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Membrane Fusion Techniques

Part B

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[11] Exocytotic Membrane Fusion as Studied in Toxin-Permeabilized Cells

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Introduction

Permeabilized cells have been widely used in the analysis of exocytotic membrane fusion or intracellular Ca^{2+} regulation. They allow the study of the function of intracellular organelles *in situ* under conditions that are close to the physiological situation in intact cells.

High-voltage discharges[1,2,2a] or detergents such as digitonin or saponin[3-5] have been applied to permeabilize secretory cells. The pitfalls of these techniques, such as the resealing of pores or disintegration of intracellular membranes, have been discussed.[2,6]

To overcome some of the problems inherent in the techniques mentioned above, we developed an approach that makes use of the well-defined pores generated by pore-forming toxins: Alpha-toxin from *Staphylococcus aureus* yields only small pores. Streptolysin O (SLO) from β-hemolytic streptococci yields large pores and therefore allows the diffusion of large molecules into and out of secretory cells.[6,7]

Permeabilized rat pheochromocytoma cells (PC-12)[8-11] and bovine adrenal chromaffin cells kept in culture for a short time[12-15] have been

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preferentially used to analyze exocytotic membrane fusion. In addition, alpha-toxin as well as SLO have been successfully applied to other secretory systems such as cytotoxic T lymphocytes,[16,17] mast cells,[18,19] or cortical synaptosomes.[20] Toxin-permeabilized preparations have also been instrumental in analyzing the intracellular glucose metabolism in hepatocytes,[21] the chain of events leading to smooth muscle contraction,[22] and the regulation of intracellular Ca^{2+} sequestration[23] (see also [12] in this volume).

Materials and Methods

Alpha-toxin is prepared as described[24] from the culture supernatant of *S. aureus* strain wood 46 (kindly provided by S. Bhakdi, Mainz, Germany). The purified toxin is dialyzed against KG buffer (see Table I),[24a] lyophilized, and stored at −20° for several months. Streptolysin O is purified as in Bhakdi *et al.*[25] and is kindly provided by S. Bhakdi; it can also be dialyzed against KG buffer and stored at −20° without loss of activity for several months. The activity of both pore-forming toxins is determined using 2.5% (v/v) rabbit erythrocytes and is given in hemolytic units (HU)/ml.[6,24] Rat pheochromocytoma cells (PC-12) (kindly provided by H. Thoenen, Max Planck Institut für Psychiatrie, Martinsried, Germany) are cultivated as described earlier.[8] Bovine adrenal chromaffin cells are prepared and kept in short-term cultures.[14,26]

Properties of Alpha-Toxin- and Streptolysin O-Permeabilized Cells

Alpha-toxin permeabilizes cells only for small molecules (up to 1000 Da), as tested by measuring the escape of Rb^+ or ATP, whereas cytoplasmic lactate dehydrogenase remains within the cells.[8,12,24] The free

[16] H. Schrezenmeier, G. Ahnert-Hilger, and B. Fleischer, *J. Exp. Med.* **168,** 817 (1988).
[17] H. Schrezenmeier. G. Ahnert-Hilger. and B. Fleischer, *J. Immunol.* **141,** 3785 (1988).
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TABLE I
ASSAY FOR EXOCYTOSIS FROM PC-12 CELLS[a]

1. Load cells with labeled dopamine (noradrenaline) for 1–2 hr in serum-free culture medium supplemented with 1 m*M* ascorbic acid
2. Wash the cells with Ca^{2+}-free balanced salt solutions several times
3. Suspend the cells in KG buffer (add KG buffer to cells on plates) containing 150 m*M* potassium glutamate, 10 m*M* PIPES, 5 m*M* NTA, 0.5 m*M* EGTA, pH 7.2 (plus 2 m*M* Mg^{2+}–ATP and 1 m*M* free Mg^{2+}). In some experiments 1 m*M* free Mg^{2+} was added. For permeabilization, 120 HU/ml (60 Hu/ml) alpha-toxin or 120 HU/ml (60 HU/ml) SLO corresponding to 300–500 HU/10^7 cells were used
4. Incubate the cells with pore-forming toxins diluted in KG buffer containing 0.1 BSA: alpha-toxin (20–30 min at 25, 30, or 37°) (same conditions) or SLO (5 min at 0°; 1 or 2 min at 25, 30, or 37°). For permeabilization with SLO the addition of DTT (1 m*M*) is necessary.
5. Centrifuge (3000 *g*, 30 s) and remove supernatant (removal of supernatant)
6. If desired, perform a further incubation with substances to be tested dissolved in KG buffer (plus Mg^{2+}–ATP and 1 m*M* free Mg^{2+}) for 20 to 40 min at 25, 30, or 37°
7. Repeat step 5
8. Stimulate with micromolar amounts of free Ca^{2+} in KG buffer (plus Mg^{2+}–ATP and 1 m*M*) free Mg^{2+}) for 10 min at 25, 30, or 37°
9. Centrifuge and count released catecholamines in the supernatant or perform HPLC analysis of catecholamines and their metabolites in the supernatant, extracted with 100 m*M* $HClO_4$
10. Solubilize the cells with 0.2% (w/v) sodium dodecyl sulfate (SDS) and count the catecholamines remaining in the cells. For HPLC analysis, extract cells with 100 m*M* $HClO_4$
11. Prior to HPLC analysis, dilute the supernatant or the cell extract with 50 m*M* $HClO_4$. HPLC separation and electrochemical detection (octadecylsilane, 5 μm, oxidation potential +700 mV) is performed as described,[24a] with some modifications: for separation of the catecholamines and the dopamine metabolites a mobile phase is used containing 50 m*M* sodium acetate, 20 m*M* citric acid, 2.8 m*M* octanesulfonic acid, 0.001 m*M* EDTA, 1 m*M* di-*n*-butylamine, pH 4.5, supplemented with 5% (v/v) methanol. The flow rate is usually 0.8 ml and 20-μl samples are injected

[a] Procedures for chromaffin cells in primary culture are given in parentheses.

passage of Ca^{2+}, which allowed a careful analysis of intracellular Ca^{2+} sequestration (see Ref. 23, and [12] in this volume), is also an excellent indicator of sufficient permeability. However, the pores formed in the plasma membrane by hexamerization of alpha-toxin monomers are too small to allow the free passage of toxin monomers.[7,27] Thus the attack of the toxin is restricted to the plasma membrane.

Streptolysin O enables not only small molecules but also proteins to escape from or enter cells. This has been demonstrated by measuring the release of lactate dehydrogenase[8] or the access of antibodies to intracellular

[27] R. Füssle, S. Bhakdi, A. Sziegoleit, J. Tranum-Jensen, T. Kranz, H.-J., and Wellensiek, *J. Cell Biol.* **91**, 83 (1981).

proteins.[9] The large SLO pores also allow the study of the intracellular action of clostridial neurotoxins and their active fragments on exocytosis.[9,11,13-15,28,29]

Damage of intracellular membranes by SLO can be avoided in two ways: by a short incubation of the cells with SLO (1–2 min) at 25, 30, or 37°, or by an incubation at 0°, a condition under which all the SLO monomers present bind to the plasma membrane, followed by warming to trigger pore formation.[7,30] In contrast to the action of SLO,[30] membrane permeabilization by digitonin is insensitive to temperature and therefore is more difficult to control.[6]

Assay for Exocytosis in Permeabilized PC-12 or Bovine Adrenal Chromaffin Cells

In most of the studies dealing with exocytosis from permeabilized cells, an "intracellular medium" containing potassium as a main cation and glutamate as an anion[1] (see also Table I) was used. Because the free Ca^{2+} concentration within the cells under resting conditions, as well as during stimulation, is in the micromolar range, this ion must be carefully controlled in the buffers used. A combination of chelators for divalent cations is suitable to buffer the free Ca^{2+} concentration from 0.1 to 100 μM under experimental conditions. Thus a typical buffer for permeabilization contains 150 mM potassium glutamate, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 mM ethylene diamine tetraacetic acid (EDTA), 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 7.2 (no differences were found using pH between 6.6 and 7.2). Added Mg^{2+} and ATP, as well as the pH of the medium, must be carefully considered because they alter the equilibrium between Ca^{2+} and the chelators present. The free Ca^{2+} and Mg^{2+} concentrations are calculated by a computer program and controlled by Ca^{2+}- and Mg^{2+}-specific electrodes (see [12] in this volume). Each Ca^{2+} buffer is prepared separately from stock solutions with a final check of pH, pCa, or pMg.[31] Buffers can be stored at −20° but should be thawed only once because decomposition of ATP may occur.

[28] B. Stecher, G. Ahnert-Hilger, U. Weller, T. P. Kemmer, and M. Gratzl, *Biochem. J.* **283,** 899 (1992).

[29] B. Stecher, J. Hens, U. Weller, M. Gratzl, W. H. Gispen, and P. De Graan, *FEBS Lett.* **312,** 192 (1992).

[30] F. Hugo, J. Reichweiss, M. Arvand, S. Krämer, and S. Bhakdi, *Infect. Immun.* **54,** 641 (1986).

[31] U. Wegenhorst, M. Gratzl, K. J. Föhr, and G. Ahnert-Hilger, *Neurosci. Lett.* **106,** 300 (1989).

Adrenal chromaffin cells in culture[26] or rat pheochromocytoma cells[32] take up labeled catecholamines and store them within secretory vesicles from which they can be released on stimulation. The released catecholamines can be detected either directly by high-performance liquid chromatography (HPLC) or the intracellular stores can be labeled with tritiated dopamine or noradrenaline. Although the different labeled catecholamines can be taken up by both types of cells, we generally used [^{3}H]dopamine to preload PC-12 cells and [^{3}H]noradrenaline to label bovine adrenal chromaffin cells. After permeabilization of the plasma membrane, release of stored labeled or endogenous catecholamines can be triggered by micromolar concentrations of Ca^{2+}. Table I summarizes the assay of exocytosis for both PC-12 and adrenal chromaffin cells, using alpha-toxin or SLO to permeabilize the plasma membrane.

Proof of Exocytotic Release of Catecholamines from Toxin-Permeabilized PC-12 or Adrenal Chromaffin Cells

The observed release of secretory product from permeabilized cells may occur by exocytosis or may be due to an unspecific leakiness of secretory vesicles. Even the loss of intact vesicles from the cells was observed in digitonin-permeabilized adrenal chromaffin cells.[12] The parallel release of low and high molecular weight secretory products can be taken as a proof for an exocytotic event, if under the same conditions large cytoplasmic constituents remain within the cells. Parallel release of catecholamines and dopamine β-hydroxylase (dopamine β-monooxygenase) from electrically permeabilized chromaffin cells,[33] or of catecholamines and chromogranin A from alpha-toxin-permeabilized chromaffin cells,[12] has been reported. Similarly, the release of vesicular serine esterase from alpha-toxin-permeabilized cytotoxic T lymphocytes was not accompanied by leakage of lactate dehydrogenase.[16,17]

Another approach to distinguish between unspecific release and exocytosis requires a precise analysis of the endogenous catecholamines and their metabolites in permeabilized PC-12 cells. The discharge of vesicular dopamine into the cytoplasm (e.g., by nigericin) results in its enzymatic oxidation, mainly to 3,4-dihydroxyphenylacetic acid (DOPAC). By contrast the direct exocytotic release of catecholamines avoids the cytoplasm and, therefore, metabolic oxidation. The pattern of catecholamines and their metabolites released by alpha-toxin- or SLO-permeabilized PC-12 cells is in accordance with these predictions[10] (Figs. 1 and 2). Calcium ions

[32] L. Greene and A. Tischler, *Adv. Cell. Neurobiol.* **3,** 373 (1982).
[33] D. Knight and P. Baker, *J. Membr. Biol.* **68,** 107 (1982).

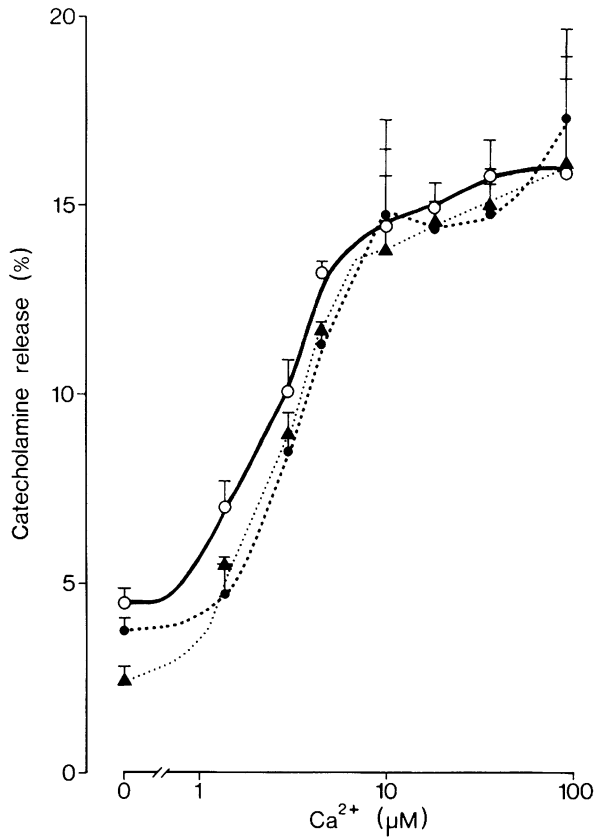

FIG. 1. Calcium ion dependence of catecholamine release from SLO-permeabilized PC-12 cells. Prelabeled ([^{3}H]dopamine) (○) PC-12 cells were washed three times with a Ca^{2+}-free balanced salt solution. The washed cells were suspended in ice-cold ATP-free KG buffer as described in Table I, but containing 1 m*M* EGTA, 1 m*M* HEDTA, and 1 m*M* NTA (instead of 0.5 m*M* EGTA and 5 m*M* NTA) adjusted to pH 7.0, for optimal buffering of the free Ca^{2+} concentration between 1 and 10 μM (see also K. J. Föhr, W. Warchol, and M. Gratzl, this volume [12]), and SLO (120 HU/ml), as outlined in Table I. The cells were incubated for a further 25 min at 25° before they were stimulated for 10 min with the various free Ca^{2+} concentrations given on the abscissa. The supernatant was collected. The released radioactive and endogenous catecholamines were detected by β counting and HPLC, respectively. The total (100%) endogenous content of catecholamines was 0.57 ± 0.1 μg dopamine and 0.43 ± 0.1 μg noradrenaline per 10^6 cells ($n = 24$, SD). Each sample contained about 2.5×10^5 cells.

result in the release of dopamine and noradrenaline that parallels that of [^{3}H]dopamine (Fig. 1). In contrast, the release of DOPAC is not stimulated. However, DOPAC is released in considerable amounts from permeabilized PC-12 cells on treatment with nigericin, which discharges the vesicular content directly into the cytoplasm (Fig. 2).[10] Besides Ca^{2+},

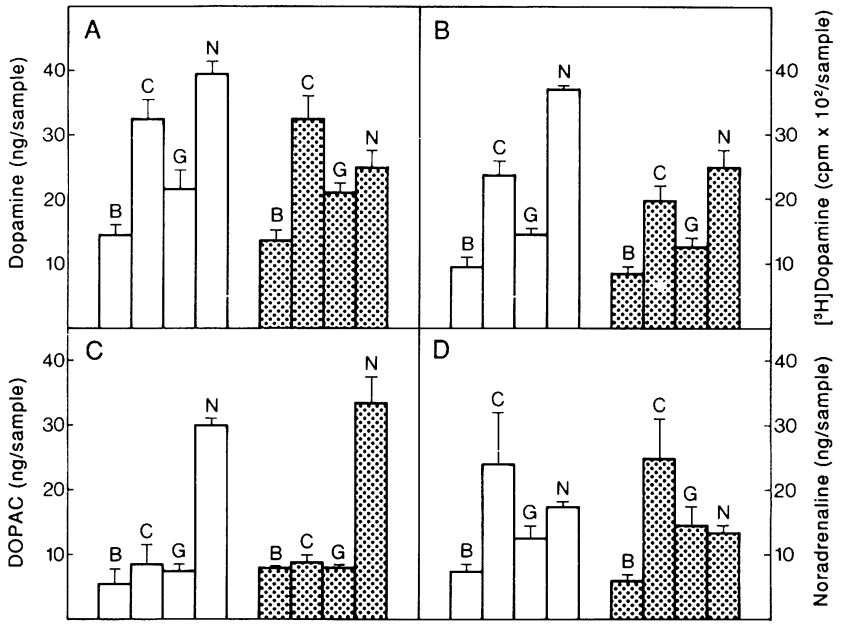

FIG. 2. Basal and stimulated release of catecholamines and DOPAC from SLO-permeabilized PC-12 cells. Prelabeled PC-12 cells were treated with SLO as described in Table I. After removal of the supernatant, the cells were suspended in fresh KG buffer containing either no additive (B), 14 μM free Ca^{2+} (C), 200 μM GMPPNHP (G), or 2 μM nigericin (N). After 20 min at 25°, the released catecholamines [(A) dopamine, (B) [^{3}H]dopamine, (C) DOPAC, and (D) noradrenaline] were determined in the supernatant either by their radioactivity or by HPLC. Cells were incubated in KG buffer without ATP (open bars) or in KG buffer containing 2 mM ATP (dotted bars). The free Mg^{2+} concentration in all buffers was 1 mM. Note that ATP does not substantially alter exocytosis. An increased release of DOPAC was detected only after nigericin treatment, as previously found for alpha-toxin-permeabilized PC-12 cells.[10] The endogenous content of catecholamines was as follows: 0.44 ± 0.09 μg dopamine and 0.35 ± 0.06 μg noradrenaline per 10^6 cells in the absence of ATP, and 0.42 ± 0.04 μg dopamine and 0.26 ± 0.05 μg noradrenaline per 10^6 cells in the presence of ATP ($n = 12$, SD). Each sample contained 2×10^5 cells.

GMPPNHP (an activator of G proteins)[34] also triggers release of catecholamines by these cells. The unchanged values of DOPAC indicate an exocytotic event (Fig. 2). Thus PC-12 cells permeabilized either with alpha-toxin or with SLO release their catecholamines by exocytosis when stimulated with Ca^{2+} or GMPPNHP. These data also demonstrate that toxin-permeabilized PC-12 cells are able to metabolize catecholamines and thus are suitable to study the metabolism of catecholamines under well-defined conditions. Also, in adrenal chromaffin cells Ca^{2+} and GMPPNHP

[34] A. Gilman, *Annu. Rev. Biochem.* **56,** 615 (1987).

cause a parallel release of noradrenaline and adrenaline, which parallels the release observed with [^{3}H]noradrenaline (Fig. 3). Metabolites of catecholamines after nigericin treatment cannot, however, be distinguished clearly from the large amounts of noradrenaline and adrenaline present in these cells (see captions to Figs. 1–3).

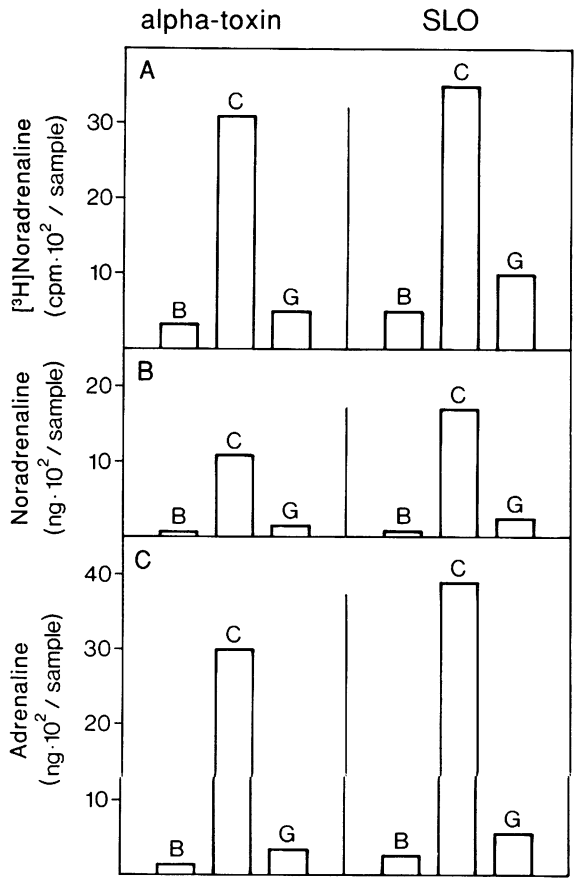

FIG. 3. Basal and stimulated release of catecholamines from alpha-toxin- and SLO-permeabilized adrenal chromaffin cells. Preloaded bovine adrenal chromaffin cells were treated either with alpha-toxin or with SLO as described in Table I. After removal of the supernatant, the cells were suspended in fresh KG buffer supplemented with 1 mM free Mg^{2+} and 2 mM Mg^{2+}–ATP containing no additive (B), 14 μM Ca^{2+} (C), or 100 μM GMPPNHP (G). After 20 min at 25° the released catecholamines [(A) [^{3}H]noradrenaline, (B) noradrenaline, and (C) adrenaline] were detected in the supernatant by determining the radioactivity or by HPLC. The endogenous content of catecholamines was 3.6 ± 0.5 μg noradrenaline and 11.5 ± 1.5 μg adrenaline per 10^6 cells ($n = 24$, SD). Each sample contained 8×10^5 cells.

TABLE II
EFFECT OF ATP ON EXOCYTOSIS FROM STREPTOLYSIN O-PERMEABILIZED ADRENAL CHROMAFFIN CELLS[a]

Incubation time (min)	Ca^{2+}-stimulated [^{3}H]noradrenaline release (%)	
	−ATP	+ATP
2	3.4	13.1
25	0.8	4.5

[a] Bovine adrenal chromaffin cells were permeabilized with SLO for 2 min at 37° in KG buffer containing 1 m*M* free Mg^{2+}, and either no ATP or 2 m*M* ATP (see Table I). After removal of the supernatant the cells were incubated for the times indicated in Ca^{2+}-free KG buffer with or without ATP. Stimulation was performed for 10 min in KG buffer with or without ATP and supplemented with 20 μM free Ca^{2+}. Basal release under both conditions was subtracted [minus ATP (3.3 ± 0.3) and plus ATP (2.3 ± 0.2), $n = 6$, SD]. Values represent the mean of two samples.

Exocytotic Membrane Fusion: An ATP-Dependent Process

The two types of chromaffin cells used in the authors' laboratory differ in their molecular requirements for exocytosis.

In PC-12 cells permeabilized with alpha-toxin or SLO, Ca^{2+} alone is sufficient to release the stored catecholamines (see also Figs. 1 and 2).[9-11] In contrast, bovine adrenal chromaffin cells require additional Mg^{2+}–ATP[1,12,31] (see also Fig. 3 and Tables II and III). Provided that the molecular mechanism of exocytotic membrane fusion as analyzed in various secretory cells is a common process, it should occur under the same conditions. Thus in permeabilized adrenal chromaffin cells an ATP-dependent step, which is probably responsible for the vesicle transport to the plasma membrane, operates in addition to the exocytotic membrane fusion between the vesicular and the plasma membrane. The former is presumably not necessary within PC-12 cells because most of the vesicles are already located near the plasma membrane.[35] In permeabilized adrenal chromaffin cells, a small fraction of chromaffin vesicles is also near the

[35] O. Watanabe, M. Torda, and J. Meldolesi, *Neuroscience* **10,** 1011 (1983).

TABLE III
MAGNESIUM ION REQUIREMENT OF ATP-DRIVEN STEP DURING EXOCYTOSIS FROM STREPTOLYSIN O-PERMEABILIZED ADRENAL CHROMAFFIN CELLS[a]

Condition	Ca^{2+}-stimulated [^{3}H]noradrenaline release (%)	
	−ATP	+ATP
No Mg^{2+}	3.4	3
Free Mg^{2+} (1 m*M*)	1.8	13.6

[a] The experimental procedure followed the protocol given in Table II. The Mg^{2+}-free KG buffer contained either no ATP or 2 m*M* ATP. The basal release was subtracted: no addition (4.1%), ATP alone (4.9%), Mg^{2+} alone (3.9%), Mg^{2+} plus ATP (3.2%). Values are the mean of two determinations.

plasma membrane and can be released without additional ATP. Indeed, after permeabilization with SLO, Ca^{2+} alone causes some catecholamine release, which amounts to roughly one fourth of that observed in the presence of ATP (Table II). Table III demonstrates that not ATP alone, but ATP in combination with Mg^{2+}, is required for the energy-consuming step during exocytosis. Similar results have been obtained for digitonin-permeabilized adrenal chromaffin cells.[36,37] Exocytosis as an ATP-independent step, which is inhibited by the light chains of tetanus toxin and botulinum A toxin, was also observed in permeabilized neurosecretosomes[38-41] and is thus not unique in PC-12 cells.

[36] T. Schäfer, U. Karli, E. Gratwohle, F. Schweizer, and M. Burger, *J. Neurochem.* **49,** 1697 (1987).
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[40] G. Daganithi, G. Ahnert-Hilger, U. Weller, J. J. Nordmann, and M. Gratzl, *Neuroscience* **39,** 711 (1990).
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