

Stimulus-Secretion Coupling in Chromaffin Cells

Volume I

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Chapter 5

UPTAKE AND RELEASE OF Ca^{2+} BY CHROMAFFIN VESICLES

Manfred Gratzl

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I. INTRODUCTION

Intracellular free Ca^{2+} in eukaryotic cells is regulated by Ca^{2+} transport systems present in the plasma membrane, as well as in membranes of subcellular structures. In secretory cells, neurons, and muscle, the control of intracellular Ca^{2+} levels is pivotal, since Ca^{2+} triggers specific functions of these cells.

In the cell membrane, besides potential-dependent or receptor-operated Ca^{2+} channels which allow influx of Ca^{2+} into the cells, two systems, $\text{Na}^+/\text{Ca}^{2+}$ exchange and Ca^{2+} ATPase, participate in the extrusion of Ca^{2+} from the cell. Likewise, mitochondria are endowed with two transport systems for Ca^{2+} , one driven by the membrane potential and another operating in exchange for monovalent cations. Ca^{2+} uptake by another intracellular compartment, the endoplasmic reticulum, is essentially dependent on ATP.¹ All these systems involved in the cellular Ca^{2+} metabolism have been found in many different secretory cells² and have also been investigated in some detail in chromaffin cells.³

In gland cells, in addition to the structures mentioned above, secretory vesicles participate in the metabolism of Ca^{2+} . The Ca^{2+} transport system present within the chromaffin vesicle membrane is discussed in this chapter.

II. Ca^{2+} TRANSPORT BY INTACT CHROMAFFIN VESICLES

As early as 1965 attention was drawn to chromaffin vesicles by the detection of high amounts of Ca^{2+} (60 nmol/mg of protein) within isolated chromaffin vesicles.⁴ Later on this observation was confirmed by means of cytochemical techniques.⁵ From the fact that the Ca^{2+} content of chromaffin vesicles increases upon stimulation of the adrenal gland, a regulatory function of these organelles for the intracellular Ca^{2+} concentration has been envisaged.⁶ $^{45}\text{Ca}^{2+}$ uptake has been observed by a crude fraction of secretory vesicles (i.e., one isolated by differential centrifugation) which, after incubation with labeled Ca^{2+} , were separated by density gradient centrifugation. Ca^{2+} uptake was independent of ATP, but Sr^{2+} was inhibitory.⁷ On the contrary, using the same technique, various degrees of stimulation of Ca^{2+} uptake by ATP have been reported.⁸⁻¹¹ However, since ATP does not modify Ca^{2+} uptake by purified chromaffin vesicles nor by chromaffin vesicle ghosts, the reported stimulating effects of the nucleotide should rather be ascribed to the presence of a different (contaminating) organelle.

The paramount importance of Na^+ on the Ca^{2+} uptake by intact purified chromaffin vesicles has been recognized when observing inhibition of Ca^{2+} uptake by this ion.¹² In fact, it turned out that Ca^{2+} uptake by intact purified chromaffin vesicles was exclusively due to a Na^+ gradient across the vesicular membrane (see Figure 1¹³). Decreasing this gradient by increasing the extravesicular Na^+ concentration concomitantly led to a decrease in Ca^{2+} uptake. Half maximal inhibition was observed with 34 mM Na^+ . Further analysis revealed that about two Na^+ can be exchanged for one Ca^{2+} across the chromaffin vesicle membrane. Moreover, $\text{Na}^+/\text{Ca}^{2+}$ exchange was reversible so that Ca^{2+} can move in either direction across the membrane, depending on the orientation of the electrochemical gradients for Na^+ (Figure 2). Furthermore, dissipation of the driving force by treatment with the Na^+ ionophore monensin abolished transport activity. In contrast to studies using less pure vesicle preparations, no effect of ATP (nor of inhibitors such as dicyclohexylcarbodiimide, *N*-ethylmaleimide, or Ruthenium red) on Ca^{2+} uptake has been noticed. Interestingly enough, a Na^+ -dependent Ca^{2+} uptake system, very similar to that described in chromaffin vesicles, has been found also in secretory vesicles isolated from the neurohypophysis.^{14,15} Two observations with intact chromaffin vesicles have stressed the importance of the state of intravesicular Ca^{2+} for the Ca^{2+} transport across the chromaffin vesicle membrane. Both the failure of Na^+ to completely release Ca^{2+} from the vesicles by high extravesicular Na^+

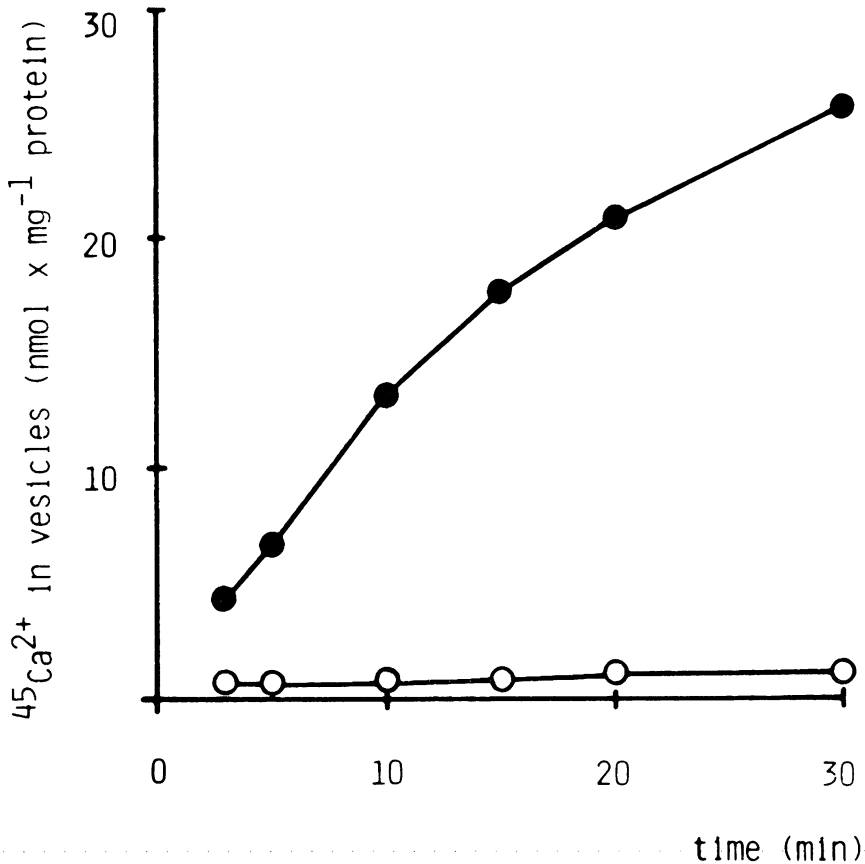


FIGURE 1. Time dependence of $^{45}\text{Ca}^{2+}$ uptake by secretory vesicles isolated from bovine adrenal medulla. The incubation medium contained 15 mM K $^{+}$ (Σ — Σ) or 210 mM Na $^{+}$ (O—O), 100 μM free Ca^{2+} , 0.5 mM EGTA, and 20 mM Mops (pH 7.3). (From Krieger-Brauer, H. and Gratzl, M., *Biochim. Biophys. Acta*, 691, 61, 1982, With permission.)

(Figure 2) as well as the massive uptake of Ca^{2+} mediated by the ionophore A23187 (Figure 3) pointed to the possibility that the majority of Ca^{2+} inside the vesicles is not in a free state.

III. $\text{Na}^{+}/\text{Ca}^{2+}$ EXCHANGE STUDIED WITH CHROMAFFIN VESICLE GHOSTS

Ca^{2+} transport by chromaffin vesicle ghosts is the subject of two different publications.^{16,17} The kinetic parameters, as well as the data characterizing the dependence of Ca^{2+} transport on the ion gradients reported in one of the studies,¹⁷ will be referred to in the following. This is because in this investigation the amount of monovalent cations present on both sides of the membrane as well as the amount of free Ca^{2+} were strictly controlled. Despite this, similar conclusions can be drawn from the investigations mentioned.

Using chromaffin vesicle ghosts loaded with different types of monovalent cations, it was shown that only a Na^{+} gradient can act as a driving force for Ca^{2+} uptake¹⁷ (Figure 4). Neither K^{+} and Li^{+} nor choline could replace Na^{+} . In addition, Ca^{2+} uptake was directly correlated with the magnitude of the Na^{+} gradient and ceased, when the concentration of intravesicular Na^{+} was in the range of that outside the vesicles. Loss of transport activity also paralleled the collapse of the Na^{+} gradient by monensin.

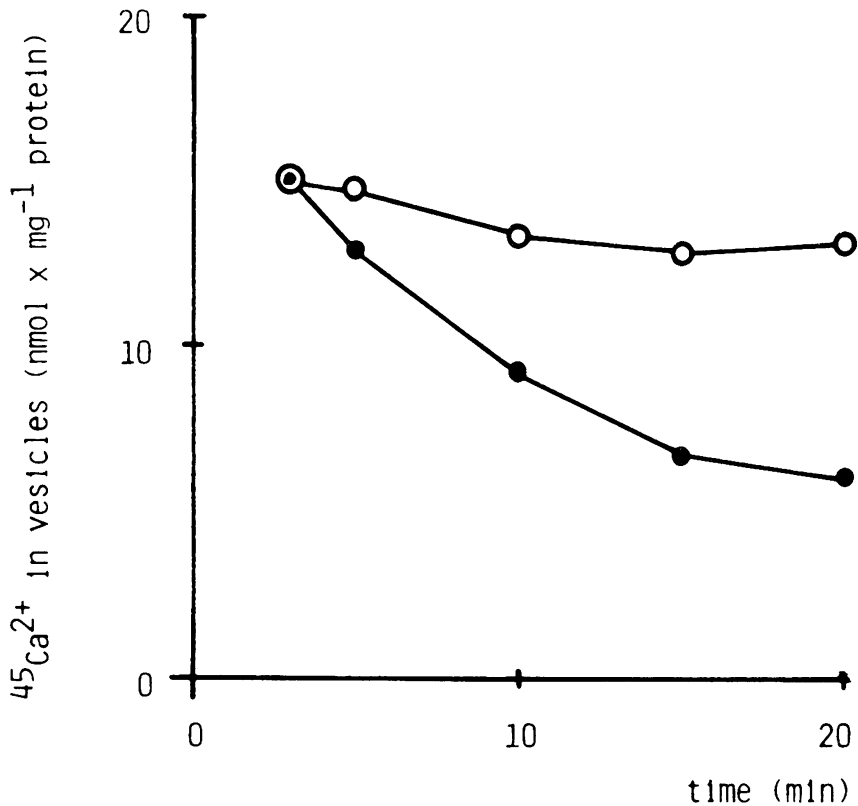


FIGURE 2. Effect of K^{+} and Na^{+} on the release of $^{45}\text{Ca}^{2+}$ from secretory vesicles isolated from adrenal medulla. Secretory vesicles which had taken up $^{45}\text{Ca}^{2+}$ for 10 min at 37°C were spun down at $12,000 \times g$ for 20 min. The pellet was resuspended in media containing 220 mM Na^{+} (●—●) or 200 mM K^{+} (○—○), 20 mM Mops (pH 7.3), 0.5 mM EGTA. The $^{45}\text{Ca}^{2+}$ content in the vesicles was determined after incubation (37°C) at different times. (From Krieger-Brauer, H. and Gratzl, M., *Biochim. Biophys. Acta*, 691, 61, 1982. With permission.)

Uptake of Ca^{2+} by intact vesicles in the presence of the ionophore A23187 has been discussed as being indicative of intravesicular Ca^{2+} binding (see Chapter 4). The capability of this substance to release Ca^{2+} from the ghosts, which are largely devoid of other intravesicular components, further supported this contention (Figure 5). The release of Ca^{2+} by inversion of the Na^{+} gradient (Figure 6) indicated that the direction of the Ca^{2+} movement is determined by the orientation of the Na^{+} gradient. The incomplete release of Ca^{2+} under these conditions, furthermore, demonstrated the importance of Ca^{2+} binding within the vesicles (compare Figure 2 and Figure 6). The functional role of intravesicular Ca^{2+} binding in the Ca^{2+} transport by chromaffin vesicles will be discussed in more detail in the following chapter.

Besides the $\text{Na}^{+}/\text{Ca}^{2+}$ exchange described above, chromaffin vesicle membranes also catalyze $\text{Ca}^{2+}/\text{Ca}^{2+}$ exchange. Only the latter process was completely inhibited by Mg^{2+} . Thus, in the presence of Mg^{2+} a kinetic characterization of the $\text{Na}^{+}/\text{Ca}^{2+}$ exchange could be undertaken. Under these conditions, an apparent K_m for Ca^{2+} uptake of $0.3 \mu\text{M}$ and a V_{max} of 14.5 nmol/mg of protein per minute were obtained.¹⁷ This K_m value is among the lowest values reported for $\text{Na}^{+}/\text{Ca}^{2+}$ exchange systems. The V_{max} is considerably lower than that reported for sarcolemmal vesicles,¹⁸ but may be readily compared with the $\text{Na}^{+}/\text{Ca}^{2+}$ exchange activity in cell membrane vesicles prepared from pituitary cells.¹⁹

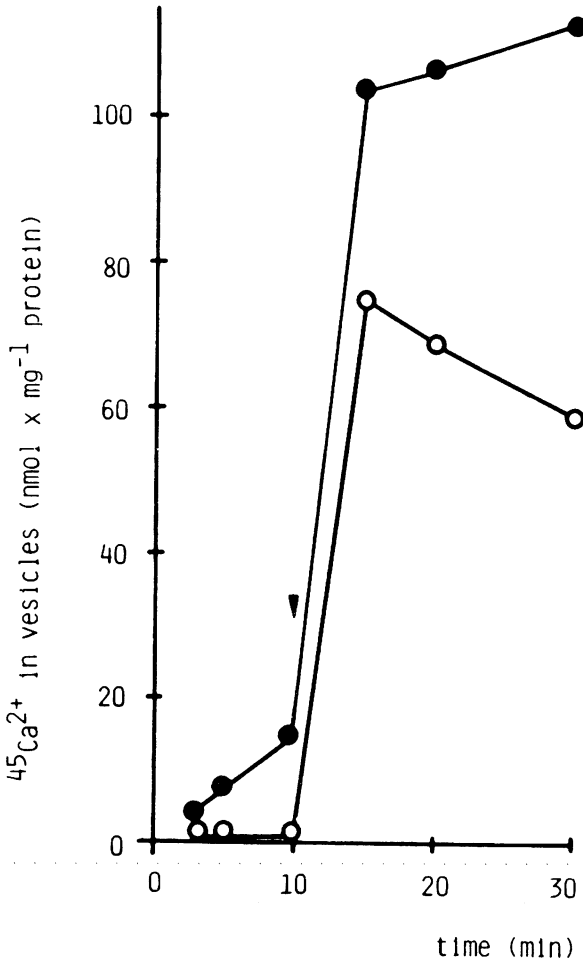


FIGURE 3. Effect of the Ca^{2+} ionophore A23187 on $^{45}\text{Ca}^{2+}$ uptake by intact secretory vesicles isolated from bovine adrenal medulla. The vesicles were incubated at 37°C with $100\ \mu\text{M}$ free Ca^{2+} along with $18\ \text{mM}$ K^+ (●—●) or $212\ \text{mM}$ Na^+ (○—○). After 10 min of incubation (arrowhead) A23187 ($2\ \mu\text{g}/\text{ml}$ final) was added. (From Krieger-Brauer, H. and Gratzl, M., *Biochim. Biophys. Acta*, 691, 61, 1982, With permission.)

For subcellular structures to be physiologically important as a Ca^{2+} buffer, the rate of the process as well as the capacity of the structures must be sufficiently high to be capable to remove cytoplasmic Ca^{2+} . In addition, transport should occur at free Ca^{2+} concentrations found within resting or stimulated chromaffin cells.²⁰ All these criteria are met by the Ca^{2+} transport system present in chromaffin vesicles. The importance of these structures is even more obvious if one considers that chromaffin vesicles are by far the most abundant subcellular organelles in chromaffin cells (about 25,000 per cell), occupying about 10% of the total cell volume.²¹

IV. FREE AND BOUND Ca^{2+} IN CHROMAFFIN VESICLES

Two lines of evidence support the idea that Ca^{2+} within intact chromaffin vesicles is mainly bound. First, this can be concluded from the difference in the extent of release of

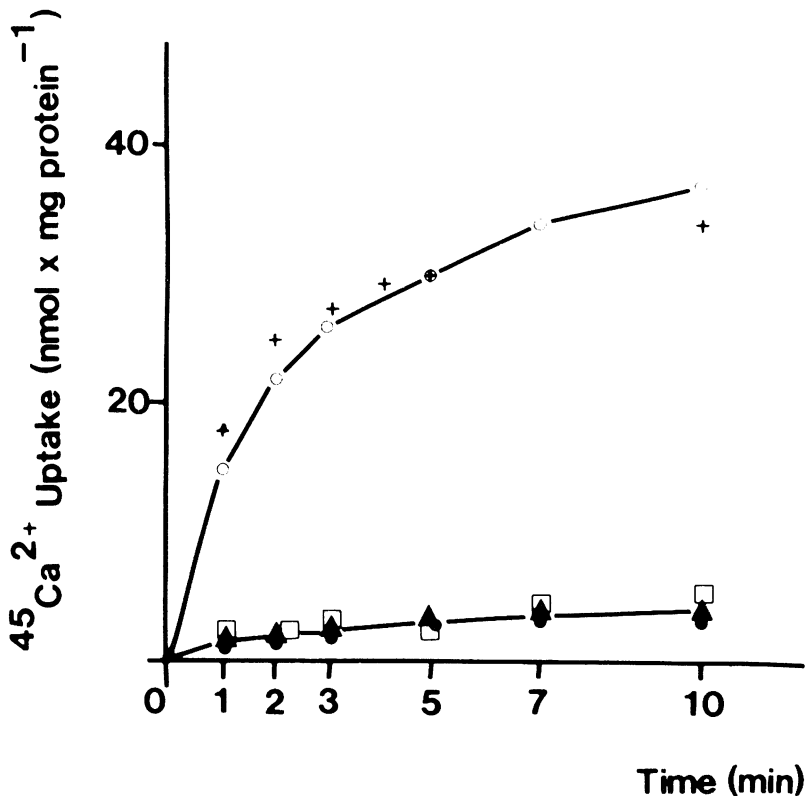


FIGURE 4. Na^+ -dependent $^{45}\text{Ca}^{2+}$ uptake by chromaffin secretory vesicle ghosts. NaCl-loaded ghosts (○), sodium isethionate-loaded ghosts (+), KCl-loaded ghosts (□), LiCl-loaded ghosts (●), and choline chloride-loaded ghosts (▲) were incubated with $1.4 \mu\text{M}$ free Ca^{2+} in 100 mM KCl, 0.5 mM EGTA, and 20 mM Mops (pH 7.3). (From Krieger-Brauer, H. and Gratzl, M., *J. Neurochem.*, 41, 1269, 1983. With permission.)

Ca^{2+} by Na^+ from intact vesicles as compared to ghosts^{13,17} (compare Figures 2 and 6). Whereas a rapid and complete release of Ca^{2+} occurs from chromaffin vesicle ghosts (which are devoid of other intravesicular components) via the $\text{Na}^+/\text{Ca}^{2+}$ exchange system when operating in the reverse mode, under the same conditions, intact chromaffin vesicles release only half of total Ca^{2+} . Secondly, incorporation of the Ca^{2+} ionophore A23187 into the membrane of intact chromaffin vesicles leads to a further massive uptake of Ca^{2+} . By contrast, upon addition of the ionophore, Ca^{2+} is released from vesicle ghosts (compare Figures 3 and 5).

The ionophore A23187 catalyzes transport of Ca^{2+} across biological membranes in exchange for protons.²² The proton gradient existing across the chromaffin vesicle membrane can be abolished by NH_4Cl . Under these conditions, Ca^{2+} movement only depends on the Ca^{2+} gradient. The intravesicular matrix-free Ca^{2+} equals the extravesicular-free Ca^{2+} at a Ca^{2+} concentration, at which no net flux of Ca^{2+} and H^+ occurs.²³ In fact, Ca^{2+} flux ceased at a free concentration of Ca^{2+} of about $4 \mu\text{M}$ (Figure 7).

The chromaffin vesicles used in these experiments contained about 80 nmol Ca^{2+} per milligram of protein. Assuming an internal volume of $2 \mu\ell/\text{mg}$ of vesicle protein, the apparent intravesicular concentration would be 40 mM , which exceeds the free intravesicular Ca^{2+} concentration by a factor of 10^4 . Binding of Ca^{2+} within subcellular structures is not unique to chromaffin vesicles. It has also been observed in mitochondria,^{24,25} sarcoplasmic reticulum,²⁶ as well as in secretory vesicles of thrombocytes.²⁷ Thus, it seems to be a general

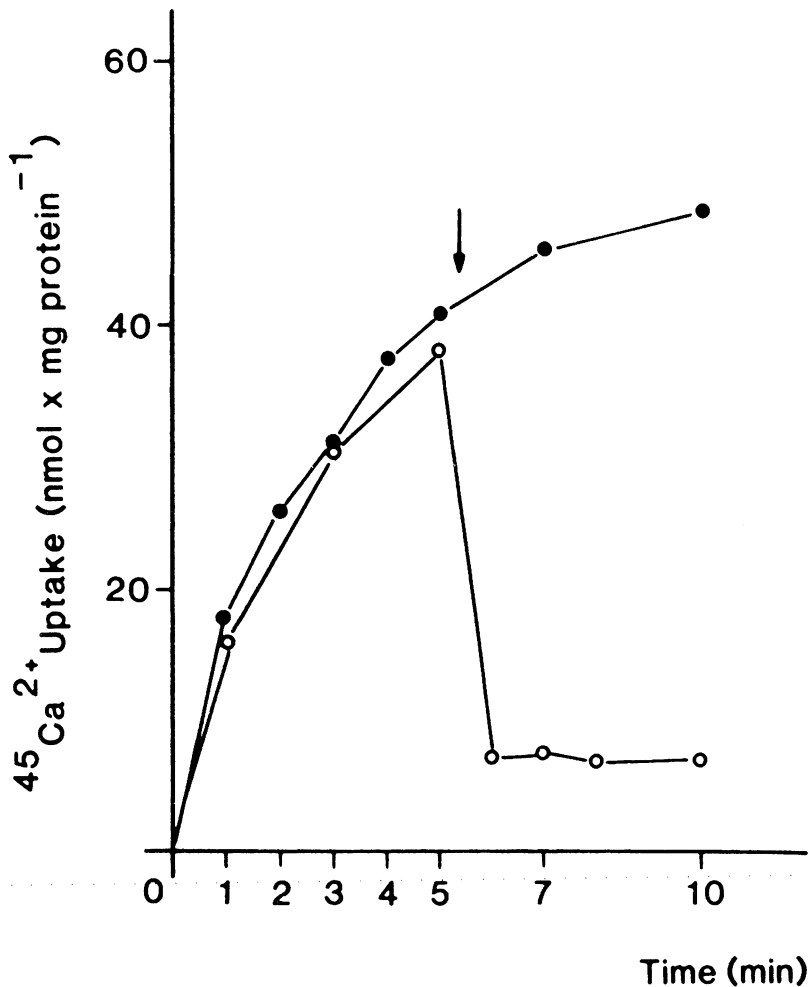


FIGURE 5. Release of Ca^{2+} by the ionophore A23187 from chromaffin secretory vesicle ghosts. Na^+ -loaded ghosts were incubated with $1.4 \mu\text{M}$ free Ca^{2+} in 160 mM sucrose, 0.5 mM EGTA, and 20 mM Mops (pH 7.3). After 5 min of $^{45}\text{Ca}^{2+}$ uptake, either A23187 (arrow) in ethanol ($8 \mu\text{g}/\text{mg}$ protein final concentration) (○) or an equivalent volume of ethanol (●) was added. (From Krieger-Brauer, H. and Gratzl, M., *J. Neurochem.*, 41, 1269, 1983. With permission.)

property of Ca^{2+} sequestering organelles to enhance both their capacity as well as their transport efficiency by binding Ca^{2+} inside these compartments.

Given an extracellular free Ca^{2+} concentration of 1 to 2 mM , a gradient of 10^4 or greater would exist across the cell membrane of the chromaffin cell. Previous determination of Ca^{2+} within chromaffin secretory vesicles suggested a similar steep Ca^{2+} gradient across the secretory vesicle membrane. The fact that Ca^{2+} within these structures exists largely in a bound form, resulting in a low concentration of free Ca^{2+} in the intravesicular space, bears important implications as to the energetics of the Ca^{2+} transport system in the chromaffin vesicle membrane. In other words, a higher stoichiometry than two Na^+ transported for one Ca^{2+} is probably not necessary. This was supported by the insensitivity of Na^+ -dependent Ca^{2+} transport against the nature of the anion,¹⁷ as well as by an evaluation of the inhibition by Na^+ of Ca^{2+} uptake in Hill plots¹³ and by direct determination of Na^+ and Ca^{2+} fluxes in chromaffin vesicle ghosts.¹⁶ Secretory vesicles from the neurohypophysis also did not transport more than two Na^+ in exchange for one Ca^{2+} .¹⁵

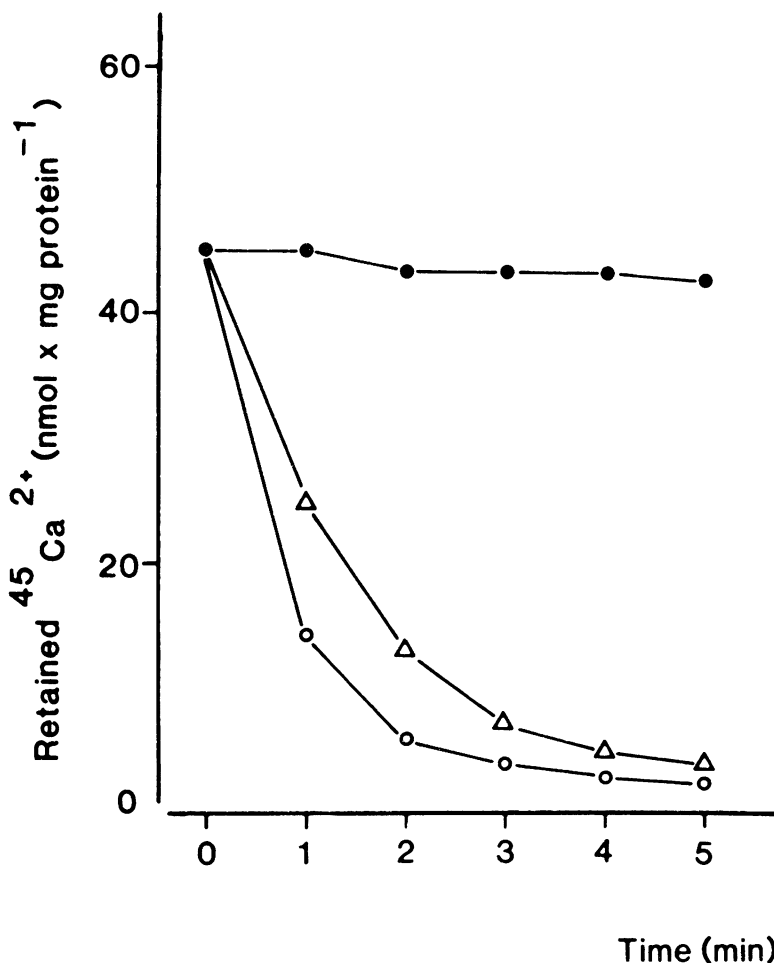


FIGURE 6. Release of Ca^{2+} by Na^+ from chromaffin secretory vesicle ghosts. $^{45}\text{Ca}^{2+}$ -loaded ghosts were incubated in 100 mM KCl (●), 100 mM NaCl (○), or in 100 mM NaCl with 1 mM free Mg^{2+} (Δ). The media contained 0.5 mM EGTA and 20 mM Mops, pH 7.3. (From Krieger-Brauer, H. and Gratzl, M., *J. Neurochem.*, 41, 1269, 1983. With permission.)

V. Ca^{2+} BINDING COMPONENTS IN CHROMAFFIN VESICLES

As discussed in the previous chapters, most of the Ca^{2+} within chromaffin vesicles is in a bound state. Then the question arises as to the nature of the binding substances.

Proteins are prominent constituents of the chromaffin vesicle matrix. About 80% of the total vesicle proteins are intravesicular and 60% are chromogranin A.²⁸ This protein has recently been found to bind Ca^{2+} .^{29,30} As shown in Figure 8, the capacity of total matrix proteins to bind Ca^{2+} is high (160 nmol Ca^{2+} can be bound per milligram of protein). The dissociation constant was around 50 μM . Taking into account that Ca^{2+} is also bound to intravesicular ATP, the vesicle matrix has an enormous binding capacity. Ca^{2+} binding to chromogranin A, as well as to ATP, is influenced by Mg^{2+} (Figure 8), pH, and ionic strength and may also be influenced by further substances present in the chromaffin vesicles which were not yet investigated as having an effect on the Ca^{2+} /protein or Ca^{2+} /ATP equilibria. For instance, it remains to be established how the intravesicular Ca^{2+} chelators discussed above cooperate in Ca^{2+} binding, a process which markedly lowers the energy requirements and enlarges the capacity for Ca^{2+} uptake.

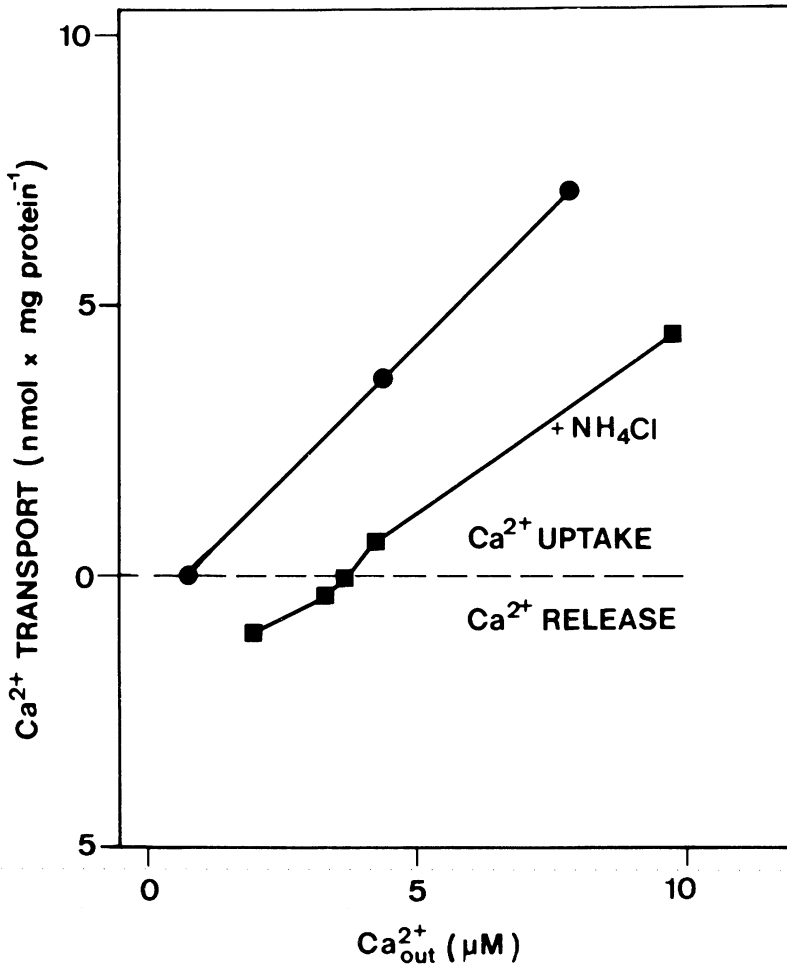


FIGURE 7. A23187-mediated uptake and release of Ca^{2+} by chromaffin vesicles. In the presence of NH_4Cl (30 mM) above 4 μM free Ca^{2+}_{out} the vesicles take up Ca^{2+} . Below 4 μM free Ca^{2+} upon addition of A23187 Ca^{2+} is even released from the vesicles. With no NH_4Cl present, the transmembrane proton gradient acts as a further driving force for Ca^{2+} uptake.²³

It is worth noting that chromogranin A exists not only in chromaffin cells, but also in other endocrine cells: it has been found in pancreatic islet cells, in the anterior pituitary, in the C cells of the thyroid gland, as in the chief cells of the parathyroid gland, as well as in the central nervous system.³¹⁻³⁵ Within the pancreatic islet it seems to coexist with insulin, glucagon, and somatostatin, not only within the same cell, but even within the same vesicle³⁶ (Figure 9).

The content of ATP in other secretory vesicles is lower than that in the chromaffin vesicle³⁷⁻³⁹ by two or three orders of magnitude. Thus, within these vesicles, Ca^{2+} binding proteins will probably be the only substance to secure low intravesicular free Ca^{2+} . This situation is very reminiscent of the role of calsequestrin in Ca^{2+} binding within the sarcoplasmic reticulum.⁴⁰

VI. CONCLUDING REMARKS

It has long been recognized that secretory vesicles contain high amounts of Ca^{2+} . For chromaffin secretory vesicles, there exists convincing evidence that the energy for the uptake

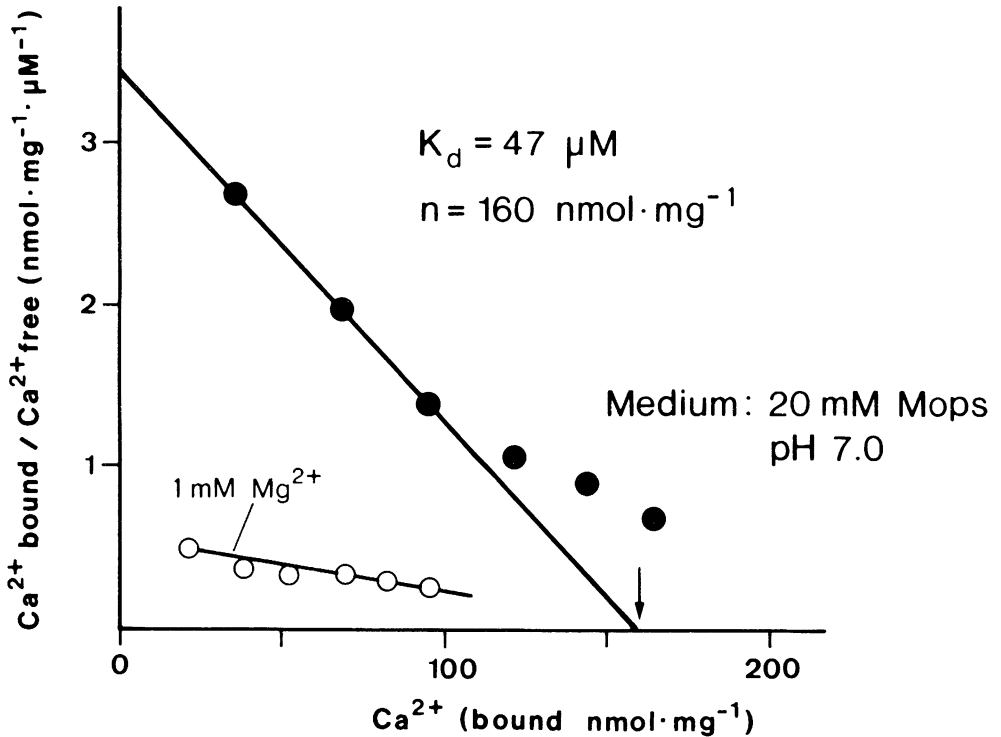


FIGURE 8. Binding of Ca^{2+} to chromaffin vesicle matrix proteins. The dissociation constant ($47 \mu\text{M}$) decreases in the presence of 1 mM Mg^{2+} , whereas the number of binding sites is unaffected.^{29,30}

of Ca^{2+} is provided by a Na^+ gradient. Once inside the vesicle, the cation is converted to a bound stage.

The $\text{Na}^+/\text{Ca}^{2+}$ exchange system present in the vesicle membrane is also capable of transporting Ca^{2+} in the reverse mode, so that Ca^{2+} can be released again via the same system. In contrast to intracellular free Ca^{2+} , up to now secretagogue-induced fluctuations of cytoplasmic Na^+ concentrations have not been described. If such transient phenomena occurred in an order of magnitude sufficient to mobilize Ca^{2+} from the secretory vesicles, one could envision sodium as a factor in the control of the exocytotic process. The mechanism of Na^+ uptake by chromaffin vesicles is not known yet. An elegant procedure for the vesicles would be to gain Na^+ from the immense extracellular reservoir concomitantly with their loss of Ca^{2+} during exocytosis. The latter process, in contrast to the situation seen with other subcellular structures, allows to by-pass the cytoplasm during release of Ca^{2+} .

It may very well turn out that chromogranin A and other intravesicular Ca^{2+} binding proteins are present in all endocrine cells, except the follicular cells of the thyroid gland and the steroid-secreting cells of the adrenals and the gonads, where hormone secretion may not occur by exocytosis or where Ca^{2+} does not appear to play a direct role in the control of secretion. Thus, intravesicular Ca^{2+} binding proteins as a functional principle may be restricted to a fraction of endocrine organs already described as "diffuse endocrine epitheliale Organe" about 50 years ago.⁴¹ For these, the intravesicular Ca^{2+} binding proteins may prove to be a valuable cytochemical marker.

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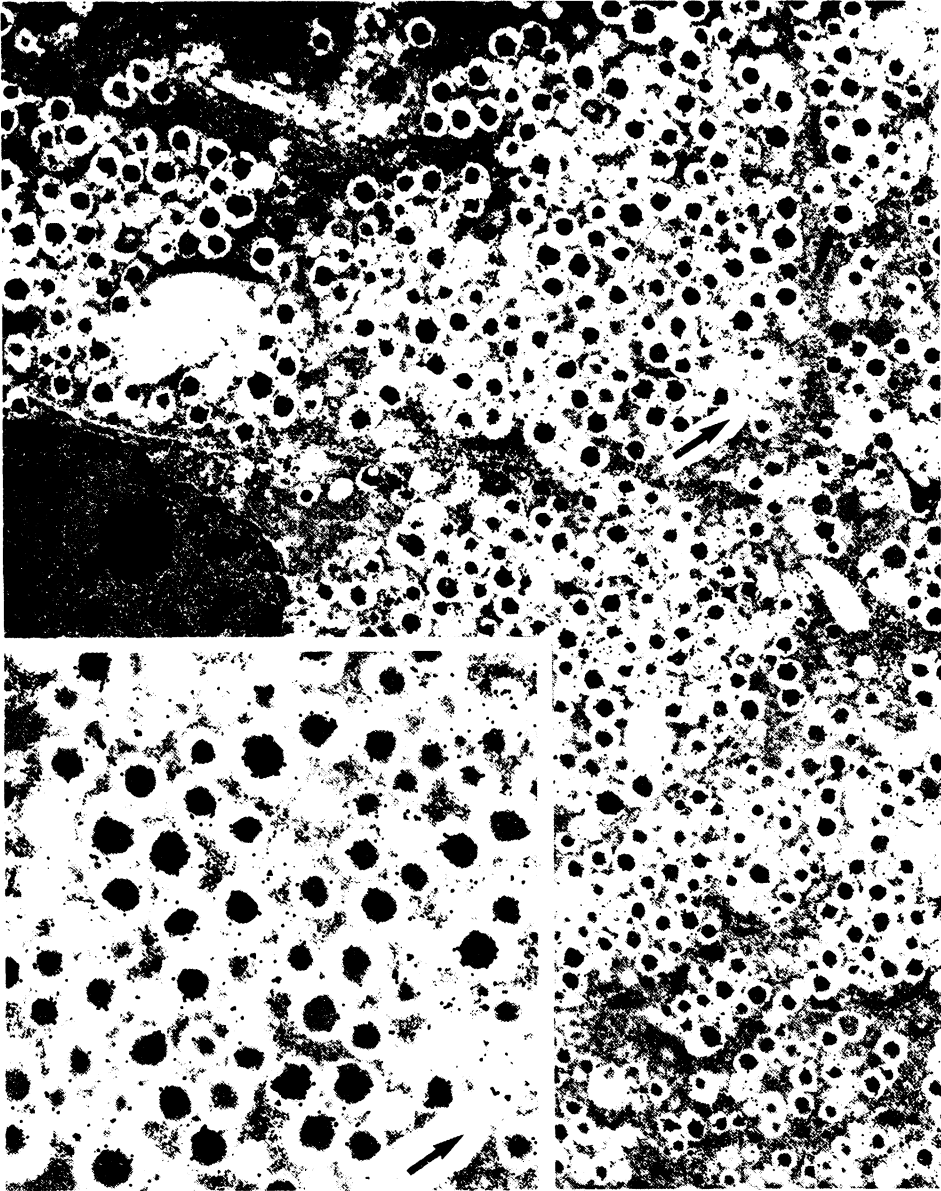


FIGURE 9. Distribution of chromogranin A immunoreactivity in pancreatic B cells. Immunostaining was carried out with the protein-A-gold technique. (Magnification $\times 10,300$; inset: $\times 16,500$.)³⁶

described in this chapter. The author's work was supported by Deutsche Forschungsgemeinschaft (Gr 681) as well as by Forschungsschwerpunkt 24 of the State of Baden-Württemberg. The skillful help of Mrs. B. Mader during preparation of this manuscript is appreciated.

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