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Matrix Free Ca^{2+} in Isolated Chromaffin Vesicles

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ABSTRACT: Isolated secretory vesicles from bovine adrenal medulla contain 80 nmol of Ca^{2+} and 25 nmol of Mg^{2+} per milligram of protein. As determined with a Ca^{2+}-selective electrode, a further accumulation of about 160 nmol of Ca^{2+}/mg of protein can be attained upon addition of the Ca^{2+} ionophore A23187. During this process protons are released from the vesicles, in exchange for Ca^{2+} ions, as indicated by the decrease of the pH in the incubation medium or the release of 9-aminoacridine previously taken up by the vesicles. Intravesicular Mg^{2+} is not released from the vesicles by A23187, as determined by atomic emission spectroscopy. In the presence of NH_4Cl, which causes the collapse of the secretory vesicle transmembrane proton gradient (ΔpH), Ca^{2+} uptake decreases. Under these conditions A23187-mediated influx of Ca^{2+} and efflux of H^+ cease at Ca^{2+} concentrations of about 4 μM. Below this concentration Ca^{2+} is even released from the vesicles. At the Ca^{2+} concentration at which no net flux of ions occurs the intravesicular matrix free Ca^{2+} equals the extravesicular free Ca^{2+}. In the absence of NH_4Cl we determined an intravesicular pH of 6.2. Under these conditions the Ca^{2+} influx ceases around 0.15 μM. From this value and the known pH across the vesicular membrane an intravesicular matrix free Ca^{2+} concentration of about 24 μM was calculated. This is within the same order of magnitude as the concentration of free Ca^{2+} in the vesicles determined in the presence of NH_4Cl. Calculation of the total Ca^{2+} present in the secretory vesicles gives an apparent intravesicular Ca^{2+} concentration of 40 mM, which is a factor of 10^4 higher than the free intravesicular concentration of Ca^{2+}. It can be concluded, therefore, that the concentration gradient of free Ca^{2+} across the secretory vesicle membrane in the intact chromaffin cells is probably small, which implies that less energy is required to accumulate and maintain Ca^{2+} within the vesicles than was previously anticipated.

The intracellular free Ca^{2+} concentration in nucleated cells is controlled by Ca^{2+} transport systems in the plasma membrane, the endoplasmic reticulum, and the mitochondria. In secretory cells the secretory vesicles also participate in the cellular Ca^{2+} metabolism. Concerning chromaffin cells, this statement is based on (i) the histochemical visualization of Ca^{2+} in subcellular structures (Ravazzola, 1976), (ii) the direct determination of Ca^{2+} in isolated secretory vesicles (Borowitz et al., 1965; Phillips et al., 1977; Krieger-Brauer & Gratzl, 1982), (iii) the observation of an increased Ca^{2+} content within these structures after stimulation of the cells (Borowitz, 1969; Serck-Hanssen & Christiansen, 1973), and (iv) the identification of carrier systems that catalyze the transport of Ca^{2+} across the vesicular membrane (Phillips, 1981; Krieger-Brauer & Gratzl, 1981, 1982, 1983).

Ca^{2+} uptake by the chromaffin and neurosecretory vesicles depends upon the presence of Na^+ within the vesicles, and it has been suggested that the energy needed to take up Ca^{2+} is provided by the Na^+ electrochemical gradient across the secretory vesicle membrane (Krieger-Brauer & Gratzl, 1982, 1983; Saermark et al., 1983a,b). High amounts of Ca^{2+} (40–100 nmol/mg of protein) have been found in isolated adrenal medullary secretory vesicles. Assuming a vesicle volume of 2 μL/mg of protein (Johnson & Scarpa, 1976), it can be calculated that the apparent intravesicular Ca^{2+} concentration may be as high as 20–50 mM. This implies that the vesicular Ca^{2+} concentration is about 10^5 times higher than the cytoplasmic free Ca^{2+} concentration in chromaffin cells (Knight & Kesteven, 1983). When comparing the magnitude of the Na^+ gradient with the magnitude of apparent Ca^{2+} gradient across the secretory vesicle membrane, one can

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calculate that Ca^{2+} uptake according to the mechanism as outlined above only occurs if the transmembrane Ca^{2+} concentration gradient is markedly lowered by intravesicular Ca^{2+} binding. In the present work, using a null-point titration technique, we present evidence that, within chromaffin vesicles, Ca^{2+} is in fact largely bound.

Experimental Procedures

Materials

The ionophore A23187 was a generous gift of Eli Lilly Co. Percoll was purchased from Pharmacia, Freiburg, and MOPS and MES were from Serva, Heidelberg. All other reagents used were of analytical grade.

Methods

Isolation of Secretory Vesicles. Bovine adrenal glands were obtained from the local slaughterhouse. Homogenization of the tissue in the laboratory was finished within 1 hour of slaughtering. Purified chromaffin secretory vesicles were prepared essentially as described previously (Gratzl et al., 1981) with the following modifications: Homogenization was carried out in isolation medium containing 20 mM MOPS[1] (pH 7.3, adjusted with KOH), 0.5 mM EGTA, and sucrose to give a final osmolality of 420 mosm/kg. For density gradient centrifugation, 3 mL of the crude chromaffin vesicle fraction obtained by differential centrifugation (Gratzl et al., 1981) was put onto 23 mL of Percoll (50%) in isolation medium and centrifuged at $56800g_{av}$ for 30 min in a fixed-angle rotor (Beckman 50.2 Ti). Purified secretory vesicles were recovered from the Percoll gradient by collecting a volume of 10 mL from the bottom of each gradient with a peristaltic pump. The absence of mitochondrial contamination in this fraction was assessed by measuring glutamate dehydrogenase as a marker enzyme (Gratzl et al., 1981). In order to remove Percoll and EGTA, the pooled vesicle fractions were washed twice with isolation medium without EGTA and centrifuged at $12000g_{av}$ for 30 min.

The pellets of highly purified chromaffin vesicles were resuspended in isolation buffer without EGTA or in a medium containing 20 mM MOPS (pH 7.3, adjusted with KOH), 50 mM sodium chloride, 35 mM potassium chloride, and sucrose to give a final osmolality of 420 mosm/kg (MSPS buffer). The final K^+ concentration, due to the pH adjustment with KOH, was 50 mM.

Measurements of the Free Ca^{2+} Concentration. Changes in free Ca^{2+} after addition of the ionophore A23187 (5 μg/mg of protein) to suspensions of chromaffin vesicles (1 mg of protein/mL) were quantified with a Ca^{2+}-selective minielectrode, operating with a neutral carrier incorporated in a poly(vinyl chloride) membrane (Simon et al., 1978). The potential difference between the Ca^{2+} electrode and a reference electrode was recorded with a Knick pH meter, connected to a chart recorder. For calibration of the electrode in the low micromolar range, we used standards of MSPS or isolation buffer containing 0.5 mM EGTA supplemented with various amounts of calcium. The actual free Ca^{2+} concentration was calculated by means of a computer program (Flodgaard & Fleron, 1974) that takes into account the known stability constants of the Ca^{2+}–EGTA complex (Sillen & Martell, 1971). In this way a linear calibration curve down to 7.5×10^{-8} M free Ca^{2+} was obtained. The Ca^{2+} electrode was not affected by NH_4Cl or A23187 at the concentrations used. The ion-selective membrane was kindly supplied by W. Simon, ETH Zürich, Switzerland. All experiments were carried out at room temperature under constant stirring.

Ion Determinations by Atomic Emission Spectroscopy. The total amount of Ca^{2+} and Mg^{2+} in chromaffin secretory vesicles was measured by atomic emission spectroscopy (AES) on a Perkin-Elmer inductively coupled plasma (ICP) spectrometer (type 5500). Chromaffin vesicles were resuspended in isolation buffer (0.6–1.2 mg of protein/mL), and the Ca^{2+} and Mg^{2+} content of the vesicles was determined in the same sample. For Ca^{2+} and Mg^{2+} flux measurements, 750-μL aliquots were taken from the Ca^{2+} electrode experiments before and after addition of the ionophore A23187. Vesicles were separated from the incubation medium by centrifugation at $12000g_{av}$ for 5 min in an Eppendorf minifuge. The pellets were washed 3 times with 1.5 mL of incubation buffer and resuspended in the same volume of water. No chemical or spectral interference could be detected upon measuring the Ca^{2+} emission at 393.36 nm and that of Mg^{2+} at 279.55 nm. Merck Titrisol standards served for calibration.

Fluorometric Determinations of ΔpH and Proton Fluxes. For measurements of the transmembrane ΔpH, a chromaffin vesicle suspension (1–3 mg of protein/mL) was diluted 10-fold with MSPS buffer, containing 2–4 μM 9-aminoacridine. Changes in fluorescence, before and after addition of the ionophore A23187 (5 μg/mg of protein, final), were determined in a Jobin Yvon 3 D spectrofluorometer (λ_{ex} = 400 nm, λ_{em} = 455 nm, 10-nm slits).

For evaluation of proton fluxes mediated by A23187, secretory vesicles were diluted in a low-buffered medium containing 0.5 mM MOPS, 200 mM sucrose, 50 mM NaCl, and 35 mM KCl. The pH was adjusted with KOH. Changes in the pH of the suspension caused by uptake or release of protons by the secretory vesicles were monitored with a pH electrode (N 60, Schott, Mainz).

Protein Determination. Protein was measured according to the method of Bradford (1976) or by the method of Lowry (1951) with bovine serum albumin as a standard. In the latter method, protein was measured after precipitation of the samples with trichloroacetic acid (10% w/v) and dissolution of the protein pellet with deoxycholate/sodium hydroxide (2%/3% w/w).

Results

Ca^{2+} Uptake by Isolated Chromaffin Vesicles Mediated by the Ionophore A23187. Chromaffin vesicles, prepared by isoosmotic self-generating Percoll gradients, contain 80 ± 15 nmol of Ca^{2+} and 25 ± 2 nmol of Mg^{2+} per milligram of total vesicle protein (mean of nine preparations ± SD), as determined by atomic emission spectroscopy. In the presence of sufficient extravesicular Ca^{2+}, addition of the ionophore A23187 to a vesicle suspension results in a further uptake of Ca^{2+}. As determined with a Ca^{2+}-selective electrode, the free Ca^{2+} concentration in the medium is rapidly lowered upon addition of A23187 (Figure 1). Ca^{2+} uptake decreases when decreasing the extravesicular Ca^{2+} concentration and ceases around 0.2 μM free Ca^{2+}.

The initial velocity of Ca^{2+} uptake in the presence of ionophore increases with the Ca^{2+} concentration. At approximately 200 μM Ca^{2+} the uptake velocity reaches a plateau.

[1] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; A23187, 5-(methylamino)-2-[[3,9,11-trimethyl-8-[1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]undec-2-yl]methyl]-4-benzoxazolecarboxylic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetate; ICP-AES, inductively coupled plasma atomic emission spectroscopy; MSPS buffer, 50 mM NaCl, 50 mM KCl, and 200 mM sucrose buffered with 20 mM MOPS, pH 7.3; 9-AA, 9-aminoacridine.

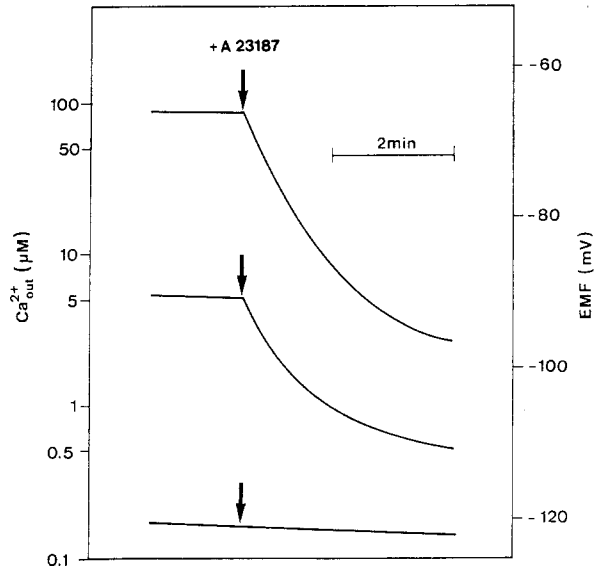

FIGURE 1: A23187-mediated Ca^{2+} uptake by chromaffin vesicles. The free Ca^{2+} concentration (the corresponding EMF's are given on the right side) was monitored by a Ca^{2+}-selective electrode in a suspension of secretory vesicles (1 mg of protein/mL) in MSPS buffer. Ca^{2+} free was titrated to the desired Ca^{2+} concentration by addition of small amounts of EGTA or $CaCl_2$. A23187 was added to give a final concentration of 5 μg/mg of vesicle protein.

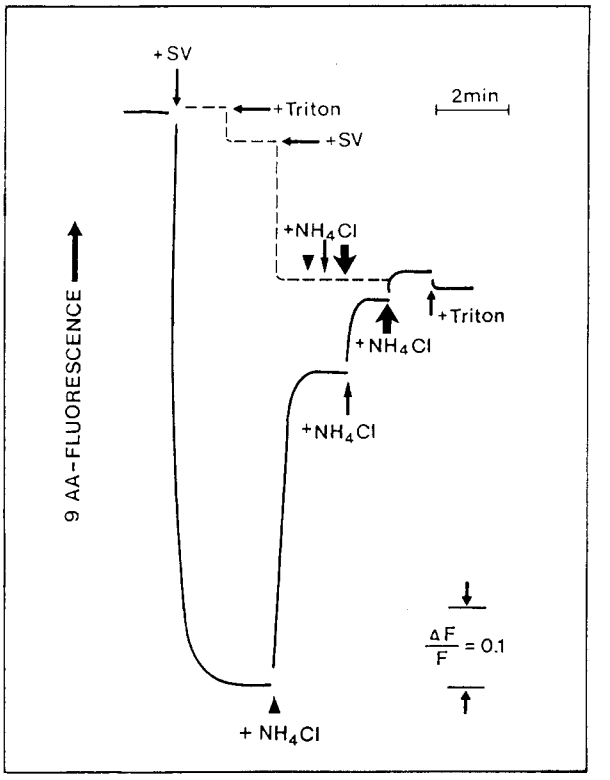

FIGURE 2: Effect of NH_4Cl on the transmembrane proton gradient. A total of 200 μL of chromaffin vesicle suspension (2.6 mg of protein/mL of MSPS buffer) was added to 1.8 mL of the same buffer containing 2 μM 9-aminoacridine. NH_4Cl (10, 20, or 30 mM final, as indicated by (▲), thin arrow, or fat arrow) was added from a stock solution of 2 M NH_4Cl in MSPS buffer. Triton X-100 was added at a final concentration of 0.1%. In control experiments, the vesicles were first lyzed by addition of Triton X-100 (--).

At this Ca^{2+} concentration the chromaffin vesicles accumulate 160 nmol of Ca^{2+}/mg of protein within 5 min (data not shown). In order to balance the Na^+ and K^+ ion gradients across the chromaffin vesicle membrane and to prevent any interference of monovalent ions transported by natural carrier systems or the ionophore A23187, the experiments described in Figure 1 were carried out in buffered sucrose medium (420 mosm/kg) containing 50 mM Na^+ and 50 mM K^+ (MSPS buffer). Omission of both salts and replacement by sucrose did not change the properties of A23187-mediated Ca^{2+} uptake by the vesicles. Thus, it can be concluded that chromaffin vesicles contain a large amount of Ca^{2+} and are able to take up rapidly twice that amount when the ionophore A23187 is added.

Chromaffin vesicles also contain considerable amounts of Mg^{2+} (see above), and since the ionophore A23187 may transport Mg^{2+} as well, although with a lower affinity (Debano et al., 1981; Grinstein et al., 1983), a Ca^{2+}/Mg^{2+} antiport catalyzed by A23187 must also be taken into consideration. However, from measurements of the Ca^{2+} and Mg^{2+} content in isolated secretory vesicles after incubation with different concentrations of Ca^{2+} (0.1–50 μM), a participation of intravesicular Mg^{2+} in the Ca^{2+} uptake process mediated by A23187 can be excluded (not shown). In these experiments intravesicular Ca^{2+} increased with the free Ca^{2+} concentrations in the medium, but the amount of intravesicular Mg^{2+} remained essentially the same; i.e., exchange of intravesicular Mg^{2+} for extravesicular Ca^{2+} does not take place.

Intravesicular pH. It is known that the ionphore A23187 catalyzes an exchange of Ca^{2+} for H^+ across biological membranes (Reed & Lardy, 1972a,b). Therefore, the Ca^{2+} uptake seen in the experiments described above might be coupled with a release of intravesicular H^+, since chromaffin vesicles are characterized by an acidic interior (Johnson & Scarpa, 1976; Pollard et al., 1976, 1979; Bashford et al., 1976). As a consequence, weak bases like 9-aminoacridine are accumulated by these vesicles, when suspended in media buffered at physiological pH values, causing a quenching of its fluorescence. When the intravesicular pH is raised by addition of increasing amounts of NH_4Cl, the intact secretory vesicles release again the accumulated 9-AA. At a concentration of approximately 30 mM NH_4Cl, the same level of fluorescence is found as that obtained when permeabilizing the vesicles with the detergent Triton X-100 (Figure 2). This indicates that 30 mM NH_4Cl (i.e., an excess of a weak base) causes a complete breakdown of the transmembrane proton gradient (ΔpH).

Thus, in further experiments the difference in the 9-AA fluoresence signals during incubation of chromaffin vesicles in media of different pH values, containing no NH_4Cl or 30 mM NH_4Cl, can be used to determine the intravesicular pH. As opposed to higher pH values, NH_4Cl did not exhibit an effect at pH 6.2 (Figure 3), indicating that the intravesicular pH of chromaffin vesicles used is 6.2.

Ionophore-Mediated Proton Fluxes. Addition of A23187 to vesicles, suspended in a medium containing 100 μM free Ca^{2+}, apparently results in a release of most of the 9-AA previously taken up (Figure 4). This indicates that the pH inside the vesicles reaches almost that of the incubation medium (7.3), a fact that can be interpreted as a transport of H^+ in exchange for Ca^{2+} due to A23187. With 10 μM free Ca^{2+} in the incubation medium, the release of 9-aminoacridine from the vesicle interior was smaller (Figure 4). After further reduction of the extravesicular free Ca^{2+} to 0.1 μM, the release of 9-AA after addition of the ionophore was negligible (Figure 4).

The proton fluxes linked to the Ca^{2+} transport can also be followed with a pH electrode. The amount of protons released decreases when the free Ca^{2+} in the medium is lowered and ceases around 0.1 μM free Ca^{2+} (Figure 5).

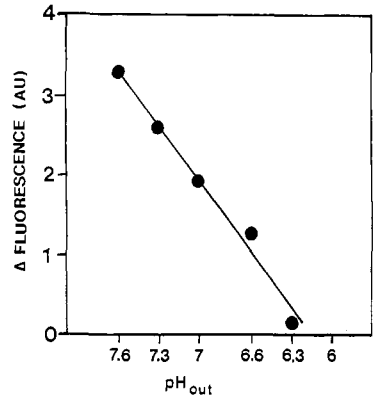

FIGURE 3: Internal pH of isolated secretory vesicles. Chromaffin vesicles were resuspended in 20 mM MOPS or MES and 360 mM sucrose with pH values between 7.6 and 6.3, adjusted with KOH; 200 μL of a vesicle suspension containing 1 mg of protein/mL was added to 2 mL of buffer, with or without NH_4Cl (30 mM). The difference in the fluorescence signals after addition of 10 μL of 9-AA (200 μM) is plottet as a function of the extravesicular pH.

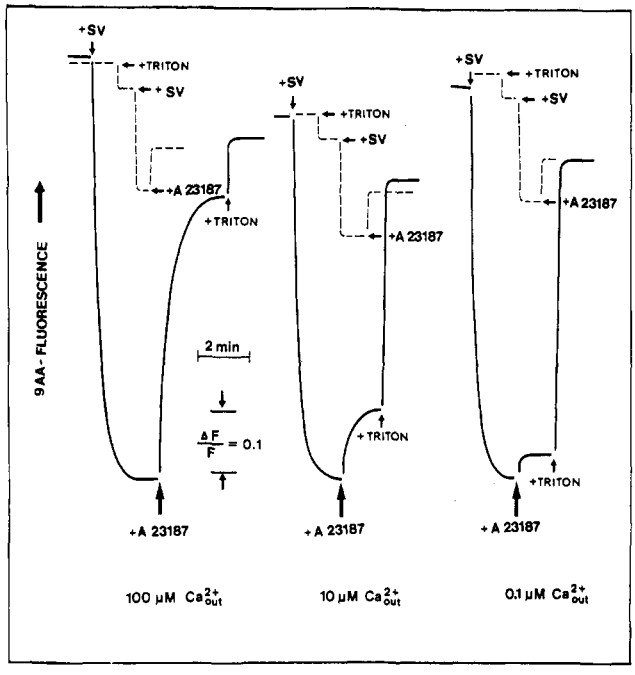

FIGURE 4: A23187-mediated proton transport. Suspensions of secretory vesicles containing 2.6 mg of protein/mL of MSPS buffer (pH 7.3) were incubated with 100, 10, or 0.1 μM free Ca^{2+} as established by the Ca^{2+} electrode. A total of 200 μL of these suspensions was added to 1.8 mL of MSPS buffer containing 2 μM 9-AA and identical free Ca^{2+} concentrations; 5 μg of A23187/mg of protein was used. As a control, the fluorescence of vesicles, lyzed with 0.1% of the detergent Triton X-100, was determined under identical conditions (--).

Effect of pH on Ca^{2+} Uptake. The experiments described so far indicate that A23187-mediated Ca^{2+} uptake is exclusively linked to H^+ fluxes. To further substantiate this fact, we decreased the transmembrane pH and determined the capacity of the vesicles to take up Ca^{2+} in the presence of 30 mM NH_4Cl, i.e., the amount of NH_4Cl sufficient to collapse the ΔpH across the vesicle membrane (Figure 2). Under these conditions the ionophore-mediated Ca^{2+} uptake amounts to 80 nmol/mg of protein, whereas in absence of NH_4Cl 160 nmol/mg of protein is taken up by the vesicles.

The effect of NH_4Cl at low extravesicular free Ca^{2+} concentrations on the Ca^{2+} uptake is shown in Figure 6. In the presence of NH_4Cl, the Ca^{2+} uptake ceases at a free extravesicular Ca^{2+} concentration of 4 μM. This is in marked

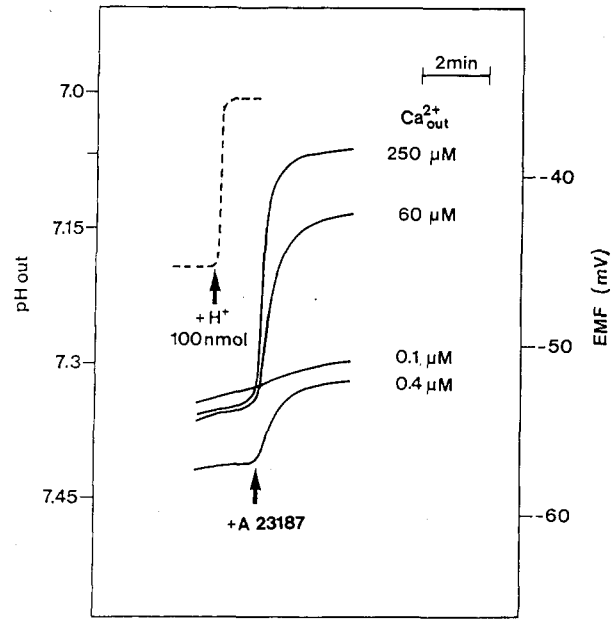

FIGURE 5: A23187-mediated H^+ release from chromaffin vesicles. Secretory vesicles were resuspended in lightly buffered medium containing 0.5 mM MOPS and sucrose to give a final osmolarity of 420 mosm/kg. Ca^{2+} free was adjusted by addition of $CaCl_2$ or EGTA; 5 μg of A23187/mg of vesicle protein was used. HCl was added for calibration (--).

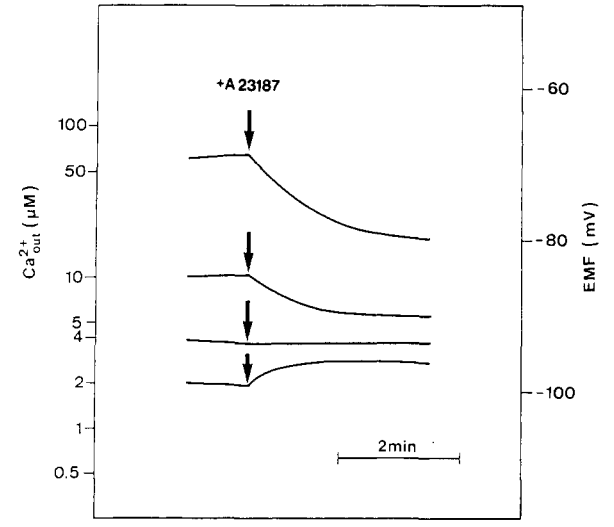

FIGURE 6: A23187-mediated Ca^{2+} transport in the presence of NH_4Cl. Chromaffin vesicles were incubated as described in the legend to Figure 1; 5 μg of A23187/mg of vesicle protein was used. In addition, 30 mM NH_4Cl was present in the incubation medium.

contrast to the Ca^{2+} uptake in the absence of NH_4Cl, which ceases only at 0.2 μM (cf. Figure 1). Furthermore, in the presence of NH_4Cl, A23187 even elicited an release of Ca^{2+} from the vesicles, when the external Ca^{2+} concentration was lower than 4 μM.

When ionophore-mediated Ca^{2+} transport is plotted as a function of the low extravesicular free Ca^{2+} concentration in the presence and absence of NH_4Cl, the Ca^{2+} concentrations at which a net flux of neither Ca^{2+} nor H^+ occurs, the null point, can be determined (Figure 6, compare Figures 5 and 1). The null point shifted from 0.15 ± 0.07 μM (mean of 10 preparations ± SD) to 3.72 ± 1.62 μM (16 preparations ± SD) when the proton gradient across the vesicle membrane was collapsed by 30 mM NH_4Cl.

In further experiments the effect of the transmembrane ΔpH on the null point was examined. Addition of increasing

amounts of NH_4Cl (5, 10, or 30 mM final) to a suspension of chromaffin secretory vesicles causes a decrease in the ΔpH, which concomitantly results in a shift of the null point to higher values (0.6, 1.6, or 4.8 μM), indicating that less Ca^{2+} can be accumulated at low free Ca^{2+} concentrations when the ΔpH was lowered. Also, decreasing the transmembrane ΔpH by lowering the extravesicular pH to 6.3 resulted in a shift of the null point for Ca^{2+} to 3.6 μM. In contrast to the marked effect of the transmembrane proton gradient on the null point, we found no significant shift of the null point when 50 μM Mg^{2+} was present. The extensive analysis of ion fluxes mediated by the ionophore A23187 has revealed that under appropriate conditions only Ca^{2+} and H^+ are transported across the membrane of chromaffin vesicles. In the absence of a proton gradient across the vesicular membrane, the extravesicular Ca^{2+} concentration at which no net flux of Ca^{2+} occurs directly gives the intravesicular free Ca^{2+} concentration at the pH of the medium used (see above). In the presence of the natural proton gradient, the intravesicular free Ca^{2+} in the acidic interior of chromaffin vesicles can be calculated from the analysis of the Ca^{2+} and H^+ fluxes and the known magnitude of the transmembrane proton gradient.

Discussion

It is generally accepted that Ca^{2+} plays a regulatory role in secretion by exocytosis. Obviously, one of the structures involved in the control of intracellular free Ca^{2+} concentrations in stimulated and resting cells is the secretory vesicle (Phillips, 1981; Krieger-Brauer & Gratzl, 1981–1983; Saermark et al., 1983a,b). A total of 25000 chromaffin vesicles, which occupy about 10% of the cell volume, participates in this process (Nordmann, 1984). Compared to other intracellular compartments involved in Ca^{2+} metabolism like mitochondria and endoplasmic reticulum, the secretory vesicles bear the advantage that Ca^{2+} taken up can be transferred directly into the extracellular space during exocytosis. In fact, secretion of Ca^{2+} upon nicotinic stimulation has been recently described (Izumi et al., 1979; Wada et al., 1984).

The large amount of 80 nmol of Ca^{2+} we found to be present in the chromaffin vesicles, which is in the same order of magnitude described previously (Borowitz et al., 1965; Phillips et al., 1977; Krieger-Brauer & Gratzl, 1982), would result in a steep Ca^{2+} gradient across the vesicle membrane, considering the low free Ca^{2+} concentration found in chromaffin cells (Knight & Kesteven, 1983). Thus, uphill transport of Ca^{2+} would be expensive for the cell in terms of energy. That at least part of the Ca^{2+} within the chromaffin vesicles is bound can be deduced from earlier experiments with intact vesicles, which take up Ca^{2+} (Johnson & Scarpa, 1976; Krieger-Brauer & Gratzl, 1982, 1983) and secretory vesicle ghosts, devoid of intravesicular substances, which release Ca^{2+} upon addition of the ionophore A23187 (Krieger Brauer & Gratzl, 1983).

The ionophore A23187 catalyzes electroneutral transport of Ca^{2+} and H^+ (Reed & Lardy, 1972a,b; Pfeiffer et al., 1974, 1976; Debano et al., 1981). The experiments presented in this study clearly show that Ca^{2+} fluxes across the membranes of intact chromaffin vesicles are coupled with H^+ fluxes (Figures 4 and 5). Intravesicular Mg^{2+} or Mg^{2+} added to the medium does not modify the Ca^{2+} fluxes. Ca^{2+} flux into intact chromaffin vesicles at high concentrations of this ion (>4 μM) obviously proceeds along its concentration gradient, and the excess of H^+ present in the acidic vesicle interior provides a further driving force. If the ΔpH is abolished (by addition of NH_4Cl), Ca^{2+} uptake still occurs because $Ca^{2+}_{out} > Ca^{2+}_{in}$. Under these conditions Ca^{2+} uptake ceases at approximately 4 μM, indicating that $Ca^{2+}_{out} = Ca^{2+}_{in}$; i.e., both the H^+ and the Ca^{2+} gradients are balanced. When applying lower free Ca^{2+} concentrations, Ca^{2+} is even released from the vesicles because $Ca^{2+}_{out} < Ca^{2+}_{in}$. Concomitantly, the vesicles take up H^+.

With no NH_4Cl present, i.e., when there exists a proton gradient across the chromaffin vesicle membrane, more Ca^{2+} in exchange for H^+ is transported into the vesicles by the ionophore. In this case Ca^{2+} influx ceases at approximately 0.15 μM free Ca^{2+}. At the null point the concentration of free Ca^{2+} inside the vesicles can even be calculated from the ratio of the H^+ concentrations, anticipating that the ionophore catalyzes an electroneutral exchange of Ca^{2+} for protons across the chromaffin vesicle membrane. Thus, with the determined intravesicular pH of 6.2, which is similar to pH values determined previously by other groups (5.5–6.25) with radiochemical and fluorometrical procedures (Johnson & Scarpa, 1976; Pollard et al., 1976, 1979; Bashford et al., 1976), the matrix free Ca^{2+} within the vesicles can be calculated, which gives a value of 23.8 ± 11 μM. This value is somewhat higher than that obtained by quenching the transmembrane ΔpH with NH_4Cl or decreasing the extravesicular pH. In this way a matrix-free Ca^{2+} concentration of 3.7 and 3.6 μM was determined, respectively (see results). Thus, it can be concluded that in chromaffin vesicles exhibiting either a transmembrane proton gradient or a balanced H^+ gradient the matrix-free Ca^{2+} is essentially the same.

Similar low free Ca^{2+} concentrations have also been found in mitochondria (Coll et al., 1982; Hansford & Castro, 1982; Joseph et al., 1983; Reinhardt et al., 1984) and α-granules isolated from platelets (Grinstein et al., 1983). Thus, internal binding of Ca^{2+} seems to be involved in the transport of this ion in several subcellular compartments. Another example may be the sarcoplasmic reticulum containing the Ca^{2+} binding protein calsequestrin (MacLennan & Wang, 1971). Ca^{2+} within the chromaffin vesicle may be bound to low or high molecular weight compounds. Particulary interesting in this respect would be the involvement of chromogranins, which comprise about 50% of the vesicle protein content and which have recently been shown to bind Ca^{2+} (F. U. Reiffen and M. Gratzl, unpublished data).

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Registry No. Ca, 7440-70-2; Mg, 7439-95-4; H^+, 12408-02-5; NH_4Cl, 12125-02-9.

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Modulation in Proportions of H1 Histone Subfractions by Differential Changes in Synthesis and Turnover during Butyrate Treatment of Neuroblastoma Cells†

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ABSTRACT: Mouse neuroblastoma cells treated with millimolar concentrations of butyrate adjusted their recipe of histone H1 subfractions over the course of several days, eventually attaining an enrichment of about 5-fold in $H1^0$. This adjustment in the proportions of the H1's, which was essentially complete by 4 days, was brought about by changes in synthesis and turnover that were different for each of the three H1 subfractions. As the cells stopped dividing, the synthesis of all histones slowed substantially, but core histones were affected more than the H1's. Transiently, therefore, there was an overproduction of H1's relative to core histones, but the excess H1 was eventually removed by turnover. The very slow turnover of $H1^0$ and the rapid turnover of H1c were not substantially affected by butyrate treatment, but the turnover of H1ab was greatly accelerated by butyrate. Acetylation of the core histones was not necessary for maintenance of elevated $H1^0$ levels in the nondividing cells, although we did not rule out its involvement in the initial accumulation of $H1^0$.

The relative proportions of histone H1 subtypes vary according to the state of differentiation of the tissue from which the histones are derived [for review, see Cole (1984)]. In earlier work aimed at understanding how the recipe for H1 subtypes is regulated, we showed that the H1 subtypes differed among themselves in the responsiveness of their synthesis to hormones (Stellwagen & Cole, 1969; Hohmann & Cole, 1971), and there were analogous studies on shifting patterns of synthesis of H1 variants in developing sea urchins (Ruderman et al., 1974). The former experiments, by relating H1 synthesis to core histone synthesis, indicated that H1 synthesis was not strictly coordinated with DNA replication [see also Gurley & Hardin (1969)], and the latter study, by including measurements of mRNA (Ruderman et al., 1974), showed that at least some regulation of the recipe of H1 subtypes occurs pretranslationally. More recently (Pehrson & Cole,

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