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Further Characterization of Dopamine Release by Permeabilized PC12 Cells

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Abstract: Rat pheochromocytoma cells (PC12) permeabilized with staphylococcal α-toxin release [^{3}H]dopamine after addition of micromolar Ca^{2+}. This does not require additional Mg^{2+}-ATP (in contrast to bovine adrenal medullary chromaffin cells). We also observed Ca^{2+}-dependent [^{3}H]-dopamine release from digitonin-permeabilized PC12 cells. Permeabilization with α-toxin or digitonin and stimulation of the cells were done consecutively to wash out endogenous Mg^{2+}-ATP. During permeabilization, ATP was removed effectively from the cytoplasm by both agents but the cells released [^{3}H]dopamine in response to micromolar Ca^{2+} alone. Replacement by chloride of glutamate, which could sustain mitochondrial ATP production in permeabilized cells, does not significantly alter catecholamine release induced by Ca^{2+}. However, Mg^{2+} without ATP augments the Ca^{2+}-induced release. The release was unaltered by thiol-, hydroxyl-, or calmodulin-interfering substances. Thus Mg^{2+}-ATP, calmodulin, or proteins containing -SH or -OH groups are not necessary for exocytosis in permeabilized PC12 cells. **Key Words:** Exocytosis—PC12—Ca^{2+}—Mg^{2+}—ATP—Permeabilized cells. **Ahnert-Hilger G. and Gratzl M.** Further characterization of dopamine release by permeabilized PC12 cells. *J. Neurochem.* **49,** 764–770 (1987).

Fusion of secretory vesicle membranes with the plasma membrane is the final event in stimulus–exocytosis coupling. This process is not easy to investigate in intact cells because the interacting membrane surfaces are not accessible. Various procedures have therefore been developed to permeabilize the plasma membrane without affecting the exocytotic machinery and it has been shown that micromolar Ca^{2+} as well as Mg^{2+}-ATP are necessary to elicit exocytosis in the bovine adrenomedullary chromaffin cell permeabilized by high-voltage discharge (Baker and Knight, 1978, 1981; Knight and Baker, 1982), saponins (Brooks and Treml, 1983*a*; Dunn and Holz, 1983; Wilson and Kirshner, 1983), or α-toxin (Bader et al., 1986). By contrast, rat pheochromocytoma cells (PC12) permeabilized by α-toxin require only Ca^{2+} for the release reaction (Ahnert-Hilger et al., 1985*a,b*). This lack of dependence on exogenous Mg^{2+}-ATP indicates that Ca^{2+} and Mg^{2+}-ATP may catalyze different steps in secretory cells prior to the final event of membrane fusion.

However, endogenous stores of Mg^{2+}-ATP might still be available for the release reaction in α-toxin-permeabilized PC12 cells. To exclude this possibility the cells were depleted of endogenous ATP before they were stimulated with Ca^{2+}. Glutamate, present during previous experiments, was exchanged for chloride to exclude production of ATP for the Ca^{2+}-induced release by enhancement of mitochondrial ATP production. Calmodulin-activated release in intact chromaffin cells (Kenigsberg et al., 1982; Brooks and Treml, 1983*b*) was studied using trifluoperazine (TFP). *N*-Ethylmaleimide (NEM), which interferes with exocytosis in electrically permeabilized bovine chromaffin cells (Knight and Baker, 1982), and 4-(hydroxymercuri)benzoic acid (PHMB), which inhibits fusion of isolated vesicles (Gratzl and Dahl, 1978), were used to test if proteins containing -SH or -OH groups are involved in dopamine release from permeabilized PC12 cells.

MATERIALS AND METHODS

PC12 cells were kept in culture as described by Ahnert-Hilger et al. (1985*b*). Permeabilized cells were studied either in suspension or grown on poly-L-lysine 70–40,000 MW-

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Abbreviations used: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DTNB, 2,2′-dinitro-*S,S*′-dithiobenzoic acid; NEM, *N*-ethylmaleimide; NTA, nitriloacetic acid; PHMB, 4-(hydroxymercuri)benzoic acid; PIPES, 1,4-piperazinediethanesulfonic acid; TFP, trifluoperazine.

precoated 16-mm multiwell plates. Cells (5×10^5 cells/well or $2-3 \times 10^5$ cells in suspension corresponding to about 100 μg of protein/sample) were loaded with [^{3}H]dopamine in Dulbecco's modified Eagle's medium (DMEM) (serum-free) supplemented with 1 m*M* ascorbic acid for 2 h at 37°C. After three washes with Ca^{2+}-free potassium glutamate buffer [mmol/L: potassium glutamate 150, 1,4-piperazine-diethanesulfonic acid (PIPES) 10, EGTA 0.5, nitriloacetic acid (NTA) 5, pH 7.2 titrated with KOH] cells were preincubated for 10–15 min in the same buffer supplemented with 0.2% bovine serum albumin (BSA) at 37°C. After removal of the preincubation buffer the given amount of Ca^{2+} and Mg^{2+} in potassium glutamate medium plus 0.2% BSA with or without α-toxin (150–200 U/ml) was added to the cells. The toxin batch used contained 30,000 hemolytic units/mg of protein (Lind et al., 1987). Incubation was stopped after 20 min by centrifugation and the radioactivity in the supernatant medium and in the cells (after lysis with 0.2% sodium dodecyl sulfate) was determined by liquid scintillation counting. In another experimental design cells were treated first with α-toxin dissolved in Ca^{2+}-free potassium glutamate buffer. After 20 min at 37°C or 30°C the medium was completely removed and cells were stimulated with the desired Ca^{2+}, Mg^{2+}, and Mg^{2+}-ATP concentrations in potassium glutamate buffer. Cells stimulated in suspension or on plates gave similar results. This holds also for experiments carried out at 30°C instead of 37°C.

The free Ca^{2+} and Mg^{2+} concentrations were calculated by a computer program according to Flodgaard and Fleron (1974), kindly provided by T. Saermark, University of Copenhagen, Denmark, using the stability constants given by Sillen and Martell (1971). The media were also controlled with a Ca^{2+}-sensitive electrode (Simon et al., 1978). The neutral carrier incorporated into a polyvinyl chloride membrane was kindly provided by Dr. W. Simon, ETH Zürich, Switzerland. α-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 by Wadström (1968). Protein was determined according to Lowry et al. (1951) using BSA as standard. ATP was measured in the cells (extracted with Tris, 50 mmol/L, pH 7.8; magnesium acetate, 10 mmol/L, EGTA, 1.5 mmol/L) as well as in the supernatants after heating to 95°C for 5 min using the firefly assay (Boehringer, Mannheim, F.R.G.) as described by Lind et al. (1987).

Materials

BSA was obtained from Sigma, München, F.R.G., PHMB and NEM from Serva Heidelberg, F.R.G., 2,2'-dinitro-*S,S'*-dithiobenzoic acid (DTNB) from Roth, Karlsruhe, F.R.G. DMEM dried medium, horse serum, and fetal calf serum were supplied by Seromed Biochrom KG, Berlin, F.R.G., culture dishes by Falcon, Becton Dickinson, Heidelberg, F.R.G., and [^{3}H]dopamine (30 Ci/mmol) from New England Nuclear, Dreieich, F.R.G.

RESULTS

Effects of Ca^{2+} and Mg^{2+}-ATP on dopamine release from permeabilized PC12 cells

In permeabilized PC12 cells release of [^{3}H]-dopamine can be triggered by elevating the free Ca^{2+} concentration in the medium above 0.1 μ*M* (see also Ahnert-Hilger et al., 1985*a,b*). In previous experiments, permeabilization and addition of Ca^{2+} were carried out simultaneously. When cells were first permeabilized with α-toxin in the absence of Ca^{2+}, the subsequent addition of micromolar Ca^{2+} was sufficient to elicit [^{3}H]dopamine release (Fig. 1). The Ca^{2+} dependency under these experimental conditions strongly resembled that found with PC12 cells described earlier (Ahnert-Hilger et al., 1985*a,b*). An increase plateauing at 4–10 μ*M* free Ca^{2+} was followed by a second rise at approximately 10 μ*M* and becoming maximal at about 100 μ*M*. The first rise was half maximal at about 1 μ*M* Ca^{2+} (Fig. 1). Additional Mg^{2+}-ATP was not present in this experiment. Since catecholamine release from adrenal medullary chromaffin cells permeabilized by different techniques depends on Mg^{2+}-ATP (see introduction) we investigated in more detail the effect of Mg^{2+}-ATP during release from PC12 cells.

A comparison of the effects of Ca^{2+} in the presence or absence of Mg^{2+}-ATP is given in Fig. 2a–c. Addition of ATP was not necessary to sustain Ca^{2+}-induced [^{3}H]dopamine release. In the absence of Ca^{2+} the release from α-toxin treated cells resembled that of control cells. However, micromolar levels of free Ca^{2+} induced release of [^{3}H]dopamine independent of the presence of ATP, whether Ca^{2+} stimulation occurred simultaneously with α-toxin treatment (Fig. 2a) or after the buffer was replaced following permea-

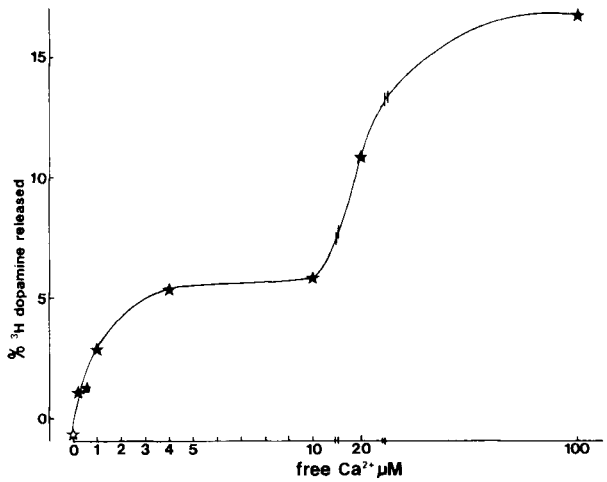

FIG. 1. Ca^{2+} dependency of [^{3}H]dopamine release from PC12 cells previously permeabilized with α-toxin. Cells grown on plates and preloaded with [^{3}H]dopamine were washed three times with potassium glutamate buffer (see Materials and Methods). Then they were treated with α-toxin (170 U/ml) in the same buffer supplemented with 0.2% BSA for 20 min at 30°C. The permeabilization medium was then exchanged for fresh potassium glutamate buffer containing the amount of the free Ca^{2+} concentration given. After a further 10 min at 30°C the radioactivity was counted in the supernatant and in the cell lysate. Each point represents the net release above control of one representative experiment. Release in control samples (in the absence of α-toxin) was 8.3 ± 0.7%, n = 6 (SD).

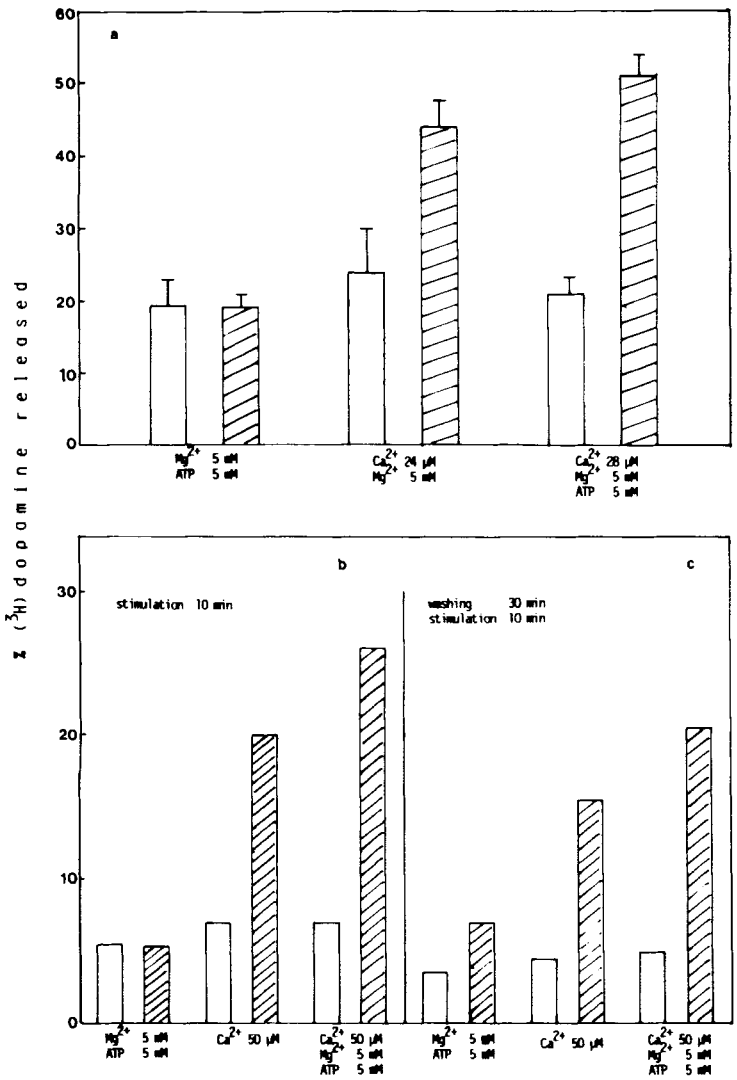

FIG. 2. Failure of Mg^{2+}-ATP to modify Ca^{2+}-induced [^{3}H]dopamine release by α-toxin-permeabilized PC12 cells. **a:** Preloaded PC12 cells on plates were treated for 20 min with α-toxin (150 U/ml) with or without ATP and in the presence of the free Ca^{2+} concentrations given. The hatched bars represent the permeabilized cells, the open ones controls. The potassium glutamate buffer contained 11 m*M* glucose, 5 m*M* EGTA, and 5 m*M* Mg^{2+}. **b:** PC12 cells were first treated with α-toxin (150 U/ml) in potassium glutamate buffer (5 m*M* EGTA) supplemented with 2.5 m*M* magnesium acetate for 20 min at 37°C. The permeabilization medium was replaced by potassium glutamate buffer supplemented with the ions indicated and incubated for a further 10 min at 37°C. **c:** Cells were further washed for 30 min in the permeabilization buffer without toxin and Ca^{2+} and then stimulated under the same conditions with the given amount of Ca^{2+}. The hatched bars represent the α-toxin-treated, the open bars control samples. The bars represent two (b, c) or three (a) experiments (SD).

bilization (Fig. 2b and c). Even after prolonged washing (Fig. 2c) Mg^{2+}-ATP only slightly augmented the Ca^{2+}-induced release of [^{3}H]dopamine.

Indeed, α-toxin renders PC12 cells permeable to ATP as does digitonin, which also causes loss of cytoplasmic macromolecules (Ahnert-Hilger et al., 1985*b*). As outlined in Table 1 both agents cause comparable releases of ATP. The subsequent substitution of fresh buffer supplemented with 20 μM free Ca^{2+} triggered [^{3}H]dopamine release in both permeabilized cell preparations. This clearly indicates that the permeabilization procedure is sufficient to deplete cellular cytoplasmic ATP and that Ca^{2+} alone leads to secretion.

To rule out the possibility that glutamate in the buffer stimulated ATP production by mitochondria and thus caused apparent ATP independence of dopamine release in previous investigations it was replaced by chloride. However, the release of dopamine as a function of the free Ca^{2+} concentration was very similar under both conditions (Fig. 3), which confirms

TABLE 1. *Comparison of ATP depletion and Ca^{2+}-induced [^{3}H]dopamine release in PC12 cells permeabilized either with digitonin or with α-toxin*

Treatment of cells	ATP released (%)	[^{3}H]Dopamine released (%)	
		No Ca^{2+}	20 μM Ca^{2+}
—	20/17	6.0/7.1	8.0/7.3
Digitonin (15 μM)	65/78	7.8/7.4	17.1/19.0
—	22/18	4.8/7.2	6.4/8.6
α-Toxin (200 U/ml)	78/73	4.2/4.8	20.0/17.5

PC12 cells were permeabilized either for 20 min at 30°C with digitonin or for 30 min at 30°C with α-toxin (see Materials and Methods). The total ATP content of the cells was about 17 nmol/mg protein (100%), which is in the range of values reported earlier (Reynolds et al., 1982). ATP was determined as described in Materials and Methods. For dopamine release the buffer was exchanged for a fresh one with or without 20 μM free Ca^{2+}. Experiments were in duplicate; each value represents one sample containing 6×10^5 cells for the ATP determination and 3×10^5 cells for the determination of [^{3}H]dopamine released.

earlier observations of the insensitivity of dopamine release from α-toxin-permeabilized PC12 cells to the anion present (Ahnert-Hilger et al., 1985*b*). The increase in the Ca^{2+}-independent basal release in chloride media indicates that chromaffin vesicles are probably less stable in chloride than in glutamate media. Hence, the α-toxin-permeabilized PC12 cells differ from identically treated primary cultures of bovine chromaffin cells, as after prolonged washing these required Mg^{2+}-ATP for the Ca^{2+}-induced release of noradrenaline and chromogranin A (Bader et al., 1986).

Effects of free Mg^{2+} on Ca^{2+}-induced dopamine release from permeabilized PC12 cells

To define more closely the small effect of Mg^{2+}-ATP observed above and to see whether Mg^{2+}-ATP can modulate exocytosis in PC12 cells, we studied the release of dopamine as a function of the free Ca^{2+} concentration in the absence or presence of 5 m*M* Mg^{2+}-ATP (Fig. 4a). Free Mg^{2+} concentration in the latter buffer is about 0.2 m*M*. Under these conditions, Ca^{2+}-induced release of secretory product increased. In the parallel experiment (Fig. 4b) 1 m*M* free Mg^{2+} without ATP was added. The marked increase of dopamine release at low (1–4 μ*M*) Ca^{2+} concentrations with Mg^{2+} alone suggests that the effect of Mg^{2+}-ATP (Fig. 4a) is due to Mg^{2+} rather than to ATP. At higher Ca^{2+} concentrations (above 20 μ*M*) Mg^{2+} did not augment dopamine release (see also Fig. 2a).

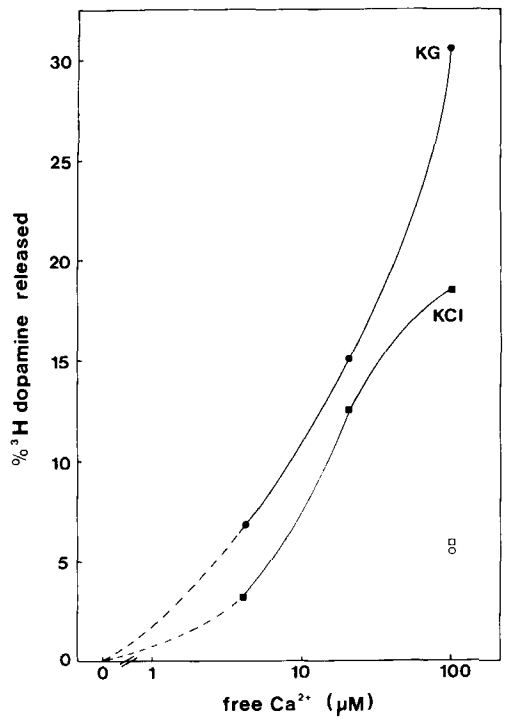

FIG. 3. Comparison of [^{3}H]dopamine secretion induced by Ca^{2+} in glutamate- or chloride-containing medium. Preloaded PC12 cells were permeabilized in the absence of Ca^{2+} with α-toxin in potassium glutamate (KG) buffer or in the same buffer containing chloride instead of glutamate. After 30 min at 30°C the buffer was completely exchanged for fresh one supplemented with the given free Ca^{2+} concentrations. After 10 min radioactivity was measured in the supernatant and in the cell lysate. The release observed in the absence of Ca^{2+} (in potassium glutamate buffer 8.9% for controls and 8.1% in α-toxin-treated samples, and in KCl buffer 15% and 19%, respectively, n = 2) was subtracted. Results are means of two experiments.

When cells first permeabilized with α-toxin in buffer with glutamate replaced by chloride were stimulated with 10 μ*M* Ca^{2+} with or without 5 m*M* Mg^{2+}-ATP in addition to 1 m*M* free Mg^{2+}, Mg^{2+} alone was much more effective than Mg^{2+}-ATP in augmenting the Ca^{2+}-elicited release (Fig. 5).

Effects of TFP and group-specific reagents on Ca^{2+}-induced dopamine release from permeabilized PC12 cells

A possible involvement of calmodulin in the release process was studied using TFP as an antagonist. Table 2 shows that 1 or 10 μ*M* TFP had little or no effect on [^{3}H]dopamine release, induced by 2 or 20 μ*M* free Ca^{2+} alone. However, 10 μ*M* TFP itself increased basal release in cells not treated with α-toxin and in permeabilized cells (Table 2). Thus the increased release of dopamine observed with 10 μ*M* TFP by 2 μ*M* Ca^{2+} is due to an additive effect of both substances (Table 2). Preincubation with 1 μ*M* TFP 20 min before the permeabilization and stimulation procedure also failed to modify [^{3}H]dopamine release by 2 and 20 μ*M* free Ca^{2+} (Table 2).

Table 3 shows that drugs that block -SH (PHMB, DTNB, NEM) and -OH groups (NEM) did not affect Ca^{2+}-induced [^{3}H]dopamine release by permeabilized PC12 cells. Two hundred micromolar NEM doubled the [^{3}H]dopamine release in nonpermeabilized cells as well as in permeabilized cells in the absence of Ca^{2+}, probably due to leakage of catecholamines.

DISCUSSION

Permeabilization of secretory cells is a promising tool for elucidating the final events in stimulus–exocytosis coupling. Permeabilization not only by high-voltage discharge (Baker and Knight, 1978) or by saponins (Dunn and Holz, 1983; Brooks and Treml, 1983*a*; Wilson and Kirshner, 1983) but also by α-toxin from *Staphylococcus aureus* can be used to gain access to the cytoplasm (Ahnert-Hilger et al., 1985*a,b*; Bader et al., 1986; Lind et al., 1987). The toxin produces stable pores in the plasma membrane that are too small to let the monomer pass and attack subcellular organelles (Füssle et al., 1981; for review see Bhakdi et al., 1981; Bhakdi and Tranum-Jensen, 1987). Micromolar concentrations of Ca^{2+} are an absolute requirement for exocytotic release in most, if not all, permeabilized secretory cells. ATP is also essential for catecholamine release from primary cultures of bovine adrenal medullary chromaffin cells but PC12 cells do not require additional substances. This was shown by the present experiments in which ATP and other small endogenous compounds were effec-

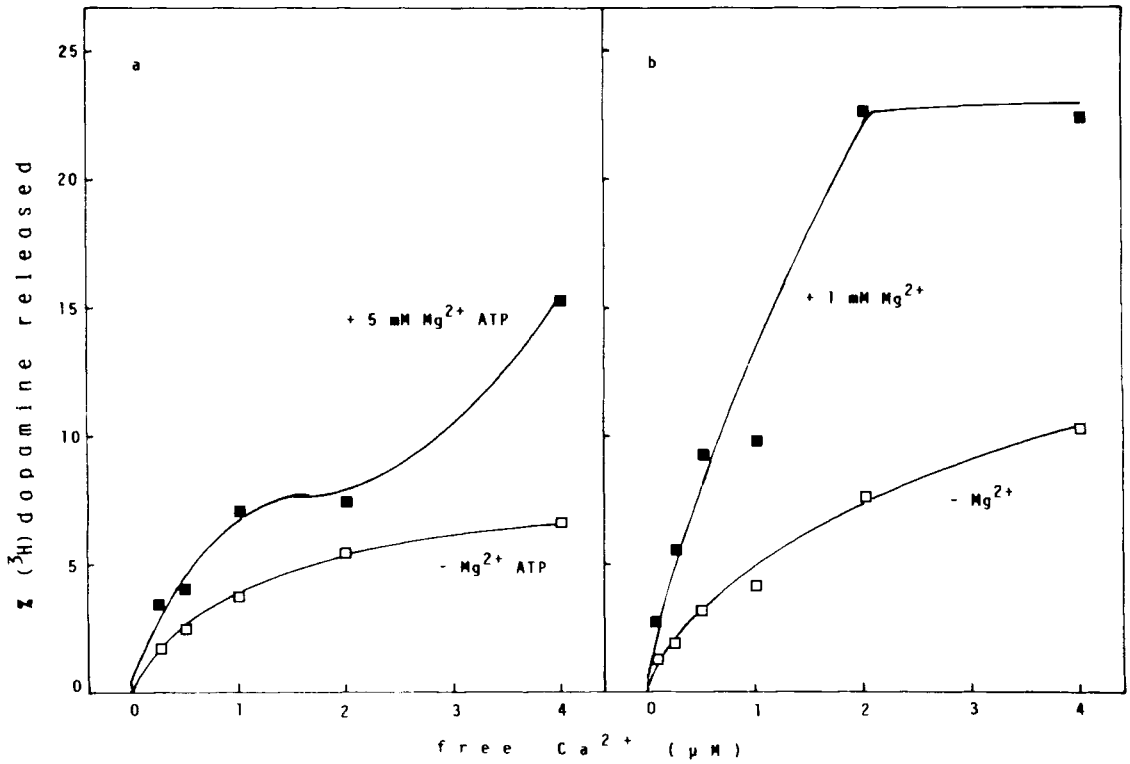

FIG. 4. Effect of free Mg^{2+} on Ca^{2+}-induced [^{3}H]dopamine release by permeabilized PC12 cells. Preloaded PC12 cells in suspension were treated with α-toxin in potassium glutamate buffer plus or minus Mg^{2+}-ATP (a) or Mg^{2+} (b). The abscissa gives the free Ca^{2+} concentration. Release in the absence of Ca^{2+} in α-toxin-treated cells was subtracted. α-Toxin treatment in the absence of Ca^{2+} does not result in a significant increase as compared to untreated controls. **a:** Ca^{2+}-induced release in the absence or presence of 5 m*M* Mg^{2+}-ATP. Addition of 5 m*M* ATP to potassium glutamate buffer containing 5 m*M* Mg^{2+} resulted in a free Mg^{2+} concentration of about 0.2 m*M*. α-Toxin-permeabilized cells without Ca^{2+} released 13.3% and 13.6% (n = 2) dopamine in the absence or presence of Mg^{2+}-ATP, respectively. **b:** Ca^{2+}-induced release in the absence or presence of 1 m*M* free Mg^{2+}. α-Toxin-permeabilized cells without Ca^{2+} released 15.6% and 16% (n = 2) dopamine in the absence or presence of Mg^{2+}, respectively.

tively washed out from the α-toxin-permeabilized PC12 cells by a procedure previously applied to adrenal medullary chromaffin cells (compare Bader et al., 1986, and Results, this article). Even after more vigorous washout procedures, or removal of glutamate from the media, as a potential source for mitochondrial ATP production, the PC12 cells still did not depend on ATP for their dopamine release. Similarly, in earlier experiments on intact PC12 cells, inhibition of ATP production by glycolysis or oxidative phosphorylation did not decrease dopamine release (Reynolds et al., 1982). On the other hand, the permeabilized adrenal medullary chromaffin cell (for references see above) and the intact cell both need ATP for catecholamine release (Rubin, 1969; Kirshner and Smith, 1969).

ATP-independent exocytosis has also been shown in sea urchin eggs, using a preparation of cortical vesicles attached to the inner surface of the plasma membrane (isolated cortices) or electrically permeabilized eggs (Vacquier, 1975; Baker and Whitaker, 1978). Similarly, isolated cortices from paramecia do not require ATP for exocytosis of trichocysts (Vilmart-Seuwen et al., 1986). In these cells the vesicles are aligned to the plasma membrane (eggs) or even docked to it (paramecia) to allow immediate release. A preferential location of secretory vesicles near the plasma membrane has also been observed in PC12 cells (Watanabe et al., 1983) whereas in adrenal medullary chromaffin cells the secretory vesicles are distributed throughout the cytoplasm (for references see Bader et al., 1981). This clearly indicates that intracellular vesicle transport may not be necessary in cells that do not depend on ATP during stimulation for exocytosis. The ATP requirement for intracellular vesicle transport is well established (Adams, 1982; Allen et al., 1985; Vale et al., 1985). The absence of a requirement for exocytotic membrane fusion agrees with the finding that isolated secretory vesicles undergo Ca^{2+}-induced fusion without added ATP (Gratzl et al., 1980; Ekerdt et al., 1981). Thus in ATP-dependent cells, vesicle movement driven by ATP most likely precedes exocytotic membrane fusion.

The target for Ca^{2+} during exocytotic membrane fusion is probably proteinaceous in nature. Antibodies to calmodulin inhibit exocytosis in isolated cortices of sea urchin eggs (Steinhardt and Alderton, 1982) and block catecholamine secretion when transferred

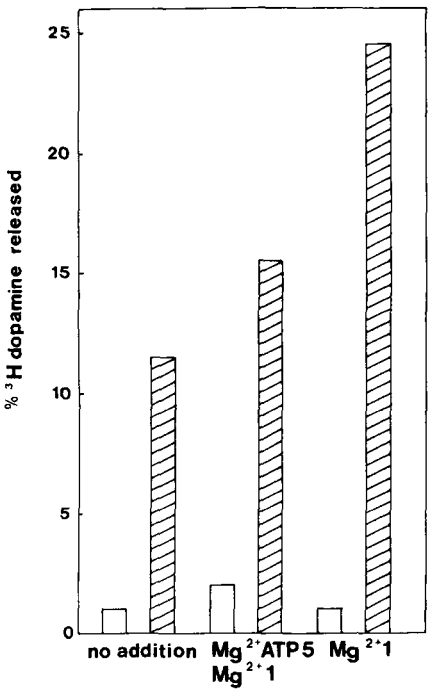

FIG. 5. Effects of Mg^{2+} with and without ATP on Ca^{2+}-induced dopamine release by permeabilized PC12 cells. Preloaded PC12 cells were permeabilized with α-toxin (180 U/ml) in KCl buffer (glutamate replaced by chloride) in the absence of Ca^{2+} either without further addition or in the presence of 1 m*M* free Mg^{2+} plus 5 m*M* Mg^{2+}-ATP or 1 m*M* free Mg^{2+} for 30 min at 30°C. The buffer was exchanged for a fresh one with the same constituents and supplemented with 10 μ*M* free Ca^{2+}. After 10 min [^{3}H]dopamine was estimated in the medium and in the cell lysate. Release in the absence of Ca^{2+} (about 20%) was subtracted. Cells treated with α-toxin in KCl buffer exhibited an increased release in the absence of Ca^{2+} as compared to cells in potassium glutamate buffer (see also Fig. 3). The release was lower in the presence of either Mg^{2+}-ATP or Mg^{2+}. The open bars represent controls, the hatched bars α-toxin-treated cells (n = 2).

TABLE 2. *Effect of TFP on Ca^{2+}-induced release of dopamine by permeabilized PC12 cells*

	Treatment of cells	[^{3}H]Dopamine released (%)		
		No Ca^{2+}	2 μ*M* Ca^{2+}	20 μ*M* Ca^{2+}
(a)	—	0.4	5.2	12.2
	1 μ*M* TFP	ND	6.2	10.8
	10 μ*M* TFP	5.8	7.0	15.2
(b)	—	1.9 ± 0.07	5.3 ± 0.2	18.1 ± 0.7
	1 μ*M* TFP	0.2 ± 0.02	4.4 ± 0.1	16.3 ± 0.6

In (a), cells preloaded on plates were permeabilized with α-toxin (150 U/ml) with or without the indicated free Ca^{2+} concentration (20 min, 37°C) in the presence or absence of the given amount of TFP. Release in untreated samples (without toxin and TFP) was subtracted (13.4% ± 1.4%, SD, n = 4).

In (b) the experiment was carried out as described under (a) but 1 μ*M* TFP was present during the last 20 min of the loading period. Release in the absence of α-toxin (16.4% ± 1%, SD, n = 6) was subtracted. Values are the means of two (a) or three ± SD (b) experiments.

TABLE 3. *Effects of PHMB, DTNB, and NEM on [^{3}H]dopamine release induced by 20 μM free Ca^{2+} in permeabilized PC12 cells*

Treatment of cells	[^{3}H]Dopamine released (%)	
	No Ca^{2+}	20 μ*M* Ca^{2+}
—	8.0	9.3
α-Toxin	9.9	22.9
200 μ*M* PHMB	ND	10.7
α-Toxin + 200 μ*M* PHMB	11.6	22.5
200 μ*M* DTNB	ND	9.7
α-Toxin + 200 μ*M* DTNB	13.4	26.9
200 μ*M* NEM	ND	21.1
α-Toxin + 200 μ*M* NEM	30.3	31.8

Preloaded cells were treated with α-toxin (150 U/ml) with or without 200 μ*M* of the indicated drug in the presence or absence of 20 μ*M* free Ca^{2+}. Values are means of duplicate determinations.

to chromaffin cells using erythrocytes as a vehicle (Kenigsberg and Trifaró, 1984). Experiments with TFP also suggested that calmodulin or related proteins are involved in catecholamine release from chromaffin cells (Kenigsberg et al., 1982; Brooks and Treml, 1983*b*). However, Ca^{2+}-induced exoytosis from permeabilized PC12 cells (this study) was not affected by TFP. This agrees with similar observations using electrically or detergent-permeabilized cells (Baker and Knight, 1981; Brooks and Treml, 1984). The findings neither prove nor exclude participation of the Ca^{2+}-binding calmodulin in secretion but they exclude its involvement in the final step of exocytosis in chromaffin cells. The involvement of other proteins with functional -OH or -SH groups is ruled out by the use of group-specific reagents (Baker and Knight, 1981; this study).

An effect of Mg^{2+} on Ca^{2+}-induced hormone release from permeabilized chromaffin cells is difficult to obtain since these cells depend on Mg^{2+}-ATP for release. Yet Mg^{2+} in high amounts inhibited Ca^{2+}-induced release in chromaffin cells permeabilized by high-voltage discharge (Knight and Baker, 1982). The experiments in this study were initially undertaken to look for inhibitory effects of Mg^{2+} in α-toxin-permeabilized PC12 cells. It is interesting to note that in the permeabilized PC12 cells Mg^{2+} enhanced secretion induced by low concentrations of Ca^{2+}. The possibility that Mg^{2+} facilitates the attachment of the secretory vesicles to the cell membrane before fusion and that exocytotic hormone release is subsequently triggered by Ca^{2+} will be investigated in future work.

Note added in proof: After this paper was submitted it was reported by S. C. Peppers and R. W. Holz [*J. Biol. Chem.* **261,** 14665–14669 (1986)] that when PC12 cells were permeabilized for 6 min with digitonin and then stimulated for 18 min with micromolar Ca^{2+}, the absence of Mg^{2+}-ATP during permeabilization only slightly inhibited Ca^{2+}-dependent secretion but its absence also during permeabilization

and incubation with Ca^{2+} resulted in a further decline in norepinephrine release. This disparity with the present study cannot at present be assessed but the rapid decrease in the secretory response of digitonin-treated cells and the inhibitory effect of digitonin as observed in electrically permeabilized cells (Baker and Knight, 1981) could be involved.

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