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Effects of Monovalent and Divalent Cations on Ca^{2+} Fluxes Across Chromaffin Secretory Membrane Vesicles

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Abstract: Bovine chromaffin secretory vesicle ghosts loaded with Na^+ were found to take up Ca^{2+} when incubated in K^+ media or in sucrose media containing micromolar concentrations of free Ca^{2+}. Li^+- or choline$^+$-loaded ghosts did not take up Ca^{2+}. The Ca^{2+} accumulated by Na^+-loaded ghosts could be released by the Ca^{2+} ionophore A23187, but not by EGTA. Ca^{2+} uptake was inhibited by external Sr^{2+}, Na^+, Li^+, or choline$^+$. All the $^{45}Ca^{2+}$ accumulated by Na^+-dependent Ca^{2+} uptake could be released by external Na^+, indicating that both Ca^{2+} influx and efflux occur in a Na^+-dependent manner. Na^+-dependent Ca^{2+} uptake and release were only slightly inhibited by Mg^{2+}. In the presence of the Na^+ ionophore Monensin the Ca^{2+} uptake by Na^+-loaded ghosts was reduced. Ca^{2+} sequestered by the Na^+-dependent mechanism could also be released by external Ca^{2+} or Sr^{2+} but not by Mg^{2+}, indicating the presence of a Ca^{2+}/Ca^{2+} exchange activity in secretory membrane vesicles. This Ca^{2+}/Ca^{2+} exchange system is inhibited by Mg^{2+}, but not by Sr^{2+}. The Na^+-dependent Ca^{2+} uptake system in the presence of Mg^{2+} is a saturable process with an apparent K_m of 0.28 μM and a V_{max} = 14.5 nmol · min^{-1} · mg protein^{-1}. Ruthenium red inhibited neither the Na^+/Ca^{2+} nor the Ca^{2+}/Ca^{2+} exchange, even at high concentrations. **Key Words:** Secretory vesicle—Na^+/Ca^{2+} exchange—Ca^{2+}/Ca^{2+} exchange—Bovine adrenal medulla. **Krieger-Brauer H. I. and Gratzl M.** Effects of monovalent and divalent cations on Ca^{2+} fluxes across chromaffin secretory membrane vesicles. *J. Neurochem.* **41**, 1269–1276 (1983).

Intact secretory vesicles isolated from bovine adrenal medulla are able to take up Ca^{2+} against an obvious concentration gradient in the presence of K^+ but not in the presence of Na^+ (Krieger-Brauer and Gratzl, 1981). Further studies with intact secretory vesicles have shown that the Ca^{2+} transport system operates in either direction depending on the Na^+ gradient. The data are in accordance with an exchange system of 1 Ca^{2+} to 2 Na^+ (Krieger-Brauer and Gratzl, 1982). Investigations with chromaffin secretory vesicle ghosts provided evidence that in addition to the Na^+/Ca^{2+} exchange, a Ca^{2+}/Ca^{2+} exchange system exists within its membrane (Phillips, 1981). Since Na^+-dependent Ca^{2+} uptake could also be demonstrated in the membrane of secretory vesicles from neurohypophysis (Saermark et al., 1982), such transport systems seem to be of general importance for the Ca^{2+} metabolism of secretory cells.

Intact chromaffin secretory vesicles contain large amounts of Na^+ and Ca^{2+} (Krieger-Brauer and Gratzl, 1982). However, it is not known to what extent Na^+ or Ca^{2+} are actually free in the vesicular content and thus participate in the ion transport. For this reason we loaded chromaffin secretory vesicles with selected monovalent cations during ghost formation and studied the effects of different gradients of monovalent cations on Ca^{2+} uptake. Experiments concerning the effects of different divalent cations on Ca^{2+} uptake allowed selective measurement of Na^+/Ca^{2+} exchange and Ca^{2+}/Ca^{2+} exchange activities.

MATERIALS AND METHODS

Ghost preparation

Highly purified chromaffin secretory vesicles were prepared as described previously (Gratzl et al., 1981). To remove vesicle contents and to load the secretory vesicle preparation with different monovalent cations, isolated

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Abbreviations used: HEPES, *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; MOPS, 3-(*N*-Morpholino) propanesulphonic acid.

secretory vesicles were lysed at 0°C by addition of a 30-fold excess of hypotonic media (~120 mosmol/kg) containing 50 m*M* NaCl, KCl, choline chloride, or LiCl, respectively. The media were buffered with 20 m*M* 3-(*N*-morpholino)propanesulphonic acid (MOPS), pH 7.0, containing 0.5 m*M* EGTA where indicated. For the experiment carried out with Monensin, the NaCl medium was buffered with 20 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), pH 8.6. The secretory vesicle ghosts were harvested by centrifugation at 100,000 *g* for 30 min at 4°C. The pellet was resuspended in 30 ml of 100 m*M* NaCl, KCl, choline chloride, or LiCl medium and recentrifuged (twice). The washing media were supplemented with 0.5 m*M* EGTA and 20 m*M* MOPS, pH 7.3.

Measurement of $^{45}Ca^{2+}$ uptake

Unless otherwise stated, secretory vesicle ghosts (0.5 mg protein/ml) loaded with monovalent cations as described above were incubated with 1.4×10^{-6} *M* $^{45}Ca^{2+}$ (specific activity, 10 mCi/mmol Ca^{2+}) in uptake medium containing 100 m*M* KCl (or 160 m*M* sucrose), 0.5 m*M* EGTA, and 20 m*M* MOPS, pH 7.3 (adjusted with KOH). Where indicated, KCl (or sucrose) was replaced by 100 m*M* NaCl, choline chloride, or LiCl (pH 7.3, adjusted with NaOH or LiOH). In certain experiments the uptake medium contained in addition $MgCl_2$ or $SrCl_2$. The concentrations of Ca^{2+}, Mg^{2+}, and Sr^{2+} are given as their free concentrations. The total amounts of Ca^{2+}, Mg^{2+}, and Sr^{2+} to yield the concentrations desired in the presence of EGTA, at the ionic strength and pH used, were calculated by means of a computer program, as described by Flodgaard and Torp-Pedersen (1978), using the stability constants quoted by Sillen and Martell (1971). In previous experiments, the actual final concentration of Ruthenium red present in the commercial sample was determined by the method described by Reed and Bygrave (1974). After incubation for various time intervals at 33°C, samples (0.1 ml) were removed rapidly, filtered through HAWP filters (Millipore, pore size, 0.45 μm), and washed twice with 4 ml ice-cold buffer containing 160 m*M* sucrose, 1 m*M* EGTA and 20 m*M* MOPS, pH 7.3 (adjusted with KOH). The filters were dried and the radioactivity retained was determined by liquid scintillation counting.

Measurement of $^{45}Ca^{2+}$ release

Na^+-loaded ghosts were incubated with 1.4×10^{-6} *M* $^{45}Ca^{2+}$ (specific activity, 10 mCi/mmol Ca^{2+}) in the sucrose uptake medium described above. After 10 min of incubation at 33°C, the radioactive medium was diluted by addition of 30-fold excess of uptake medium containing no $^{45}Ca^{2+}$ and no Ca^{2+} and washed with the same buffer. This procedure did not cause any loss of accumulated $^{45}Ca^{2+}$. Subsequently, the $^{45}Ca^{2+}$-loaded ghosts (0.2–0.5 mg protein/ml) were incubated at 33°C in media containing 100 m*M* KCl, 100 m*M* NaCl, or 160 m*M* sucrose as well as 20 m*M* MOPS (pH 7.3) and 0.5 m*M* EGTA. Where indicated, various amounts of Sr^{2+}, Mg^{2+}, and Ca^{2+} (unlabeled) were added. After incubation, $^{45}Ca^{2+}$ release was measured by determination of the $^{45}Ca^{2+}$ retained within ghosts. Filtration of 0.1-ml samples and washing of the filters as well as determination of $^{45}Ca^{2+}$ content were carried out as described for uptake.

Protein determination

Protein was determined (Lowry et al., 1951) by precipitating the samples with trichloroacetic acid (10% wt/vol) and dissolving the protein pellet with desoxycholate/sodium hydroxide (2%/3%, wt/wt).

Materials

Substances were purchased from the following manufacturers: Ruthenium red from Merck; ionophore A23187 and Monensin from Calbiochem; $^{45}Ca^{2+}$ (20–34.1 mCi/mg) from New England Nuclear. All other chemicals were of the purest grade commercially available.

RESULTS

Na^+-dependent Ca^{2+} uptake

To characterize further the Na^+-dependent Ca^{2+} transport system present in chromaffin secretory vesicles, we prepared chromaffin secretory vesicle ghosts containing different concentrations of monovalent cations. When the ghosts were loaded with Na^+ (see Materials and Methods) and resuspended in uptake medium containing 100 m*M* K^+ and 1.4×10^{-6} *M* Ca^{2+}, a rapid $^{45}Ca^{2+}$ uptake during the first 5 min of incubation was observed (Fig. 1).

In contrast, when Na^+ was replaced by K^+, Li^+, or choline$^+$ during the loading procedure, Ca^{2+} uptake was substantially reduced when ghosts were incubated in uptake medium containing 100 m*M* K^+ (Fig. 1). To load the ghosts with Li^+ or choline$^+$, 0.5 m*M* EGTA was present to avoid a Ca^{2+}/Ca^{2+} exchange caused by the Ca^{2+} content of commercial LiCl and choline chloride used. The results show that a Na^+ gradient (in > out) is required for

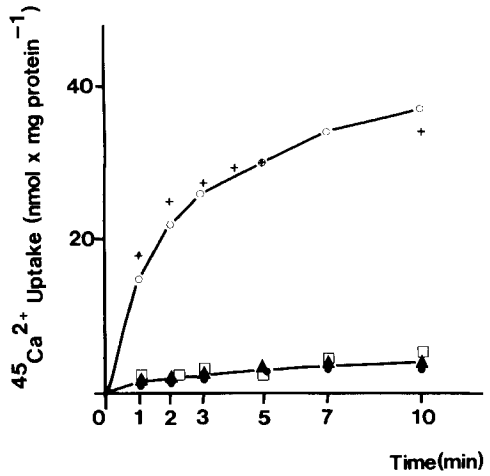

FIG. 1. Na^+-dependent $^{45}Ca^{2+}$ uptake by chromaffin secretory vesicle ghosts. NaCl-loaded ghosts (○), sodium isethionate-loaded ghosts (+), KCl-loaded ghosts (□), LiCl-loaded ghosts (●), and choline chloride-loaded ghosts (▲) were incubated with 1.4×10^{-6} *M* free $^{45}Ca^{2+}$ in 100 m*M* KCl. The incubation media were supplemented with 0.5 m*M* EGTA and 20 m*M* MOPS (pH 7.3). The transport assays were performed as described in Materials and Methods.

Ca^{2+} uptake and Li^+ or choline$^+$ could not replace Na^+ to drive Ca^{2+} uptake over chromaffin secretory membrane vesicles.

In further control experiments we investigated the influence of the anion on the Na^+-dependent Ca^{2+} transport. For this purpose we used the more impermeable anion isethionate rather than chloride for loading of the ghosts. As shown in Fig. 1, the nature of anion does not change the capacity of the secretory membrane vesicles to take up Ca^{2+}. To investigate the influence of the external ions on the Na^+-dependent Ca^{2+} uptake, the Na^+-loaded ghosts were incubated in $^{45}Ca^{2+}$ uptake media containing different amounts of monovalent cations. As shown in Fig. 2, the initial velocity of Na^+-dependent Ca^{2+} uptake is reduced in the presence of external choline$^+$ or Li^+, and uptake is almost completely abolished in the presence of 100 m*M* Na^+. In this context it is interesting to note that Ca^{2+} uptake occurred only as a consequence of a Na^+ gradient (and not of Li^+ or choline$^+$ gradients). However, an inhibition of Ca^{2+} uptake could be observed with external Na^+ as well as external Li^+ or choline$^+$ but not with K^+. Decreasing the K^+ concentration in the uptake medium by partial replacement with sucrose did not affect Ca^{2+} uptake (see, for example, Fig. 5). From these results we concluded that K^+ does not influence the Na^+-dependent Ca^{2+} uptake. For this reason usually KCl media or sucrose media were used for Ca^{2+} uptake experiments.

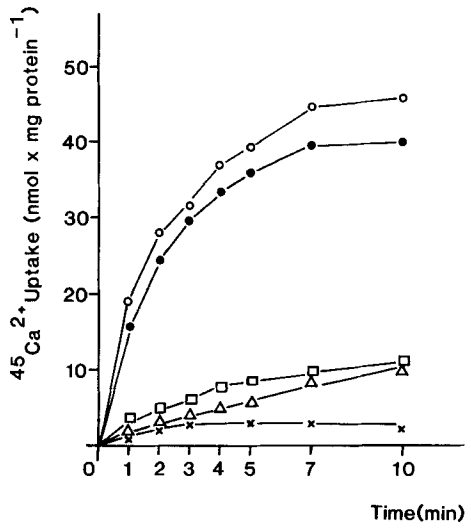

FIG. 2. Na^+-dependent $^{45}Ca^{2+}$ uptake by Na^+-loaded chromaffin secretory vesicle ghosts. Na^+-loaded ghosts were incubated with 1.4×10^{-6} *M* free $^{45}Ca^{2+}$ in media containing 100 m*M* K^+ (○), 100 m*M* K^+ and 12 m*M* Na^+ (●), 100 m*M* Li^+ (△), 100 m*M* choline$^+$ and 12 m*M* Na^+ (□), or 100 m*M* Na^+ (x). The media were supplemented with 0.5 m*M* EGTA and 20 m*M* MOPS (pH 7.3).

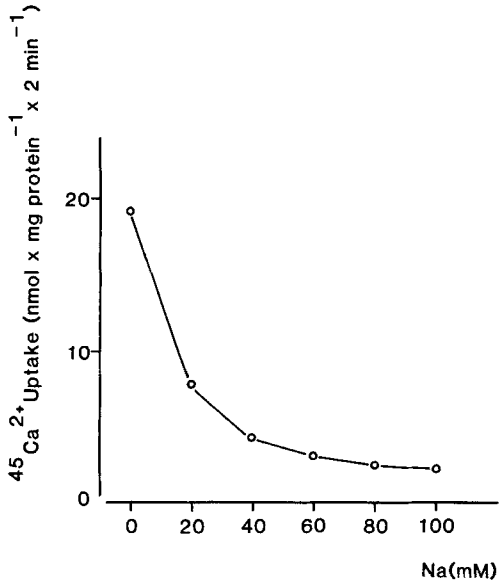

FIG. 3. $^{45}Ca^{2+}$ uptake by chromaffin secretory vesicle ghosts in the presence of different Na^+ concentrations in the incubation media. Na^+-loaded ghosts were incubated with 1.4 $\times 10^{-6}$ *M* free $^{45}Ca^{2+}$ in media containing different concentrations of Na^+. The media were supplemented with 20 m*M* MOPS, pH 7.3; 0.5 m*M* EGTA; 1 m*M* free Mg^{2+}; and sucrose to give final osmolarity of 220 mosmol/kg. $^{45}Ca^{2+}$ uptake was terminated after 2 min of incubation.

Ca^{2+} influx in the presence of external Na^+ and Monensin

To investigate further the dependence of Ca^{2+} uptake on external Na^+, Na^+-loaded ghosts were incubated in media containing increasing concentrations of Na^+. As shown in Fig. 3 the initial velocity of $^{45}Ca^{2+}$ uptake is inversely related to the external Na^+ concentration. In the presence of 50 m*M* Na^+ (the concentration of Na^+ used during loading), the Na^+ gradient is balanced and the Ca^{2+} uptake is almost completely inhibited. A similar effect on the Ca^{2+} uptake could also be demonstrated with the Na^+ ionophore Monensin (Fig. 4). For these experiments we lysed the chromaffin secretory vesicles in 50 m*M* NaCl and 20 m*M* HEPES, pH 8.6, and incubated the resealed ghosts in the sucrose uptake medium, pH 7.3, in the presence of Monensin (5 μg/mg protein). Under these conditions the ionophore Monensin is able to exchange the intravesicular Na^+ for external H^+ (Sandeaux et al., 1982). Consequently the Na^+-driven Ca^{2+} uptake system of chromaffin secretory membrane vesicles ceases.

Effect of A23187 on Ca^{2+} transport

The addition of A23187 (8 μg/mg protein) after 5 min of incubation with $^{45}Ca^{2+}$ caused the release of 82% of the $^{45}Ca^{2+}$ accumulated by ghosts (Fig. 5). Thus the chromaffin secretory vesicle ghosts can concentrate Ca^{2+} internally, and this Ca^{2+} is re-

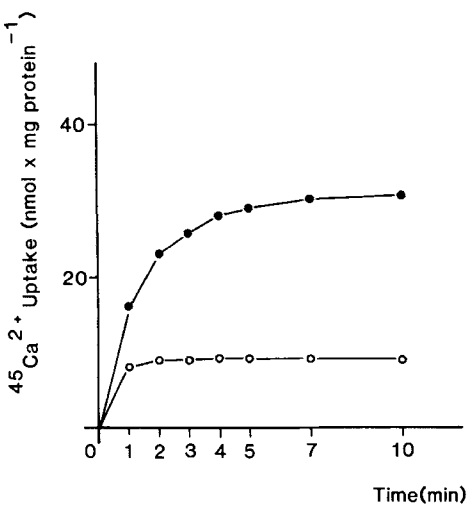

FIG. 4. $^{45}Ca^{2+}$ uptake by chromaffin secretory vesicle ghosts in the presence of the Na^+ ionophore Monensin. Ghosts loaded with 50 m*M* NaCl and 20 m*M* HEPES, pH 8.6, were incubated in 160 m*M* sucrose, 0.5 m*M* EGTA, and 20 m*M* MOPS, pH 7.3, containing 1.4×10^{-6} *M* free $^{45}Ca^{2+}$ and 2 μl ethanol (●) or 2 μl Monensin dissolved in ethanol (○). (The final concentration of Monensin was 5 μg/mg protein.)

leased in response to a selective increase of the Ca^{2+} permeability. Further, the release of Ca^{2+} by A23187 from ghosts also demonstrated that the Na^+/Ca^{2+} exchange system is able to transport Ca^{2+} against a Ca^{2+} concentration gradient, as the ionophore equilibrates the intravesicular Ca^{2+} with the external Ca^{2+}.

Na^+-induced Ca^{2+} efflux

To study the Na^+-dependent Ca^{2+} release from the chromaffin secretory vesicle ghosts, the ghosts were first allowed to take up $^{45}Ca^{2+}$ in a Na^+-dependent manner. After removal of extravesicular $^{45}Ca^{2+}$ (see Materials and Methods), Na^+ was added in high concentrations (100 m*M*) to reverse the Na^+ gradient across the vesicular membrane. As shown in Fig. 6, $^{45}Ca^{2+}$ was almost completely released (>95%) from the ghosts by the addition of Na^+ with or without Mg^{2+}. In the presence of Mg^{2+} the Na^+-dependent release was slower, but after 5 min almost all the accumulated $^{45}Ca^{2+}$ (>95%) was released. In contrast, in the control experiment no Ca^{2+} release could be measured in the presence of 100 m*M* KCl and 0.5 m*M* EGTA, indicating that neither EGTA nor K^+ is able to induce the release of intravesicular Ca^{2+}. The results are in agreement with those obtained with intact secretory vesicles (Krieger-Brauer and Gratzl, 1982) indicating that in secretory vesicle membranes there exists a Na^+/Ca^{2+} exchange system, which is able to catalyse Ca^{2+} fluxes in both directions. As seen in Fig. 6 the Ca^{2+} permeability of the membrane was not influenced by EGTA or K^+. A parallel finding has been described with intact secretory vesicles (Krieger-Brauer and Gratzl, 1982).

Influence of divalent cations on Ca^{2+} transport

The Na^+-dependent Ca^{2+} uptake by secretory vesicle ghosts was diminished by ~20% only in the

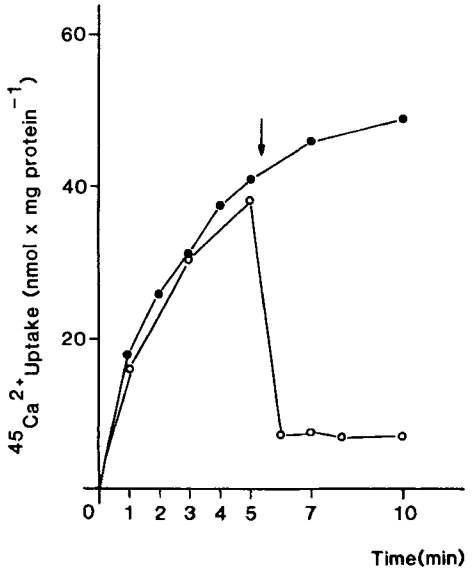

FIG. 5. Release of Na^+-dependent accumulated $^{45}Ca^{2+}$ by the ionophore A23187. Na^+-loaded chromaffin secretory vesicle ghosts were incubated with 1.4×10^{-6} *M* free $^{45}Ca^{2+}$ in 160 m*M* sucrose, 0.5 m*M* EGTA, and 20 m*M* MOPS (pH 7.3). After 5 min of $^{45}Ca^{2+}$ uptake (arrow), either A23187 in ethanol (8 μg/mg protein final concentration) (○) or an equivalent volume of ethanol (●) was added. $^{45}Ca^{2+}$ uptake was determined as described in Materials and Methods.

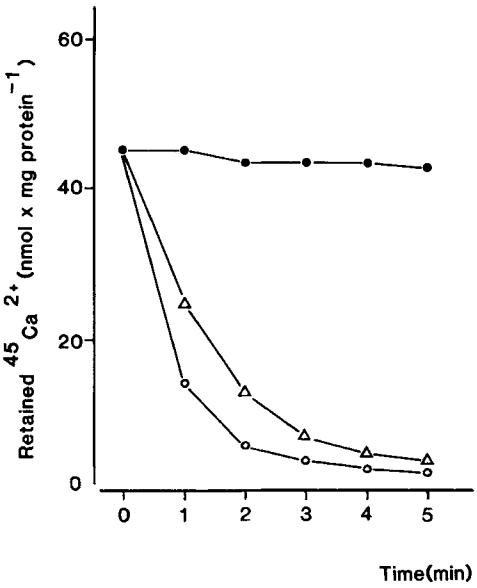

FIG. 6. Release of accumulated $^{45}Ca^{2+}$ by Na^+. Na^+-dependent $^{45}Ca^{2+}$-loaded chromaffin secretory vesicle ghosts were incubated in 100 m*M* KCl (●), 100 m*M* NaCl (○), or in 100 m*M* NaCl with 1 m*M* free Mg^{2+} (△). The media contained 0.5 m*M* EGTA and 20 m*M* MOPS, pH 7.3. The $^{45}Ca^{2+}$ retained within the ghosts was determined as described in Materials and Methods.

presence of 1 m*M* Mg^{2+}, using 1.4×10^{-6} *M* Ca^{2+} in the external medium (Fig. 7). In contrast, at the same Ca^{2+} concentration 1 m*M* Sr^{2+} caused a complete inhibition of Ca^{2+} uptake by the chromaffin secretory vesicle ghosts (Fig. 7). Decreasing the Sr^{2+} concentration to 1 μM at the same Ca^{2+} concentration inhibited Ca^{2+} uptake by 50% (data not shown). Ca^{2+} influx into intact secretory vesicles was likewise insensitive to Mg^{2+}, but Sr^{2+} was strongly inhibitory (Krieger-Brauer and Gratzl, 1982).

The different effects of Mg^{2+} and Sr^{2+} on the Na^+-dependent Ca^{2+} influx into chromaffin secretory vesicles stimulated our interest in investigating the influence of the divalent cations on Ca^{2+} efflux. The presence of a Ca^{2+}-dependent Ca^{2+} efflux system was first observed with chromaffin secretory vesicle ghosts (Phillips, 1981).

The existence of Ca^{2+}-induced Ca^{2+} release could be clearly demonstrated using $^{45}Ca^{2+}$ preloaded secretory vesicle ghosts. Na^+-loaded ghosts were allowed first to accumulate $^{45}Ca^{2+}$. The extravesicular $^{45}Ca^{2+}$ was removed (as described in Materials and Methods) and the $^{45}Ca^{2+}$-loaded ghosts were incubated with 1.4×10^{-6}*M* Ca^{2+} (unlabeled) in a Na^+-free buffer. As shown in Fig. 8, $^{45}Ca^{2+}$ was rapidly released from the secretory vesicle ghosts, indicating a Ca^{2+}/Ca^{2+} exchange system in the chromaffin secretory vesicle membranes.

As observed with Ca^{2+} in medium devoid of Na^+, Ca^{2+} release from the secretory vesicle ghosts could also be elicited by 1 m*M* Sr^{2+}. The initial velocity of $^{45}Ca^{2+}$ efflux caused by low external Ca^{2+} concentration (1.4×10^{-6}*M*) could not be stimulated by adding 1 m*M* Sr^{2+} (Fig. 8).

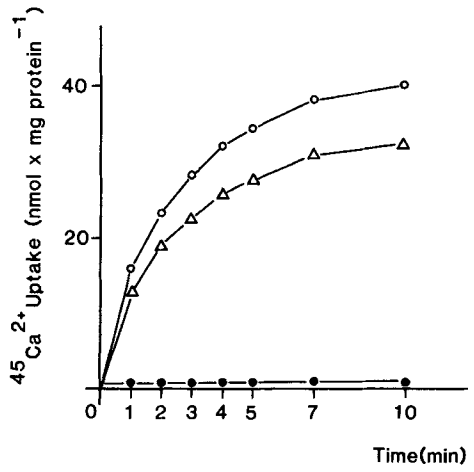

FIG. 7. $^{45}Ca^{2+}$ uptake by chromaffin secretory vesicle ghosts in the presence of divalent cations. Na^+-loaded ghosts were incubated with 1.4×10^{-6} *M* free $^{45}Ca^{2+}$ in media containing 100 m*M* KCl (○), 100 m*M* KCl with 1 m*M* free Mg^{2+} (△), or 100 m*M* KCl with 1 m*M* free Sr^{2+} (●). The media were supplemented with 0.5 m*M* EGTA and 20 m*M* MOPS, pH 7.3.

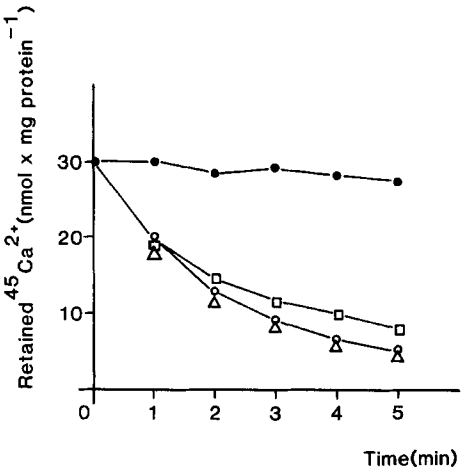

FIG. 8. Release of accumulated $^{45}Ca^{2+}$ by Sr^{2+} and Ca^{2+}. Na^+-dependent $^{45}Ca^{2+}$-loaded chromaffin secretory vesicle ghosts were incubated in 160 m*M* sucrose, 0.5 m*M* EGTA, and 20 m*M* MOPS, pH 7.3 (●); in the same medium supplemented with 1 m*M* free Sr^{2+} (○); with 1.4×10^{-6} *M* free Ca^{2+} (□); or with 1 m*M* free Sr^{2+} and 1.4×10^{-6} *M* free Ca^{2+} (△). The $^{45}Ca^{2+}$ retained was determined as described in Materials and Methods.

The Na^+-dependent Ca^{2+} influx as well as efflux is not affected by Mg^{2+} (Figs. 6 and 7). The action of Mg^{2+} on the Ca^{2+}/Ca^{2+} exchange activity is demonstrated in Fig. 9. A 1 m*M* concentration of Mg^{2+} does not induce $^{45}Ca^{2+}$ release from $^{45}Ca^{2+}$-loaded ghosts. This result would be expected from

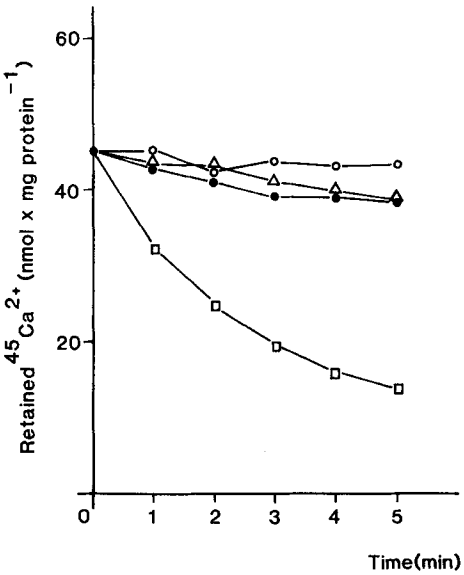

FIG. 9. Inhibition of Ca^{2+}-initiated $^{45}Ca^{2+}$ release by Mg^{2+}. Na^+-dependent $^{45}Ca^{2+}$-loaded chromaffin secretory vesicle ghosts were incubated in 160 m*M* sucrose, 0.5 m*M* EGTA, and 20 m*M* MOPS, pH 7.3 (●) or the same medium supplemented with 1 m*M* free Mg^{2+} (○), 1.4×10^{-6} *M* Ca^{2+} (□), or 1 m*M* free Mg^{2+} and 1.4×10^{-6} *M* free Ca^{2+} (△). The $^{45}Ca^{2+}$ retained within ghosts was determined as described in Materials and Methods.

the lack of influence of Mg^{2+} on Ca^{2+} fluxes driven by Na^+ (Figs. 6 and 7). However, the Ca^{2+}-induced $^{45}Ca^{2+}$ efflux can be completely inhibited by the addition of 1 m*M* Mg^{2+} (Fig. 9). In these experiments the free Ca^{2+} concentration was 1.4×10^{-6} *M* for initiating $^{45}Ca^{2+}$ release. By decreasing the Mg^{2+} concentration from 1 m*M* to 1 μ*M* at constant Ca^{2+} (1.4×10^{-6} *M*) concentration, the inhibitory effect of Mg^{2+} could no longer be observed (data not shown). The fact that Mg^{2+} in millimolar concentrations is able to prevent completely the Ca^{2+}/Ca^{2+} exchange, without a major effect on the Na^+/Ca^{2+} exchange activity (Fig. 6), can serve as a tool to characterize the Ca^{2+} transport across the chromaffin secretory vesicle membrane and to measure selectively Ca^{2+}/Ca^{2+} and Na^+/Ca^{2+} exchange activities. An example is given in the following experiment. In the presence of 1 m*M* Mg^{2+} we examined the effect of the Ca^{2+} concentration on the initial rate of the Na^+-dependent Ca^{2+} uptake. Lineweaver-Burk plots of the experimental data obtained with secretory vesicle ghosts fitted a straight line obtained by linear regression analysis ($r = 0.97$) and showed saturation kinetics. The uptake saturates between 0.5 and 1 μ*M* Ca^{2+} (Fig. 10A). An analysis of the uptake rates from Fig. 10A by a Lineweaver-Burk plot is shown in Fig. 10B. An apparent K_m of 0.12 μ*M* was obtained in this experiment, and the V_{max} was 14.3 nmol min^{-1} · mg $protein^{-1}$. Using three different chromaffin vesicle ghost preparations, we determined an apparent K_m of 0.28 ± 0.16 μ*M* for the Na^+-dependent Ca^{2+} uptake (in the presence of Mg^{2+}), and the maximal Ca^{2+} uptake rate was 14.5 ± nmol · min^{-1} · mg $protein^{-1}$ (mean ± SD).

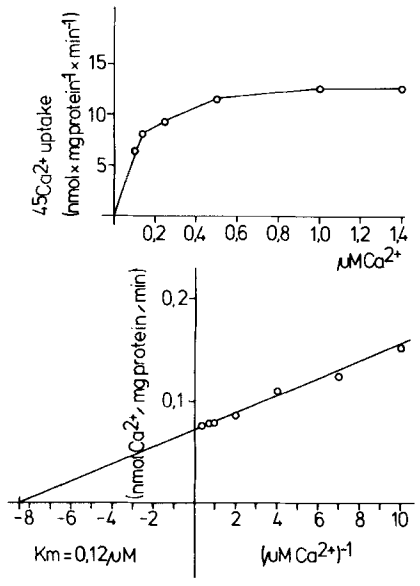

FIG. 10. (A, top) $^{45}Ca^{2+}$ uptake by chromaffin secretory vesicle ghosts in the presence of 1 m*M* Mg^{2+}. Na^+-loaded ghosts were incubated with increasing concentrations of $^{45}Ca^{2+}$ in media containing 1 m*M* Mg^{2+}, 160 m*M* sucrose, 0.5 m*M* EGTA, and 20 m*M* MOPS, pH 7.3. **(B, bottom)** Lineweaver-Burk plot for $^{45}Ca^{2+}$ uptake into chromaffin secretory vesicle ghosts in the presence of Mg^{2+}. The values used for this plot were obtained as described in A.

Influence of Ruthenium red on Ca^{2+} transport

Since the sensitivity to Ruthenium red allowed characterization of different mechanisms for Ca^{2+} movement across the mitochondrial inner membrane of various tissues (Crompton et al., 1976; Crompton et al., 1978; Goldstone and Crompton, 1982), we investigated whether this substance also affects Ca^{2+} transport by secretory vesicle ghosts. The addition of 4.5 nmol Ruthenium red to 1 mg chromaffin secretory vesicle ghosts had no effect on either the Na^+-dependent Ca^{2+} influx or on the Na^+-dependent Ca^{2+} efflux. Further, we could not observe a modification of the Ca^{2+}-induced Ca^{2+} efflux in the presence of Ruthenium red (data not shown).

DISCUSSION

In agreement with previous publications (Krieger-Brauer and Gratzl, 1981, 1982; Phillips, 1981), the results in this report clearly demonstrated the presence of a Ca^{2+}/Na^+ exchange transport system in secretory vesicle membranes of adrenal medulla. In contrast with earlier studies (Phillips, 1981), secretory vesicle ghosts with artificial ion gradients were used to study Ca^{2+} uptake. With Na^+-loaded ghosts we could show that Ca^{2+} uptake can be driven only by an outwardly directed Na^+ gradient. The experiments with K^+-, $choline^+$- or Li^+-loaded ghosts show that Na^+ could not be replaced by K^+, $choline^+$, or Li^+ to drive Ca^{2+} uptake. It can also be concluded that our ghost fraction was free of internal ionized Ca^{2+}, because we could not observe any $^{45}Ca^{2+}$ uptake to K^+-loaded ghosts in the absence of external Mg^{2+} induced by the Ca^{2+}/Ca^{2+} exchange activity discussed later. The initial Ca^{2+} uptake rate of Na^+-loaded ghosts is based on the action of the Na^+-dependent Ca^{2+} transport system, which can be blocked (by ~70%) by adding external Na^+ or Monensin. Under both conditions, the Na^+ gradient across the secretory vesicle membrane is abolished, resulting in the loss of Na^+-dependent Ca^{2+} uptake. Similar properties have been found in other systems in which Na^+/Ca^{2+} exchange has been examined. This holds also for the striking inhibition of the Na^+-dependent Ca^{2+} uptake by the presence of extravesicular choline or Li^+ (Schellenberg and Swanson, 1982; Saermark et al., 1983).

When the Na^+ gradient is reversed, the Na^+-dependent Ca^{2+} transport process mediates Ca^{2+} efflux from ghosts. The initial rate of Ca^{2+} efflux depends on the external sodium concentration and after 5 min, >90% of the accumulated $^{45}Ca^{2+}$ can

be released in the presence of external Na^+ concentrations greater than 50 m*M*. However, the addition of higher Na^+ concentrations to intact secretory vesicles caused the release of only ~50% of the accumulated $^{45}Ca^{2+}$ (Krieger-Brauer and Gratzl, 1982). Accumulated $^{45}Ca^{2+}$ in secretory vesicle ghosts could also be released by the ionophore A21387 in the presence of external Ca^{2+}. In contrast, the addition of the ionophore A21387 to intact secretory vesicles resulted in a rapid further uptake of $^{45}Ca^{2+}$ (Krieger-Brauer and Gratzl, 1982; see also Johnson and Scarpa, 1976). From the effects of external Na^+ and of the ionophore A23187 on intact secretory vesicles compared with secretory vesicle ghosts it can be concluded that most of the Ca^{2+} in secretory vesicle is bound to the intravesicular components, which can be released during hypotonic lysis.

The experiments described in this report as well as in earlier studies (Krieger-Brauer and Gratzl, 1981, 1982; Phillips, 1981) point to the fact that Ca^{2+} transport by chromaffin secretory vesicles occurs via Na^+/Ca^{2+} exchange as well as Ca^{2+}/Ca^{2+} exchange. For further characterization of the Ca^{2+} transport systems it is important to define conditions for selectively measuring Na^+/Ca^{2+} exchange activity and Ca^{2+}/Ca^{2+} exchange activity. As shown in this study, Ca^{2+}-induced Ca^{2+} release is specifically inhibited by Mg^{2+} and not by Sr^{2+}, but Na^+-dependent Ca^{2+} influx and efflux are only slightly affected by Mg^{2+}. The small inhibition of the Na^+-dependent Ca^{2+} transport by Mg^{2+} (Figs. 6 and 7) is probably based on the inhibition of Ca^{2+}/Ca^{2+} exchange taking place during Na^+/Ca^{2+} exchange. Further, in initiation of Ca^{2+}-induced $^{45}Ca^{2+}$ release, Ca^{2+} can be replaced by Sr^{2+}, but not by Mg^{2+}. This Ca^{2+} loss produced by Sr^{2+} seems to be the reason for the observed inhibition of Na^+-dependent Ca^{2+} uptake, indicating that Sr^{2+} does not affect Na^+-dependent Ca^{2+} uptake directly. These results clearly demonstrate the different targets of Mg^{2+} and Sr^{2+} by influencing Ca^{2+} transport across the membrane of chromaffin secretory vesicles, and give further information on the underlying mechanisms of the inhibitory effects of Sr^{2+} and Mg^{2+} on Ca^{2+} uptake, which were described in earlier reports (Kostron et al., 1977; Phillips, 1981; Krieger-Brauer and Gratzl, 1982).

The specific inhibition of the Ca^{2+}-induced Ca^{2+} efflux by Mg^{2+} demands further experiments concerning the stoichiometry of the Ca^{2+}/Na^{2+} exchange system to improve earlier experiments, suggesting that 2 Na^+ will be exchanged for 1 Ca^{2+} (Phillips, 1981; Krieger-Brauer and Gratzl, 1982). During measurement of the transmembrane Na^+/Ca^{2+} exchange in EGTA-free and Mg^{2+}-free buffer (Phillips, 1981) the release of Ca^{2+} or its uptake would be the sum of the Ca^{2+}/Ca^{2+} exchange and of the Na^+/Ca^{2+} exchange process as well as the noncarrier-mediated Ca^{2+} efflux due to the Ca^{2+} leakage across the membrane.

It has been reported (Phillips, 1981) that the Ca^{2+}- and the Na^+-dependent Ca^{2+} influx across chromaffin secretory membrane vesicles is sensitive to Ruthenium red. We could not find any effect of Ruthenium red on Ca^{2+} movement across the membrane of secretory vesicles. This discrepancy cannot be resolved at present.

The selective inhibition of Ca^{2+}/Ca^{2+} exchange by Mg^{2+} allowed determination of the apparent K_m for the Na^+-dependent Ca^{2+} transport system. The system is characterized by its high affinity for Ca^{2+} (0.28 μM). For the Ca^{2+}/Ca^{2+} exchange system an apparent K_m of 38 μM has been described (Phillips, 1981).

Considering the function of Na^+/Ca^{2+} exchange and Ca^{2+}/Ca^{2+} exchange system *in vivo*, it is of interest to compare the cytosolic ion concentrations with that in the secretory vesicles. Measurements of cytosolic Mg^{2+} and Na^+ concentrations in resting cells gave values of ~1 m*M* Mg^{2+} and ~15 m*M* Na^+ (cf. Carafoli and Crompton, 1978; Borle, 1981), and the measurement of the apparent intravesicular concentrations gave values in the range of 50 m*M* for Na^+ and 9 m*M* for Mg^{2+}, i.e., in the same order as above (Krieger-Brauer and Gratzl, 1982). Therefore, in resting cells the Na^+/Ca^{2+} exchange system preferably transports Ca^{2+} into secretory vesicle from the cytosol because Ca^{2+}/Ca^{2+} exchange is inhibited by Mg^{2+}. The cytosolic ionized Ca^{2+} concentration in the chromaffin cell is ~0.1 μM (Baker and Knight, 1981), which is in the range of the apparent K_m for Ca^{2+} of the Na^+-dependent Ca^{2+} transporter. Thus the Ca^{2+} uptake by chromaffin secretory vesicles described in this paper might contribute to the buffering of the cytoplasmic Ca^{2+} concentration.

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