

# Ca<sup>2+</sup>-Stimulated Catecholamine Release from $\alpha$ -Toxin-Permeabilized PC12 Cells: Biochemical Evidence for Exocytosis and Its Modulation by Protein Kinase C and G Proteins<sup>†</sup>

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**ABSTRACT:** Two possible cellular pathways of catecholamines from the chromaffin vesicles of PC12 cells to the surrounding medium are explored in this study. The direct one circumventing the cytoplasm can be activated in  $\alpha$ -toxin-permeabilized cells with micromolar levels of free Ca<sup>2+</sup>. Catecholamine metabolites formed in the cytoplasm (i.e., 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylethanol) are neither formed nor released from the cells under these conditions. However, when vesicular catecholamines were discharged into the cytoplasm by addition of the ionophore nigericin, such metabolites are formed and released into the medium independent of Ca<sup>2+</sup>. Both types of experiments provide direct evidence for the operation of Ca<sup>2+</sup>-induced exocytosis of dopamine and noradrenaline in permeabilized PC12 cells. The Ca<sup>2+</sup> dependence of dopamine or noradrenaline release, as measured by the determination of the endogenous catecholamines using the high-performance liquid chromatography technique, exhibits two different phases. One is already activated below 1  $\mu$ M free Ca<sup>2+</sup> and plateaus at 1–5  $\mu$ M free Ca<sup>2+</sup>, while a second occurs in the presence of larger amounts of free Ca<sup>2+</sup> (10–100  $\mu$ M). Ca<sup>2+</sup>-induced catecholamine release from the permeabilized cells can be modulated in different ways: It is enhanced by the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate and the diacylglycerol 1-oleyl-2-acetyl-glycerol provided Mg<sup>2+</sup>/ATP is present, and it is inhibited by guanosine 5'-*O*-(3-thiotriphosphate). The latter effect is abolished by pretreatment of the cells with pertussis toxin but not by cholera toxin. Thus, it appears that Ca<sup>2+</sup>-induced exocytosis can be modulated via the protein kinase C system, as well as via GTP binding proteins.

Permeabilized secretory cells are often used to investigate the molecular mechanism underlying exocytosis. Different techniques have been introduced in the past few years to overcome the plasma membrane as a permeability barrier and thus influence the exocytotic machinery inside the cells [cf. Knight and Baker (1982), Wilson and Kirshner (1983), Dunn and Holz (1983), and Brooks and Trembl (1983)]. However, it is often not clear in these investigations to what extent exocytosis, leakiness, or even release of intact secretory vesicles contributes to the observed release of secretory product from permeabilized cells. Leakiness of the secretory vesicle membrane and escape of intact secretory vesicles through large membrane lesions (Brooks & Carmichael, 1983) are serious problems in studies using detergents as permeabilizing agents (Bader et al., 1986).

Parallel release of large and small molecular weight substances from secretory vesicles is certainly a convincing indication for exocytosis, provided that cytoplasmic substances similar in size to the larger ones do not leak out from the cells. While it may be possible to exclude leakiness and release of secretory vesicles as such by proper controls, positive evidence for exocytosis is provided only in few investigations with permeabilized cells. Especially in cells permeabilized by detergents, it has not been possible to convincingly demonstrate exocytosis and simultaneously exclude other likely release mechanisms.

Staphylococcal  $\alpha$ -toxin creates transmembrane pores in the plasma membrane [Bhakdi et al., 1981, 1984; Füssle et al., 1981; for a review, see Bhakdi and Tranum-Jensen (1987)]. This toxin has been successfully applied to PC12 cells (Ahnert-Hilger et al., 1985a,b; Ahnert-Hilger & Gratzl, 1987; Lind et al., 1987) and bovine chromaffin cells in primary culture (Bader et al., 1986) to selectively permeabilize the plasma membrane. Whereas small molecules can be exchanged, larger ones [i.e., cytoplasmic lactate dehydrogenase (LDH)<sup>1</sup>] are retained by these cells.

As shown for chromaffin cells in primary culture (Bader et al., 1986), the size of the pores allows one to take the release of the small vesicular catecholamines simultaneously with the large molecule chromogranin A as evidence for exocytosis, since cytoplasmic LDH is not released concomitantly from these cells. Here we report on a different approach to investigate the mode of secretion from  $\alpha$ -toxin-permeabilized PC12 cells by taking the metabolism of catecholamines in the cytoplasm as an indication of whether or not the secretory products avoid the cytoplasm. In this way, we obtained direct evidence for Ca<sup>2+</sup>-induced exocytosis in  $\alpha$ -toxin-permeabilized PC12 cells.

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<sup>1</sup> Abbreviations: BSA, bovine serum albumin; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium; KG buffer, potassium glutamate buffer; NTA, nitriloacetic acid; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DOPAC, 3,4-dihydroxyphenylacetic acid; DOPET, 3,4-dihydroxyphenylethanol; HPLC, high-performance liquid chromatography; GTP $\gamma$ S, guanosine 5'-*O*-(3-thiotriphosphate); OAG, 1-oleyl-2-acetyl-glycerol; ChT, cholera toxin; PTT, pertussis toxin; LDH, lactate dehydrogenase; EDTA, ethylenediamine-tetraacetic acid; SDS, sodium dodecyl sulfate.

We also investigated the effects of TPA and OAG as activators of protein kinase C, and of GTP $\gamma$ S, cholera toxin, and pertussis toxin on this process. The data support the conclusion that Ca<sup>2+</sup>-induced exocytosis can be modulated by the protein kinase C system as well as by GTP binding proteins.

## MATERIALS AND METHODS

**Materials.** 12-*O*-Tetradecanoylphorbol 13-acetate (TPA), 1-oleyl-2-acetyl glycerol (OAG), guanosine 5'-*O*-(3-thiotriphosphate (GTP $\gamma$ S), nigericin, bovine serum albumin (BSA), and cholera toxin (ChT) were purchased from Sigma (Deisenhofen); pertussis toxin was from List Biological Laboratories, Campbell. DMEM dried medium, horse serum, and fetal calf serum were supplied by Seromed Biochrom KG, Berlin. Culture dishes were bought from Becton Dickinson, Heidelberg. [<sup>3</sup>H]Dopamine (30 Ci/nmol) was supplied from New England Nuclear, Dreieich.

**Methods.** Rat pheochromocytoma cells (clone PC12, kindly provided by H. Thoenen, München, FRG) were cultivated in flasks as described elsewhere (Bräutigam et al., 1984). For permeabilization, they were washed twice with buffered saline [NaCl (150 mmol/L), Pipes (10 mmol/L), and EGTA (1 mmol/L) titrated to pH 7.2 with NaOH] and once with KG buffer [potassium glutamate (150 mmol/L), Pipes (10 mmol/L), NTA (5 mmol/L), and EGTA (0.5 mmol/L) titrated to pH 7.2 with KOH]. After they were suspended in KG buffer supplemented with 0.1% BSA, they were permeabilized with  $\alpha$ -toxin (200 units/mL, diluted in the same buffer) at 30 °C for 30 min. After a short centrifugation (Eppendorf, 0.5 min at 10000g), the supernatant was removed. For stimulation, the cells were incubated in KG medium containing the given free Ca<sup>2+</sup> concentrations for a further 10 min at 30 °C. Each sample contained about 600  $\mu$ g of cell protein per 200  $\mu$ L.

The content of noradrenaline, dopamine, and the metabolites of dopamine (DOPAC and DOPET) was estimated in the supernatants (during permeabilization and stimulation) as well as in the cells at the end of the experiment. For HPLC, each supernatant (200  $\mu$ L) received 20  $\mu$ L of 4 M perchloric acid containing 0.5% EDTA and 0.13% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> supplemented with 50 ng of adrenaline as internal standard. Cells were extracted with 400  $\mu$ L of 0.24 M perchloric acid supplemented with the same constituents as above by sonication. The supernatant of the cell extract was subjected to HPLC.

HPLC separation and electrochemical detection (Waters M 460 electrochemical detector; oxidation potential, +800 mV) of catecholamines were done as described elsewhere (Bräutigam et al., 1982), but for better separation of the dopamine metabolites, the following mobile phase was used: Solutions of 0.1 M sodium acetate and 0.1 M citric acid in bidistilled water, both containing 0.5 mM 1-octanesulfonic acid, 0.5 mM di-*n*-butylamine, and 0.1 mM EDTA, were mixed to yield a final pH of 3.4; after filtration, 3.5% (v/v) methanol was added. The flow rate was usually 0.8 mL/min, and 20- $\mu$ L samples were injected into the HPLC.

For studies using tritiated dopamine, cells were cultivated and treated as described earlier (Ahnert-Hilger et al., 1985b; Ahnert-Hilger & Gratzl, 1987). The experimental protocol followed the one with the nonlabeled cells (see above) except that radioactivity was counted in the media and in the SDS lysate of the cells. Each sample contained about 100  $\mu$ g of protein. Modifications and further details are given in the figure legends.

The free Ca<sup>2+</sup> and Mg<sup>2+</sup> concentrations were calculated by the aid of a computer program according to Flodgaard and Fleron (1974), kindly provided by T. Saermark, University

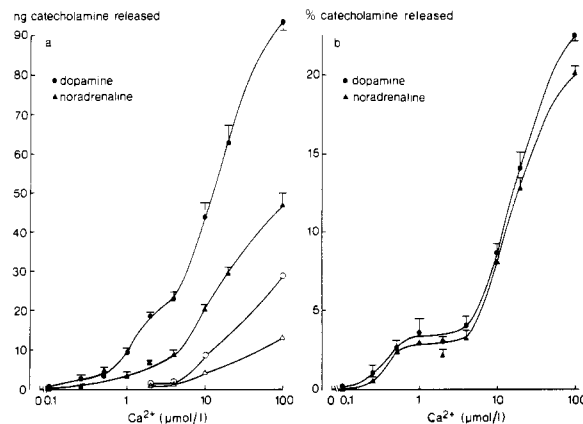


FIGURE 1: Corelease of dopamine and noradrenaline in  $\alpha$ -toxin-permeabilized PC12 cells as a function of the free Ca<sup>2+</sup> concentration. Samples of 600  $\mu$ g of protein of  $\alpha$ -toxin-treated cells were incubated in fresh KG buffer supplemented with the indicated Ca<sup>2+</sup> concentrations. The cells contained about twice as much dopamine as noradrenaline at the beginning of the experiment (523  $\pm$  63 ng vs 233  $\pm$  25 ng, SD,  $n$  = 48). During permeabilization, the cells released 167  $\pm$  24 ng of dopamine and 45.5  $\pm$  14.4 ng of noradrenaline; control cells 160  $\pm$  28 ng and 47  $\pm$  6.9 ng, respectively. (a) The Ca<sup>2+</sup>-stimulated release (10 min at 30 °C) is given in nanograms per sample [dopamine (●); noradrenaline (▲)]. The release in the absence of Ca<sup>2+</sup> was subtracted (28.2  $\pm$  1 ng of dopamine and 12.6  $\pm$  0.8 ng of noradrenaline for toxin-treated cells and 26.3  $\pm$  1.9 ng and 9.6  $\pm$  0.8 ng, respectively, for intact cells;  $n$  = 3). The closed symbols represent permeabilized cells, the open ones intact cells. (b) Values from (a) were expressed as percent of total catecholamine content in order to show the close correlation between dopamine and noradrenaline release.

of Copenhagen, Denmark, using the stability constants given by Sillen and Martell (1971). The media were also controlled with a Ca<sup>2+</sup>-sensitive electrode (Simon et al., 1978). The neutral carrier incorporated into a poly(vinyl chloride) membrane was kindly provided by Dr. W. Simon, ETH Zürich, Switzerland.  $\alpha$ -Toxin was purified from culture supernatants of *Staphylococcus aureus* strain Wood 46 using the FPLC technique (Lind et al., 1987). The toxicity was checked with 2.5% rabbit erythrocytes and calculated as described (Wadström, 1968). Protein was determined according to Lowry et al. (1951) using BSA as standard.

## RESULTS

PC12 cells, permeabilized with  $\alpha$ -toxin, release endogenous dopamine and noradrenaline (measured by the HPLC technique) as a function of the free Ca<sup>2+</sup> concentration (between 0.2 and 100  $\mu$ mol/L) in the medium (Figure 1a). The close similarity of the percentages of both catecholamines released indicates that none of the catecholamines were released preferably (Figure 1b). The Ca<sup>2+</sup> dependency of the release of both endogenous catecholamines was biphasic and thus resembled the situation seen earlier using [<sup>3</sup>H]dopamine-preloaded PC12 cells (Ahnert-Hilger et al., 1985a,b; Ahnert-Hilger & Gratzl, 1987). In order to prove that the Ca<sup>2+</sup>-induced catecholamine release in  $\alpha$ -toxin-permeabilized cells occurs via exocytosis and not by an unspecific leakiness of the vesicles, the main cytoplasmic metabolites of dopamine, 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylethanol (DOPAC and DOPET), were determined in the same samples.

Figures 2–4 show the release of dopamine, noradrenaline, DOPAC, and DOPET from intact and  $\alpha$ -toxin-permeabilized cells. Figure 2 gives an example of representative HPLC elution profiles from both intact and  $\alpha$ -toxin-permeabilized PC12 cells subjected to different treatments. In the absence

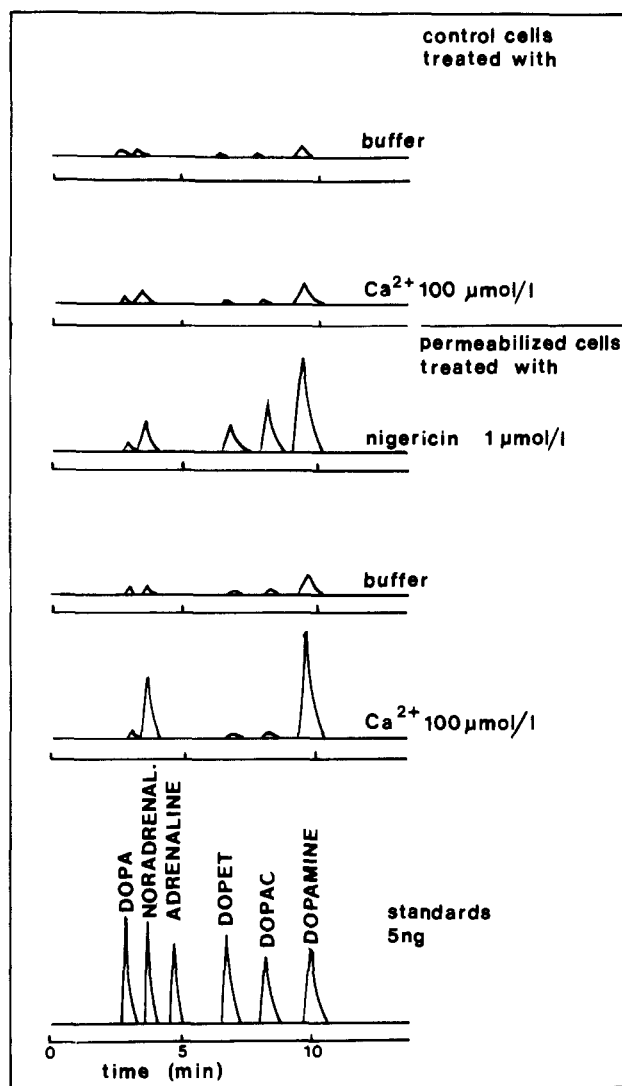


FIGURE 2: HPLC elution profiles of supernatants from differently treated control and  $\alpha$ -toxin-permeabilized PC12 cells. PC12 cells were treated either with  $\text{Ca}^{2+}$ -free KG buffer (first two traces on the top) or with the same buffer supplemented with  $\alpha$ -toxin (following traces) as given under Materials and Methods. The trace at the bottom represents the standard mixture. After removal of the permeabilization medium, the cells were subjected to the treatments indicated for 10 min at 30 °C, and the supernatant was subjected to HPLC. Under these conditions, the metabolites of noradrenaline eluted before DOPA and could not be discriminated with certainty from the front peak (not shown in the figure).

of free  $\text{Ca}^{2+}$ , the intact and the permeabilized cells released low amounts of catecholamines and hardly detectable amounts of metabolites. Addition of 100  $\mu\text{mol/L}$  free  $\text{Ca}^{2+}$  markedly increased the dopamine and noradrenaline peaks in the permeabilized cells when compared to intact cells. By contrast, the amount of DOPAC and DOPET did not change. However, upon addition of nigericin, a lipophilic ionophore which exchanges  $\text{H}^+$  for monovalent cations across the chromaffin vesicle membrane and thus abolishes or inverts the proton gradient necessary for catecholamine transport, an increased release of DOPAC and DOPET was observed due to metabolism of the released dopamine. Addition of 4, 20, or 100  $\mu\text{mol/L}$   $\text{Ca}^{2+}$  resulted in an increased release of both dopamine and noradrenaline, but not of the cytoplasmic metabolite DOPAC, from the permeabilized cells. By contrast, nigericin results in an enhanced release of both catecholamines and DOPAC in both intact and permeabilized PC12 cells (Figure 3). This indicates that noradrenaline and dopamine are effectively discharged into the cytoplasm after treatment with

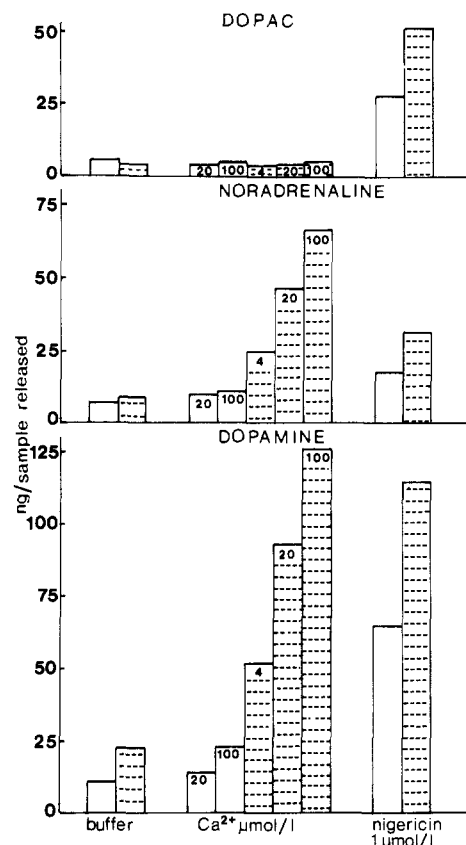


FIGURE 3: Effect of  $\text{Ca}^{2+}$  and nigericin on the release of dopamine, the metabolite of dopamine (DOPAC), and noradrenaline by permeabilized PC12 cells and control cells. PC12 cells were first permeabilized with  $\alpha$ -toxin in KG buffer and then incubated either with buffer (controls), with 4, 20, or 100  $\mu\text{mol/L}$  free  $\text{Ca}^{2+}$ , or with 1  $\mu\text{mol/L}$  nigericin for 10 min at 30 °C. The release given in nanograms per sample is the mean of two determinations. The hatched bars represent permeabilized cells, the open ones intact cells. The samples contained  $560 \pm 32 \mu\text{g}$  of protein (SD,  $n = 8$ ).

the ionophore. Part of the catecholamines are metabolized to DOPAC and are released together with the amines into the extracellular space independent of  $\text{Ca}^{2+}$ .

Figure 4 shows the kinetics of the release of dopamine, of its cytoplasmic metabolites DOPAC and DOPET, and of noradrenaline after treatment with either  $\text{Ca}^{2+}$  (20  $\mu\text{mol/L}$ ) or nigericin (1  $\mu\text{mol/L}$ ) from  $\alpha$ -toxin-permeabilized PC12 cells. As can be seen, dopamine released into the cytoplasm by nigericin (1  $\mu\text{mol/L}$ ) is subsequently metabolized to DOPAC and DOPET. By contrast, metabolites were neither formed during the incubation period in KG buffer alone nor released in KG buffer supplemented with 20  $\mu\text{mol/L}$  free  $\text{Ca}^{2+}$ . In the absence of nigericin, dopamine and noradrenaline were released from the  $\alpha$ -toxin-permeabilized PC12 cells in a  $\text{Ca}^{2+}$ -dependent way. This clearly indicates that the chromaffin vesicles within the permeabilized cells are stable in the media used and provides convincing evidence for exocytosis because a cytoplasmic pathway would inevitably lead to the formation of catecholamine metabolites.

The phorbol ester TPA enhanced  $\text{Ca}^{2+}$ -induced exocytosis in  $\alpha$ -toxin-permeabilized PC12 cells. To be effective, TPA required a further 30 min of incubation (see legend to Figure 4). Due to this long preincubation (total of 60 min), the permeabilized cells hardly respond to low amounts of free  $\text{Ca}^{2+}$  (2  $\mu\text{mol/L}$ ). However, addition of TPA (between 10 and 500 nmol/L) caused enhanced secretion in a dose-dependent manner, which could clearly be distinguished from the effects of TPA on intact cells or on  $\alpha$ -toxin-treated cells in the absence of  $\text{Ca}^{2+}$  (Figure 5). In contrast to the induction of exocytosis

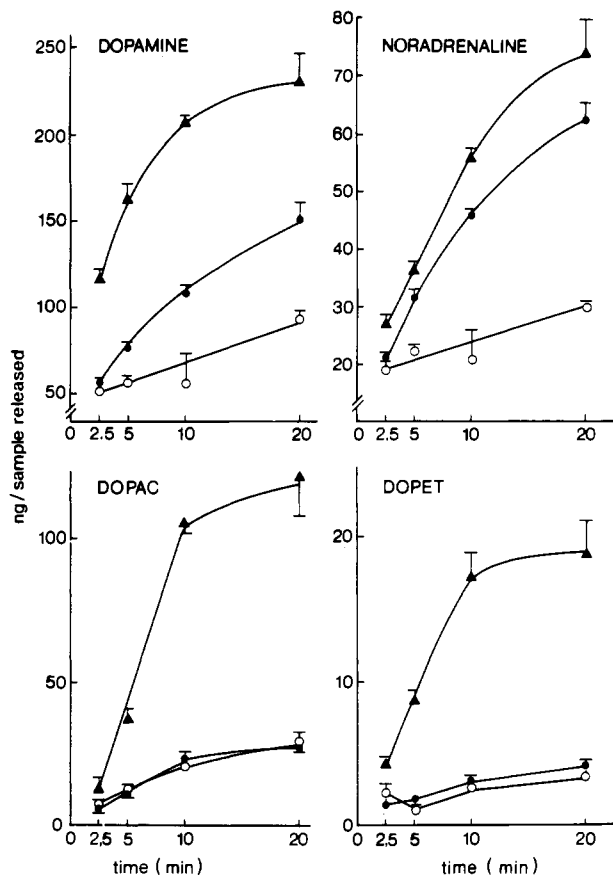


FIGURE 4: Release of noradrenaline, dopamine, and metabolites of dopamine in  $\alpha$ -toxin-permeabilized cells as a function of time. Permeabilized cells were incubated either with KG buffer ( $\circ$ ), with KG buffer supplemented with  $20 \mu\text{mol/L}$  free  $\text{Ca}^{2+}$  ( $\bullet$ ), or with  $1 \mu\text{mol/L}$  nigericin ( $\blacktriangle$ ) for the indicated time at  $30^\circ\text{C}$ . The ordinate gives the release (nanograms per sample) of catecholamines. Each value represents the mean of three determinations ( $\pm\text{SD}$ ). The samples contained  $573 \mu\text{g}$  of protein  $\pm 41.3 \mu\text{g}$  ( $n = 8$ ).

Table I:  $\text{Mg}^{2+}/\text{ATP}$  Dependence of the Stimulating Effect of TPA on  $\text{Ca}^{2+}$ -Induced Exocytosis in Permeabilized PC12 Cells<sup>a</sup>

treatment	$\text{Ca}^{2+}$ -stimulated release (%)	
	A	B
	( $4 \mu\text{mol/L}$ $\text{Ca}^{2+}$ , $1 \text{ mmol/L}$ $\text{Mg}^{2+}$ )	( $10 \mu\text{mol/L}$ $\text{Ca}^{2+}$ )
$100 \text{ nmol/L}$ TPA	$5.2 \pm 1.7$	$4.8 \pm 0.5$
$5 \text{ mmol/L}$ $\text{Mg}^{2+}/\text{ATP}$	$4.5 \pm 0.3$	$8.0 \pm 0.7$
$100 \text{ nmol/L}$ TPA + $5 \text{ mmol/L}$ $\text{Mg}^{2+}/\text{ATP}$	$5.4 \pm 0.7$	$6.3 \pm 0.6$
	$9.0 \pm 0.1$	$28.0 \pm 1.5$

<sup>a</sup> Cells were treated as in Figure 5. One fraction of the cells was incubated either with  $1 \text{ mmol/L}$  free  $\text{Mg}^{2+}$  (A) or without  $\text{Mg}^{2+}$  (B) and the other fraction in buffer with an additional  $5 \text{ mmol/L}$   $\text{Mg}^{2+}/\text{ATP}$ . TPA ( $100 \text{ nmol/L}$ ) was present during the whole experiment (70 min). Stimulation was carried out for 10 min with the given free  $\text{Ca}^{2+}$  concentrations in the indicated buffer. Values represent the mean of three determinations  $\pm \text{SD}$ ; the release in the absence of  $\text{Ca}^{2+}$  was subtracted.

by  $\text{Ca}^{2+}$ , in the absence of TPA [see Ahnert-Hilger et al. (1985b) and Ahnert-Hilger and Gratzl (1987)], the stimulating effect of TPA itself absolutely depends on the presence of  $\text{Mg}^{2+}/\text{ATP}$  (Table I). The presence of additional free  $\text{Mg}^{2+}$  was not necessary. Addition of  $1 \text{ mM}$  free  $\text{Mg}^{2+}$  in order to facilitate  $\text{Ca}^{2+}$ -induced exocytosis (Ahnert-Hilger & Gratzl, 1987) reduced the  $\text{Mg}^{2+}/\text{ATP}$ -dependent TPA effect (Table I). OAG, a synthetic analogue of diacylglycerol and a direct activator of purified protein kinase C, also increased  $\text{Ca}^{2+}$ -stimulated release. In contrast to TPA, relatively high amounts

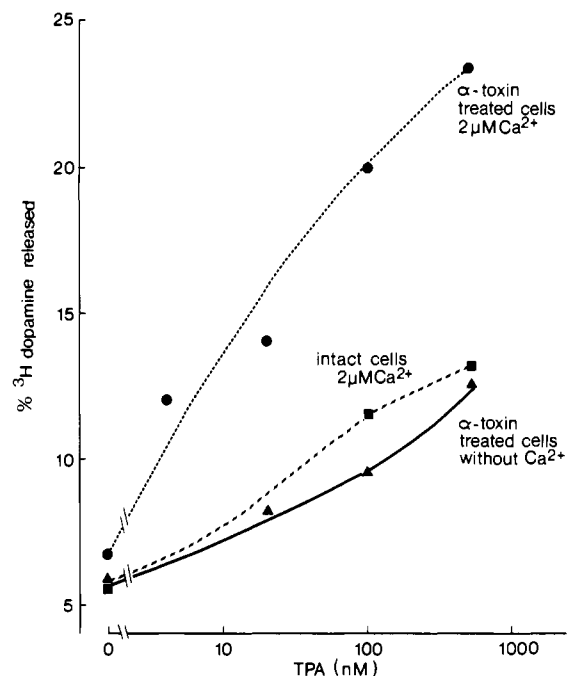


FIGURE 5: Effect of TPA on  $[^3\text{H}]$ dopamine release by permeabilized PC12 cells. Cells on plates preloaded with  $[^3\text{H}]$ dopamine (see Methods) were permeabilized with  $180 \text{ units/mL}$   $\alpha$ -toxin in KG buffer supplemented with  $5 \text{ mM}$   $\text{Mg}^{2+}/\text{ATP}$  and the given amount of TPA for 30 min at  $30^\circ\text{C}$ . This buffer was replaced by the same one, containing no toxin. After further incubation for 30 min at  $30^\circ\text{C}$ , the medium was replaced by fresh one containing in addition  $2 \mu\text{mol/L}$  free  $\text{Ca}^{2+}$ . Release of  $[^3\text{H}]$ dopamine was determined after 10 min in the supernatant and in the cell lysate. TPA was present during the whole experiment (70 min). Values are the mean of two determinations. The circles represent permeabilized cells stimulated with  $2 \mu\text{mol/L}$   $\text{Ca}^{2+}$ , the squares identically treated intact cells, and the triangles permeabilized cells with no  $\text{Ca}^{2+}$  present.

Table II: ATP-Dependent Effect of OAG and TPA on  $\text{Ca}^{2+}$ -Induced Exocytosis by Permeabilized PC12 Cells<sup>a</sup>

	$[\text{Ca}^{2+}]$ ( $\mu\text{mol/L}$ )	$\text{Ca}^{2+}$ -stimulated release (%)		
		control	OAG (200 $\mu\text{mol/L}$ )	TPA (50 $\text{nmol/L}$ )
A	3	2	1.5	2.4
	10	4.6	4.9	5.3
	30	12.6	14.6	13.9
B	3	3.8	6.2	8.2
	10	8.8	13.1	12.1
	30	17.4	19.2	22.3

<sup>a</sup> PC12 cells preloaded with  $[^3\text{H}]$ dopamine were permeabilized with  $\alpha$ -toxin ( $180 \text{ units/mL}$ ) in the absence (A) or presence (B) of  $5 \text{ mmol/L}$   $\text{Mg}^{2+}/\text{ATP}$  (with or without the indicated stimulators of kinase C). After 30 min, the buffer was replaced by a fresh one containing the same constituents except  $\alpha$ -toxin. This buffer was removed 30 min later, and the cells were stimulated with the given  $\text{Ca}^{2+}$  concentrations with or without ATP. The values (mean of two experiments) in the absence of  $\text{Ca}^{2+}$  (between 6% and 8%) were subtracted.

of OAG ( $200 \mu\text{mol/L}$ ) were necessary to be effective. As observed with TPA, the effect of OAG was absolutely dependent on the presence of  $\text{Mg}^{2+}/\text{ATP}$ . Both TPA and OAG were stronger stimuli in media containing low  $\text{Ca}^{2+}$  concentrations (Table II).

We also investigated the possible involvement of G proteins in the final steps of exocytosis using  $\text{GTP}\gamma\text{S}$  as a probe.  $\text{GTP}\gamma\text{S}$  in concentrations between  $10$  and  $100 \mu\text{mol/L}$  blocked about half the  $[^3\text{H}]$ dopamine release elicited by  $10 \mu\text{mol/L}$  free  $\text{Ca}^{2+}$  in the presence of  $1 \text{ mM}$   $\text{Mg}^{2+}$  from permeabilized cells. A complete inhibition of  $\text{Ca}^{2+}$ -induced exocytosis could never be achieved, even when concentrations above  $100 \mu\text{mol/L}$   $\text{GTP}\gamma\text{S}$  were used. To be effective,  $\text{GTP}\gamma\text{S}$  obviously

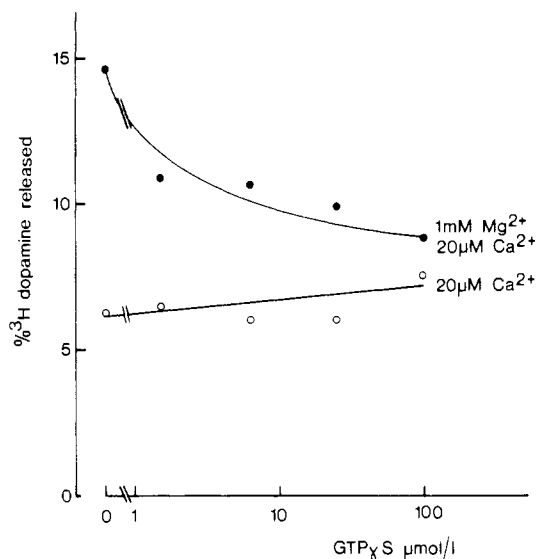


FIGURE 6: Inhibition of  $\text{Ca}^{2+}$ -induced  $[^3\text{H}]$ dopamine release in permeabilized PC12 cells. After  $\alpha$ -toxin permeabilization for 30 min without  $\text{Ca}^{2+}$  in the presence of the given (abscissa)  $\text{GTP}\gamma\text{S}$  concentrations, the cells were stimulated (10 min) with or without  $20 \mu\text{mol/L}$   $\text{Ca}^{2+}$  plus the indicated amount of  $\text{GTP}\gamma\text{S}$ . Values expressed as percent (the release in the absence of  $\text{Ca}^{2+}$  was subtracted) are the mean of duplicate determinations. The symbols represent the  $\text{Ca}^{2+}$ -stimulated  $[^3\text{H}]$ dopamine release in the absence (open) and in the presence (closed) of  $1 \text{ mM}$  free  $\text{Mg}^{2+}$ .

Table III: Effects of Cholera Toxin Pretreatment on the Inhibitory Action of  $\text{GTP}\gamma\text{S}$ <sup>a</sup>

[ $\text{Ca}^{2+}$ ] ( $\mu\text{mol/L}$ )	% [ $^3\text{H}$ ]dopamine released			
	control	$\text{GTP}\gamma\text{S}$ (100 $\mu\text{mol/L}$ )	cholera toxin (100 $\mu\text{g/mL}$ )	
			no $\text{GTP}\gamma\text{S}$	$\text{GTP}\gamma\text{S}$ (100 $\mu\text{mol/L}$ )
2	7	6.2	10.5	11.8
10	18.6	12.6	30.8	22.8
10	27.2	16.7	36.3	27.1
50	33.9	25.7	41.3	34.6

<sup>a</sup>PC12 cells were preloaded with  $[^3\text{H}]$ dopamine either in the presence or in the absence of cholera toxin ( $100 \mu\text{g/mL}$ ) for 4 h. The cells were washed and then permeabilized with  $\alpha$ -toxin plus or minus  $\text{GTP}\gamma\text{S}$ . After 30 min, the buffer was changed to a fresh one supplemented with the given free  $\text{Ca}^{2+}$  concentrations plus or minus  $\text{GTP}\gamma\text{S}$ . The values represent the mean of two experiments.

requires the presence of  $\text{Mg}^{2+}$ , since in its absence no effect of this substance on  $\text{Ca}^{2+}$ -induced exocytosis was observed (Figure 6). Inhibition by  $\text{GTP}\gamma\text{S}$  was half-maximal at about  $2 \mu\text{mol/L}$  and remained constant, irrespective of the free  $\text{Ca}^{2+}$  concentration used for stimulation (Table III). Pretreatment of the cells with cholera toxin (up to  $200 \mu\text{g/mL}$ ) which ADP-ribosylates the GTP binding protein ( $G_s$ ) increased both the basal and the  $\text{Ca}^{2+}$ -stimulated release. However,  $\text{GTP}\gamma\text{S}$  was still inhibitory after treatment with cholera toxin (Table III). By contrast, pretreatment of the cells with pertussis toxin which ADP-ribosylates different GTP binding proteins ( $G_i$ ,  $G_o$ ) abolished the inhibitory effects of  $\text{GTP}\gamma\text{S}$  on  $\text{Ca}^{2+}$ -induced dopamine release (Figure 7). Thus, it can be concluded that GTP binding proteins ribosylated by pertussis toxin participate in the modulation of  $\text{Ca}^{2+}$ -induced exocytosis in PC12 cells.

## DISCUSSION

Permeabilization of cells has become a widely used approach to study intracellular events "in situ". Chromaffin cells permeabilized by different techniques are favorite objects for studying the requirements for exocytosis [for a review, see

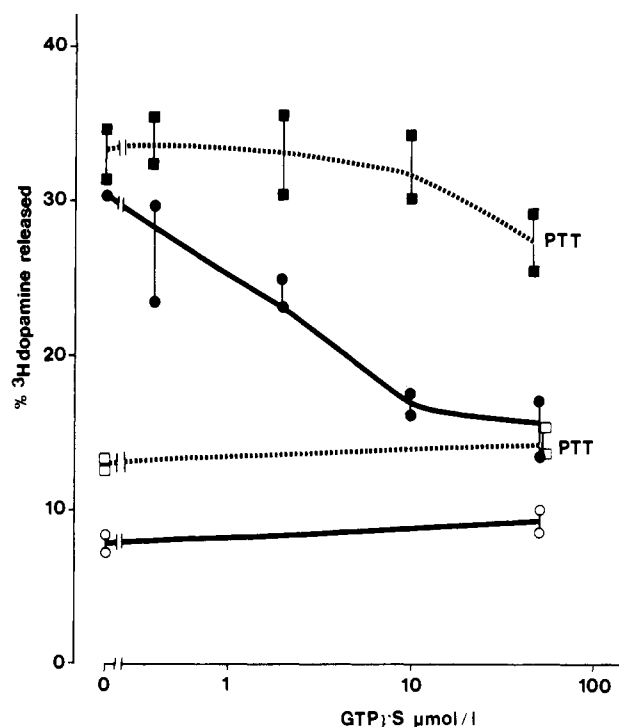


FIGURE 7: Pretreatment of PC12 cells with pertussis toxin overcomes the inhibitory effects of  $\text{GTP}\gamma\text{S}$  on exocytosis. PC12 cells were treated with or without  $11 \mu\text{g/mL}$  pertussis toxin during the 4-h  $[^3\text{H}]$ dopamine loading. Following the usual washings, the cells were permeabilized with  $\alpha$ -toxin ( $180 \text{ units/mL}$ ) for 30 min in KG buffer containing  $1 \text{ mmol/L}$  free  $\text{Mg}^{2+}$  with or without the given amounts of  $\text{GTP}\gamma\text{S}$ . This buffer was changed to a fresh one with the same constituents plus an additional  $10 \mu\text{mol/L}$  free  $\text{Ca}^{2+}$ . The experiment was run in duplicate. Each point represents one sample. The absence gives the final concentration of  $\text{GTP}\gamma\text{S}$  in micromoles per liter. (○) No  $\text{Ca}^{2+}$ , no PTT pretreatment; (●)  $10 \mu\text{mol/L}$   $\text{Ca}^{2+}$ , no PTT; (□) no  $\text{Ca}^{2+}$ , PTT pretreatment; (■)  $10 \mu\text{mol/L}$   $\text{Ca}^{2+}$ , PTT pretreatment.

Gratzl (1987)]. In the presence of micromolar concentrations of  $\text{Ca}^{2+}$ , permeabilized chromaffin cells release endogenous or labeled catecholamines previously taken up by the cells. From the parallel release of vesicular marker proteins such as dopamine  $\beta$ -hydroxylase or chromogranin A, it was deduced that an exocytotic process occurs. This conclusion can only be drawn (1) if the lesions caused by the permeabilization procedure are restricted to the plasma membrane and (2) if they are small enough to prevent the escape of high molecular weight compounds or even of complete organelles from the cells.

High-voltage permeabilization apparently meets these criteria. It elicits small pores which allow diffusional control of molecules below  $1000 \text{ daltons}$ . By contrast, larger molecules, i.e., lactate dehydrogenase, equilibrate only very slowly. The  $\text{Ca}^{2+}$ -stimulated release of both dopamine  $\beta$ -hydroxylase and catecholamines, therefore, is in accordance with an exocytotic event (Baker & Knight, 1978; Knight & Baker, 1982; Baker et al., 1985).

Staphylococcal  $\alpha$ -toxin has become an equipotent tool for the selective permeabilization of secretory cells which likewise meets the above-mentioned criteria. The soluble protein monomer is too large to pass through the pores formed by its hexamerization in the plasma membrane and thus does not attack intracellular organelles. The pore formed is permanent and can hardly be removed from the plasma membrane under physiological conditions (Bhakdi et al., 1983; Bhakdi & Tranum-Jensen, 1984, 1987). This is an advantage compared to "nonstabilized pores" obtained by high-voltage charge techniques or the use of detergents which may be subject to

some resealing. As observed with the electrically permeabilized cells, small molecules (e.g., ATP and cytoplasmic catecholamine metabolites) can be removed by diffusion from the  $\alpha$ -toxin-permeabilized cells (Ahnert-Hilger & Gratzl, 1987; Lind et al., 1987). On the other hand, large molecules like lactate dehydrogenase remain within the cells (Ahnert-Hilger et al., 1985b; Bader et al., 1986). In  $\alpha$ -toxin-treated chromaffin cells, it has been clearly demonstrated that micromolar concentrations of  $\text{Ca}^{2+}$  induce a parallel release of catecholamines and the vesicular marker chromogranin A, giving strong evidence for an exocytotic event (Bader et al., 1986). With detergents such as digitonin, the permeabilization process is difficult to control. Vesicles may undergo lysis during detergent treatment, and intravesicular substances may leak out of the cells. Even entire vesicles can apparently escape through the big holes generated by the attack of detergents (Bader et al., 1986).

For  $\alpha$ -toxin-permeabilized PC12 cells, comparable experiments are lacking. Evidence for exocytosis showing a parallel release of catecholamines and dopamine  $\beta$ -hydroxylase or chromogranin A is difficult to obtain, since these cells contain small amounts of these markers which will not be released in sufficient amounts upon stimulation (Sabban et al., 1983). However, evidence for exocytosis in  $\alpha$ -toxin-permeabilized PC12 cells can be obtained by another experimental approach. Since cytoplasmic enzymes such as lactate dehydrogenase remain entrapped in these cells after permeabilization, the enzymes involved in the metabolism of catecholamines are also retained. Thus, discharge of the vesicular catecholamines into the cytosol results in the enzymatic oxidation to DOPAC and DOPET. By contrast, if catecholamines are released by exocytosis, the cytoplasm is circumvented, and an increase of metabolites will not occur. The pattern of catecholamines and their metabolites released by  $\alpha$ -toxin-permeabilized PC12 cells is in accordance with these predictions.  $\text{Ca}^{2+}$  only causes the release of dopamine and noradrenaline but not of metabolites. The relation between the free  $\text{Ca}^{2+}$  concentration and the amount of released catecholamines is similar to the one observed earlier with cells loaded with [ $^3\text{H}$ ]dopamine (Ahnert-Hilger et al., 1985a,b; Ahnert-Hilger & Gratzl, 1987). If catecholamines are artificially discharged into the cytosol (by addition of nigericin), enzymatic degradation occurs. Nigericin is known to dissipate the proton gradient across the chromaffin granule membrane, thereby leading to rapid efflux of amines from their vesicular store (Rebois et al., 1980). As demonstrated earlier (Bräutigam et al., 1985), depletion of dopamine into the cytoplasm is accompanied by increased levels of dopamine metabolites, mainly DOPAC, as found also for other tissues (Zumstein et al., 1981). Apparently, the efflux is more rapid than the metabolizing capacity of the responsible enzymes, and some catecholamines escape from the cytosol also via the  $\alpha$ -toxin pores before degradation occurs. From the fact that metabolism only takes place when the catecholamines are discharged into the cytoplasm and that  $\text{Ca}^{2+}$  stimulation results in the release of nonmetabolized catecholamines, it can be concluded that  $\alpha$ -toxin-permeabilized cells release their stored catecholamines by exocytosis upon stimulation.

Using the HPLC technique, we have also demonstrated here that the storage vesicles are fairly stable in the permeabilized cells. This fact opens the possibility to study drugs which may modulate exocytosis. The phorbol ester TPA has been shown to increase the  $\text{Ca}^{2+}$  sensitivity in electrical-permeabilized chromaffin cells (Knight & Baker, 1983) as well as in digitonin-permeabilized PC12 cells (Peppers & Holz, 1986). Our observation that TPA in the nanomolar range increases the

$\text{Ca}^{2+}$ -stimulated catecholamine release in  $\alpha$ -toxin-permeabilized PC12 cells is in general agreement with these findings.

In contrast to the situation seen with chromaffin cells, where exocytosis strongly depends on  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ /ATP (Knight & Baker, 1982; Bader et al., 1986), secretion by PC12 cells does not require ATP (Ahnert-Hilger et al., 1985a,b; Ahnert-Hilger & Gratzl, 1987; Reynolds et al., 1982). Even when the cells were kept open for 1 h, in order to deplete them from cytoplasmic ATP,  $\text{Ca}^{2+}$  alone is sufficient to cause exocytosis. In this study, we found that  $\text{Ca}^{2+}$ -induced secretion from  $\alpha$ -toxin-permeabilized cells can be further enhanced by TPA and to a lesser extent by OAG provided  $\text{Mg}^{2+}$ /ATP is present. In permeabilized chromaffin cells, an ATP-dependent effect of TPA was also described (Yee & Holz, 1986). However, since both exocytosis and the effect of TPA are dependent on ATP in these cells, the ATP dependency of the TPA effect could not convincingly be worked out. By contrast, since exocytosis in  $\alpha$ -toxin-permeabilized PC12 cells is independent of ATP, we were able to obtain direct evidence for a modulatory role of the protein kinase C system during exocytosis. Following the arguments of Nishizuka (1984), two distinct pathways, namely, one involving  $\text{Ca}^{2+}$  and the other one the protein kinase C system, can lead to the activation of cells. In permeabilized PC12 cells, TPA in the absence of  $\text{Ca}^{2+}$  is hardly effective (Peppers & Holz, 1986; this study). Since TPA-induced catecholamine release is  $\text{Ca}^{2+}$  dependent, it may thus be concluded that both pathways are linked in these cells. The synergistic action of  $\text{Ca}^{2+}$  with TPA has also been observed in chromaffin cells in primary culture (Frye & Holz, 1985; Knight & Baker, 1983; Brocklehurst & Pollard, 1985). On the other side, the effects of  $\text{Ca}^{2+}$  and TPA seem to be completely independent from each other in the parathyroid cells (Muff & Fischer, 1986) or in platelets (Rink et al., 1983).

How exactly protein kinase C exerts its effects still remains to be investigated. A phosphorylation step may occur at the vesicle membrane or nearby to prime the vesicle for exocytosis. In accordance with this, phosphorylation of various cytoplasmic proteins (Brocklehurst et al., 1985) and microtubule associated proteins [cf. Nishizuka (1984)] has been observed in response to TPA. Thus, with the few exceptions given above, most of the studies agree that  $\text{Ca}^{2+}$  and TPA exert a synergistic role in exocytosis. This is also in accordance with protein kinase C itself being a  $\text{Ca}^{2+}$ -activated enzyme (Nishizuka, 1984).

GTP $\gamma$ S is a poorly hydrolyzable analogue of GTP that binds with high affinity to the regulatory site of G proteins, thereby causing persistent activation (Stryer & Borne, 1986; Gilman, 1984, 1986). Our finding that GTP $\gamma$ S inhibits the  $\text{Ca}^{2+}$ -triggered exocytosis in  $\alpha$ -toxin-permeabilized PC12 cells is in agreement with previous observations for electrically permeabilized bovine chromaffin cells (Knight & Baker, 1985). Yet the effect of GTP $\gamma$ S is not consistent in the different secretory cells studied. In chicken, chromaffin cell  $\text{Ca}^{2+}$ -induced catecholamine release is stimulated by GTP $\gamma$ S (Knight & Baker, 1985) as is secretion in human platelets (Haslam & Davidson, 1984), parathyroid cells (Oetting et al., 1986), and sea urchin eggs (Turner et al., 1986). A  $\text{Ca}^{2+}$ -independent stimulatory effect by GTP analogues was reported for permeabilized rat insulinoma cells (Vallar et al., 1987) and bovine chromaffin cells in primary culture (Bittner et al., 1986). In bovine chromaffin cells, on the other hand, GTP $\gamma$ S plays an inhibitory role in  $\text{Ca}^{2+}$ -induced catecholamine release (Knight & Baker, 1985).

To elucidate in more detail the nature of the G protein involved in exocytosis, the cells were pretreated with either cholera toxin or pertussis toxin. These toxins cause specific

ADP-ribosylations of subclasses of G proteins. Cholera toxin is known to ribosylate the  $\alpha$ -subunit of the stimulatory G protein ( $G_s$ ), thereby causing persistent activation of enzymes regulated by  $G_s$  as described for adenylate cyclase [Cassel & Pfeuffer, 1978; cf. references cited in Gilman (1984)]. Pertussis toxin, on the other hand, inactivates different G proteins by ADP-ribosylation (e.g.,  $G_i$  and  $G_o$ ) (Gilman, 1984, 1986). With the aid of both cholera toxin and pertussis toxin, we obtained information about the nature of the G proteins involved in the modulation of exocytosis in PC12 cells. Cholera toxin, even at high concentrations, failed to modify the inhibitory action of  $GTP\gamma S$ . In contrast, pretreatment with pertussis toxin (11  $\mu g/mL$ ) abolished the effects of  $GTP\gamma S$ . These findings lead to the conclusion that exocytosis in PC12 cells can be modulated by a pertussis toxin sensitive G protein, probably  $G_i$  or  $G_o$ . The recent detection of three pertussis toxin substrates and  $G_o$  immunoreactivity in both plasma membrane and chromaffin vesicle membranes (Toutant et al., 1987) supports our findings on the participation of G proteins in the regulation of exocytosis.

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**Registry No.** Ca, 7440-70-2; dopamine, 51-61-6; noradrenaline, 51-41-2; protein kinase, 9026-43-1.

#### REFERENCES

- Ahnert-Hilger, G., & Gratzl, M. (1987) *J. Neurochem.* **49**, 764-770.
- Ahnert-Hilger, G., Bhakdi, S., & Gratzl, M. (1985a) *Neurosci. Lett.* **58**, 107-110.
- Ahnert-Hilger, G., Bhakdi, S., & Gratzl, M. (1985b) *J. Biol. Chem.* **260**, 12730-12734.
- Bader, M. F., Thiersé, D., Aunis, D., Ahnert-Hilger, G., & Gratzl, M. (1986) *J. Biol. Chem.* **261**, 5777-5783.
- Baker, P. F., & Knight, D. E. (1978) *Nature (London)* **276**, 620-622.
- Baker, P. F., Knight, D. E., & Umbach, J. A. (1985) *Cell Calcium* **6**, 5-14.
- Bhakdi, S., & Tranum-Jensen, J. (1984) *Philos. Trans. R. Soc. London, B* **306**, 311-324.
- Bhakdi, S., & Tranum-Jensen, J. (1987) *Rev. Physiol., Biochem. Pharmacol.* **107**, 147-223.
- Bhakdi, S., Füssle, R., & Tranum-Jensen, J. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5475-5479.
- Bhakdi, S., Tranum-Jensen, J., Utermann, G., & Füssle, R. (1983) *J. Biol. Chem.* **258**, 5899-5904.
- Bhakdi, S., Muhly, M., & Füssle, R. (1984) *Infect. Immun.* **46**, 318-323.
- Bittner, M. A., Holz, R. W., & Neubig, R. R. (1986) *J. Biol. Chem.* **261**, 10182-10188.
- Bräutigam, M., Dreesen, R., & Herken, A. (1982) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **320**, 85-89.
- Bräutigam, M., Dreesen, R., & Herken, H. (1984) *J. Neurochem.* **42**, 390-396.
- Bräutigam, M., Laschinski, G., Kittner, B., & Herken, H. (1985) *Biochem. Pharmacol.* **34**, 941-947.
- Brocklehurst, K. W., & Pollard, H. B. (1985) *FEBS Lett.* **183**, 107-111.
- Brocklehurst, K. W., Morita, K., & Pollard, H. B. (1985) *Biochem. J.* **228**, 35-42.
- Brooks, J. C., & Carmichael, S. W. (1983) *Mikroskopie* **40**, 347-356.
- Brooks, J. C., & Trembl, S. (1983) *J. Neurochem.* **40**, 468-474.
- Dunn, L. A., & Holz, R. W. (1983) *J. Biol. Chem.* **258**, 4983-4993.
- Flodgaard, H., & Fleron, P. (1974) *J. Biol. Chem.* **249**, 3465-3474.
- Frye, R. A., & Holz, R. W. (1985) *J. Neurochem.* **44**, 265-273.
- Füssle, R., Bhakdi, S., Sziegoleit, A., Tranum-Jensen, J., Kranz, T., & Wellensiek, H. J. (1981) *J. Cell Biol.* **91**, 83-94.
- Gilman, A. G. (1984) *Cell (Cambridge, Mass.)* **36**, 577-579.
- Gilman, A. G. (1986) *Trends Neurosci. (Pers. Ed.)* **9**, 460.
- Gratzl, M. (1987) in *Cellular Membrane Fusion: Fundamental Mechanisms and Applications of Membrane Fusion Techniques* (Wilschut, J., Hoekstra, D., & Dekker, M., Eds.) New York Publications (in press).
- Haslam, R. J., & Davidson, M. M. L. (1984) *FEBS Lett.* **174**, 90-95.
- Knight, D. E., & Baker, P. F. (1982) *J. Membr. Biol.* **68**, 107-140.
- Knight, D. E., & Baker, P. F. (1983) *FEBS Lett.* **160**, 98-100.
- Knight, D. E., & Baker, P. F. (1985) *FEBS Lett.* **189**, 345-349.
- Lee, S. A., & Holz, W. (1987) *J. Biol. Chem.* **261**, 17089-17098.
- Lind, I., Ahnert-Hilger, G., Fuchs, G., & Gratzl, M. (1987) *Anal. Biochem.* **164**, 84-89.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275.
- Muff, R., & Fischer, J. A. (1986) *Biochem. Biophys. Res. Commun.* **139**, 1233-1238.
- Nishizuka, Y. (1984) *Science (Washington, D.C.)* **225**, 1365-1370.
- Oetting, M., LeBoff, M., Swistan, L., Preston, J., & Brown, E. (1986) *FEBS Lett.* **208**, 4170.
- Peppers, S. C., & Holz, R. W. (1986) *J. Biol. Chem.* **261**, 14665-14670.
- Rebois, R. V., Reynolds, E. E., Toll, L., & Howard, B. D. (1980) *Biochemistry* **19**, 1240-1248.
- Reynolds, E. E., Melega, W. P., & Howard, B. D. (1982) *Biochemistry* **21**, 4795-4799.
- Rink, T. J., Sanchez, A., & Hallam, T. J. (1983) *Nature (London)* **305**, 317-319.
- Sabban, E. L., Greene, L. A., & Goldstein, M. (1983) *J. Biol. Chem.* **258**, 7812-7819.
- Sillen, L. G., & Martell, A. D. (1971) *Suppl. 1, The Chemical Society, London.*
- Simon, W., Ammann, D., Oehme, M., & Morf, W. E. (1978) *Ann. N.Y. Acad. Sci.* **307**, 52-70.
- Stryer, L., & Bourne, H. R. (1986) *Annu. Rev. Cell. Biol.* **2**, 389-417.
- Toutant, M., Aunis, O., Bockaert, J., Homburger, V., & Rouot, B. (1987) *FEBS Lett.* **215**, 339-344.
- Turner, P. R., Jaffe, L. A., & Flin, A. (1986) *J. Cell Biol.* **102**, 70-76.
- Vallar, L., Biden, T. J., & Wollheim, C. B. (1987) *J. Biol. Chem.* **262**, 5049-5056.
- Wadström, T. (1968) *Biochim. Biophys. Acta* **168**, 228-242.
- Wilson, S. P., & Kirshner, N. (1983) *J. Biol. Chem.* **258**, 4994-5000.
- Zumstein, A., Karduck, W., & Starke, K. (1981) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **316**, 205-217.