MOLECULAR MECHANISMS IN SECRETION

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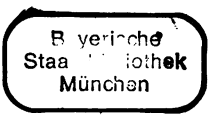

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Metabolism and Function of Ca^{2+} in Secretory Cells

Manfred Gratzl

Interest in the metabolism of Ca^{2+} by secretory cells has increased since the proposal of a key role for Ca^{2+} in the regulation of exocytosis. The Ca^{2+} transport systems and channels present in the plasma membrane have been explored in particular detail because many secretory cells make use of extracellular Ca^{2+} during the stimulation period. In addition, intracellular systems such as mitochondria or endoplasmic membranes may act either as a source of regulatory Ca^{2+} or as its sink. In any case the collaboration of all the aforementioned systems assures the precise control of intracellular free Ca^{2+} and consequently the timing and extent of exocytosis.

The hormone-containing vesicles are the most abundant intracellular structures within the secretory cells. They have also been found to participate in the Ca^{2+} metabolism of these cells. During the secretory event these vesicles temporarily face the extracellular fluid. Thus, unlike the other subcellular structures involved in cellular Ca^{2+} metabolism, they can discharge accumulated Ca^{2+} into the extracellular space. The mechanisms as well as the components of vesicular Ca^{2+} transport systems have been analyzed in the past few years and will be described first.

The investigation of the function of Ca^{2+} in exocytosis has been greatly facilitated by the development of permeabilized cell preparations. These systems fill the gap between simple liposomes and biological membranes as models for exocytotic membrane fusion and the complex situation seen in intact cells. Alpha-toxin produced by *Staphylococcus aureus* is a valuable tool for cell permeabilization because it forms a stable pore in the plasma membrane. This toxin does not impair the exocytotic processes itself and allows definition of the specific

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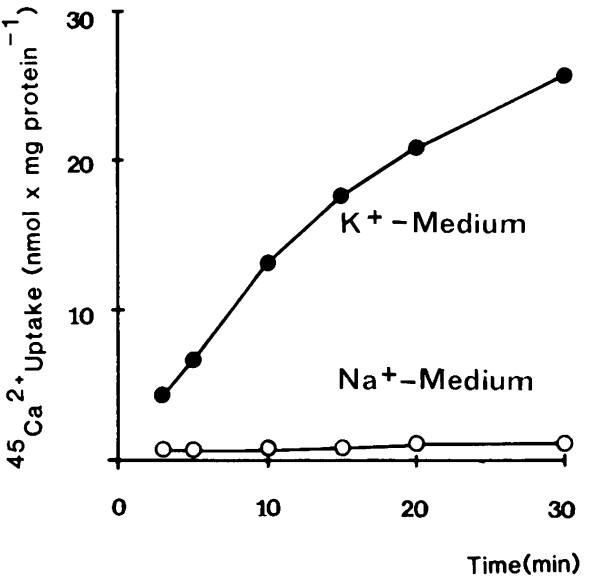

Figure 1. Ca^{2+} uptake by secretory vesicles isolated from adrenal medulla. $^{45}Ca^{2+}$ uptake was observed only in a medium containing potassium (●–●) and not in a medium containing sodium (○–○). (From Krieger-Brauer & Gratzl 1982, with permission).

intracellular requirements for exocytosis in different secretory cells. The role played by regulatory and modulatory substances during exocytosis is the subject of the second part of this communication.

ON THE MECHANISM AND THE COMPONENTS OF THE CALCIUM TRANSPORT SYSTEM PRESENT IN SECRETORY VESICLES

The crucial observation in the analysis of the mechanism of Ca^{2+} uptake by chromaffin vesicles was its complete inhibition by extravesicular sodium (Fig. 1; Krieger-Brauer & Gratzl 1981, 1982). These vesicles normally contain large amounts of sodium (about 50 mM). Reduction of the sodium gradient by addition of increasing amounts of sodium to the extravesicular medium gradually decreases Ca^{2+} uptake by these vesicles. The data were found to be consistent with a carrier system exchanging 2 sodium ions against 1 calcium ion during one cycle (Krieger-Brauer & Gratzl 1982). The ion carrier in the chromaffin vesicle membrane was further analyzed using chromaffin vesicle ghosts (Phillips 1981, Krieger-Brauer & Gratzl 1983). When these ghosts were loaded with sodium a rapid ($V_{max} = 14.5$

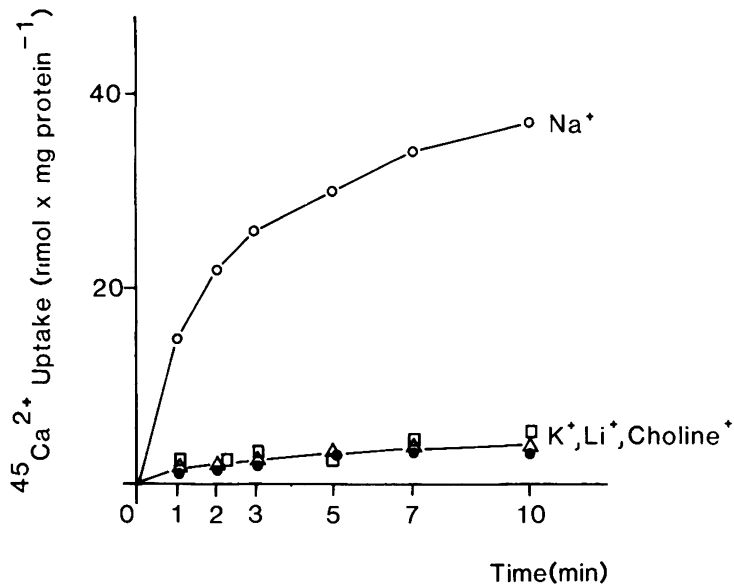

Figure 2. Ion specificity of the Ca^{2+} uptake across the chromaffin vesicle membrane. Vesicles loaded with sodium (O) take up $^{45}Ca^{2+}$ from a medium containing 1.4 μM free Ca^{2+}. Potassium. lithium or choline can not replace sodium (From Krieger-Brauer & Gratzl 1983, with permission).

nmol × min^{-1} × mg protein^{-1}) uptake of Ca^{2+} was observed. Since other ions such as Li^+, K^+, or choline could not replace Na^+, it was concluded that only a Na^+ gradient can serve as a driving force for Ca^{2+} uptake (Fig. 2; Krieger-Brauer & Gratzl 1983). A further activity, perhaps catalyzed by the same carrier, is a Ca^{2+}/Ca^{2+} exchange or Ca^{2+}/Sr^{2+} exchange. This may be the reason for the inhibition of Ca^{2+} uptake by Sr^{2+} described by several groups (Kostron *et al.* 1977, Phillips 1981, Krieger-Brauer & Gratzl 1981, 1982). The Ca^{2+}/Ca^{2+} exchange was found to be completely abolished by extravesicular Mg^{2+} at normal intracellular concentrations. This indicates that vesicular uptake of Ca^{2+}, rather than Ca^{2+}/Ca^{2+} exchange, is the physiologically relevant activity of this carrier system. Since the Km for Ca^{2+} uptake is about 0.3 μM, the vesicles can take up Ca^{2+} from media containing Ca^{2+} in concentrations found in resting or stimulated secretory cells. A Ca^{2+} transport system with almost identical properties has been described for the secretory vesicles from the neurohypophysis (Saermark *et al.* 1983a, b). This carrier exchanges 2 Na^+ for 1 Ca^{2+}, and takes up Ca^{2+} half maximal from media containing 0.7 μM free Ca^{2+}.

Inversion of the Na^+ gradient across the secretory vesicle membrane results in

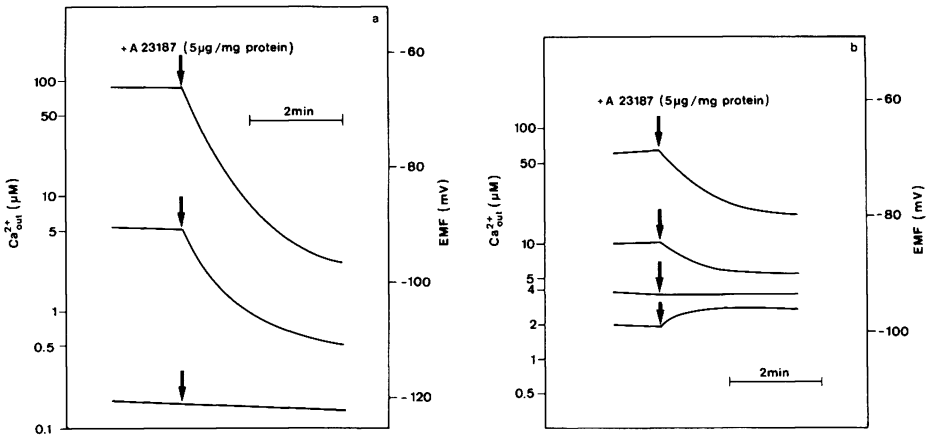

Figure 3. Determination of matrix free Ca^{2+} in isolated secretory vesicles from bovine adrenal medulla. Addition of the ionophor A23187 to isolated chromaffin vesicles suspended in media containing free Ca^{2+} higher than 0.2 μM results in a immediate Ca^{2+} uptake (a). The proton gradient across the chromaffin vesicle membrane which acts as a driving force for ionophore-mediated Ca^{2+} uptake can be collapsed by NH_4Cl. Under these conditions Ca^{2+} uptake ceases at approximately 4 μM (b), indicating that free Ca^{2-} is the same within the vesicles and in the surrounding medium (Reprinted with permission from Biochemistry, Bulenda & Gratzl, 24, 7760–7765, Copyright 1985, American Chemical Society).

release of Ca^{2+} from the vesicles. It is interesting to note that intact secretory vesicles never completely release Ca^{2+}, whereas secretory vesicle ghosts can release total Ca^{2+} (Krieger-Brauer & Gratzl 1982, 1983). One would expect that addition of the Ca^{2+} ionophor A23187 would result in the release of Ca^{2+} from any vesicular structure. However, while Ca^{2+} efflux from vesicle ghosts does indeed occur upon addition of A23187, intact chromaffin vesicles instead act to take up large amounts of Ca^{2+} (Fig. 3a; Krieger-Brauer & Gratzl 1982, 1983). The latter observation is in accordance with the incomplete release of Ca^{2+} from intact chromaffin vesicles upon inversion of the Na^+ gradient, suggesting that Ca^{2+} may be in a bound state within these vesicles. Finally, calculations on the energetics of the Na^+/Ca^{2+} exchange point to the possibility that only a small amount of Ca^{2+} present in the chromaffin vesicles is in the free state.

Binding of Ca^{2+} within chromaffin vesicles has been proved during Ca^{2+} flux experiments (Bulenda & Gratzl 1985). After balancing all gradients of ions which may be transported by the ionophor A23187, Ca^{2+} uptake should occur when the free Ca^{2+} concentration is higher outside the vesicles and release should occur when the vesicle Ca^{2+} concentration is higher than in the medium. At the Ca^{2+}

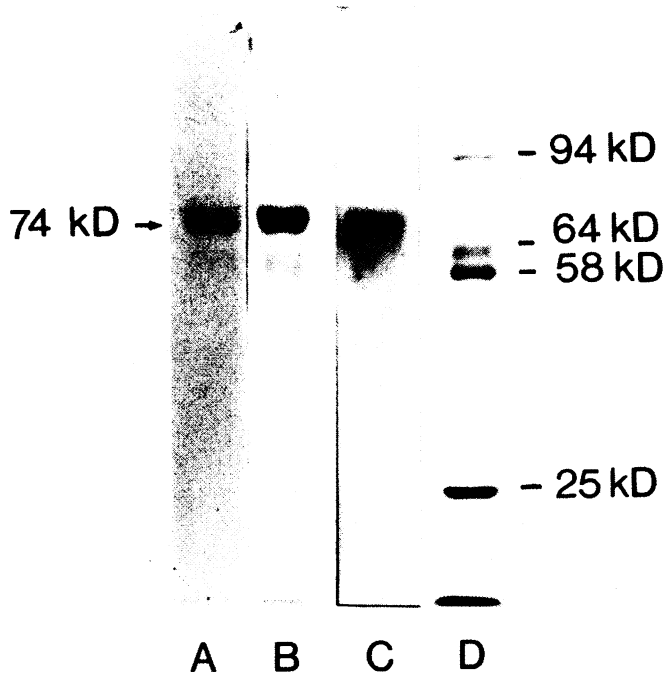

Figure 4. Ca^{2+} binding to purified chromogranin A. Chromogranin A after sodiumdodecyl sulfate-polyacrylamide gel electrophoresis was stained with a carbocyanine dye ("Stains-all") which interacts also with other Ca^{2+}-binding proteins (lane A). Following blotting onto nitrocellulose and incubation with $^{45}Ca^{2+}$ the 74 kD band of chromogranin A was labelled (lane C). Lane B gives the protein stain with amido black and lane D the molecular weight standards (Reprinted with permission from Biochemistry, Reiffen & Gratzl, 25, 4402–4406, Copyright 1986, American Chemical Society).

concentration at which no net flux occurs, measurements with a Ca^{2+}-specific electrode indicate that the intravesicular free Ca^{2+} equals the extravesicular free Ca^{2+} (Fig. 3b). In these and additional experiments it was found that most of the Ca^{2+} present within chromaffin vesicles is bound (usually 80 nmol/mg of protein which amounts to about 40 mM Ca^{2+}) leaving only 4 μM free Ca^{2+} in the secretory vesicle matrix (Bulenda & Gratzl 1985).

Within the chromaffin vesicles Ca^{2+} can be bound to ATP and/or chromogranin A. Chromogranin A specifically interacts with a carbocyanin dye ("stains all"), which binds also to other well known Ca^{2+} binding proteins, as well as with Ca^{2+} itself (Fig. 4; Reiffen & Gratzl 1986a, b). In media mimicking the intravesicular fluid (with respect to ionic strength, presence of Mg^{2+}, pH 6) the affinities of

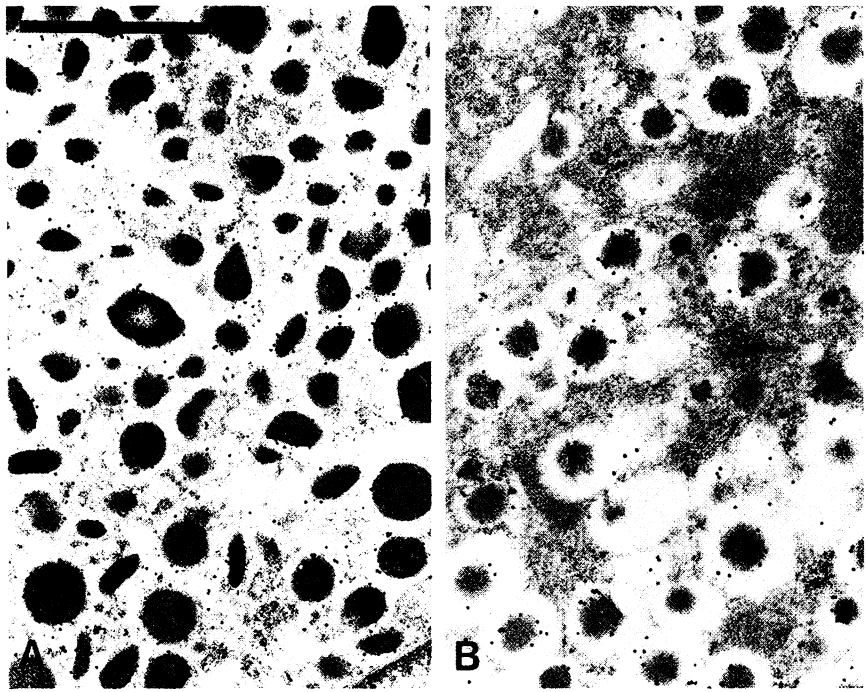

Figure 5. Presence of chromogranin A in the adrenal medulla and the pancretic islet. Both the catecholamine-containing vesicles of chromaffin cells (A) and the insulin-containing vesicles of the B cell of the pancreatic islet (B) exhibit chromogranin A immunoreactivity (protein A-gold technique). Scale bar indicates 1 μm. (Courtesy of M. Ehrhart, Abteilung Anatomie und Zellbiologie der Universität Ulm, GFR).

ATP and chromogranin A for Ca^{2+} indeed are very similar. Thus it appears that small changes in the matrix composition (e.g. in the intravesicular pH due to the proton translocating ATPase) can determine whether Ca^{2+} is bound preferentially to ATP or to the matrix protein chromogranin A.

Interestingly enough, chromogranin A also occurs in other endocrine cells as well as the chromaffin cells (see Fig. 5, c.f. Cohn *et al.* 1982, O'Connor *et al.* 1983). In the pancreatic islet cells chromogranin A has been detected not only within the same cells but also within the same vesicles as the established hormones (Ehrhart *et al.* 1986). The presence of chromogranin A and related proteins has been confirmed in immunoblots of pancreas extracts (Yoshie *et al.* 1987, Ehrhart *et al.* 1988). It is not clear whether chromogranin A fulfills the same Ca^{2+}-binding function in other endocrine cells. However, the observation that Ca^{2+} and

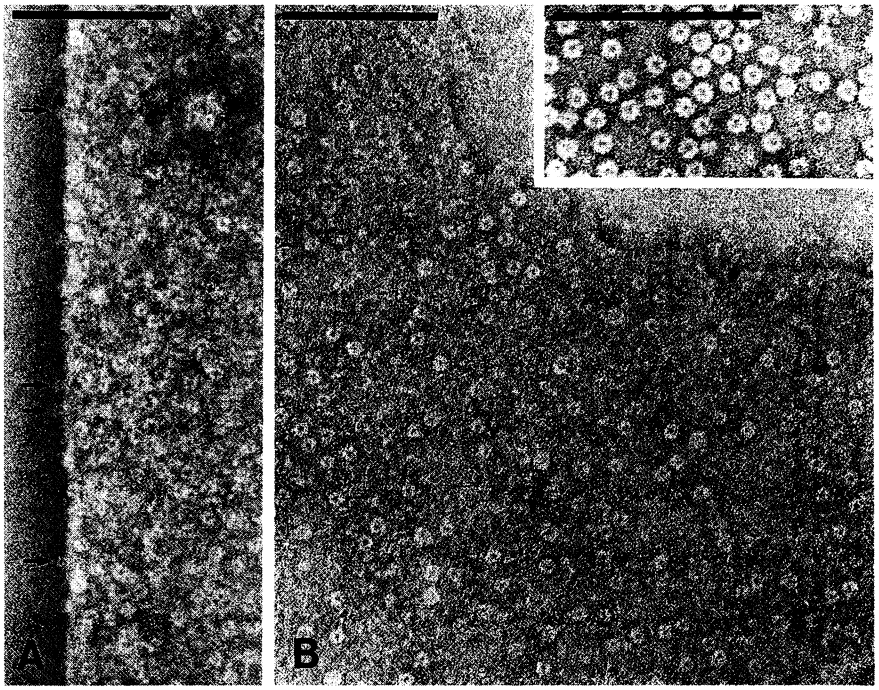

Figure 6. Electron micrographs of negatively stained fragments of rabbit erythrocytes with incorporated *Staphylococcus aureus* alpha-toxin. The cylindrical hexameric structure of the toxin is seen in profiles along the bend edge a ghost membrane in frame A (arrows) and in axial projection in frame B. The inset of frame B depicts pure, isolated toxin hexamers. Scale bar indicates 100 nm. (Courtesy of J. Tranum-Jensen, Institute of Anatomy, Department C, University of Cophenhagen, Denmark).

chromogranin A display a colocalization in the periphery of the secretory vesicles of the pancreatic islet cells is interesting in this context (c.f. Ehrhart *et al.* 1986, Ravazzola *et al.* 1976).

Thus it appears that one of the physiological functions of chromogranin A is the formation of a Ca^{2+} complex within the secretory vesicles. In addition, chromogranin A, when secreted together with the hormones, may play a further role elsewhere in the body.

CALCIUM AND THE REGULATION OF EXOCYTOTIC MEMBRANE FUSION

The process of membrane fusion during exocytosis is difficult to analyze mainly because the interacting membrane surfaces are not accessible from the outside of the cell. Recently, several procedures have been reported which permeabilize the

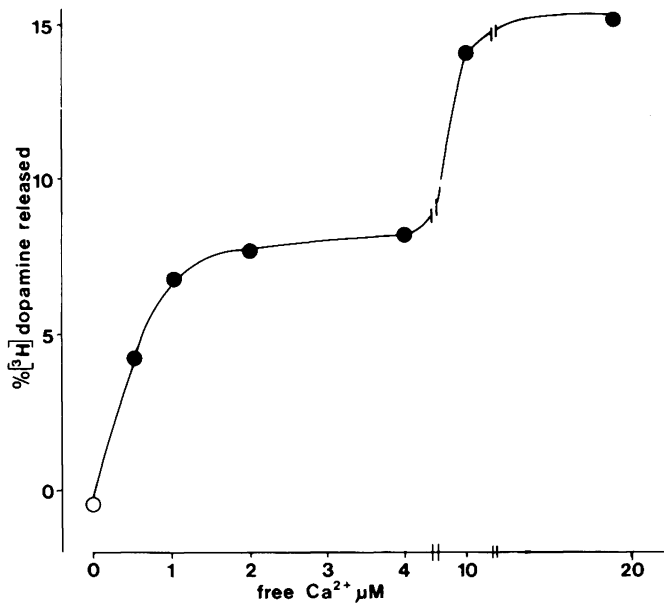

Figure 7. Dopamine release from alpha-toxin permeabilized pheochromocytoma cells as a function of the free Ca^{2+} centration. The cells were first loaded with (^{3}H)dopamine. Then they were permeabilized with alpha-toxin and incubated for 20 min at 37°C in a medium containing the indicated amounts of free Ca^{2+} (From Ahnert-Hilger *et al.* 1985, with permission).

plasma membrane of secretory cells while leaving the process of exocytosis intact. An ideal tool for cell permeabilization is the alpha-toxin produced by *Staphylococcus aureus*. This toxin is a water soluble protein of a molecular weight of about 32 KD. It inserts into the membrane and hexamerizes, forming a stable transmembrane pore (see Fig. 6; c.f. Bhakdi & Tranum-Jensen 1987). The size of the pore allows replacement of molecules up to a molecular mass of 1 KD. Thus most of the substances thought to have a regulatory role in exocytosis can be easily transferred into the cytoplasm.

Pheochromocytoma and insulinoma cell lines from rat and bovine adrenal medullary chromaffin cells in primary culture have been successfully permeabilized with alpha-toxin (Ahnert-Hilger *et al.* 1985a, b, Ahnert-Hilger & Gratzl 1987, Bader *et al.* 1986, Lind *et al.* 1987). An absolute requirement for the release of secretory product observed in alpha-toxin permeabilized cell preparations is the presence of μM concentrations of free Ca^{2+}. With rat pheochromocytoma cells (PC12) the Ca^{2+} activation curve was biphasic (see Fig. 7, Ahnert-Hilger *et*

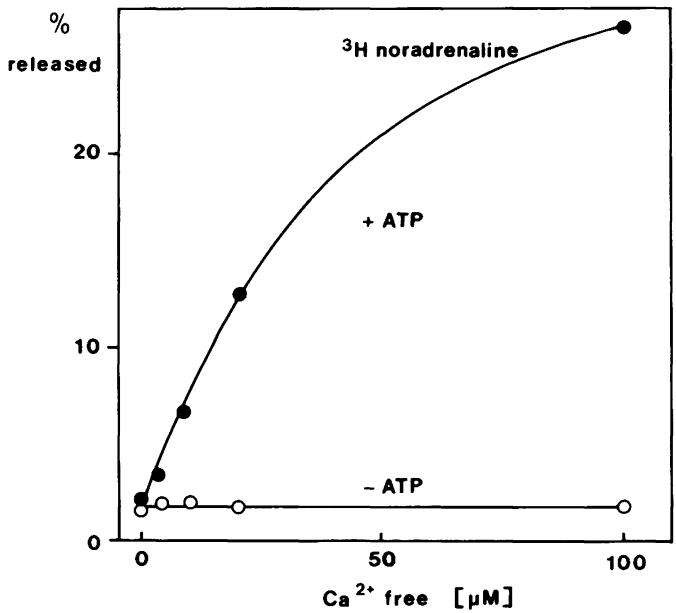

Figure 8. ATP requirement of Ca^{2+}-induced noradrenaline release from alpha-toxin permeabilized chromaffin cells in primary culture. The cells were first permeabilized with alpha-toxin and then incubated with a medium containing the indicated amounts of free Ca^{2+} plus or minus 5 mM ATP and 1 mM free Mg^{2+} (From Bader *et al.* 1986, with permission).

al. 1985a, b, Ahnert-Hilger & Gratzl 1987). A first increase of dopamine release plateaued at 1–4 μM free Ca^{2+}, followed by a second rise responding to higher Ca^{2+} concentrations. Thus the release of secretory product occurred with the same concentrations of Ca^{2+} observed in stimulated secretory cells.

Adrenal medullary chromaffin cells were found to require ATP in addition to Ca^{2+} in order to release catecholamines (Fig. 8; Bader *et al.* 1986). ATP could not be replaced by any of the other nucleotides tested (Fig. 9; Bader *et al.* 1986). The precise role of ATP in these cells is not known, but it may function as an energy source for translocation of secretory vesicles towards the plasma membrane. This translocation is not necessary within the pheochromocytoma cells due to the subplasmalemmal location of their secretory vesicles (see Bader *et al.* 1986).

In the permeabilized pheochromocytoma cells the Ca^{2+}-induced release of dopamine, while insensitive to ATP, can be potentiated by the presence of Mg^{2+} (Fig. 10; Ahnert-Hilger & Gratzl 1987). This suggests that, besides the specific effect of Ca^{2+} ions during the interaction of the secretory vesicles with the plasma

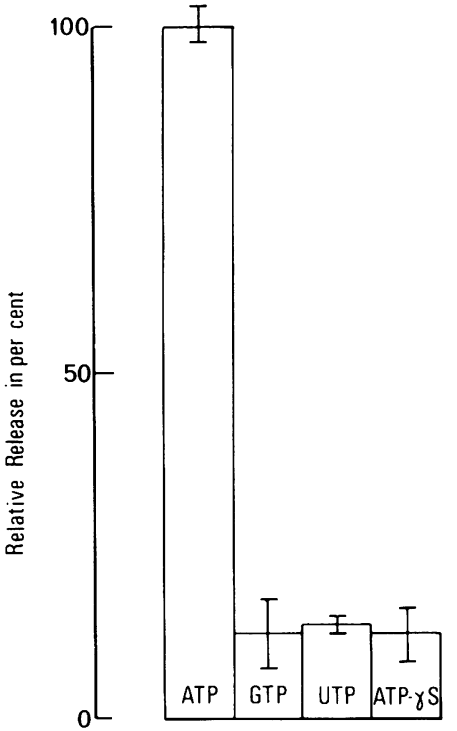

Figure 9. Nucleotide specificity of Ca^{2+}-induced catecholamine release from alpha-toxin permeabilized chromaffin cells in primary culture. From the nucleotides tested only ATP was effective in supporting Ca^{2+}-induced release of secretory product (From Bader *et al.* 1986, with permission).

membrane, several further modulatory processes not necessarily linked to the process of exocytotic membrane fusion may be important. This is in accordance with the finding that secretory vesicle fusion as a model for exocytosis is solely dependent on Ca^{2+} and requires no additional substances (Dahl & Gratzl 1976, Ekerdt *et al.* 1981, Gratzl & Dahl 1976, 1978, Gratzl *et al.* 1977).

Ca^{2+}-induced release of catecholamines from alpha-toxin permeabilized pheochromocytoma cells can be modulated via the protein kinase C as well as GTP-binding proteins (Ahnert-Hilger *et al.* 1987). The phorbol ester TPA and the diacylglycerol OAG, which both activate purified protein kinase C, cause an enhancement of secretion from permeabilized pheochromocytoma cells. GTP-gamma-S on the other hand inhibits Ca^{2+}-induced release from the same cells. Since the latter effect is abolished by pretreatment of the cells with pertussis toxin

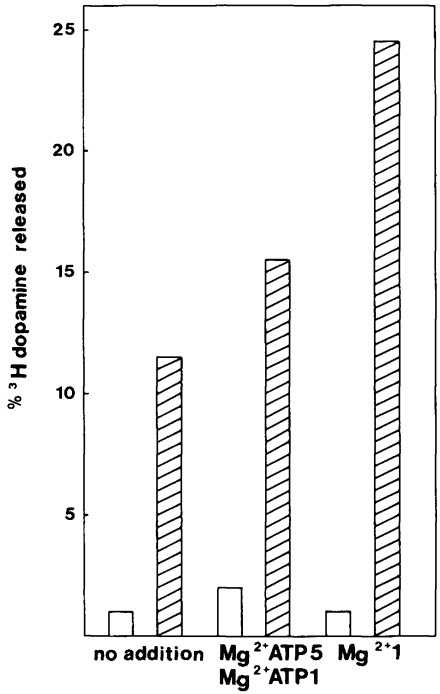

Figure 10. Effect of Mg^{2+} on Ca^{2+}-indcued dopamine release from alpha-toxin permeabilized pheochromocytoma cells. (^{3}H)dopamine release induced by 10 μM free Ca^{2+} is observed when 5 mM ATP is present in addition to 1 mM free Mg^{2+}. However, 1 mM free Mg^{2+} alone is more potent (From Ahnert-Hilger & Gratzl 1987, with permission).

G-proteins, probably of the type Gi or Go participate in the regulation of exocytosis.

Further investigation of the release of secretory product in permeabilized cell preparations certainly will yield further information on the process of exocytotic membrane fusion and the preceeding modulatory processes.

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