A pertussis-toxin-sensitive protein controls exocytosis in chromaffin cells at a step distal to the generation of second messengers

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The role of GTP-binding proteins (G-proteins) in the secretory process in chromaffin cells was investigated by studying the effects of pertussis toxin (PTX) on catecholamine release and generation of various second messengers. PTX was found to stimulate the catecholamine secretion induced by nicotine, 59 mM-K⁺ or veratridine. PTX also potentiated Ca²⁺-evoked catecholamine release from permeabilized chromaffin cells, suggesting that PTX substrate(s) regulate the exocytotic machinery at a step distal to the rise in intracellular Ca²⁺. We have investigated the possible intracellular pathways involved in the stimulation of secretion by PTX. PTX did not modify the translocation of protein kinase C (PKC) to membranes in intact or permeabilized cells; in addition, neither inhibitors nor activators of PKC had any effect on catecholamine release induced by PTX. Thus it seems unlikely that the effect of PTX on secretion is mediated by activation of PKC. The effect of PTX is also cyclic AMP-independent, as PTX did not change cytoplasmic cyclic AMP levels. The relationship between PTX treatment and arachidonic acid release was also examined. We found that an increase in cytoplasmic arachidonic acid concentration enhanced Ca²⁺-evoked catecholamine release in permeabilized cells, but arachidonic acid did not mimic the effect of PTX on the Ca²⁺-dose-response curve for secretion. Furthermore, PTX did not significantly modify the release of arachidonic acid measured in resting or stimulated chromaffin cells, suggesting that the stimulatory effect of PTX on secretion is not mediated by an activation of phospholipase A₂. Taken together, these results suggest that PTX may modulate the intracellular machinery of secretion at a step distal to the generation of second messengers. In α -toxin-permeabilized cells, full retention of the PTX-induced activation of secretion was observed even 30 min after permeabilization. In contrast, when chromaffin cells were permeabilized with streptolysin-O (SLO), there was a marked progressive loss of the PTX effect. We found that SLO caused the rapid leakage of three G-protein α -subunits which are specifically ADP-ribosylated by PTX. We propose that a PTX-sensitive G-protein may play an inhibitory role in the final stages of the Ca²⁺-evoked secretory process in chromaffin cells.

INTRODUCTION

It is well recognized that Ca²⁺ plays a central role in the regulated exocytosis of many neurotransmitters, hormones and enzymes. More recently, the possibility has been raised that a class of GTP-binding proteins (G-proteins) called G_e-proteins may exert a direct inhibitory or activatory effect on the exocytotic machinery [1–7]. This conclusion has been favoured by studies using permeabilized secretory cell preparations, in which non-hydrolysable GTP analogues regulate secretion at a step distal to the rise in cytosolic Ca²⁺. To prove the existence of G_e and define a direct role for GTP in exocytosis, it is essential to demonstrate that no modification in the cytoplasmic concentrations of second messengers known to modulate secretion occurs when the putative G_e is activated or inhibited. We have recently reported that non-hydrolysable GTP analogues potentiate Ca²⁺-evoked catecholamine release from chromaffin cells permeabilized with α -toxin from *Staphylococcus aureus* [1]. However, three lines of evidence indicate that this effect of GTP analogues was exerted through protein kinase C (PKC) activation: (i) phorbol esters and GTP analogues modulated Ca²⁺-induced secretion in a similar manner, (ii) PKC inhibitors completely suppressed the stimulatory effects of GTP analogues, and (iii) GTP analogues were found to activate PKC, as indicated by the translocation of the enzyme from a soluble to a membrane-bound compartment. Thus the use of GTP analogues in permeabilized chromaffin cells

did not provide any information concerning the direct involvement of a putative G-protein in the exocytotic machinery of these cells.

Another experimental approach currently employed to probe the role of G-proteins in intracellular pathways is the use of the islet activating protein, pertussis toxin (PTX). PTX is known to uncouple G-proteins from their associated receptors on the plasma membrane by ADP-ribosylating the GTP-binding α -subunit [8]. For instance, the inhibitory effect of somatostatin on high K⁺-induced prolactin secretion by GH₄C₁ cells is affected by PTX [9]. In anterior pituitary cells, pretreatment with PTX potentiates secretagogue-induced growth hormone secretion [10] and gonadotropin-releasing-hormone-stimulated luteinizing hormone secretion [11]. Treatment of mast cells with PTX blocks an array of effects observed in response to compound 48/80, including breakdown of phosphatidylinositol, ⁴⁵Ca²⁺ influx, generation of arachidonate and histamine secretion [12]. Similarly, virtually all neutrophil responses to fMet-Leu-Phe, such as elevation of intracellular Ca²⁺, phosphatidylinositol turnover, protein phosphorylation and lysosomal enzyme release are blocked by PTX [13,14].

In the present study we have used PTX as a probe to investigate the role of G-proteins in the cascade of events underlying the stimulus-secretion coupling in chromaffin cells. The effects of PTX on the basal and stimulated secretory activity and the generation of second messengers was studied in both intact cells

Abbreviations used: BPB, *p*-bromophenacyl bromide; [Ca²⁺]_i, cytosolic free Ca²⁺ concentration; cAMP, cyclic AMP; DTT, dithiothreitol; G-protein, GTP-binding protein; GTP[S], guanosine 5'-[γ -thio]triphosphate; KG medium, 150 mM-potassium glutamate/10 mM-Pipes (pH 7.0)/5 mM-nitrotriacetic acid/0.5 mM-EGTA/5 mM-MgATP/4.5 mM-magnesium acetate/0.2% (w/v) BSA; NEM, *N*-ethylmaleimide; PTH, parathyroid hormone; SLO, streptolysin-O; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin; PKC, protein kinase C.

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and those permeabilized with *S. aureus* α -toxin. In the latter, the cytosolic proteins essential for exocytosis are retained after permeabilization [15], and therefore this model permits one to examine the complete sequence of events leading to the exocytotic release of secretory material. We show that PTX is able to induce secretion in the absence of external secretagogues by a pathway which does not involve the classical second messengers known to modulate exocytosis. Since this effect cannot be simply explained by an uncoupling of a PTX-sensitive G-protein from an associated cell surface receptor, we suggest that a PTX-sensitive protein may directly control the exocytotic machinery in chromaffin cells.

MATERIALS AND METHODS

Culture of chromaffin cells

Chromaffin cells were isolated from fresh bovine adrenal glands by retrograde perfusion with collagenase and purified on self-generating Percoll gradients [16,17]. They were suspended in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum containing streptomycin (50 μ g/ml) and penicillin (50 units/ml). Growth of non-chromaffin cells was inhibited by the addition of cytosine arabinoside (10 μ M) and 5-fluorodeoxyuridine (10 μ M) to the culture medium.

[³H]Noradrenaline release from intact chromaffin cells

For catecholamine release experiments, 3–6-day-old cultured chromaffin cells, grown on 24-well 16 mm Costar plates (Costar, Cambridge, MA, U.S.A.) at a density of 2.5×10^5 cells/well, were loaded with [³H]noradrenaline (16 Ci/mmol; Amersham, Les Ulis, France) and subsequently washed with Locke's solution (140 mM-NaCl, 4.7 mM-KCl, 2.5 mM-CaCl₂, 1.2 mM-MgCl₂, 1.2 mM-KH₂PO₄, 11 mM-glucose, 0.56 mM-ascorbic acid and 15 mM-Hepes, pH 7.2) as previously described [16]. Cells were then stimulated for 10 min with Locke's solution containing 10 μ M-nicotine, 30 μ M-veratridine or 59 mM-K⁺ (54.3 mM-NaCl replaced by KCl). [³H]Noradrenaline release after stimulation was determined by measuring the radioactivity present in the incubation medium after centrifugation for 5 min at 12000 *g* and in cells after precipitation with 10% (w/v) trichloroacetic acid. The amount of released [³H]noradrenaline is expressed as a percentage of total radioactivity present in the cells before stimulation.

[³H]Noradrenaline release from permeabilized chromaffin cells

Chromaffin cells loaded with [³H]noradrenaline were washed four times with Locke's solution and twice with Ca²⁺-free Locke's solution (containing 1 mM-EGTA). Cells were subsequently permeabilized for 30 min with α -toxin (40 units/ml) or for 2 min with streptolysin-O (SLO; 15 units/ml) in 200 μ l of Ca²⁺-free KG medium [150 mM-glutamate (potassium salt) 10 mM-Pipes, pH 7.0, 5 mM-nitrilotriacetic acid, 0.5 mM-EGTA, 5 mM-MgATP, 4.5 mM-magnesium acetate and 0.2% (w/v) BSA]. Extracellular fluids were then removed and cells were stimulated with 200 μ l of KG medium containing various amounts of CaCl₂, to yield the indicated free Ca²⁺ concentrations, and magnesium acetate, to maintain a final free Mg²⁺ concentration of 1 mM. The exact free Ca²⁺ and Mg²⁺ concentrations in the KG medium were calculated as described by Flodgaard & Fleron [18] using the stability constants given by Silen & Martell [19]. Release of [³H]noradrenaline is expressed as a percentage of total radioactivity present in the cells before Ca²⁺-induced stimulation.

S. aureus α -toxin and SLO

S. aureus α -toxin (Calbiochem) was purified by (NH₄)₂SO₄ precipitation at 75% saturation and stored as a suspension in

75%-satd. (NH₄)₂SO₄ solution at 4 °C. SLO (Institut Pasteur, Paris, France) was activated with 4 mM-dithiothreitol (DTT), purified by precipitation with 75%-satd. (NH₄)₂SO₄ and stored as a suspension in 75%-satd. (NH₄)₂SO₄ solution at 4 °C. The haemolytic activity of the toxins was determined with a 2.5% rabbit erythrocyte suspension. The dilution of toxin haemolysing 50% of red cells (EC₅₀) was estimated and the reciprocal of this value was taken as the number of haemolytic units/ml of the non-diluted toxin solution.

Measurement of PKC activity

PKC activity was measured in cultured chromaffin cells grown on 50 mm-diam. plastic dishes at a density of 2.5×10^6 cells. Cells were scraped off and homogenized in 600 μ l of ice-cold buffer composed of 20 mM-Tris/HCl (pH 7.5), 2 mM-EDTA, 2 mM-DTT, 2 mM-phenylmethanesulphonyl fluoride, 20 μ g of leupeptin/ml, 20 μ g of aprotinin/ml and 20 μ g of pepstatin/ml. The cell solution was sonicated twice for 5 s at setting 9 on an Ultrasonic Cell Disruptor (MSE Scientific Instruments, Crawley, W. Sussex, U.K.). The homogenate (500 μ l) was centrifuged at 100000 *g* for 20 min, the supernatant (cytosol) was removed and the pellet (particulate fraction) was resuspended in 500 μ l of homogenizing solution. Triton X-100 was added to the particulate fraction to give a final concentration of 0.1%. A 40 μ l sample of the cytosolic fraction (soluble PKC) or particulate fraction (membrane-bound PKC) was added to the PKC assay medium [20], which contained 1.75 mM-CaCl₂, 16 μ g of phosphatidylserine/ml and 100 μ M-diacylglycerol. PKC was assayed by measuring, over 4 min at 30 °C, the incorporation of ³²P into histone H₁ from [γ -³²P]ATP as described by Castagna *et al.* [21]. PKC activity, defined as Ca²⁺- and phosphatidylserine/diacylglycerol-dependent activity, was expressed in terms of pmol of ³²P incorporated/min per mg of protein.

PTX-catalysed ADP-ribosylation

Soluble proteins released from digitonin-treated cells and proteins from plasma membranes and chromaffin granules purified from continuous sucrose-density gradients prepared as described by Toutant *et al.* [22] were ADP-ribosylated with PTX (List Biological Laboratories, Campbell, CA, U.S.A.) using the protocol described by Ribeiro-Neto *et al.* [23] with minor modifications [24]. The reaction was stopped by the addition of 2% SDS and BSA (100 μ g/ml) and proteins were precipitated with 10% (w/v) trichloroacetic acid. After centrifugation for 10 min at 10000 *g*, the pellets were washed twice with diethyl ether, dried and analysed by SDS/PAGE [10% (w/v) acrylamide/0.13% (w/v) bisacrylamide] in the presence of 100 mM-N-ethylmaleimide (NEM).

Modification of PTX with NEM

PTX (List Biological Laboratories) was treated with NEM essentially as described by Singh Banga *et al.* [25]. Purified toxin (50 μ g/ml) was incubated for 3 h at room temperature with 3 M-urea, 2 mM-DTT, 0.1 mM-ATP, 1% CHAPS, 0.05 M-sodium phosphate, pH 7.5. NEM (10 mM) was then added and incubation was continued for a further 1 h. The reaction was terminated by adding 10 mM-DTT. The toxin was then diluted in culture medium and used at the indicated concentrations.

Assay of cyclic AMP (cAMP)

For the assay of cAMP levels, chromaffin cells grown on 24-well 16 mm-diam. Costar plates at a density of 10^6 cells/well were incubated without or with cholera toxin (1 μ g/ml) or PTX (1 μ g/ml) for 4 h or various concentrations of forskolin for 20 min. Cells were then scraped off in 5 mM-Tris/HCl (pH 7.5)/4 mM-EDTA (250 μ l for 6 wells), frozen and thawed

three times and centrifuged, and the supernatant was taken for the assay of cAMP using the cAMP assay kit (Amersham), as described in the manufacturer's instructions.

Arachidonic acid release

Cultured chromaffin cells grown on 24-well 16 mm-diam. Costar plates at a density of 2.5×10^5 cells/well were incubated for 5 h with culture medium (400 μ l/well) containing 0.2 μ Ci of [^{14} C]arachidonic acid/ml (Amersham; 55 mCi/mmol). Cells were then washed four times with Locke's solution and twice with Ca^{2+} -free Locke's solution, and permeabilized for 10 min with SLO (15 units/ml) in KG medium in the presence or absence of 10 μ M free Ca^{2+} . [^{14} C]Arachidonic acid release after stimulation was determined by measuring the radioactivity present in the incubation medium after centrifugation for 5 min at 12000 g and in cells after solubilization in 1% Triton X-100. Results of the [^{14} C]arachidonic acid release experiments were expressed as percentages of the total radioactivity present in cells before stimulation, assessed from the amount released plus the amount remaining in the cells.

Presentation of data

All experiments described were performed on at least two different cell preparations. Figures and Tables show typical experiments; unless indicated in the legend, data are given as the means of triplicate determinations on the same cell preparation $\pm$ S.E.M. Error bars smaller than the point symbols were omitted from Figures.

RESULTS

Effect of PTX treatment on secretagogue-induced catecholamine release in intact and permeabilized chromaffin cells

The effect of PTX on catecholamine release evoked by nicotine, 59 mM- K^+ or veratridine was examined in intact cells. As shown in Fig. 1, preincubation of cultured chromaffin cells with PTX induced a dose-dependent stimulation of the secretory response evoked by each of the three secretagogues. The maximal effect (approx. 150% of control) was observed by pretreating the cells with 100 ng of PTX/ml for 4 h. PTX at concentration of up to 2 μ g/ml appeared to have no deleterious effects on cell viability, as judged by the exclusion of Trypan Blue. Interestingly, PTX enhanced the basal rate of catecholamine release during the 4 h preincubation period, in agreement with the results reported by Brocklehurst & Pollard [26]. However, this spontaneous release was only significantly enhanced at PTX concentrations greater than 50 ng/ml (Fig. 1a), in contrast with the dose-response curve for the secretagogue-evoked catecholamine secretion (Fig. 1b). Since an increase in intracellular Ca^{2+} concentrations plays an essential role in catecholamine release from chromaffin cells [27,28], we examined the effect of PTX pretreatment on secretagogue-evoked $^{45}\text{Ca}^{2+}$ influx; however, neither basal $^{45}\text{Ca}^{2+}$ uptake nor nicotine- or high- K^+ -induced $^{45}\text{Ca}^{2+}$ uptake were affected by PTX (results not shown).

In α -toxin-permeabilized chromaffin cells, catecholamine secretion is triggered by increasing the Ca^{2+} concentration in the incubation medium. Pretreating these cells with increasing concentrations of PTX had no effect on basal release measured in the absence of Ca^{2+} , but potentiated secretion in response to 20 μ M free Ca^{2+} (Fig. 2). In agreement with the previous observations on intact cells, a maximal effect (150% of control) was observed by preincubating cells for 4 h with 100 ng of PTX/ml. Control experiments were done using PTX modified by treatment with NEM, which inactivates the ADP-ribosyltransferase activity without changing the binding properties of the toxin [25]. NEM-

inactivated PTX caused no significant stimulation of the secretory activity in permeabilized cells (Fig. 2).

Fig. 3 shows the Ca^{2+} -dose-response curve for α -toxin-permeabilized chromaffin cells. PTX enhanced the release of catecholamines evoked by Ca^{2+} concentrations greater than 1 μ M, but had little effect at lower Ca^{2+} concentrations, indicating that the toxin requires a minimal Ca^{2+} concentration in order to potentiate the secretory process.

Effect of PTX on the generation of second messengers

PKC-dependent pathway. Direct activation of PKC with phorbol esters enhances catecholamine release from intact (see below) and permeabilized [1] chromaffin cells. Therefore we have examined whether the effect of PTX on Ca^{2+} -evoked catecholamine secretion could be mediated by PKC activation. In both control and PTX-treated chromaffin cells, the catecholamine release evoked by either 59 mM- K^+ (Fig. 4) or 20 μ M-nicotine (results not shown) was similarly enhanced by the phorbol ester

Fig. 1. Effect of PTX pretreatment on secretagogue-induced catecholamine release from intact chromaffin cells

Chromaffin cells were labelled with [^3H]noradrenaline and then incubated for 4 h with the indicated concentrations of PTX in the culture medium. (a) Release of [^3H]noradrenaline was measured during the 4 h incubation with PTX and is expressed as a percentage of total radioactivity present in the cells before incubation with the toxin. (b) Cells were subsequently washed with Locke's solution and then stimulated for 10 min with 10 μ M-nicotine or 59 mM- K^+ or 30 μ M-veratridine. Basal release was estimated by incubating the cells for 10 min in Locke's solution. [^3H]Noradrenaline release is expressed as a percentage of total radioactivity present in the cells before stimulation.

Fig. 2. Effect of PTX pretreatment on Ca^{2+} -evoked catecholamine release from α -toxin-permeabilized chromaffin cells

Chromaffin cells were loaded with [^3H]noradrenaline and then incubated with the indicated concentration of PTX (●, ○) or PTX modified with NEM (■) in the culture medium. Cells were then washed four times with Locke's solution and twice with Ca^{2+} -free Locke's solution, and then permeabilized for 30 min with α -toxin (40 units/ml) in Ca^{2+} -free KG medium. Cells were subsequently stimulated with KG medium in the presence (●, ■) or absence (○) of $20 \mu\text{M}$ free Ca^{2+} . After a 10 min stimulation, extracellular fluids were collected and the [^3H]noradrenaline present in solutions and cells was assayed. [^3H]Noradrenaline release is expressed as a percentage of the total radioactivity present in the cells before stimulation. There was no significant difference in [^3H]noradrenaline release during the 30 min permeabilization period over the range of PTX concentrations tested ($9.7 \pm 1.2\%$ of the total radioactivity present in the cells before permeabilization).

Fig. 3. Effect of PTX on the Ca^{2+} -dose-response curve for secretion in α -toxin-permeabilized chromaffin cells

Chromaffin cells labelled with [^3H]noradrenaline were preincubated for 4 h in the presence (■) or absence (●) of 100 ng of PTX/ml. Cells were then washed, permeabilized for 30 min with α -toxin in Ca^{2+} -free KG medium and subsequently stimulated for 10 min with KG medium containing the indicated free Ca^{2+} concentration. [^3H]Noradrenaline release is expressed as a percentage of the total radioactivity present in the cells before stimulation.

Fig. 4. Effect of PMA and sphingosine on the PTX-dependent potentiation of catecholamine secretion from intact chromaffin cells

Chromaffin cells labelled with [^3H]noradrenaline were preincubated for 4 h in the presence (●, ○) or absence (■, □) of 100 ng of PTX/ml in the culture medium. Cells were then washed with Locke's solution, incubated for 10 min with the indicated concentrations of either sphingosine (○, □) or PMA (●, ■) in Locke's solution and stimulated for 10 min with 59 mM-K^+ . [^3H]Noradrenaline release is expressed as a percentage of the total radioactivity present in the cells before stimulation. PMA and sphingosine had no effect on basal release, as determined by incubating cells for 10 min in Locke's solution (results not shown).

Table 1. Effect of PTX on membrane-bound PKC activity in intact and permeabilized chromaffin cells

Chromaffin cells were treated with PTX (100 ng/ml), washed and then stimulated for 10 min with Locke's solution (intact cells) or 59 mM-K^+ in Locke's solution (intact cells) or permeabilized with SLO for 5 min in the presence or absence of $4 \mu\text{M}$ free Ca^{2+} . Cells were then immediately scraped in ice-cold homogenizing buffer and the suspension was sonicated and centrifuged to separate the membrane fraction from the soluble fraction. Data show membrane bound PKC activity in pmol/min per mg of protein.

	PKC activity (pmol/min per mg of protein)	
	Control	PTX
Intact cells		
Locke	177.3 ± 5.4	159.4 ± 16.9
59 mM-K^+	286.2 ± 7.7	285.6 ± 7.2
Permeabilized cells		
Zero Ca^{2+}	115.2 ± 1.0	115.2 ± 8.7
$4 \mu\text{M-Ca}^{2+}$	195.7 ± 9.8	199.9 ± 19.0

phorbol 12-myristate 13-acetate (PMA). In addition, neither sphingosine (Fig. 4), an inhibitor of PKC, nor 24 h of treatment with PMA (results not shown), a treatment which significantly decreases PKC activity in chromaffin cells [1,29], modified the potentiation of catecholamine secretion induced by PTX. We also examined whether PTX could affect the subcellular distribution of PKC in resting and stimulated chromaffin cells. However, the repartition of PKC between soluble and particulate fractions was not modified by pretreating chromaffin cells with PTX (Table 1), indicating that PTX apparently does not stimulate catecholamine release by activating PKC.

cAMP-dependent pathway. In chromaffin cells, the adenylate cyclase activator forskolin was observed to enhance both Ca^{2+} -

Fig. 5. Effect of forskolin on catecholamine secretion from intact and α -toxin-permeabilized chromaffin cells

Chromaffin cells labelled with [^{3} H]noradrenaline were washed with Locke's solution and then incubated for 2×10 min with Locke's solution containing the indicated concentration of forskolin. Cells were subsequently stimulated with $10 \mu\text{M}$ -nicotine ($\bullet$) or 59 mM - K^+ ($\blacksquare$) in the presence of forskolin. Cells permeabilized with α -toxin ($\blacktriangle$) were incubated for 20 min in Ca^{2+} -free KG medium containing forskolin and then stimulated with KG medium containing $20 \mu\text{M}$ free Ca^{2+} in the presence of forskolin. [^{3} H]Noradrenaline release is expressed as a percentage of the total radioactivity present in the cells before stimulation. Forskolin over the range of concentrations tested had no effect on basal release in intact and permeabilized cells (results not shown).

Table 2. Effect of PTX, cholera toxin and forskolin on cAMP levels in cultured chromaffin cells

Chromaffin cells were incubated with forskolin (20 min) or PTX as shown. Cells were then lysed and centrifuged, and the supernatant was assayed for cAMP.

	Concentration of cAMP (pmol/ 10^6 cells)	Relative concentration (%)
Basal	0.32 ± 0.10	100
Forskolin ($1 \mu\text{M}$)	1.35 ± 0.25	420 ± 78
Forskolin ($10 \mu\text{M}$)	4.10 ± 0.59	1280 ± 184
Forskolin ($50 \mu\text{M}$)	5.76 ± 0.50	1800 ± 156
PTX ($1 \mu\text{g/ml}$; 4 h)	0.26 ± 0.06	81 ± 18

Table 3. Effect of PTX on [^{14} C]arachidonic acid release in resting and stimulated chromaffin cells

Chromaffin cells were loaded for 5 h with [^{14} C]arachidonic acid in culture medium in the presence or absence of PTX (100 ng/ml ; 4 h). Cells were then washed, permeabilized with α -toxin and then stimulated for 10 min in the presence or absence of $20 \mu\text{M}$ free Ca^{2+} . [^{14} C]Arachidonic acid release is expressed as a percentage of the total radioactivity present in the cells before stimulation. Pretreatment with PTX had no significant effect on the release of [^{14} C]arachidonic acid in either resting or stimulated chromaffin cells ($P > 0.05$; $n = 10$).

[Ca^{2+}] (μM)	[^{14} C]Arachidonic acid release (%)	
	Control	PTX
0	2.71 ± 0.22	2.81 ± 0.24
10	4.18 ± 0.28	4.60 ± 0.38

Fig. 6. Effect of arachidonic acid on Ca^{2+} -evoked catecholamine release from α -toxin-permeabilized chromaffin cells pretreated with PTX

(a) Arachidonic acid dose-response curve for catecholamine secretion from control and PTX-treated chromaffin cells. [^{3} H]Noradrenaline-labelled chromaffin cells were incubated for 4 h in the presence ($\square$, $\blacksquare$) or absence ($\circ$, $\bullet$) of 100 ng/ml of PTX/ml. Cells were then washed, permeabilized with α -toxin and stimulated for 10 min with KG medium containing arachidonic acid at the indicated concentration in the presence ($\blacksquare$, $\bullet$) or absence ($\square$, $\circ$) of $20 \mu\text{M}$ free Ca^{2+} . (b) Effect of PTX and arachidonic acid (AA) on the Ca^{2+} -dose-response curve for secretion in α -toxin-permeabilized chromaffin cells. Chromaffin cells were preincubated in culture medium in the presence ($\blacksquare$) or absence ($\bullet$, $\blacktriangle$) of 100 ng of PTX/ml. Cells were then permeabilized for 30 min with α -toxin (40 units/ml) and subsequently stimulated for 10 min with KG medium containing the indicated free Ca^{2+} concentrations, in the presence ($\blacktriangle$) or absence ($\bullet$, $\blacksquare$) of $250 \mu\text{M}$ -arachidonic acid.

evoked catecholamine release from α -toxin-permeabilized cells and nicotine- and high- K^+ -induced release from intact cells (Fig. 5). The inhibitory effect of higher concentrations of forskolin on the nicotinic response may be related to a non-specific blockade of the cholinergic-receptor-coupled ion channel, as reported for PC12 cells and rat sympathetic ganglia [30,31]. In order to determine whether the activatory effect of PTX may be mediated by an elevation in cAMP, we measured cellular cAMP levels in control cells and in cells pretreated with forskolin or PTX. However, in contrast with forskolin, PTX did not modify the intracellular level of cAMP (Table 2), rendering unlikely the possibility that PTX increases secretion through cAMP-dependent mechanisms.

Phospholipase A_2 . We also investigated the possibility that PTX may enhance catecholamine secretion in chromaffin cells by activating phospholipase A_2 and arachidonic acid release. Stimulation of α -toxin-permeabilized chromaffin cells for 10 min with $20 \mu\text{M}$ free Ca^{2+} caused almost a 2-fold increase in the amount of [^{14} C]arachidonic acid released into the medium (Table 3), in agreement with the values previously reported with digitonin-permeabilized cells [32]. Pretreatment with 100 ng of PTX/ml did not significantly modify the basal [^{14} C]arachidonic acid release estimated in the absence of Ca^{2+} , nor did it affect the [^{14} C]arachidonic acid release evoked by $20 \mu\text{M}$ free Ca^{2+} (Table 3). We examined the effect of introducing exogenous arachidonic acid on catecholamine release from untreated and PTX-treated cells. In α -toxin-permeabilized cells, arachidonic acid enhanced in a dose-dependent manner the catecholamine secretion induced by $20 \mu\text{M}$ free Ca^{2+} (Fig. 6). Pretreatment of chromaffin cells with 100 ng of PTX/ml potentiated [^{3} H]noradrenaline release over

Fig. 7. Effect of PTX on GTP[S]-induced potentiation of secretion from α -toxin-permeabilized chromaffin cells

Chromaffin cells labelled with [^{3} H]noradrenaline were preincubated for 4 h with the indicated concentration of PTX in the culture medium. Cells were subsequently washed, permeabilized with α -toxin in Ca^{2+} -free KG medium and then stimulated for 10 min with KG medium containing $20 \mu\text{M}$ free Ca^{2+} , in the presence (■) or absence (○) of $50 \mu\text{M}$ -GTP[S]. Basal release was determined by incubating cells for 10 min in Ca^{2+} -free KG medium, in the presence (□) or absence (○) of $50 \mu\text{M}$ -GTP[S]; no modification of the basal release was observed.

the range of arachidonic acid concentrations tested (Fig. 6a), suggesting that arachidonic acid and PTX modulate the Ca^{2+} -evoked secretory process by distinct mechanisms. Moreover, arachidonic acid induced mainly a leftward shift in the Ca^{2+} activation curve for secretion, whereas PTX enhanced both Ca^{2+} sensitivity and the extent of release at high Ca^{2+} concentrations (Fig. 6b).

Effect of guanosine 5'-[γ -thio]triphosphate (GTP[S]) on catecholamine release from PTX-treated chromaffin cells

We have recently reported that non-hydrolysable guanine nucleotide analogues potentiate Ca^{2+} -evoked catecholamine release from α -toxin-permeabilized chromaffin cells in a dose-dependent manner [1]. The possibility that PTX may interfere with the stimulatory effect of GTP[S] was therefore considered. Chromaffin cells pretreated with various concentrations of PTX were permeabilized with α -toxin and subsequently stimulated with $20 \mu\text{M}$ free Ca^{2+} in the presence of $50 \mu\text{M}$ -GTP[S]. As illustrated in Fig. 7, GTP[S] enhanced the secretory response to a similar extent in cells pretreated with either submaximal or maximal concentrations of PTX, suggesting that GTP[S] and PTX modulate the secretory process by acting on two distinct sites.

Loss of chromaffin-cell responsiveness to PTX after permeabilization by SLO

The effect of PTX on the Ca^{2+} -evoked secretory response from chromaffin cells permeabilized with SLO was also examined. Fig. 8 shows that PTX potentiated catecholamine release evoked by Ca^{2+} , provided that SLO-permeabilized cells were stimulated immediately with Ca^{2+} . When a lag time was introduced between permeabilization and the Ca^{2+} -induced stimulation, there was a marked progressive loss of the PTX effect on secretion (Fig. 8). Similar results were observed with digitonin-treated chromaffin cells (results not shown). In contrast, with α -toxin-permeabilized

Fig. 8. Effect of varying the incubation period between permeabilization and Ca^{2+} -induced stimulation on the PTX-induced potentiation of secretion from α -toxin- and SLO-permeabilized chromaffin cells

Chromaffin cells were loaded with [^{3} H]noradrenaline and then incubated for 4 h in the presence (□, ■) or absence (○, ●) of 100 ng of PTX/ml. Cells were subsequently washed four times with Locke's solution, twice with Ca^{2+} -free Locke's solution and permeabilized with α -toxin (40 units/ml) (○, □, △) or SLO (15 units/ml) (●, ■, ▲) in Ca^{2+} -free KG medium. Cells were then incubated for the indicated times in Ca^{2+} -free KG medium before stimulation for 10 min with KG medium containing $20 \mu\text{M}$ free Ca^{2+} . Basal release was estimated by stimulating cells for 10 min with Ca^{2+} -free KG medium (△, ▲). [^{3} H]Noradrenaline release is expressed as a percentage of the total radioactivity present in the cells before stimulation.

cells, full retention of the PTX-induced activation was observed even at 30 min after permeabilization (Fig. 8). The major difference between these cell preparations is that only small metabolites ($< 5 \text{ kDa}$) leak out from α -toxin-treated cells, whereas large cytoplasmic proteins such as lactate dehydrogenase diffuse out from SLO- or digitonin-permeabilized cells [15,16,33–36]. Thus substrates for PTX may be lost from these latter preparations.

To test this possibility, soluble proteins released from digitonin-permeabilized cells were collected, concentrated and tested for the presence of target proteins for PTX. After gel electrophoresis, PTX specifically ADP-ribosylated three proteins of molecular masses of 39, 40 and 41 kDa (Fig. 9, lane C). All of these three PTX substrates were present in chromaffin cell plasma membrane preparations (Fig. 9, lane E), but the 39 kDa and 40 kDa ADP-ribosylated bands were more intensely labelled in fractions containing highly purified chromaffin granule membranes (Fig. 9, lane F). Interestingly, the addition of G-protein $\beta\gamma$ -subunits to the incubation medium was required to detect the 39 kDa ADP-ribosylated band among the cytosolic proteins released from digitonin-treated cells (Fig. 9, lane C), in agreement with the fact that $\beta\gamma$ has a higher affinity for the 40 and 41 kDa α_i -subunits than for the 39 kDa α_o -subunit [37].

Effects of alkylating agents

Pretreatment of chromaffin cells with *p*-bromophenacyl bromide (BPB) or NEM, two protein alkylating agents, results in a dose-dependent inhibition of Ca^{2+} -stimulated exocytosis in

Fig. 9. Autoradiogram of PTX-catalysed [³²P]ADP-ribosylated proteins present among the cytosolic proteins released from digitonin-permeabilized chromaffin cells

Chromaffin cells were permeabilized for 10 min with 10 μ M-digitonin in Ca²⁺-free KG medium and then incubated for 30 min in Ca²⁺-free KG medium without detergent; proteins released from the cytosol during this 30 min incubation period were collected. Chromaffin-cell plasma membranes and chromaffin-granule membranes were purified on sucrose-density gradients [22]. Proteins were ADP-ribosylated with [³²P]NAD⁺ in the presence of PTX by the method described by Toutant *et al.* [22]. Lanes A-C, cytosolic proteins released from digitonin-treated cells (60 μ g of protein); lane E, purified chromaffin-cell plasma membrane proteins (28 μ g of protein); lane F, chromaffin granule membrane proteins (63 μ g of protein). In lanes C and D, 0.5 μ g of $\beta\gamma$ -subunits from purified G_o and G_i G-proteins (purified as described in ref. [62]) were included in samples. As controls, no labelling was found when PTX was not included in the incubation sample (lane A) or when cytosolic proteins leaked from digitonin-permeabilized cells were absent (lane D). In addition, no labelling was detected when ADP-ribosylation was carried out on extracellular fluid from α -toxin-treated cells (results not shown).

Fig. 10. Effect of BPB and NEM on catecholamine secretion from control and PTX-treated chromaffin cells

Chromaffin cells labelled with [³H]noradrenaline were preincubated in the presence (□, ■) or absence (○, ●) of 100 ng of PTX/ml, washed and then incubated for 15 min in Ca²⁺-free Locke's solution containing the indicated concentration of either BPB (a) or NEM (b). Cells were subsequently permeabilized and stimulated with SLO (15 units/ml) in KG medium in the presence (■, ●) or absence (□, ○) of 10 μ M free Ca²⁺. Catecholamine release was determined after 5 min.

digitonin-permeabilized chromaffin cells [32]. The effects of BPB and NEM on catecholamine release from SLO-permeabilized chromaffin cells pretreated with PTX are shown in Fig. 10. BPB and NEM caused a marked decrease in the Ca²⁺-induced catecholamine secretion, with IC₅₀ (concn. causing half-maximal

inhibition) values of 5 μ M and 65 μ M respectively. Both agents inhibited the PTX-dependent enhancement of catecholamine release at lower concentrations, particularly NEM, which induced a half-maximal inhibition of the PTX effect at 18 μ M. Thus PTX apparently enhances catecholamine secretion in chromaffin cells by a mechanism which is sensitive to alkylating agents.

DISCUSSION

The results presented here demonstrate that pretreatment of cultured bovine chromaffin cells with PTX facilitates the release of catecholamines evoked by nicotine, KCl and veratridine, confirming a recent report by Tanaka *et al.* [38]. Furthermore, we observed that the ⁴⁵Ca²⁺ uptake evoked by either nicotine or KCl was not altered by PTX, in agreement with the observation that neither basal nor carbamoylcholine- or KCl-induced rises in [Ca²⁺]_i are sensitive to PTX [39]. These results suggest that PTX may increase catecholamine release by acting on a common process of the depolarization-mediated and the receptor-mediated secretory mechanisms, distal to the rise in [Ca²⁺]_i, such as the final events in the exocytotic process. Accordingly, PTX facilitates the Ca²⁺-dependent secretory process in α -toxin-permeabilized cells, and also in SLO-permeabilized cells if the latter are permeabilized in a medium containing Ca²⁺.

GTP-binding proteins have been implicated in the regulation of the synthesis of second messengers such as cAMP, phosphoinositide breakdown products and arachidonic acid [40,41]. Since these agents are known to modulate catecholamine secretion in chromaffin cells, it is necessary to ascertain whether the facilitation of catecholamine release provoked by PTX involves changes in the cytoplasmic levels of these compounds.

An increase in cAMP synthesis stimulates catecholamine secretion from intact chromaffin cells [42,43]. Here we show that the elevation of cAMP with forskolin also potentiated Ca²⁺-evoked secretion from permeabilized cells, indicating that cAMP can modulate stimulus-secretion coupling downstream of Ca²⁺ entry. Since PTX is known to ADP-ribosylate the G_i-protein which inhibits adenylate cyclase [44,45], it could theoretically cause an increase in cytoplasmic cAMP which might account for the PTX-induced facilitation of secretion. However, treatment of the cells with a concentration of PTX which caused maximal catecholamine release had no effect on the cytoplasmic levels of cAMP, indicating that the stimulatory effect of PTX is probably not mediated by an increase in the cytoplasmic concentration of cAMP.

In many systems membrane-bound phosphoinositides are degraded into a number of inositol phosphates and diacylglycerol by a phosphoinositide-specific phospholipase C. The breakdown of phosphoinositides results in mobilization of intracellular Ca²⁺ and activation of PKC. Guanine nucleotides can activate phospholipase C, supporting the idea that a G-protein controls phospholipase C activity [41,46]. Tumour-promoting phorbol esters (e.g. PMA), which can substitute for diacylglycerols and activate PKC, enhance Ca²⁺-dependent catecholamine secretion from intact (the present paper) and permeabilized [1,34] chromaffin cells. Moreover, a nicotinic stimulation causes the activation of PKC, as indicated by the translocation of the enzyme from a soluble to a membrane-bound compartment [36]. Thus the PTX-induced enhancement of catecholamine release could conceivably be mediated by the activation of PKC. This mechanism seems unlikely, for the following reasons. (i) Inhibition of PKC by sphingosine or by long-term incubation with PMA did not interfere with the facilitation of catecholamine release induced by PTX. (ii) Pretreatment with PTX did not affect the translocation of PKC observed in intact cells stimulated with high concentration of K⁺ or in permeabilized cells stimulated

with Ca^{2+} . We have recently reported that guanine nucleotides potentiate Ca^{2+} -evoked secretion in α -toxin-permeabilized chromaffin cells by activating PKC [1]. As illustrated here, this effect was still observed in PTX-treated cells, indicating that the G-protein which probably modulates the activity of PKC through phospholipase C is not sensitive to PTX in chromaffin cells. In agreement, a G-protein which is not a substrate for PTX has been reported to be involved in phosphoinositide metabolism in several cell types [47,48], including chromaffin cells [49]. Moreover, the observation that GTP[S] enhanced to a similar extent the secretory response in control cells and in cells pretreated with PTX is in line with the proposition that PTX and PKC do not act at the same site of the secretory process. Finally, neomycin, a phospholipase C inhibitor [3], does not change the facilitating action of PTX on secretion (M.-F. Bader, J.-M. Sontag & D. Aunis, unpublished work), and high-[K^+]-induced inositol trisphosphate accumulation is not affected in PTX-treated cells [39], providing further evidence that phospholipase C and phosphoinositide breakdown products do not play a role in the action of PTX.

It has been reported that phospholipase A_2 may be regulated by both stimulatory and inhibitory PTX-sensitive G-proteins [50–53]. Thus another possibility that may explain the facilitatory effect of PTX on the secretory process is that the toxin could modify the activity of phospholipase A_2 and thereby the production of arachidonic acid. Indeed, we observed that an increase in cytoplasmic arachidonic acid enhanced the Ca^{2+} -evoked secretory process in α -toxin-permeabilized chromaffin cells, in agreement with the data recently obtained using digitonin-treated cells [54]. However, arachidonic acid did not mimic the effect of PTX on the Ca^{2+} -dose-response curve for secretion, and pretreatment of the cells with PTX had no significant effect on the release of arachidonic acid from either resting or stimulated chromaffin cells. These observations rule out the possibility that the stimulatory effect of PTX on secretion may be mediated by activation of phospholipase A_2 .

In summary, our data indicate that PTX facilitates the exocytotic release of catecholamines from chromaffin cells by a mechanism which acts at a step distal to Ca^{2+} entry, independently of changes in the levels of cAMP, arachidonic acid and InsP_3 . In addition, the effect of PTX cannot be attributed to the activation of PKC. This raises the question of the identity of the effector systems mediating the action of PTX. To our knowledge, there is only one report indicating that PTX can activate platelets by a mechanism which does not involve the ADP-ribosylation of a G-protein [25]. However, this activating effect was also observed with NEM-inactivated toxin and was attributed to the B-oligomer of PTX [25]. In contrast, NEM-inactivated PTX was unable to potentiate the secretory response in chromaffin cells. Moreover, permeabilization with SLO progressively decreased the facilitating effect of PTX on the secretory response and produced a concomitant leakage of three G-protein α -subunits that are specifically ADP-ribosylated by PTX. Thus we propose that PTX potentiates the secretory response in chromaffin cells by modulating the activity of a G-protein. This G-protein may be coupled to the production of an as yet unidentified second messenger which is necessary for secretion. Alternatively, this PTX-sensitive G-protein may regulate secretion in chromaffin cells independently of all known intracellular signals. In fact, a class of G-proteins (G_e) which directly activates [2,4,7] or inhibits [5,6,55] the secretory process at the exocytotic sites has been described. Interestingly, the inhibitory G_e proteins that have been identified in several secretory systems seem to be substrates for PTX [5,6].

Our finding that PTX potentiates catecholamine release from permeabilized cells stimulated by Ca^{2+} in the absence of any

exogenous receptor agonist is somewhat difficult to reconcile 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. This effect of PTX on permeabilized cells may be analogous to the PTX-induced catecholamine release observed in intact chromaffin cells in the absence of any stimulatory or inhibitory agonists (the present paper; [26]). These observations raise the possibility that two types of G-protein control the secretory process in chromaffin cells: the 'classic' G-proteins, which are under the direct control of plasma membrane receptors, and G-proteins which may be controlled by intracellular factors and can be uncoupled from them by PTX. Interestingly, PTX-sensitive G-proteins have been described in intracellular organelles such as chromaffin secretory granules [22]. The recent finding that proteins of the G_e family seem to be regulated by intracellular as well as extracellular signals [56] also offers strong support for our hypothesis.

Koffer & Gomperts [57] have recently postulated the existence of a cytosolic protein which reduces the affinity for Ca^{2+} of the exocytotic reaction in mast cells. They indicate that this inhibitory protein leaks immediately from cells permeabilized with SLO. Similarly, we find here that the stimulatory effect that PTX exerts on secretory activity is rapidly lost when chromaffin cells are treated with SLO. If PTX uncouples G-proteins from intracellular or extracellular regulators, then our results argue for the occurrence of a G-protein playing an inhibitory role in the Ca^{2+} -dependent exocytotic process in chromaffin cells. This inhibitory G-protein appears to behave like the inhibitory cytosolic protein described in mast cells [57], also being lost within minutes of treatment with SLO. The effector coupled to this putative inhibitory G-protein remains to be characterized. An attractive speculation is that this G-protein inhibits a docking protein [58] or a putative fusion protein of the plasma membrane. Our observation that the effect of PTX was specifically abolished by treatment of the cells with NEM before stimulation seems to indicate that this target protein may be sensitive to NEM. In this context, it is of interest that a NEM-sensitive fusion protein has been purified on the basis of its ability to catalyse fusion of intracellular membranes in both secretory and endocytotic pathways [59–61].

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