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CALCIUM/SODIUM EXCHANGE IN PURIFIED SECRETORY VESICLES FROM BOVINE NEUROHYPOPHYSES

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ABSTRACT

Purified secretory vesicles isolated from bovine neurohypophyses take up Na^+ under the same circumstances where an efflux of Ca^{2+} takes place, suggesting a Na^+/Ca^{2+} exchange. Potassium cannot substitute for Na^+ in this process. Also, a Ca^{2+}/Ca^{2+} exchange can occur. Inhibiting the latter process by Mg^{2+} allowed to estimate an apparent K_M of 0.7 µM free Ca^{2+} and a maximal uptake of 1.5 nmol x mg protein^{-1} x min^{-1} Ca^{2+} in exchange for Na^+.

The vesicles did not contain plasma membrane marker (Na^+/K^+ ATPase) as shown by distribution analyses on the density gradients on which they were purified. Similarly, distribution studies also showed that no other ATPase activity could be detected in the purified vesicle fraction.

It is concluded that a Na^+/Ca^{2+} exchange is operating across the secretory vesicle membrane and that it is not directly dependent on ATP hydrolysis.

INTRODUCTION

The existence in nerve terminals of non mitochondrial Ca^{2+} sequestering systems with high affinity has been proposed by several authors (1-5). The systems described up to now, e.g. in brain synaptosomes (3) and in the neurohypophysis (5) seem to be dependent on adenosine 5'-triphosphate (ATP).

The hormone-containing neurohypophyseal secretory vesicles within the terminals also contain Ca^{2+}, as shown both by electron probe analysis of intact tissue (6) as well as by analyses of vesicles isolated by subcellular fractionation (7, 8).

Secretory vesicles therefore could contribute to the sequestration of Ca^{2+}. We have previously shown that purified isolated intact secretory vesicles from the neurohypophysis take up Ca^{2+} (7). Ca^{2+} influx was inhibited by Na^+ and Ca^{2+} previously taken up by the vesicles could be released again by external Na^+. It was not affected by ATP, oligomycin or ruthenium red. These properties are in accordance with a Na^+/Ca^{2+} exchange system present in the secretory vesicle membranes operating in either direction depending on the electrochemical gradients for Na^+ and Ca^{2+} across this membrane. A similar uptake system for Ca^{2+} in adrenal medulla secretory vesicles has previously been established (9, 33).

In the present paper we describe that under the same circumstances where Ca^{2+} efflux occurs, Na^+ is taken up by the vesicles. This was demonstrated by measurements of $^{22}Na^+$ uptake as well as by changes in the optical density of the vesicles. Also, we report that vesicular Ca^{2+} can be released by addition of external Ca^{2+}. Thus secretory vesicles obviously are able to carry out Ca^{2+}/Ca^{2+} exchange as well. The finding that this latter process could be completely inhibited by Mg^{2+} allowed us to determine the apparent K_M for the Ca^{2+} uptake in exchange for Na^+. The low value obtained (7×10^{-7} M free Ca^{2+}) would be in agreement with a physiological role of the vesicular Ca^{2+} uptake system in neurohypophysial nerve endings. The Ca^{2+}/Na^+ exchange and Ca^{2+} uptake cannot be directly correlated to ATP hydrolysis, since no ATPase activity copurified with vasopressin when two different procedures were used for isolation of the secretory vesicles.

METHODS

Isolation of Secretory Vesicles. Bovine hypophyses were obtained immediately after slaughtering at Københavns Eksportslagteri and Slagelse Andelsslagteri. The neural lobes were isolated by dissection and placed in icecold medium containing 190 mM sucrose, 25 mM ethyleneglycol bis (β-amino-ethylether) N,N'-tetraacetic acid (EGTA), 20 mM 2([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino ethane-sulfonic acid (TES), pH 7.0. KOH was used to adjust pH. Homogenization of the tissue in the laboratory was finished not later than two hours from slaughtering.

Purified secretory vesicles were obtained by a newly developed method employing differential and density gradient centrifugation using Percoll (50%) as gradient material (in isotonic media) (10), which allows to separate the secretory vesicles from mitochondria, microsomes (10), and lysosomes (this study). Such vesicles are stable on incubation in isosmolar media. Secretory vesicles recovered from the Percoll gradients (fractions 1-8) and sedimented by centrifugation at 100,000 x g for 1 h were resuspended and submitted to gel filtration on Bio-Gel A 150 (BioRad) to remove Percoll.

The specific activity of vasopressin and oxytocin in the band of vesicles selected was 37.4 ± 8.5 (mean, SD of 5 preparations) and 20.8 ± 7.5 (mean, SD of 9 preparations) μg x mg^{-1} protein, respectively.

In a second series of experiments, the basic medium used throughout the purification procedure was 260 mM sucrose, 0.1 mM EGTA, 20 mM TES, pH 7.0. In this case 30% Percoll was used for density gradient centrifugation (60,000 g_{av} for 30 min). A similar procedure (except for use of 0.1 mM EGTA) has been employed by Russell (11).

In a series of experiments the secretory vesicle preparation obtained from 30% Percoll/0.1 mM EGTA gradients was further purified on a discontinuous sucrose gradient by a modification of the procedure of Vilhardt and Hope (12). The gradients were made up of 1 ml of 2.0 M, 1.4 M, 1.35 M and 1.3 M sucrose in 0.1 mM EGTA, 2 mM TES, pH 7.0. The material was layered in 1 ml on top of each gradient and centrifuged in a Beckman rotor at 100,000 x g_{av} x 150 min (4°C). Fractions of 500 μl were collected from the gradients by means of a peristaltic pump (Dich Instruments, DK.).

Measurement of Ca^{2+} Uptake. After removal of Percoll by gel filtration the secretory vesicles were centrifuged at 60,000 x g for 20 min and resuspended in 260 mM sucrose, 20 mM KCl, 0.5 mM EGTA, 20 mM TES, pH 7.3 (adjusted with KOH) "uptake medium". Resuspension was carried out by hand in a loose--fitting Teflon-to-glass homogenizer (vol. 1 ml). In one set of experiments 5 mM nitrilo-tri-acetic acid (NTA), 0.5 mM EGTA, 1 mM Mg^{2+} (free), 100 mM KCl, 20 mM TES, pH 7.3, was used.

Ca^{2+} was included in the "uptake medium" to give various free Ca^{2+} concentrations calculated as decribed (13), taking into account the known stability constants (14). The calculated free Ca^{2+} concentrations were checked by use of a calcium specific electrode, donated by Professor W.Simon, Zürich, and the calcium binding constant and EGTA concentration were corrected by the use of a Scatchard plot according to the method of Bers (15). $^{45}Ca^{2+}$ was added to the solutions to give a specific activity of 5 μCi/mmole Ca^{2+}. The uptake medium (150 μl) was kept at 30°C for 5 min before addition of secretory vesicles (50 μg) in a volume of 50 μl. After incubation (10 min unless otherwise stated) the vesicles were separated from the medium by filtration (Millipore cellotate filters, pore size 0.2 μm). The filters were washed with 2 x 5 ml of icecold uptake medium described above, containing no Ca^{2+}, placed in counting vials, dried at 80°C for 1 h, and cooled to room temperature. The filters were counted in 10 ml of Lumagel scintillation fluid. The samples were left for 24 h at 4°C before counting. In certain experiments 130 mM KCl or various concentrations of NaCl were used in the incubation medium to replace sucrose.

Measurement of Ca^{2+} Efflux. Secretory vesicles preloaded with Ca^{2+} were prepared in the following way: Secretory vesicles were incubated to take up calcium in a medium containing 10^{-4} M free Ca^{2+}, $^{45}Ca^{2+}$ (5 μCi/mmol), 130 mM KCl, 0.5 mM EGTA and 20 mM TES (pH 7.3) for 15 min at 30°C. These vesicles were then diluted tenfold in a medium containing either 130 mM NaCl, 130 mM KCl, or 10^{-4} M free Ca^{2+} plus 130 mM KCl. In addition, these media contained 0.5 mM EGTA and 20 mM TES pH 7.3. The pH was adjusted with KOH and the temperature during further incubation was 30°C. Filtration and washing of the filters as well as the determination of $^{45}Ca^{2+}$ was done as described for uptake. In a series of experiments free Mg^{2+} (10^{-6}-10^{-3} M) (calculated as described for free Ca^{2+}) was present in the medium during efflux.

Measurement of Na^+ Uptake. Samples preloaded by Ca^{2+} as described for measurement of Ca^{2+} efflux were diluted 1:10 in a medium containing various concentrations of NaCl labelled with $^{22}Na^+$. The activity of $^{22}Na^+$ in the medium was 0.2 μCi/ml. In addition KCl was included to obtain a final total concentration of 130 mM salt and 1 mM $MgCl_2$. The filters were washed 5 times with the same medium (without isotope) to obtain blanks of 0.05 % of totals. Using this procedure the amount of protein needed was 500 μg to obtain a doubling of the background. $^{22}Na^+$ was counted in a γ-counter (Searle).

During all isolation and incubation procedures the total osmolality of the buffers and media was kept between 315 and 320 mosmol/kg. All experiments were finished within 16 h from slaughtering of the animals.

Measurements of Vasopressin, Oxytocin, and Protein. Vasopressin was measured by radioimmunoassay as described in (16), oxytocin by a similar assay. Protein was determined by the use of fluorescamine (Fluram, Roche (17)).

Enzyme Assays. ATPases were measured as described in (18). GLDH was assayed as described in (10), and beta glucuronidase by the method of Price and Dance (19). Acid phosphatase was measured as described in (20), using β-glycerophosphate as substrate. Malakit green (Fluka) was used for determination of inorganic phosphate liberated after precipitation of protein in 5% H_2SO_4 (21).

MATERIALS

$^{22}Na^+Cl$ and $^{45}Ca^{2+}$ Cl^2 were obtained from Amersham. Lumagel was from Lumac System AG, Basel. Calmodulin was from Fluka, batch No. 233745 682. Percoll was a product of Pharmacia, Sweden, which was dialyzed for 2 x 12 h against the appropriate medium before use.

All other reagents were of analytical grade.

RESULTS

Effects of K^+, Na^+, and Ca^{2+} on the efflux of Ca^{2+}. The efflux of Ca^{2+} from preloaded secretory vesicles in the presence of various cations is shown in Fig. 1. In the presence of KCl no efflux was observed. Addition of Ca^{2+} to a concentration of 10^{-4} M (free) or substituting KCl by NaCl induced Ca^{2+} efflux, which was linear within 5 min. About 50% of the Ca^{2+} taken up during preloading remained in the vesicles. This experiment indicates that Na^+ as well as Ca^{2+} are able to release Ca^{2+} from preloaded vesicles, probably by exchange of intravesicular Ca^{2+} with extravesicular Ca^{2+}. Mg^{2+} had little effect on Na^+ induced Ca^{2+} efflux (Ca^{2+} content after 20 min was 48 ± 8% of control (n=4) in the presence of 1 mM Mg^{2+}).

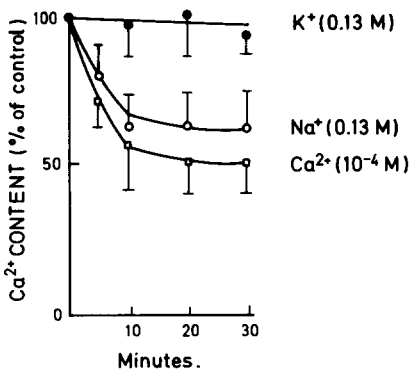

Fig. 1 Ca^{2+} and Na^+ induced Ca^{2+} efflux from secretory vesicles. Secretory vesicles were first incubated at 30°C in a medium containing 130 mM KCl, 0.5 mM EGTA, 20 mM TES, pH 7.3 and Ca^{2+} labelled with $^{45}Ca^{2+}$ (Free Ca^{+} concentration of 10^{-4} M). After 15 min of incubation, the samples were diluted 1:10 in the same medium containing no Ca^{2+} (K^+, ●), in the medium containing 10^{-4} M Ca^{2+}, but no $^{45}Ca^{2+}$ (Ca^{2+}, □) or in a medium containing no Ca^{2+} and 130 mM NaCl instead of KCl (Na^+, o). At various time points the samples were filtered and counted as described in the Methods section. The values represent the mean of 3 expts, bars=SD. 100% corresponds to 13.5 ± 2.8 nmol Ca^{2+}/mg protein.

The stability of the vesicles was the same in the different incubation procedures. This was shown by incubating the secretory vesicles under the same conditions for 1 h followed by centrifugation at 28,000 g_{av} x 15 min. Incubation in the medium containing 130 mM KCl caused a release of 23% ± 4.7

(S.D., n=3) of vesicular vasopressin. After incubation in media containing 130 mM Na^+ 26% ± 1 (S.D. n=3) of hormone was found in the supernatant. Addition of Ca^{+} to the medium did not change the stability (24% ± 3 release, independently of the monovalent cation). These figures should be compared with a release of 21% when the vesicles were incubated under similar conditions in the isolation buffer.

Inhibition of Ca^{2+} induced Ca^{2+} efflux by Mg^{2+}. Test of effect of calmodulin. Fig. 2 shows that Mg^{2+} strongly inhibited the Ca^{2+} efflux from $^{45}Ca^{2+}$ preloaded secretory vesicles induced by Ca^{2+}. The Ca^{2+} retained in the vesicles was determined after 10 or 20 minutes at 30°C in the presence of various free concentrations of Mg^{2+} (see Methods). The inhibition was almost complete above 0.1 mM free Mg^{2+}.

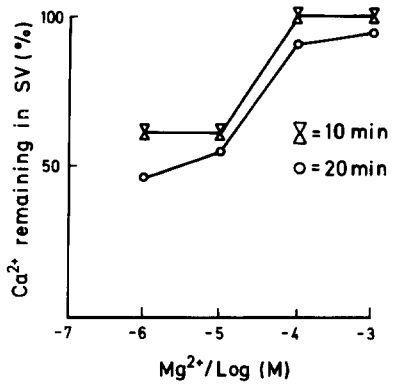

Fig. 2 Inhibition of Ca^{2+}/Ca^{2+} exchange by Mg^{2+}. Secretory vesicles (SV) were preincubated in the same way as described in legend to Fig. 1. The samles were then diluted 1:10 in the same medium containing Ca^{2+} (10^{-4} M free) without $^{45}Ca^{2+}$ and including Mg^{2+} to give the free concentrations indicated. After a further incubation for 10 or 20 min. the samples were filtered and counted as described in the Methods section.

The fact that Ca^{2+} induced Ca^{2+} release (Ca^{2+}/Ca^{2+} exchange) can be inhibited by Mg^{2+} and that Na^+/Ca^{2+} exchange is not affected by Mg^{2+} (see also 7) allowed to measure Ca^{2+} influx selectively.

Fig. 3 shows a double reciprocal plot of the Ca^{2+} concentration dependence for Ca^{2+} uptake measured in the presence of 1 mM free Mg^{2+}. The data could be fitted to a straight line (R^2=0.975), data from 3 expts.

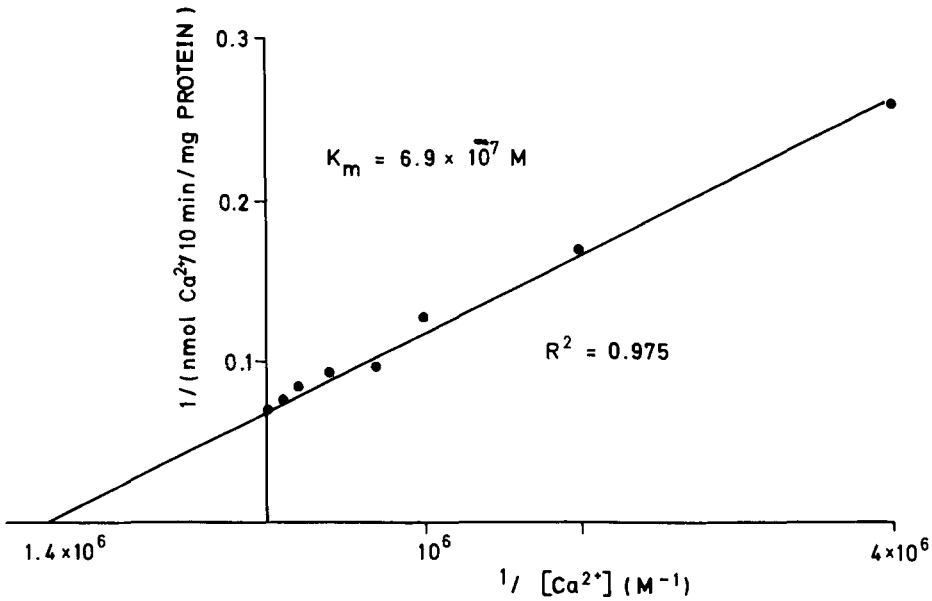

Fig. 3 Ca^{2+} uptake into secretory vesicles in the presence of 1 mM Mg^{2+}. Double reciprocal plot of initial velocity of Ca^{2+} uptake by secretory vesicles as a function of the Ca^{2+} concentration. The buffer used was the same as described for the Ca^{2+} loading procedure in Fig. 1, containing in addition 1 mM Mg^{2+} (free). The initial velocity was calculated on the basis of measurements of Ca^{2+} uptake after 5 and 8 min. Within these time periods the uptake was linear with time. It was expressed per 10 min period. Ca^{2+} uptake was measured as described in the Methods section.

Incubation was carried out at 30°C in a medium containing 130 mM KCl, 20 mM TES, 0.5 mM EGTA, pH 7.3 including 1 mM Mg^{2+} (free). The incubation was carried out not longer than 8 min, i.e. the period in which uptake was linear with time (7) and the Ca^{2+} uptake per 10 min was calculated. The K_m value was 6.9×10^{-7} and V_{max} was 1.47 nmol/mg x min. Using 5 mM NTA, 0.5 mM EGTA, 100 mM KCl and 20 mM TES, pH 7.3, a K_m value of 1.2×10^{-6} M was found ($R^2 = 0.95$). The addition of calmodulin to the incubation medium before addition of Ca^{2+} did not change the kinetic properties of the Ca^{2+} uptake. In the presence of 1 µg calmodulin/100 µg secretory vesicle protein, the K_m was 6.4×10^{-7}. The V_{max} was 1.44 nmol/mg x min (R^2= 0.970 for data from 3 expts.). This is in accordance with results of earlier experiments in which trifluoperazine, an inhibitor of calmodulin linked reactions, was also ineffective (7).

Uptake of $^{22}Na^+$. The Ca^{2+} preloaded secretory vesicles (see Methods) were diluted 1:10 in a medium containing 20 mM TES, 0.5 mM EGTA in addition to KCl and NaCl (labelled with $^{22}Na^+$) in a total concentration of 130 mM giving a final osmolality of approx. 320 mOsmol/kg. In this way NaCl and KCl concentrations were varied to give the final NaCl concentrations on the abscissa in Fig. 4 at a constant ionic strength. Na^+ uptake increased linearly up to about 20 mM Na^+, at higher concentrations the uptake levelled off.

If the data were plotted as a double reciprocal plot, the experimental points from individual experiments did not fit well using linear regression analysis (R^2 about 0.830). When data from 6 experiments were analyzed, however, half maximal Na^+ uptake occurred with 20 mM Na^+, regression coefficient 0.988.

Na^+ uptake as indicated by decrease in optical density. As further evidence for Na^+ uptake by the secretory vesicles, the optical density was measured during incubation of Ca^{2+} preloaded secretory vesicles in presence of 130 mM NaCl. A decrease should be an indication of swelling due to increased intravesicular osmotic pressure caused by more Na^+ taken up than Ca^{2+} released (7, compare 22, 23).

Neurohypophysial secretory vesicles respond to changes in the osmolality of the media. This was determined by incubation of the vesicles in 0.5 mM EGTA, 20 mM TES, pH 7.3, including various amounts of sucrose. The optical density at 560 nm decreased with sucrose media below 200 mOsmol/kg (data not shown). This is in agreement with the osmotic stability measured by vasopressin efflux published earlier (10).

Fig. 5 shows the change in optical density at 560 nm of a suspension of secretory vesicles preloaded with Ca^{2+} (see Methods) incubated at 30^0C in presence of 130 mM KCl or NaCl with or without 10^{-4} M free Ca^{2+} present. 1 mM Mg^{2+} was present in all media. In addition to the ions 20 mM TES and 0.5 mM EGTA, pH 7.3, were included in the assay medium. Air was used as reference. The optical densities were recorded at intervals of 1 min to avoid local heating. Starting from a level of 0.2 the extinction decreased when 130 mM NaCl was included in the incubation medium. The optical density in presence of 130 mM NaCl was 0.1 after 60 min, compared with 0.190 in the presence of 130 mM KCl (control). The small reduction in the optical density in the presence of KCl (130 mM) was identical in the presence or in the absence of external Ca^{2+} (10^{-4} M). 10^{-4} M Ca^{2+} slightly reduced the effect of Na^+. It should be recalled here that within 1 h no significant difference of vasopressin release could be detected for the different media (see p. [illegible]).

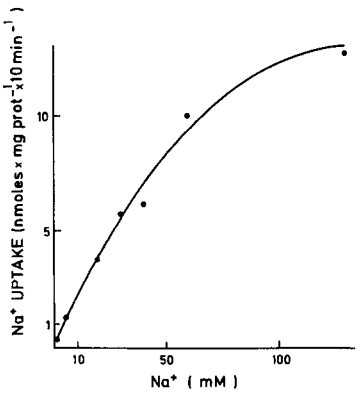

Fig. 4

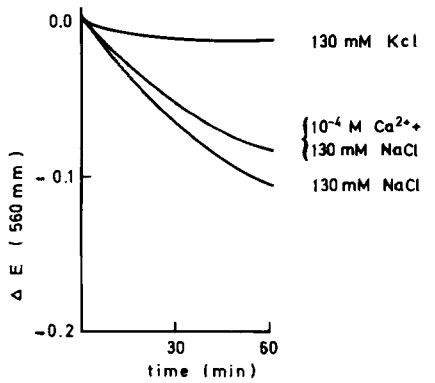

Fig. 5

Fig. 4. Na^+ uptake into SV preloaded with Ca^{2+}. SV were preincubated for 15 min in a medium containing 10^{-4} M Ca^{+}, 130 mM KCl, 0.5 mM EGTA, 20 mM TES, pH 7.3. At 15 min. the samples were diluted 1:10 in a medium containing 0.5 mM EGTA, 20 mM TES, pH 7.3, including increasing amounts of NaCl containing $^{22}Na^+$. KCl was added to give a final (NaCl + KCl) concentration of 130 mM corresponding to an osmolar concentration of 320 mOsmol/kg. After 10 min the samples were filtered, washed with 2 x 5 ml 130 mM NaCl, 0.5 mM EGTA, 20 mM TES, pH 7.3, and the radioactivity retained was determined (see Methods section) (means of 4 expts.). Na^+ uptake at 130 mM NaCl was 12.9 nmol ± 4.8 per 10 min (S.D., n=4).

Fig. 5. Na^+ uptake indicated by change in optical density. Secretory vesicles (75 µg) were preincubated at 30°C in 130 mM KCl, 10^{-4} M Ca^{2+}, 0.5 mM EGTA, 20 mM TES, pH 7.3 for 15 min in a volume of 0.5 ml. At time 0 they were diluted 1:10 in media containing 130 mM KCl, 130 M NaCl x 10^{-4} M Ca^{2+} or 130 mM NaCl. The optical density at 560 nm was read at intervals to avoid local heating of the samples. All media contained 1 mM Mg^{2+} to inhibit Ca^{2+} induced efflux of Ca^{2+}.

The incubation of secretory vesicles not preloaded with Ca^{2+} also changed their optical densities when added to 130 mM NaCl, probably due to the endogenous Ca^{2+} present in isolated secretory vesicles (7).

ATPase activities in the secretory vesicle fraction. Na^+/Ca^{2+} exchange is a well-known process taking place in the cell membranes of nerve and muscle cells (1). To exclude that the Na^+/Ca^{2+} exchange system described here might be due to presence of cell membrane fragments in our preparation we determined Na^+/K^+ ATPase distribution in the gradients used for secretory vesicle isolation. We also measured Mg^{2+} ATPase activities

which have been described to occur in neurohypophysial secretory vesicles (24) although its presence has not been found in an earlier investigation (12).

Discribution on 50% Percoll gradients containing 25 mM EGTA. In the gradients used in the main part of this work (50% Percoll in a medium containing 190 mM sucrose, 25 mM EGTA and 20 mM TES, pH 7.0, see Methods), we did not find any Mg^{2+} ATPase activity in the secretory vesicle fractions (band centered around fraction 5, see protein profile, Fig. 6B) which contains vasopressin, oxytocin, and neurophysin (see 10). The Mg^{2+} ATPase was assayed as described in the Methods section at a free Mg^{2+} concentration of 1 mM. The addition of 10^{-6} or 10^{-4} free Ca^{2+} to the assay changed neither the ATPase activity nor its pattern of distribution. Of the total Mg^{2+} ATPase activity present in the homogenate only 0.38% ± 0.19 (S.D. n=4) was found in the secretory vesicles (fractions 1-7, Fig. 6B). The relative specific activity was 0.09 ± 0.04 (S.D. n=4) (Table 1).

Table 1. Distribution of ATPases in secretory vesicle fractions recovered from 3 types of density gradients (means ± S.D.)

GRADIENT	Pooled fraction No.	n		Mg^{2+} ATPase	Na^+/K^+ ATPase
50% Percoll 25 mM EGTA	1-7	4	SA	0.24±0.3	<0.08±0.06*
			R.S.A.	0.09±0.04	<0.16±0.09
			% of Total	0.38±0.19	<0.42±0.3
			Recovery(%)	101±16	91±17
30% Percoll 0.1 mM EGTA	1-12	4	SA	2.1 ±0.6	<0.02±0.03
			R.S.A.	0.72±0.5	<0.04±0.01
			% of Total	5.1 ±2.0	<0.4 ±0.2
			Recovery(%)	113±28	126±25
2.0-1.3 M Sucrose 0.1 mM EGTA	1-10	3	SA	0.2 ±0.1	
			R.S.A.	0.1 ±0.07	
			% of Total	1.1 ±0.8	
			Recovery (%)	110±9	

*In 2 out of 4 expts. Na^+/K^+ ATPase activity could not be detected with the assay sensitivity (3 nmol ATP hydrolyzed; 21)
SA = Specific Activity (µmol/h/mg)
R.S.A. (Relative Specific Activity) = $\frac{\text{\% recovered activity}}{\text{\% recovered protein}}$
% of Total = % of total activity in homogenate recovered in secretory vesicles

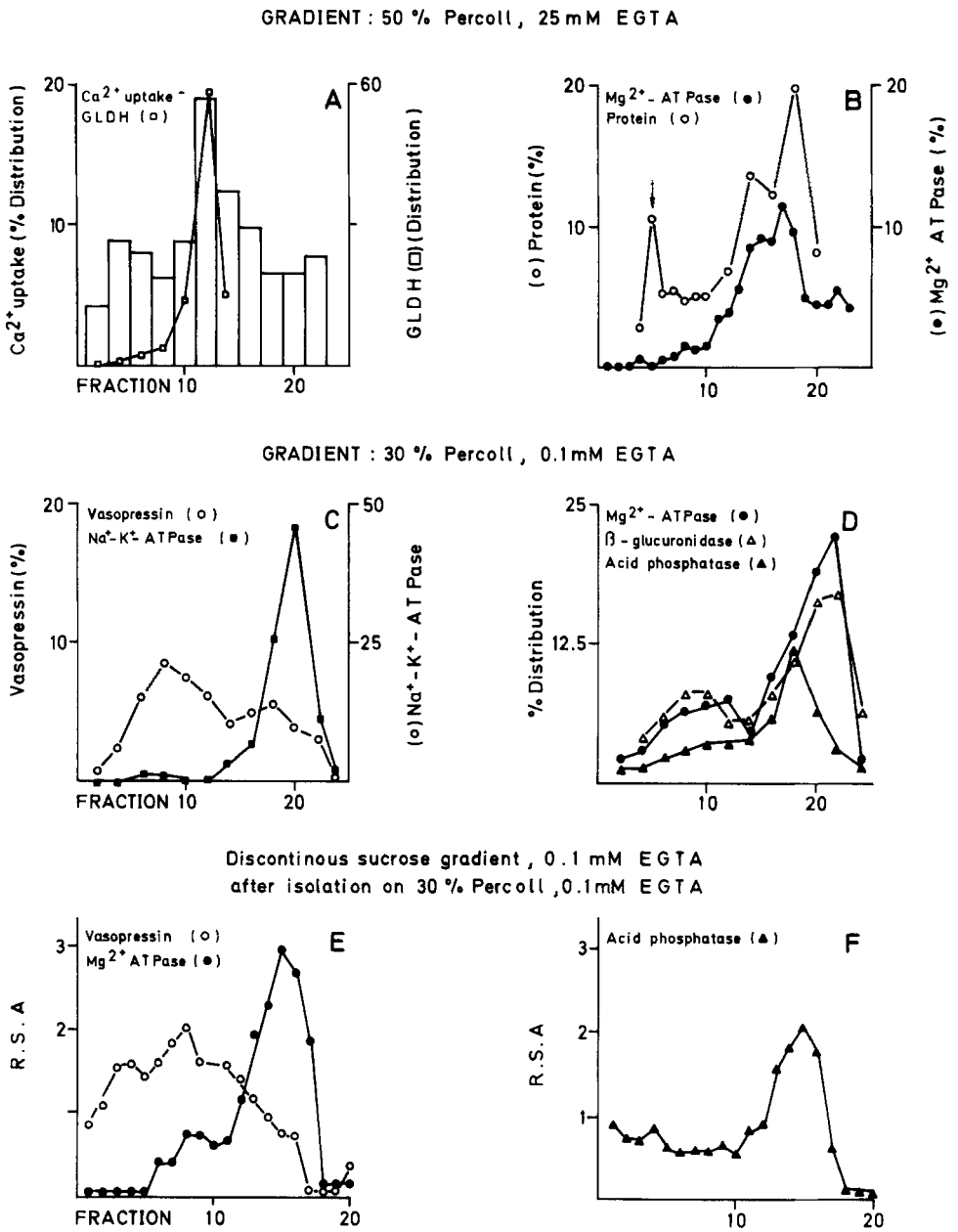

Fig. 6. Distribution of ATPases and other enzymes and markers as well as protein on gradients composed of

A, B 50% Percoll, 25 mM EGTA, 20 mM TES (pH 7.0) and 130 mM sucrose. C, D 30% Percoll, 0.1 mM EGTA, 20 mM TES (pH 7.0) and 260 mM sucrose. E, F Discontinuous sucrose (2.0-1.3 M), 0.1 mM EGTA, 20 mM TES (pH 7.0).
Material placed on 50% as well as 30% Percoll gradient: Fraction P_2 (see 10); material placed on discontinuous sucrose gradient: Fraction 1-12 from 30% Percoll gradient.

Recoveries for acid phosphatase: 86 ± 12% (n=3). Recoveries for beta glucuronidase: 68 ± 10% (n=3)

Relative specific activity (R.S.A.) is: $\frac{\% \text{ recovered activity}}{\% \text{ recovered protein}}$

The data represent means of at least 3 expts. in each figure.

Also, no Na^+/K^+ ATPase was found in the secretory vesicle region (Table I). This is in accordance with the original paper describing the isolation of secretory vesicles on isosmolar Percoll gradients (10).

The distribution of Ca^{2+} accumulation by fractions recovered from the 50% Percoll gradient is given in Fig. 6A. The incubation (30°C) was carried out for 10 min with 10^{-4} M free Ca^{2+}. Each fraction was diluted 1:20 into the assay medium containing 260 mM sucrose, 1 mM $MgCl_2$, 20 mM TES, pH 7.0. The final EGTA concentration was 1.25 mM. Two peaks appeared. One coinciding with the secretory vesicle fraction and the other with localization of the mitochondrial marker (glutamate dehydrogenase). It has been described earlier that secretory vesicles accumulate Ca^{2+} even in presence of ruthenium red and that this process is not stimulated by ATP (7). By contrast Ca^{2+} uptake into neurohypophysial mitochondrial fractions was stimulated by ATP (7). From the experiments described it can be concluded that the secretory vesicles prepared in these gradients neither contain Mg^{2+} ATPase nor Na^+/K^+ ATPase, nor exhibit an ATP stimulated Ca^{2+} uptake, but take up Ca^{2+} as a consequence of a Na^+ gradient ($[Na^+]_i > [Na^+]_o$).

Distribution on 30% Percoll gradients containing 0.1 mM EGTA.
It could be argued that the high concentration of EGTA used during subfractionation might cause inactivation of the ATPase and thus explain the extremely low amount of Mg^{2+} ATPase or Na^+/K^+ ATPase activity in the secretory vesicle fraction.

To test this possibility we reduced the concentration of EGTA to 0.1 mM in the buffered media used for the isolation of the secretory vesicles (see Methods). 30% Percoll was used instead of 50% to obtain a proper separation in the gradients at lower ionic strength (11) (see Methods).

The distribution of Na^+/K^+ ATPase on the 30% Percoll gradients containing 0.1 mM EGTA (Fig. 6C) was not different from that

reported earlier (10) using 50% Percoll as a gradient material and 25 mM EGTA in the medium. No significant activity was present in the secretory vesicle fractions (Fig. 6C, Table I). The relative specific activity (0.04) and the percentage of total Na^+/K^+ ATPase activity found in the secretory vesicles (0.4) were in the same low order of magnitude compared with the original work on secretory vesicle isolation on these gradients (10).

As opposed to the distribution of Na^+/K^+ ATPase in both types of gradients, in the presence of 0.1 mM EGTA, using a 30% Percoll gradient, a peak of Mg^{2+} ATPase activity was apparent in the secretory vesicle fraction (Fig. 6D, Table I). The specific activity in the secretory vesicle fraction (No. 1-12) was 2.1 µmol/h/mg ± 0.6 (S.D., n=3) which should be compared with the value of 0.24 µmol/h/mg ± 0.3 (S.D. n=4) for the fractions 1-7 in Fig. 6B (50% Percoll, 25 mM EGTA). The high amount of Mg^{2+} ATPase activity present in secretory vesicles when prepared on 0.1 mM EGTA/30% Percoll gradients might represent a lysosomal contamination since acid phosphatase and β-glucuronidase were distributed in the same way as the Mg^{2+} ATPase (Fig. 6D). The low amount of Mg^{2+} ATPase activity in the secretory vesicle fractions shown in Fig. 6B compared to Fig. 6D is not an effect of the high EGTA concentration employed for the preparation of the secretory vesicles. If 25 mM EGTA was added to the vesicle fractions obtained from the gradients in Fig. 6C (containing 0.1 mM EGTA) before the assay, no change in Mg^{2+} ATPase activity was observed. The specific ATPase activity for the fractions containing secretory vesicles prepared in 0.1 mM EGTA (Fig. 6C, fractions 4-12, pooled) was 2.1 µmol/mg protein per h compared to 2.0 µmol/mg protein per h, when 25 mM EGTA was added to the fractions 2 h before the assay. Similarly, there was also no change in the distribution of Mg^{2+} ATPase activity on the gradient, when 25 mM EGTA was added to the individual fractions from the gradient prepared in 0.1 mM EGTA immediately before the assay.

In secretory vesicles from adrenal medulla, FCCP (a protonophor) is known to stimulate the Mg^{2+} ATPase up to 300% (25). FCCP did not change the enzyme activity in our preparation of secretory vesicles (50% Percoll, Table I). When 25 µg of FCCP was added to 50 µg of secretory vesicles in 1.5 ml incubation medium, the enzyme activity in our preparation was 92% of the control value (2 expts.). Thus, no activation of the enzyme activity was found.

Distribution of Mg^{2+} ATPase, vasopressin, and acid phosphatase and β-glucuronidase after further purification of secretory vesicles on a sucrose gradient. Finally, we attempted to remove Mg^{2+} ATPase and acid phosphatase activities by a further purification of the vesicles, first partially purified on 30% Percoll gradients containing 0.1 mM EGTA, on sucrose density gradients. For this purpose we applied sucrose gradients used earlier to isolate secretory vesicles from the neurohypophysis (12).

In Fig. 6E the relative specific activity of vasopressin (AVP) and Mg^{2+} ATPase are shown. A difference in the distribution of vasopressin versus Mg^{2+} ATPase and acid phosphatase (Fig. 6F) is clearly seen.

The specific activity of AVP in the pooled fractions 1-10 recovered from the sucrose gradients was 54 ± 14 µg AVP x mg^{-1} protein (mean ± SD) (n=3), and that from the 30% Percoll gradients 39.3 ± 16 µg AVP x mg^{-1} protein (n=4).

From the results described above it can be concluded that the high specific activity of Mg^{2+} ATPase present in secretory vesicles isolated on 30% Percoll gradients containing 0.1 mM EGTA is a contaminant. The low Mg^{2+} ATPase activity in the vesicles obtained from 50% Percoll gradients in the presence of 25 mM EGTA reflects a high degree of purity of the vesicles and not an inactivation of the ATPase by the high EGTA concentration used.

The distribution of the acid phosphatase on the sucrose gradient (Fig. 6E, F) suggests a co-purification of Mg^{2+} ATPase and this enzyme. Therefore, the Mg^{2+} ATPase found in the secretory vesicles, when prepared on 0.1 mM EGTA/30% Percoll gradients, is present in a different compartment and could be of lysosomal origin.

DISCUSSION

The determination of kinetic data for Ca^{2+} uptake and release by secretory vesicles requires that the vesicles must be stable and do not lose intravesicular Ca^{2+}, even if large Ca^{2+} gradients exist across the vesicle membrane. Also, Ca^{2+} fluxes should only occur by the mechanism analysed (e.g. Na^{+}/Ca^{2+} exchange) and not concomitantly in other ways.

Isolated secretory vesicles, prepared under isotonic conditions on Percoll gradients, are stable and leak little intravesicular hormone (10) or Ca^{2+} even in case of steep Ca^{2+} gradients (with EGTA in the incubation medium) (Fig. 1). Since Ca^{2+} uptake by neurohypophysial secretory vesicles did not exhibit typical saturation with increasing Ca^{2+} concentrations, a second process, namely Ca^{2+}/Ca^{2+} exchange may actually have contributed to a certain extent to the observed Na^{+} dependent Ca^{2+} uptake (7). Ca^{2+} induced Ca^{2+} release from neurohypophysial secretory vesicles can be demonstrated directly (Fig. 1). This process (Ca^{2+}/Ca^{2+} exchange) is inhibited by Mg^{2+} (Fig. 2). On the other hand, Na^{+}/Ca^{2+} exchange is not affected by Mg^{2+}, neither in secretory vesicles from the neurohypophysis, nor from adrenal medulla (7, 33). These facts allowed to measure selectively Na^{+} dependent Ca^{2+} uptake (in the presence of Mg^{2+}). Under these conditions, in a double reciprocal plot of the initial velocity of the Ca^{2+} uptake as a function of the Ca^{2+}

concentration a straight line (saturation) was obtained (Fig. 3). This indicated the presence of a carrier with high affinity (K_m approx. = 7 x 10^{-7} M) transporting maximally 1.5 nmol Ca^{2+} x mg^{-1} x min^{-1} across the vesicular membrane in both NTA and EGTA buffers.

It is not likely that the Ca^{2+}/Na^{+} exchange observed occurred to cell membrane vesicles present as impurities. Neither the analysis of gradients composed of 50% Percoll/25 mM EGTA or 30% Percoll has provided any evidence for cell membrane fragments in this preparation. This is in accordance with earlier findings by Gratzl et al. (10). Særmark et al. (7) also found that Li^{+} has a much smaller effect on Ca^{2+} uptake than Na^{+} which contrasts to the effect of lithium on the Na^{+}/Ca^{2+} exchange in cell membranes. Finally, it has been reported by Særmark et al. (7) that the ratio of Na^{+}/K^{+} ATPase to Ca^{2+}/Mg^{2+} ATPase in neurosecretory vesicles is very different from the ratio in cell membranes from the same terminals. In addition, it was shown (7) that Ca^{2+} uptake to the secretory vesicle fraction was not inhibited by oligomycin in the presence of ATP. Also, no stimulation by ATP was found.

The apparent concentration of Na^{+} (24 mM) within the secretory vesicles analyzed is higher than that in the cytoplasm (7). The concentration of free cytoplasmic Ca^{2+} in several systems varies between 0.1-10 µM (26). The K_M of the Na^{+} dependent Ca^{2+} transporter (0.7 µM) is obviously of interest in this connection. In the presence of Mg^{2+} (in concentrations likely to occur in the cytoplasm) Ca^{2+}/Ca^{2+} exchange does not take place, but Ca^{2+} can be taken up in exchange for Na^{+} by the secretory vesicles. Whether Na^{+} dependent Ca^{2+} efflux from secretory vesicles may also occur in stimulated or resting cells is not easy to evaluate. It should be noted, however, that increased intracellular Na^{+}, elicited by specific inhibition of the Na^{+}/K^{+} ATPase or the Na^{+} ionophore monensin, initiates secretion even in the absence of extracellular Ca^{2+} (27). This means that the amount of Ca^{2+} present in secretory vesicles, which can be released by Na^{+}, would be easily sufficient to trigger exocytosis.

Approximately 35 mM Na^{+} are required for half maximal inhibition of Ca^{2+} uptake by the secretory vesicles. When preloaded with Ca^{2+} half maximal release of Ca^{2+} from the vesicles requires roughly the same Na^{+} concentration (7). Direct measurements of Na^{+} uptake by secretory vesicles preloaded with Ca^{2+} was half maximal at 20 mM (see Results). Since we cannot exclude Na^{+}/Na^{+} exchange to take place during the experiments, we might have underestimated the affinity of the system for Na^{+}. The data presented here do not allow to determine directly the coupling ratio between the amount of Na^{+} and Ca^{2+} transported. However, using the data from Fig. 1 an efflux rate of Ca^{2+} of 8.2 nmol x 10 min^{-1} x mg^{-1} protein was found with 130 mM NaCl in the extravesicular space. The maximal uptake rate of Na^{+} was 12.9 nmol x 10 min^{-1} x mg^{-1} protein under the same con-

ditions (Fig. 4). This suggests a coupling ratio of 1.6. We have obtained earlier a coupling ratio of 1.44 from the evaluation of the inhibition of Ca^{2+} uptake by Na^{+} (7). The precise stoichiometry of the process as well as the demonstration of possible concomitant charge shifts deserve further experimental work.

All data described above indicate that probably 2 Na^{+} will be transported across the secretory vesicle membrane in exchange for one Ca^{2+}. It could be predicted, therefore, that release of Ca^{2+} from the vesicles would result in an increase of the osmolality of the intravesicular fluid due to two moles Na^{+} taken up per mole of Ca^{2+} released. Concomitant osmotic uptake of water and vesicle swelling would change the optical density of secretory vesicles suspensions. That this actually happens is shown in Fig. 5. Addition of 130 mM NaCl to vesicles preloaded with Ca^{2+} results in a marked reduction of the optical density of the suspension, without leakage of hormone from the vesicles. This effect was specific for Na^{+} since 130 mM KCl did not cause any changes. Obviously, Na^{+} uptake linked to Ca^{2+} release can be measured both by the isotope technique described above, as well as the analysis of turbidity measurements. Similar approaches have been made in order to demonstrate coupled transport of metabolites and ions by cell membrane vesicles (22).

Using a secretory vesicle fraction isolated from the neurohypophysis a low pH has been measured in the interior and a membrane potential across the vesicle membrane (28, 29). Such gradients might provide the driving force for the Ca^{2+} accumulation and of other components present in these vesicles (e.g. Na^{+}). In analogy to findings with adrenal medullary secretory vesicles (30) the existence of a Mg^{2+} ATPase has been suggested to provide the energy for such gradients also across neurohypophysial secretory vesicle membranes (29, 31). However, we did not detect Mg^{2+} ATPase activity in the neurohypophysial secretory vesicle fractions recovered from density gradients composed of 50% Percoll (and 25 mM EGTA in the medium) (Fig. 6B).

In order to try to resolve the discrepancy on the presence of Mg^{2+} ATPase in the neurosecretory vesicles we isolated the vesicles according to a procedure described earlier (11) using 30% Percoll as a gradient material only modified in the way that we added 0.1 mM EGTA to the medium. Actually in such gradients Mg^{2+} ATPase could not be separated from the secretory vesicle fractions (Fig. 6C, D) and the specific activity of the enzyme in the secretory vesicle fraction recovered amounted to 2.1 µmol/mg/h. This is close to the value reported earlier (24) for secretory vesicle fractions made on sucrose--metrizamide gradients and similar to the activity found in a cell membrane fraction prepared from the same tissue (32).

Two possibilities were tested to explain the different distribution of the Mg^{2+} ATPase in the two types of gradients. First, the enzyme could be inactivated by the presence of 25 mM EGTA in the media used during incubation and thus not be detected on the 50% Percoll gradient; secondly, the enzyme activity could be present in another subcellular structure co-purifying with the secretory vesicles on the 30% Percoll gradient. The first possibility could be ruled out by experiments describing the unchanged enzymatic activity of Mg^{2+} ATPase after prolonged incubation in media containing 25 mM EGTA (see Results). Evidence for the presence of other subcellular structures (e.g. lysosomes) in the secretory vesicle fractions prepared on the 30% Percoll gradient is provided by the observation that acid phosphatase, β-glucuronidase, as well as Mg^{2+} ATPase activities, form a second band in the 30% Percoll gradient fractions containing the highest amounts of vasopressin (Fig, 6C, D). When these fractions were put on a discontinuous sucrose gradient, secretory vesicles could be separated from other structures exhibiting Mg^{2+} ATPase as well as acid phosphatase activity (Fig. 6E, F).

Thus, with the reservation that some unknown activator is missing in our system, we conclude that there is no Mg^{2+} ATPase in the neurosecretory vesicles. This conclusion is in accordance with previous work carried out by Vilhardt & Hope (12) in which, by means of rate zonal centrifugation for a rather long time, the distribution of vasopressin and Mg^{2+} ATPase was distinctly different.

In the light of the arguments presented, measurements of ΔpH as well as membrane potential, sensitive to Mg^{2+} ATP, carried out with isolated secretory vesicles must be interpreted with caution, since both properties are linked to a proton pumping Mg^{2+} ATPase which we have not been able to find in secretory vesicles purified by different methods. It is for future work to establish the mechanisms of forming and maintaining driving forces for the transport processes that we have described in this paper.

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