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Ca^{2+} UPTAKE TO PURIFIED SECRETORY VESICLES FROM BOVINE NEUROHYPOPHYSES

TORBEN SÆRMARK [a], HEIDEMARIE I. KRIEGER-BRAUER [b], NIELS A. THORN [a] and MANFRED GRATZL [b]

[a] *Institute of Medical Physiology C, University of Copenhagen, DK-2200 Copenhagen (Denmark) and* [b] *Department of Physiological Chemistry, University of Saarland, D-665 Homburg/Saar (F.R.G.)*

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Purified secretory vesicles isolated from bovine neurohypophyses were found to take up Ca^{2+} when incubated at 30°C in media containing 10^{-7} to 10^{-4} M free Ca^{2+}. At 10^{-4} free Ca^{2+} 19 nmol/mg protein were taken up within 30 min. The initial uptake at this Ca^{2+} concentration was about 2 nmol/mg protein per min. The uptake of Ca^{2+} to secretory vesicles was not affected by ATP, oligomycin, ruthenium red, trifluoperazine, Mg^{2+} or K^{+}, but was inhibited by Na^{+} and Sr^{2+}. From these characteristics it can be concluded that the uptake system does not utilize directly ATP (as the Ca^{2+}-ATPases known to be present in the cell membrane and the endoplasmic reticulum) and is different from the mitochondrial Ca^{2+} uptake system driven by respiration and/or ATP hydrolysis. However, Ca^{2+}-Na^{+} exchange may well operate: In experiments using different concentrations of Na^{+} we found half-maximal inhibition of Ca^{2+} uptake with 33.3 mM Na^{+}. An analysis of the data in a Hill plot indicated that at least 2 Na^{+} would be exchanged for 1 Ca^{2+}. Also, it was found that Ca^{2+} previously taken up could be released again by external Na^{+} but not by K^{+}.

Introduction

It is generally accepted that the intracellular concentration of free Ca^{2+} is maintained at a low value by processes in the cell membrane, by the endoplasmic reticulum and microvesicles, as well as by the mitochondria [1–3]. In secretory cells the regulatory systems also reduce the elevated concentrations of free Ca^{2+} that occur after stimulation to resting levels.

Several of the Ca^{2} transporting systems just mentioned have been studied in some detail with subcellular fractions from the neurohypophysis that contains the nerve terminals of a 'classical' neurosecretory system (cf. Ref. 4).

The hormone-containing neurohypophysial secretory vesicles within the terminals also contain Ca^{2+}, as shown both by electron microprobe analysis of intact tissue [5] as well as by analysis of vesicles isolated by subcellular fractionation [6]. Secretory vesicles therefore could contribute to the sequestration of Ca^{2+}. These vesicles, in contrast to other intracellular structures present within the nerve endings, would have the advantage that accumulated Ca^{2+} can be released again from the vesicles into the extracellular space during exocytosis.

In the present investigation we describe an influx of Ca^{2+} into isolated secretory vesicles from the neurohypophysis, as well as effects of monovalent and divalent cations and a variety of substances known to influence Ca^{2+} transport in other systems on this process. The experiments indicate that the Ca^{2+} influx into secretory vesicles proba-

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulfonic acid.

bly does not derive its energy directly from the hydrolysis of ATP. The data presented are in accordance with an Na^+-Ca^{2+} exchange system present in isolated neurohypophysial secretory vesicles.

Experimental procedures

Isolation of secretory vesicles. Bovine hypophyses were obtained immediately after slaughtering at Københavns Eksportslagteri and Slagelse Andelsslagteri, and the neural lobes were isolated by dissection and placed in icecold buffer containing 190 mM sucrose, 25 mM EGTA, 20 mM Tes (pH 7.0). KOH was used to adjust the pH. Homogenization of the tissue in the laboratory was finished not later than 2 h from slaughtering.

Purified secretory vesicles were obtained by a newly developed method employing differential and density gradient centrifugation on isoosmolar Percoll®/sucrose gradients [7]. This method, in contrast to previous ones using sucrose gradients, yields vesicles that have a very small contamination with mitochondria and are stable on incubation in isosmolar media. Secretory vesicles obtained from the Percoll®/sucrose gradient and sedimented by centrifugation at $100\,000 \times g$ for 1 h were resuspended and submitted to gel filtration on BioGel A 150 (BioRad) to remove Percoll.

The specific activity of vasopressin in the band of vesicles selected was 37.4 ± 8.5 (mean ± S.D. of five preparations) μg vasopressin per mg protein, the specific activity of oxytocin was 20.8 ± 7.5 (mean ± S.D. of nine preparations) μg oxytocin per mg protein.

Measurement of Ca^{2+} uptake. After gel filtration the secretory vesicles were centrifuged at $60\,000 \times g$ for 15 min and resuspended in 260 mM sucrose, 0.5 mM EGTA, 20 mM Tes (pH 7.3). Resuspension was carried out by hand in a loose-fitting Teflon-to-glass homogenizer (volume 1 ml).

Secretory vesicles (50 μg protein) were added to the uptake medium containing 260 mM sucrose, 0.5 mM EGTA, 20 mM Tes (pH 7.3). 20 mM KCl was included if nothing else is stated. pH was adjusted with KOH. The final volume was 200 μl. Ca^{2+} was included in the uptake medium to give various free Ca^{2+} concentrations calculated as described [8] taking into account the known stability constants. $^{45}Ca^{2+}$ was added to the solutions to give a specific activity of 5 μCi/mmol Ca^{2+}. The free Ca^{2+} concentration was checked by use of a calcium specific electrode [9]. The uptake medium was kept at 30°C for 5 min before addition of secretory vesicles in a volume of 50 μl. After incubation (10 min unless otherwise stated) the vesicles were separated from the medium by filtration (Millipore EGWP 02500 0.2 μm). The filters were washed with 2×5 ml of icecold incubation buffer containing no Ca^{2+}, placed in counting vials, dried at 80°C for 1 h, and cooled to room temperature. The filters were treated with 500 μl NCS tissue solubilizer for 5 min before addition of 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 130 mM LiCl or various concentration of NaCl were used in the incubation medium instead of sucrose. In these cases the filters were washed with the same medium.

Measurement of Ca^{2+} efflux. Samples incubated under uptake conditions (10^{-4} M free Ca^{2+} + 130 mM KCl) for 15 min were diluted 10-fold in a medium containing either 130 mM NaCl or KCl. In addition, this medium 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. There was no difference in the amount of $^{45}Ca^{2+}$ measured whether the samples had been diluted immediately before filtration or not. Neither was there any difference between the solutions used for dilution. Filtration and washing of the filters as well as the determination of $^{45}Ca^{2+}$ was done as described for uptake.

In 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.

Measurement of Mg^{2+}, Ca^{2+}, Na^+, K^+, oxytocin, vasopressin, and protein. Ca^{2+} and Mg^{2+} were measured by atomic absorption spectroscopy, Na^+ and K^+ by flame photometry in a FL 9 instrument (Zeiss). Vasopressin was measured by radioimmunoassay as described in Ref. 10, oxytocin by a similar assay. Protein was determined by

the use of fluorescamine (Fluram, Roche [11]).

Materials. $^{45}Ca\ Cl_2$ and NCS tissue solubilizer were obtained from Amersham, Lumagel was from Lumac System AG, Basel. Trifluoperazine hydrochloride (Batch CA 7635900) was a gift from Dr. J. Zylber, Rhone-Poulenc Pharma Norden A/S. It was protected from light and prepared immediately before use. All other chemicals not specified were of the purest grade commercially available.

Results

Ca^{2+} uptake by isolated secretory vesicles at various concentrations of free Ca^{2+} in the presence of Na^+, K^+ or Li^+

When incubated in an Na^+-free medium containing 20 mM K^+ and 10^{-4} M Ca^{2+} secretory vesicles within 30 min took up 19 ± 3.0 nmol Ca^{2+}/mg protein (average of six preparations, mean ± S.D.) (Fig. 1).

Within 10 min Ca^{2+} uptake was linear with time. At a Ca^{2+} concentration of 10^{-4} M after 5 min 9 ± 1 nmol/mg (mean ± S.D.; $n = 5$) was taken up (Fig. 1) giving an initial Ca^{2+} uptake of approx. 2 nmol/min per mg protein. The uptake was proportional to the amount of vesicle protein added.

The uptake was found to be linearly correlated to log[Ca^{2+}] in the range between 10^{-4} and 10^{-7} M free Ca^{2+} (Fig. 2). At a concentration of

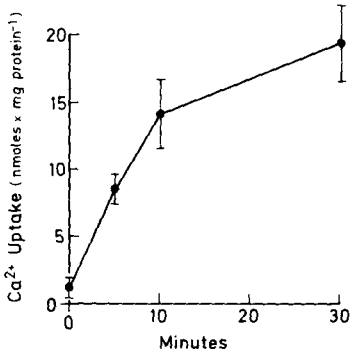

Fig. 1. Time dependence of Ca^{2+} uptake into purified secretory vesicles from ox neurohypophyses. Secretory vesicles were incubated in the presence of 10^{-4} M free Ca^{2+} in 20 mM KCl, 260 mM sucrose, 0.5 mM EGTA, 20 mM Tes (pH 7.3) at 30°C as described in Methods. Filtration and washing of the samples were complete within 30 s. Protein concentration was 50 μg in a volume of 200 μl. (5 expts., bars indicate S.D.)

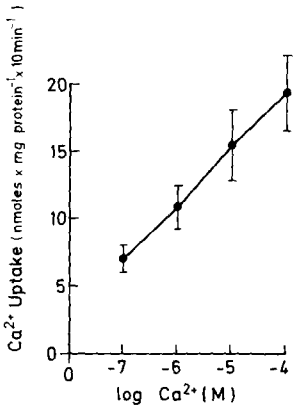

Fig. 2. Ca^{2+} uptake at various concentrations of free Ca^{2+}. Secretory vesicles were incubated at various concentrations of free Ca^{2+} in a medium containing 20 mM KCl, 260 mM sucrose, 0.5 mM EGTA, 20 mM Tes (pH 7.3) at 30°C for 10 min. Protein concentration was 50 μg in a total volume of 200 μl. The calcium concentrations were calculated and controlled by a calcium ion specific electrode, as described in Methods. (5 expts., bars indicate S.D.)

10^{-6} M, which is likely to be compatible with levels of free Ca^{2+} during stimulation in the cytosol of secretory cells, uptake was 11.4 ± 1.4 nmol Ca^{2+}/mg protein (mean ± S.D., $n = 3$) within 10 min (Fig. 2); i.e., at 10^{-6} M free Ca^{2+}, more than 50% of the uptake observed at 10^{-4} M free Ca^{2+} had occurred (Fig. 1). As opposed to low concentrations of free Ca^{2+}, Ca^{2+} uptake to secretory vesicles increased at high concentrations of free Ca^{2+} (10^{-6}–10^{-4} M) only moderately; that is, no typical 'saturation' has been observed.

A low concentration of Na^+ during the experiments was essential for Ca^{2+} influx. At 130 mM Na^+ the uptake was $12 \pm 4\%$ of the control value ($n = 4$), whereas at a KCl concentration of 130 mM Ca^{2+} uptake was $97 \pm 20\%$ of the control ($n = 4$). Lithium at a concentration of 130 mM decreased the Ca^{2+} uptake to $59 \pm 12\%$ of the control ($n = 4$).

In Fig. 3 the inhibition of Ca^{2+} uptake is shown as a function of the extravesicular Na^+ concentration. Ca^{2+} uptake decreased with an increase of the Na^+ concentration in the incubation medium. An analysis of the same data in a Hill plot suggested that 1.44 Na^+ may be exchanged for 1 Ca^{2+}. If the reciprocal uptake of Ca^{2+} was plotted as a function of $[Na^+]^{1.44}$, from the intercept on the x axis, the half-maximal inhibition of Ca^{2+}

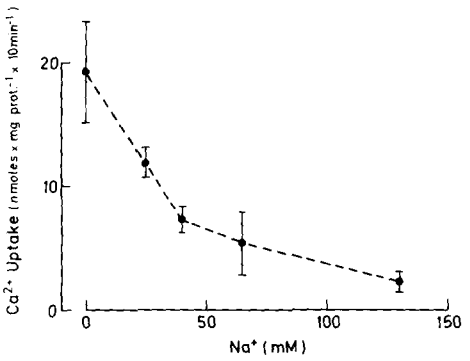

Fig. 3. Inhibition of $^{45}Ca^{2+}$ uptake by increasing concentration of Na^+ in the incubation medium. Secretory vesicles were incubated at 30°C for 10 min in the presence of 10^{-4} M free Ca^{2+}, 0.5 mM EGTA, 20 mM Tes (pH 7.3), increasing concentration of Na^+ and decreasing concentration of sucrose (total osmolarity of medium about 315 mosmol/kg) as described in Methods.

uptake by Na^+ gave a value of 33.3 mM.

2 mM Mg^{2+}, when added to the secretory vesicles during $^{45}Ca^{2+}$ uptake, had no effect but 2 mM Sr^{2+} was strongly inhibitory (Table I). We did not find K^+ within the isolated secretory vesicles but the amount of Na^+ determined in two experiments after careful removal of Percoll was 47 and 57 nmol/mg protein.

The amount of Mg^{2+} present in isolated secretory vesicles was 8.9 nmol/mg protein and that of Ca^{2+} was 12.9 nmol/mg protein (average for two preparations).

Efflux of Ca^{2+} from preloaded vesicles in the presence of various cations

The inhibition of Ca^{2+} uptake of secretory vesicles by Na^+ could be explained by the existence of an Na^+-Ca^{2+} exchange system using an Na^+ gradient across the vesicular membrane (Na^+_{in}/Na^+_{out}) as an energy source which is abolished by adding Na^+. If this were the case, by an inversion of the Na^+ gradient (by increasing the extravesicular Na^+ concentration over the intravesicular one), Ca^{2+} should be released again from the vesicles. To test this hypophysis, secretory vesicles were allowed first to take up Ca^{2+} in a medium containing 130 mM K^+ for 15 min. Then the samples were diluted 10-fold in 130 mM NaCl or KCl in the presence of 0.5 mM EGTA and the amount of $^{45}Ca^{2+}$ in the vesicles was determined with time (Fig. 4). Actually, in the presence of Na^+ an efflux of Ca^{2+} could be measured but in the presence of potassium, calcium could not be released from the vesicles (Fig. 4). This fact is also important for the evaluation of Ca^{2+} uptake with the filtration technique: By addition of an excess of EGTA (and KCl) the free external concentration of Ca^{2+} is lowered to less than 10^{-7} M and the amount of Ca^{2+} within the vesicles remains constant. The release of Ca^{2+} from the vesicles by sodium under the experimental conditions was not complete; this might be due to binding of Ca^{2+} within the vesicles as a component of the hormone storage complex. As shown in

TABLE I

EFFECT OF DIFFERENT INHIBITORS ON Ca^{2+} UPTAKE INTO SECRETORY VESICLES

Secretory vesicles were preincubated in the presence of various inhibitors at 30°C for 5 min in a medium containing 20 mM KCl, 0.5 mM EGTA, 260 mM sucrose, 20 mM Tes (pH 7.3). Then Ca^{2+} with tracer was added (final free concentration 10^{-4} M) in the same buffer with or without 2 mM Mg^{2+}/ATP as stated in the Table. Incubation was carried out for 30 min and the reaction was stopped by filtration as described in Methods. The vesicles took up 19.3 nmol Ca^{2+}/mg protein per 30 min (S.D. ±4, $n = 6$) in the medium containing 20 mM KCl. This value was set as 100 to compare in different experiments the effect of the inhibitors used. The figures represent the mean of at least five experiments (±S.D.) (n.d., not determined).

Addition to medium	$-Mg^{2+}$/ATP	$+Mg^{2+}$/ATP
10^{-4} M Ca^{2+}	100	129(±30)
10^{-4} M Ca^{2+} + Mg^{2+} 2 mM	87(±21)	n.d.
10^{-4} M Ca^{2+} + Sr^{2+} 2 mM	40(± 4)	n.d.
10^{-4} M Ca^{2+} + Ruthenium red 0.050 mM	87(±11)	n.d.
10^{-4} M Ca^{2+} + oligomycin 100 μg/mg protein	n.d.	120(± 7)
10^{-4} M Ca^{2+} + trifluoperazine, 0.010 mM	98(± 7)	n.d.

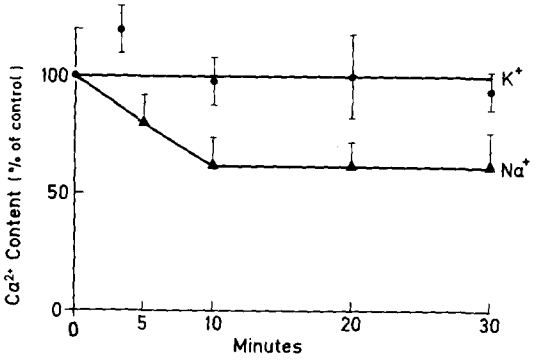

Fig. 4. Influence of K^+ and Na^+ on efflux of $^{45}Ca^{2+}$ from vesicles preloaded with the isotope. Secretory vesicles were incubated at 30°C for 15 min in 0.5 mM EGTA, 20 mM Tes (pH 7.3), 10^{-4} M free Ca^{2+} with tracer, 260 mM sucrose and 20 mM KCl. They were then transferred to media containing 130 mM KCl or NaCl with the sucrose concentration reduced to give an osmolarity of approx. 315 mosmol/kg.

Fig. 5 the efflux of $^{45}Ca^{2+}$ after preloading increased with increasing Na^+ concentration.

Effects of Mg^{2+}, ATP and various inhibitors of Ca^{2+} transport on Ca^{2+} uptake

In the presence of 2 mM Mg^{2+}/ATP Ca^{2+} uptake at a free Ca^{2+} concentration of 10^{-4} M was increased to 129% (± 30, $n = 5$) as compared to values obtained with no Mg^{2+}/ATP added (Table I). This effect probably reflects the presence of some mitochondria in the secretory vesicle fraction, since mitochondria are located above the secretory vesicle fraction on the Percoll gradient and cannot completely be removed from the secretory vesicle fraction [7]. That a crude mitochondria and microsome fraction actually takes up Ca^{2+} under the experimental conditions employed was shown in a separate series of experiments. Here,

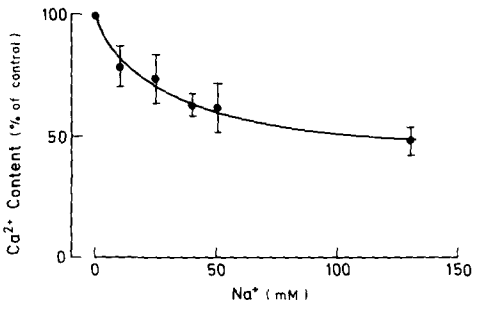

Fig. 5. Na^+ stimulation of efflux by Na^+ in increasing concentrations. Experimental conditions as in Fig. 4.

the remainder of the Percoll gradient [7] was pooled (crude mitochondria and microsomes) and Ca^{2+} uptake was determined in the presence or absence of Mg^{2+}/ATP (2 mM). These membranes took up 69.8 nmol Ca^{2+}/mg protein per 30 min (average of two experiments) at a Ca^{2+} concentration of 10^{-4} M in a medium containing 20 mM KCl. In the presence of 2 mM Mg^{2+}/ATP the uptake was increased to 196 nmol Ca^{2+}/mg protein per 30 min.

Incubation in the presence of mitochondrial inhibitors was carried out to see whether the slight stimulation of Ca^{2+} uptake by ATP of the secretory vesicles fraction could be explained by contamination from mitochondria. Oligomycin is known to inhibit the ATP dependent Ca^{2+} uptake by mitochondria [12]. In the presence of oligomycin (100 μg/mg protein) and Mg^{2+}/ATP (2 mM) Ca^{2+} uptake of the secretory vesicles appeared to be somewhat less compared to the Ca^{2+} uptake measured with Mg^{2+}/ATP in the absence of oligomycin (Table I). FCCP (carboxyl cyanide *p*-trifluoromethoxy phenylhydrazone) is a potent inhibitor of H^+ gradients in mitochondria [13]. In the presence of FCCP (100 μg/ml) the secretory vesicle Ca^{2+} uptake was 97 ± 20% (S.D. $n = 3$) of the control values. Ruthenium red is known to be a very potent inhibitor of Ca^{2+} binding and Ca^{2+} transport in mitochondria [14]. Incubation of secretory vesicles in the presence of Ruthenium red (50 μM) did not affect Ca^{2+} transport significantly. No inhibition was found, either, when concentrations of Ruthenium red up to 1 mM were used (data not shown).

Calmodulin has been found in secretory vesicle fractions isolated from the neurohypophysis [15, 16]. When trifluoperazine (which binds calmodulin and prevents Ca^{2+} calmodulin linked reactions) was added to the incubation medium, no effects were found in the concentrations thought to be specific for calmodulin-trifluoperazine interaction in other systems [17] (10^{-5} M) (Table I). Increasing the trifluoperazine concentration above 10^{-5} M caused increasing inhibition and no uptake could be detected using 1 mM trifluoperazine.

Discussion

Of the principal systems which participate in the control of intracellular free Ca^{2+} in different

cells: the cell membrane, the mitochondria and the endoplasmic reticulum [2] the latter two might be of minor importance in the neurohypophysial nerve terminals. The endoplasmic reticulum is poorly developed and only few mitochondria are present in the terminal dilatations [18]. However, as recognized recently [19], microvesicles are present in the terminals and are able to take up Ca^{2+} in vitro upon addition of ATP.

It is unlikely that the Ca^{2+} uptake observed in the present experiments occurred to plasma membrane vesicles or to microvesicles present as impurities in the secretory vesicle fraction. The secretory vesicle fraction contains no detectable ($Na^+ + K^+$)-ATPase activity [7]. Also, the plasma membrane Ca^{2+}-Na^+ exchange is more sensitive to lithium than to sodium [20] (cf. the smaller sensitivity to Li^+ described earlier). Finally, the ratio of ($Na^+ + K^+$)-ATPase/($Ca^{2+} + Mg^{2+}$)-ATPase in plasma membrane fractions from the neurohypophysis is 3 : 1 [21] as opposed to the undetectable ($Na^+ + K^+$)-ATPase versus a ($Ca^{2+} + Mg^{2+}$)-ATPase activity of 0.07 mol/h per mg protein found in the secretory vesicle fraction [7] and checked in the fraction of the present experiment. As mentioned above, the Ca^{2+} uptake to microvesicles [19] is dependent on ATP in contrast to the uptake found in the present experiments.

Until now, the possible role of secretory vesicles in the regulation of intracellular free Ca^{2+} has been studied very little. Previously a very small ATP dependent uptake of Ca^{2+} by a secretory vesicle fraction from ox neurohypophyses was found [22]. Since it was observed in the present investigation that the slight increase in Ca^{2+} uptake by ATP using highly purified secretory vesicles was inhibited by oligomycin, it is reasonable to assume that the effect of ATP in those earlier studies was mainly due to contaminating mitochondria.

Isolated, purified neurohypophysial secretory vesicles contain Ca^{2+} (as well as Mg^{2+}) and considerable amounts of Na^+ (see Results). Ca^{2+} was taken up by the vesicles when 10^{-7} M to 10^{-4} M free Ca^{2+} was added, provided no Na^+ was present in the incubation medium. In other words, Ca^{2+} uptake could proceed if an Na^+ gradient existed over the vesicular membrane. By decreasing the Na^+ gradient, i.e. when Na^+ is increased in the extravesicular space, Ca^{2+} uptake decreases concomitantly. We made a rough estimate of the intravesicular fluid Na^+ concentration. The internal water space of neurosecretory vesicles can be calculated to be approx. 2.2 μl/mg protein from a water space of vesicles of 57% [23], from an average diameter of the vesicles of 160 nm, a number of neurophysin/vasopressin (oxytocin) molecules of approx. $8 \cdot 10^4$ per vesicle [24], a percentage of soluble protein in vesicles of 65 [25] and a percentage of neurophysin of the total protein of 50 [26]. Using the Na^+ concentration of 52 nmol/mg protein (see Results) this gives an intravesicular Na^+ concentration of 24 mM. It thus appears that the Ca^{2+} uptake is greatly inhibited when the extravesicular Na^+ concentration approaches the intravesicular concentration (Fig. 3).

Mg^{2+} in concentrations found within the cytoplasm (cf. Ref. 3) did not influence Ca^{2+} influx into vesicles but Sr^{2+} was found to be inhibitory. The effect of the Sr^{2+} is shared with several Ca^{2+} transporting systems [2]. The fact that trifluoperazine elicits no effect on the Ca^{2+} influx into neurohypophysial secretory vesicles suggests that calmodulin-linked processes are probably not involved. By contrast an effect of this drug has been described on Ca^{2+} transport by a plasma membrane enriched fraction isolated from bovine neurohypophyses [21].

The following conclusions can be drawn from the observations described above:

(1) The modulation of Ca^{2+} uptake by the Na^+ gradient across the vesicular membrane is compatible with an Na^+-Ca^{2+} exchange across the membrane.

(2) The fact that Ca^{2+} efflux was induced by high concentrations of extravesicular Na^+ is also compatible with an Na^+-Ca^{2+} exchange.

(3) Influx and efflux of Ca^{2+} can be triggered with small variations of the Na^+ concentrations found in mammalian cells (cf. Refs. 2 and 27).

(4) Since secretory vesicles can take up Ca^{2+} from media containing Ca^{2+} in concentrations found in resting cells as well as in stimulated cell the process is likely to be of physiological relevance.

(5) The Hill coefficient for the influence of Na^+ on Ca^{2+} influx (1.4) suggests that at least two Na^+ may be exchanged for one Ca^{2+}. This is also in

accordance with the observation that the amount of Ca^{2+} taken up never exceeds half the amount of Na^+ (25.9 nmol/mg protein) present in isolated secretory vesicles. However, the precise stoichiometry of the Ca^{2+}-Na^+ exchange (as well as the questions concerning a concomitant charge shift across the membrane) remains to be elucidated.

The Ca^{2+} influx and efflux observed with neurohypophysial secretory vesicles in its properties is very similar to the process found in intact secretory vesicles from adrenal medulla [28,29]. The process described so far with intact secretory vesicles from neurohypophysis and adrenal medulla very much resembles the Ca^{2+} for Na^+ exchange that takes place of over the cell membrane of nerve and muscle cells [2].

Secretion of catecholamines from adrenal medulla [30] as well as of vasopressin from the neurohypophysis [31] is affected by ouabain. An increase in cytoplasmic Na^+ concentration under these conditions may not only change the Na^+-Ca^{2+} exchange across the cell membrane but also across the membrane of secretory vesicles and thus may contribute to the stimulatory effect of intracellular free Ca^{2+} on secretion. Whether Ca^{2+} is released from secretory vesicles also during physiological conditions of stimulation remains to be elucidated.

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