0.18 nM) for the indicated time intervals, and catecholamine release into the medium was measured after addition of nicotine. The released catecholamine was calculated as a percentage of total cellular content, and the results are shown as mean ± SD ($n=3$). In the absence of pertussis toxin, 10.2 ± 0.6% of cellular catecholamine was released.

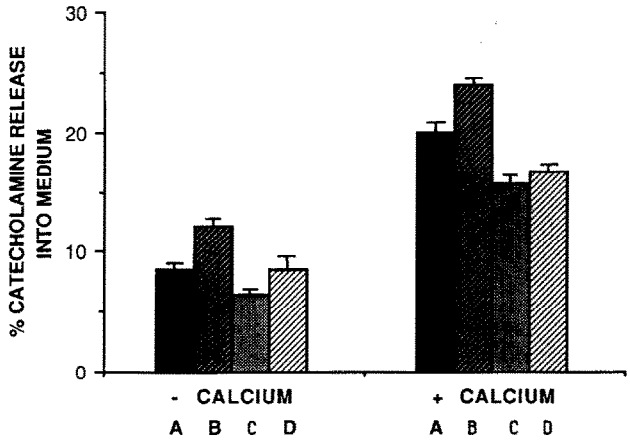

Fig. 4. The effect of tetanus toxin on pertussis-toxin-sensitive catecholamine release. Cells were incubated with 0.9 nM pertussis toxin for 20 h, then permeabilized with digitonin in the presence or absence of 0.7 μM tetanus toxin for 12 min. A solution with or without 10 μM free Ca^{2+} ions was added to the cells for a further 15 min, and the percentage catecholamine released into the medium determined. (A) control cells, no toxins; (B) pertussis toxin alone; (C) tetanus toxin alone; (D) pertussis toxin followed by tetanus toxin. The values presented here are shown as means ± SD ($n = 3$).

tetanus toxin on the stimulation of catecholamine release by pertussis toxin (Fig. 4). Cells were first incubated for 20 h with pertussis toxin (0.9 nM) and then permeabilized in the presence of tetanus toxin (0.7 μM) for 12 min. Tetanus toxin inhibited release under all conditions: Ca^{2+}-sensitive release in the absence of pertussis toxin was decreased by 50% and Ca^{2+}-sensitive pertussis-stimulated release was decreased by 40%, while Ca^{2+}-insensitive pertussis-stimulated secretion was reduced to the basal level. Tetanus toxin thus inhibits both Ca^{2+}-evoked release and the effect attributed to treatment with pertussis toxin.

4. DISCUSSION

Pertussis toxin has been found to have stimulatory effects on secretion in a variety of cells, such as insulin release induced by glucose in rat pancreatic islets [16], and growth hormone release induced by growth hormone releasing factor [17]. In this paper, we have shown that this pertussis stimulation of secretion in chromaffin cells is inhibited by tetanus toxin (a toxin that also inhibits secretion from untreated chromaffin cells).

Stimulation by pertussis toxin of catecholamine secretion from chromaffin cells is likely to involve the ADP-ribosylation of one or more of the three GTP-binding proteins that are specifically ADP-ribosylated by the toxin [8,10]. Modulation of the affinity for Ca^{2+} of the known target of pertussis toxin by a GTP-binding protein has been implicated in the direct control of exocytosis [18].

The mechanism by which tetanus toxin inhibits exocytosis is not known. However, both our work (Fig. 3) and a recent demonstration [3] that tetanus toxin internalized after permeabilizing the plasma membrane of chromaffin cells can block exocytosis favour an intracellular site of action. The inhibition of pertussis-toxin-stimulated catecholamine release by tetanus toxin reported here shows that the two toxins probably act at different sites, suggesting that tetanus toxin does not inhibit exocytosis by affecting GTP binding proteins. Experiments in many laboratories, including our own, have failed to find any ADP-ribosylation catalyzed by tetanus toxin or by the related botulinum neurotoxin (e.g. [18]). This is further supported by the finding that tetanus toxin exerts its action in a few minutes in permeabilized cells which presumably lack the nucleotides needed for ADP-ribosylation.

There is a difference in the time that the two toxins take to act in chromaffin cells. Our previous work showed that more than 16 h are required for tetanus toxin to inhibit exocytosis from these adrenal cells when they are intact (M.K. Bansal, C.A. Colville, J.H. Phillips and S. van Heyningen, manuscript in preparation), yet the maximum effect of pertussis toxin is achieved after only about 4 h of incubation. It is not, of course, known whether the rate-limiting step is internalization or the interaction of toxin with intracellular targets.

The catecholamine that is secreted from normal cells in the absence of any secretagogue or from permabilized cells in the absence of Ca^{2+} ions is probably released from those granules that are already close to the plasma membrane. As an elevation of free [Ca^{2+}] is known to regulate aspects of actin-filament organisation [19], a change in the structure of actin filaments may be required in order to allow secretory granules access to the sites of exocytosis [20]. Pertussis toxin stimulates basal release even in the absence of Ca^{2+}, indicating that its site of action may be distal to interactions between granules and the cytoskeleton, and showing that it is also possible that an as yet unidentified GTP-binding protein which acts to inhibit exocytosis may be present in chromaffin cells. The presence of substrates for pertussis toxin in secretory granules, as well as plasma membrane fractions, has been shown by Toutant et al. [10], and it is possible that a similar reaction may occur physiologically, since proteins ADP-ribosylated at cysteine residues (i.e. analogously to the action of pertussis toxin) have been found in the plasma membrane of liver cells [21]. The processing of vesicles for fusion during protein transport through the Golgi stack has been shown to involve a GTP-binding protein, suggesting a possible role for G-proteins in membrane fusion events in general [22]. The inhibition of pertussis-toxin-stimulated basal release by tetanus toxin indicates that it might be interfering with the movement of synaptic vesicles to active release sites near the plasma membrane from closely adjacent positions, as suggested by

Dreyer et al. [23], as well as affecting their fusion to the plasma membrane [24].

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