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UPTAKE OF Ca^{2+} BY ISOLATED SECRETORY VESICLES FROM ADRENAL MEDULLA

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Intact secretory vesicles isolated from bovine adrenal medulla contain 94 nmol Na^+ per mg of protein, and Ca^{2+} influx into the vesicles is inhibited by increasing concentrations of extravesicular Na^+ (but not of K^+, Li^+ or choline$^+$) or by addition of the Na^+ ionophore monensin. Thus Ca^{2+} influx is determined by the Na^+ gradient across the vesicular membrane. Half maximal inhibition of Ca^{2+} influx occurs with 34 mM Na^+ extravesicularly. The fact that Ca^{2+} can also be released from the vesicles by inversion of the Na^+ gradient provides direct evidence that an Na^+-Ca^{2+} exchange may operate. According to an analysis of the inhibition of Ca^{2+} uptake by Na^+ in a Hill plot 2 Na^+ would be exchanged for 1 Ca^{2+}. Ca^{2+} influx into the vesicles increases with temperature (energy of activation: 16 kcal/mol), can be observed already with 10^{-7} M free Ca^{2+} and increases up to 10^{-4} M Ca^{2+}. Ca^{2+} influx is not affected by Mg^{2+} but Sr^{2+} is inhibitory. Since the process is only slightly influenced by the pH of the incubation medium and is insensitive to Mg^{2+}-ATP or inhibitors of the proton translocating Mg^{2+}-ATPase the electrochemical proton gradient across the vesicular membrane does not affect directly the Ca^{2+} influx into the secretory vesicles. Ca^{2+} uptake is insensitive to ruthenium red and oligomycin.

Introduction

Recently, we have discovered that adrenal medullary secretory vesicles can actually take up Ca^{2+} against an obvious concentration gradient in the presence of K^+ but not in the presence of Na^+ [1].

Ca^{2+} plays an essential role in the regulation of exocytosis. One of the key events triggered by Ca^{2+} is the process of membrane fusion. This has been demonstrated with secretory vesicles from adrenal medulla and other types of tissue [2,3].

Exocytosis is paralleled by an increased level of intracellular free Ca^{2+}. The action of Ca^{2+} is terminated by sequestration into membrane-bounded subcellular systems such as mitochondria and endoplasmic reticulum and/or by its extrusion through the cell membrane and into the extracellular space. Another pathway to remove Ca^{2+} from the cytosol could involve the secretory vesicle itself. This process would have the advantage that accumulated Ca^{2+} could be released from the cell by exocytosis.

The membrane of adrenal medullary secretory vesicles can be regarded as an inside-out cell membrane. This conclusion can be drawn from the

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Abbreviations: EGTA, ethyleneglycol bis (β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; EDTA, ethylenediaminetetraacetate; ATP, adenosine 5'-triphosphate K^+-salt; Mops, 3-(*N*-morpholino)propanesulphonic acid; Mes, 2-(*N*-morpholino)ethanesulphonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid.

asymmetric (intravesicular) distribution of α-bungarotoxin binding sites and acetylcholinesterase [4,5]. In the present investigation we characterized the uptake and release of Ca^{2+} by intact secretory vesicles and show that the system operated in a manner which is compatible with a Ca^{2+}-Na^+ exchange system. Within the cell, the Na^+ gradient across secretory vesicle membrane allows Ca^{2+} uptake into the vesicles. Parts of this work have been presented in abstract form [6].

Materials and Methods

Secretory vesicles were isolated as described recently [4]. The pH in the isolation media was adjusted with KOH. Unless stated otherwise, the isolated secretory vesicles (1–2 mg/ml) were incubated in a volume of 200 μl for different times, temperature, and pH in media (final osmolality of 420 mosmol/kg) containing various concentrations of Na^+, K^+, Li^+, Mg^{2+}, sucrose, ATP, 0.5 mM EGTA and 20 mM Mops (pH 7.3). The total amount of Ca^{2+} required to yield the desired concentration of free Ca^{2+} and in the presence of various substances binding Ca^{2+} and at the pH and the ionic strength employed, were calculated using a computer program taking into account the known values for stability constants as described [7]. In addition the media contained trace amounts of $^{45}Ca^{2+}$. After incubation the uptake of Ca^{2+} was stopped by adding 4 ml of an ice-cold medium containing 1 mM EGTA, 18 mM K^+, 20 mM Mops (pH 7.3) and sucrose to give a final osmolality of 420 mosmol/kg. Secretory vesicles were separated from the medium by filtration (cellulose acetate, pore size: 0.45 μm). The filters were washed with 4 ml of the medium used to stop Ca^{2+} uptake. The filters were dried and $^{45}Ca^{2+}$ was determined by liquid scintillation counting.

The measure the release of $^{45}Ca^{2+}$ from adrenal medullary secretory vesicles in the presence of Na^+, the vesicles were first preloaded with $^{45}Ca^{2+}$ by incubation for 10 min at 37°C in a medium containing 100 μM free Ca^{2+}, 18 mM K^+, 20 mM Mops (pH 7.3), 0.5 mM EGTA and sucrose to give a final osmolality of 420 mosmol/kg. The suspension was diluted with the same (cold) medium containing no Ca^{2+} and spun down at $12000 \times g_{av}$ for 20 min. The pellet was resuspended in a medium containing 220 mM Na^+ (or 220 mM K^+), 20 mM Mops (pH 7.3), 0.5 mM EGTA and the $^{45}Ca^{2+}$ content was than determined in the vesicles as described above.

Na^+, Mg^{2+} and Ca^{2+} were determined in the supernatants of secretory vesicles diluted 1 : 10 with a medium containing 0.76 mM CsCl, 0.1 M HCl, 0.5 mM EDTA and 0.5 mM EGTA. Ca^{2+} and Mg^{2+} was measured by atomic absorption spectroscopy, Na^+ by flame photometry in a FL 9 instrument (Zeiss).

All other procedures used were carried out as described previously [4].

Substances were purchased from the following manifacturers: Ruthenium red from Merck; dicyclohexylcarbodiimide from Sigma; *N*-ethylmaleimide from Serva; oligomycin A from Sigma; K^+ salt of ATP, ionophore A 23187 and monensin from Calbiochem; $^{45}Ca^{2+}$ (24.6 mCi/mg) from New England Nuclear. All other chemicals were of the purest grade commercially available.

Results

A fraction containing mitochondria and secretory vesicles (P_2) has been obtained from adrenal medullary homogenates by differential centrifugation as described [4]. Secretory vesicles can be separated from the other subcellular components by the use of a self generating gradient of Percoll™ [4]. As shown in Fig. 1 (bottom) two main bands can be observed in the gradient (see protein profile). One band, centered around fraction 9, consists of secretory vesicles and is characterized by a high content of adrenalin, noradrenalin and ascorbate [4]. We have determined the absorbance at 280 nm in the fractions of the gradient shown in Fig. 1 after precipitation of the protein with 10% trichloroacetic acid. In this way, catecholamines as well as nucleotides can be located conveniently. Presumably, this measures catecholamines and nucleotides contained within adrenal medullary secretory vesicles as well as nucleotides in the mitochondria. Mitochondrial activity (glutamate dehydrogenase) exhibits a peak around fraction 24 (see Fig. 1, bottom). No glutamate dehydrogenase has been observed in the secretory vesicle fractions of high density (Fig. 1). Secretory vesicles contain latent acetylcholinesterase activity (Fig. 1). This is

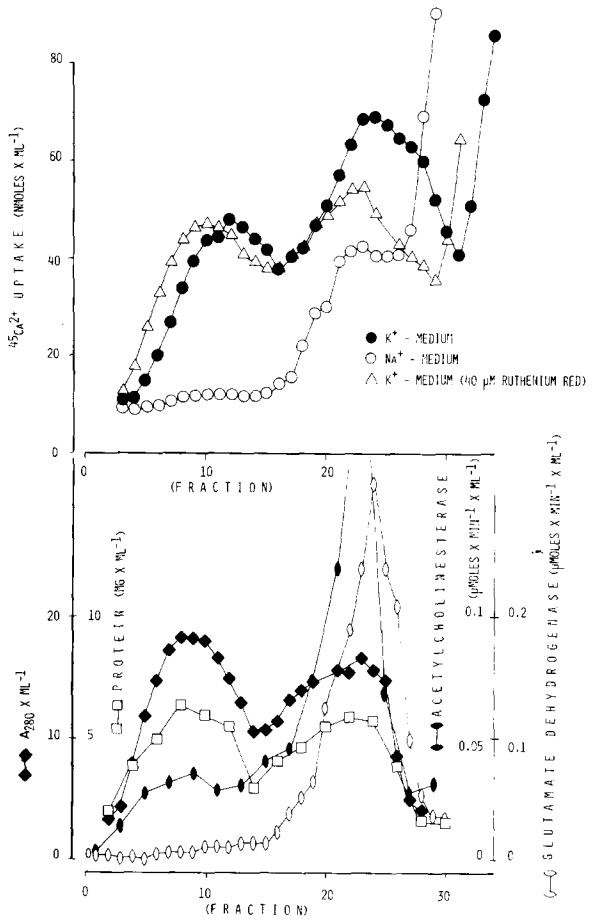

Fig. 1. $^{45}Ca^{2+}$ uptake by mitochondria and secretory vesicles of adrenal medulla. Fraction P_2 (40 mg protein/ml) of adrenal medulla homogenates [4] which contains mainly secretory vesicles and mitochondria was incubated at 37°C for 10 min in a medium containing: 20 mM Mops (pH 7.3), 210 mM Na^+ acetate (or 210 mM K^+ acetate), 0.5 mM EGTA and Ca^{2+} to give a final concentration of free Ca^{2+} of 100 μM. After incubation, the material was separated on a selfgenerating Percoll™ gradient [4] and analyzed.

Top. Distribution of $^{45}Ca^{2+}$ in the gradient after incubation in a medium containing 210 mM K^+ (●———●), 210 mM K^+ plus 40 μM Ruthenium red (△———△) or 210 mM Na^+ (○———○).

Bottom. Characterization of components present in the gradient. Distribution of protein (□———□, mg/ml), catecholamines plus nucleotides (◆———◆, A_{280}/ml), acetylcholinesterase activity (●———●, μmol acetylthiocholine hydrolyzed per min per ml in the presence of 0.2% Triton X-100) as well as glutamate dehydrogenase activity (○———○, μmol NADH oxidized per min per ml). The distribution of constituents was independent of the medium composition during incubation prior to gradient centrifugation. Fractions taken starting from the bottom of the gradient (most dense).

in accordance with previous findings that this enzyme (actually two types of it) is located within adrenal medullary secretory vesicles [4].

Fraction P_2 was incubated for 10 min with 100 μM free Ca^{2+} containing $^{45}Ca^{2+}$ as a tracer, in a medium supplemented with K^+ (210 mM), EGTA (0.5 mM) and Mops (20 mM, pH 7.3). When this preparation was then separated on the Percoll gradient, a bimodal distribution of radioactivity was observed (Fig. 1, top). According to the markers, $^{45}Ca^{2+}$ had been taken up by secretory vesilces as well as mitochondria (Fig. 1). The high radioactivity of the last few fractions comes from Ca^{2+} that is not taken up by the subcellular fractions and so stays at low densities since the material was placed on the top of the gradient material after incubation. Therefore, Fig. 1 should indicate that fractions were taken starting from the bottom of the gradient. If Na^+ (210 mM) was present during incubation with $^{45}Ca^{2+}$, no uptake of the isotope by secretory vesicles was observed (Fig. 1, top) Ruthenium red, in concentrations known to inhibit the uptake of Ca^{2+} by mitochondria (cf. Ref. 8) did not influence the uptake of Ca^{2+} into secretory vesicles but decreased the uptake of Ca^{2+} into adrenal medullary mitochondria (Fig. 1, top).

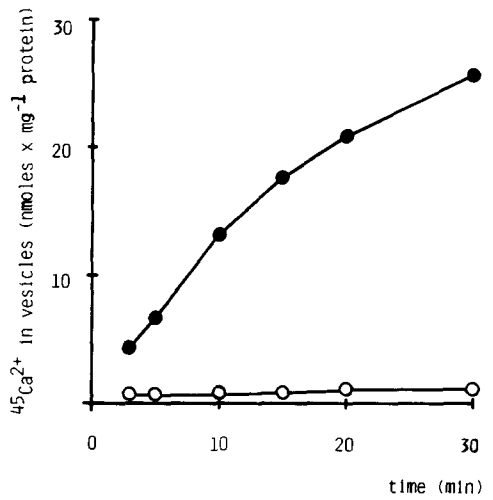

Fig. 2. Time dependence of the $^{45}Ca^{2+}$ uptake by secretory vesicles isolated from bovine adrenal medulla. (Ca^{2+}) free = 100 μM, T = 37°C. An osmolality of 420 mosmol/kg was maintained by addition of sucrose. Incubation medium contained 15 mM K^+ (●———●) or 210 mM Na^+ (○———○), 0.5 mM EGTA and 20 mM Mops (pH 7.3).

TABLE I

EFFECT OF VARIOUS SUBSTANCES ON THE Ca^{2+} UPTAKE BY SECRETORY VESICLES ISOLATED FROM ADRENAL MEDULLA

Adrenal medullary secretory vesicles take up 11.9 nmol Ca^{2+} per mg protein ($n = 12$; S.D. = 2.4) in a medium containing 18 mM K^+, 100 μM free Ca^{2+} at 37°C within 10 min. This value was set as 100 to compare in different experiments the effect of various media and substances present during incubation. The media contained 0.5 mM EGTA and 20 mM Mops (pH 7.3) as well as K^+, Na^+, Mg^{2+}, Sr^{2+} or ATP as indicated in the table, and were supplemented with sucrose to obtain a final osmolality of 420 mosmol/kg.

Medium composition (mM)	Substance added ($\times$ mg^{-1} protein)	Ca^{2+} uptake within 10 min (mean $\pm$ S.D.)	Number of preparations investigated (n)
K^+ (18)		100	
K^+ (220)		85.5 ± 8.7	4
Na^+(220)		5.1 ± 1.6	7
K^+ (18) + Sr^{2+} (2)		33.1 ± 1.4	2
K^+ (18) + Mg^{2+} (2)		90.3 ± 3.1	4
K^+ (18) + Mg^{2+}-ATP(2)		97.1 ± 14.3	8
K^+ (18)	*N*-ethylmaleimide (200 nmol)	120.5 ± 19.5	2
K^+ (18) + Mg^{2+}-ATP(2)	*N*-ethylmaleimide (200 nmol)	106.5 ± 10.5	2
K^+ (18)	dicyclohexylcarbodiimide (60 nmol)	77.5 ± 0.5	2
K^+ (18) + Mg^{2+}-ATP(2)	dicyclohexylcarbodiimide (60 nmol)	70.0 ± 3.0	2
K^+ (18) + Mg^{2+}-ATP(2)	oligomycin A (2 μg)	92.5 ± 0.5	2
K^+ (18) + Mg^{2+}-ATP(2)	Ruthenium red (2 nmol)	94.5 ± 3.5	2

In further experiments we used highly purified adrenal medullary secretory vesicles, prepared as described recently [4]. As shown in Fig. 2, the vesicles in K^+ medium take up Ca^{2+} linearly up to 10 min. After this time the process levels off. In Na^+ medium (212 mM), no Ca^{2+} uptake occurs. In most of the following experiments, Ca^{2+} uptake was followed within 10 min or less, i.e. in the range where Ca^{2+} uptake was linear with time.

To obtain information on the specificity of the inhibition by Na^+ of the uptake of Ca^{2+}, $^{45}Ca^{2+}$ uptake was followed within 10 min in media containing different concentrations of Na^+ or K^+. In the concentrations of monovalent cations used, the uptake of Ca^{2+} was unaffected by K^+ (nor by choline$^+$ or Li^+ (Table I)). In contrast, Na^+ inhibited strongly the uptake of Ca^{2+}, and almost complete inhibition was seen at 50 mM Na^+ or higher concentrations (Table I, Figs. 1, 2 and 3a). Thus, it appears that Na^+ might play an essential role in the uptake of Ca^{2+} by these secretory vesicles.

The inhibition of the uptake of Ca^{2+} by Na^+ can be rationalized if one postulates the existence of an Na^+ gradient across the vesicular membrane as well as a Na^+-Ca^{2+} exchange system. We have tested this postulate in the following ways:

Firstly we analyzed whether Na^+ is actually present within secretory vesicles and have measured the amounts of selected metal cations within the isolated vesicles. We found 94.0 ± 19.6) nmol Na^+, 42.6 ± 11.2 nmol Ca^{2+} and 18.4 ± 2.8 nmol Mg^{2+} per mg protein (mean of four preparations, S.D.). The levels of Ca^{2+} and Mg^{2+} are somewhat lower than those found by other workers (cf. Ref. 10) but this is probably due to the presence of a chelator during our subfractionation procedure [4]. The internal water space of adrenal medullary secretory vesicles has been estimated to be about 2μl/mg protein [9]. Assuming that Na^+ is free in the space within the vesicles, its apparent intravesicular concentration would be: 47 mM Na^+.

The intravesicular 'pool' of Na^+ is easily sufficient to provide an Na^+ gradient and to balance the uptake to Ca^{2+} if indeed an exchange mechanism operates. Also, the apparent intravesicular concentration of Na^+ is comparable to those concentrations of Na^+ found inhibit Ca^{2+} uptake

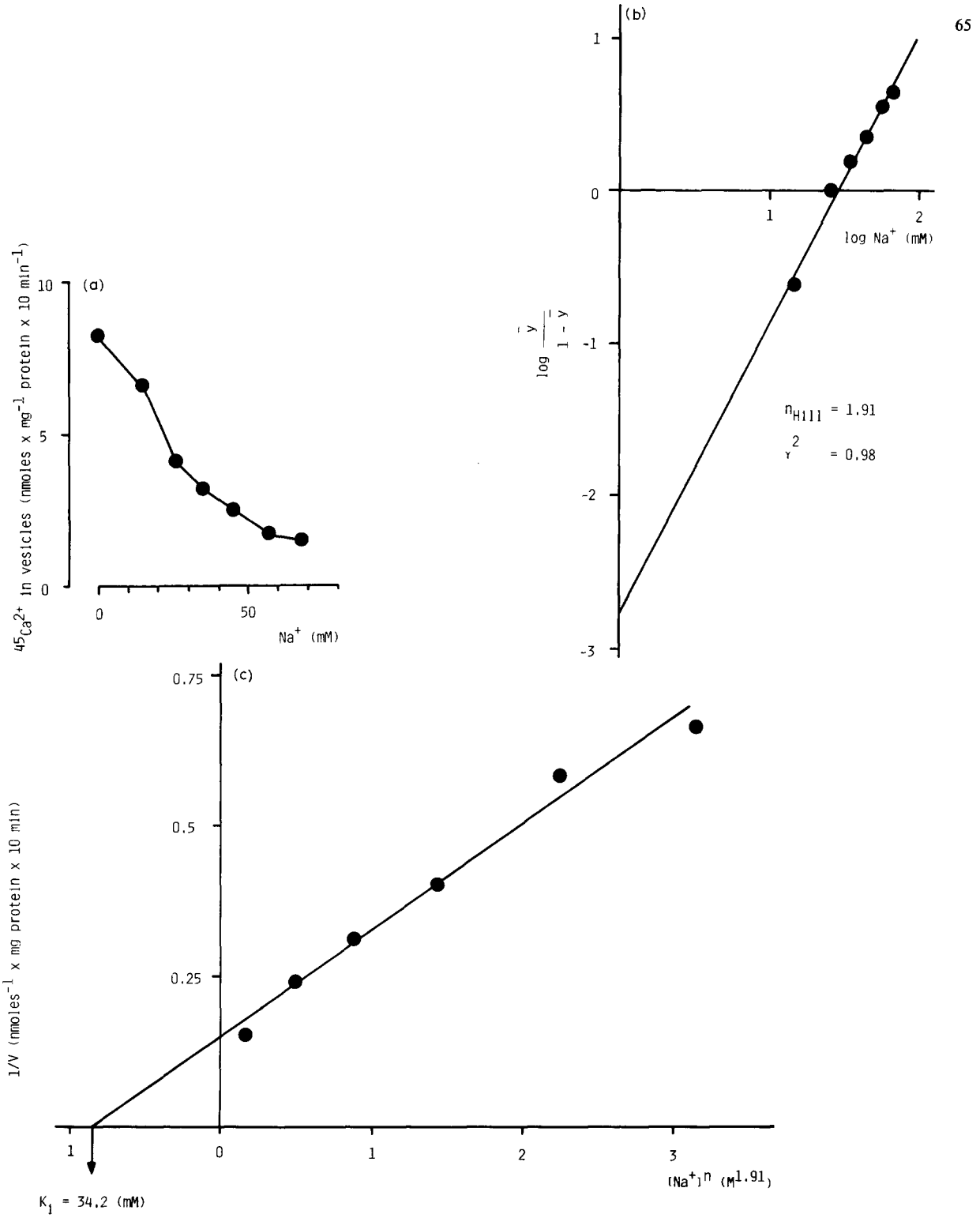

Fig. 3. (a) Effect of Na^+ on the $^{45}Ca^{2+}$ uptake by secretory vesicles isolated from bovine adrenal medulla. The vesicles were incubated for 10 min at 30°C with 100 μM free Ca^{2+} in media containing different concentrations of NaCl. An osmolality of 420 mosmol/kg was maintained by addition of sucrose.

(b) Hill plot of the data shown in Fig. 3a. $\bar{y}$ = fractional inhibition of Ca^{2+} uptake by Na^+. The linear dependence was fitted to the experimental points by linear regression analysis.

(c) Reciprocal uptake of Ca^{2+} as a function of $[Na^+]^{1.91}$ Data from Fig. 3a.

(Fig. 3a). This is as would be expected if the uptake of Ca^{2+} is inhibited by a decrease of the Na^+ gradient across the vesicular membrane. An analysis of the data concerning the inhibition of Ca^{2+} uptake by Na^+ in Hill plot suggests that 1.91 Na^+ would be exchanged for 1 Ca^{2+} (Fig. 3b). This stoichiometry is also supported by the observation that the vesicles never took up more Ca^{2+} than half the amount of Na^+ present within the vesicles. If the reciprocal uptake of Ca^{2+} is plotted as a function of $[Na^+]^{1.91}$ from the intercept on the x-axis, the half maximal inhibition of Ca^{2+} uptake by Na^+ gives a value of 34.2 mM (Fig. 3c).

Our second test of the postulate was to push the exchange into reverse by an inversion of Na^+ gradient across the vesicular membrane. Vesicles preloaded with $^{45}Ca^{2+}$ were suspended in media containing Na^+ or K^+ (220 mM) plus 0.5 mM EGTA. Only in the Na^+ medium was Ca^{2+} release observed (Fig. 4). This further supports the postulate that an Na^+-Ca^{2+} exchange system exists within chromaffin secretory vesicle membrane.

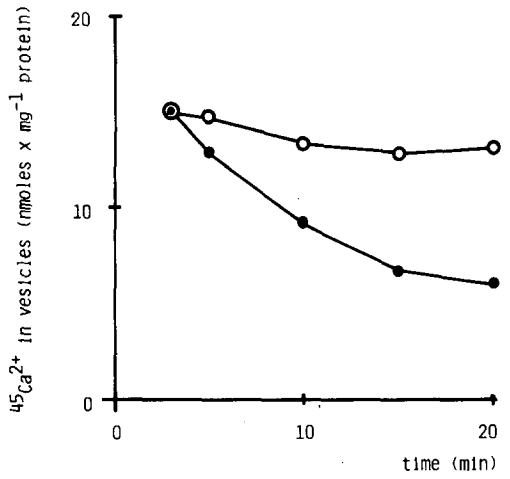

Fig. 4. Effect of K^+ and Na^+ on the release of $^{45}Ca^{2+}$ from secretory vesicles isolated from adrenal medulla. Secretory vesicles which had taken up $^{45}Ca^{2+}$ for 10 min at 37°C in a medium containing 100 μM free Ca^{2+}, 18 mM K^+, 20 mM Mops (pH 7.3), 0.5 mM EGTA and sucrose to give a final osmolality of 420 mosmol/kg, were diluted with the same medium (cold) containing no Ca^{2+} and spun down at 12000× g_{av} for 20 min. The pellet was resuspended in media containing 220 mM Na^+ (●——●) or 220 mM K^+ (○——○), 20 mM Mops (pH 7.3), 0.5 mM EGTA and the $^{45}Ca^{2+}$ content in the vesicles was determined after incubation (37°C) at different times.

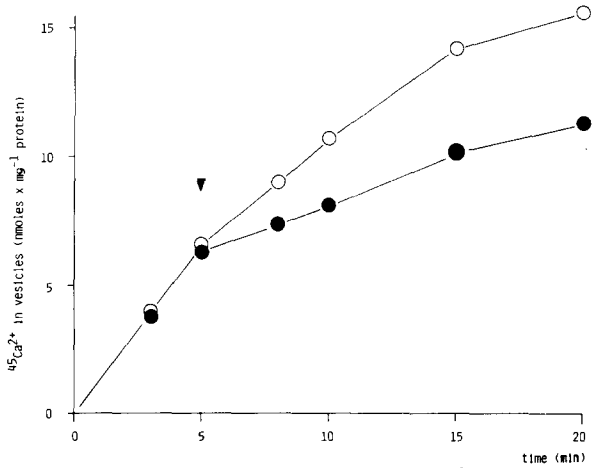

Fig. 5. Effect of the ionophore monensin on the $^{45}Ca^{2+}$ uptake by secretory vesicles isolated from adrenal medulla. The vesicles were incubated at 30°C in 0.5 mM EGTA, 20 mM Mes (pH 5.5), 100 μM free Ca^{2+}, 18 mM K^+ and sucrose to give a final osmolality of 420 mosmol/kg. To one sample monensin (11 μg/mg protein) was added (arrowhead) after 5 min of incubation (●——●), the other sample served as a control (○——○).

Thirdly we have investigated the effect of the Na^+ ionophore monensin on Ca^{2+} uptake by isolated secretory vesicles. Actually in the absence of external Na^+, Ca^{2+} uptake was decreased in the presence of the ionophore compared to the control carried out with no ionophore added (Fig. 5). Since the inhibition by monensin was not complete, this subtance at pH 7.3 obviously does not cause an immediate breakdown of the Na^+ gradient during the experiment.

Ca^{2+} is also taken up by the vesicles after addition of the Ca^{2+} ionophore A23187 (Fig. 6). In the presence of the ionophore vesicles took up Ca^{2+} almost instantaneously both in K^+ medium as well as in Na^+ medium. In K^+ medium, after a rapid uptake due to the ionophore a further slow uptake of Ca^{2+} was evident which however was slower than before addition of ionophore. In Na^+ medium, after Ca^{2+} uptake in the presence of the ionophore, Ca^{2+} leaked out from the vesicles with time (Fig. 6). The latter process is probably in part due to the Na^+ induced release of Ca^{2+} described above. The high amount of Ca^{2+} in the vesicles, when Ca^{2+} entry is aided by A23187 points to the fact that secretory vesicles are capable to store large quantities of Ca^{2+}, possibly as an internal

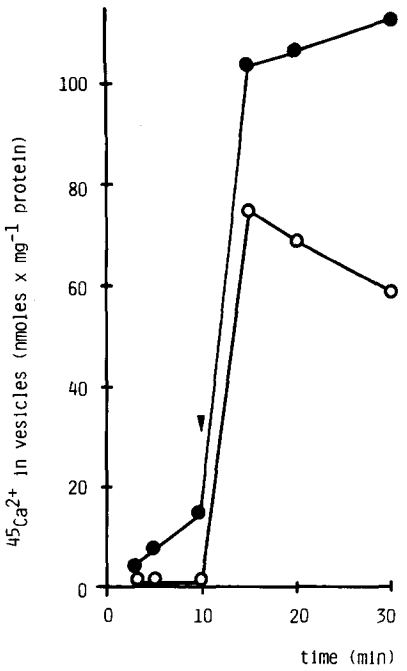

Fig. 6. Effect of the ionophore A23187 on the $^{45}Ca^{2+}$ uptake by scecretory vesicles isolated from bovine adrenal medulla. The vesicles were incubated at 37°C with 100 μM free Ca^{2+} along with 18 mM K^+ (●———●) or 212 mM Na^+ (○———○). An osmolality of 420 mosmol/kg was maintaned by addition of sucrose. After 10 min of incubation (arrowhead) A23187 (2 μg/ml final) was added.

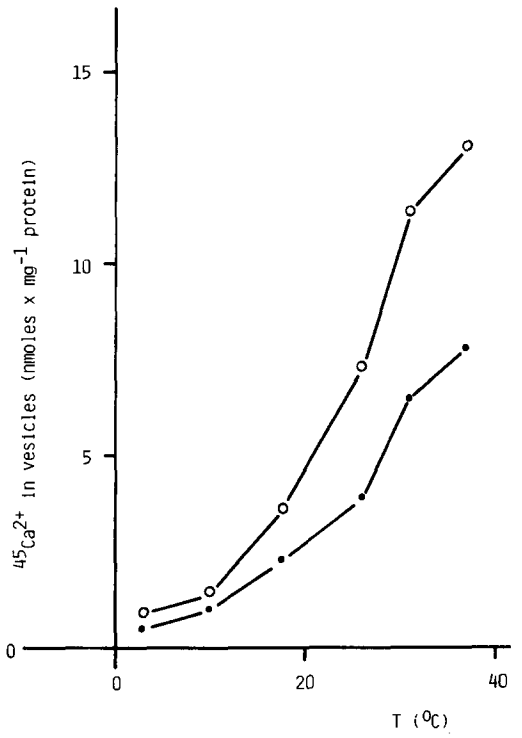

Fig. 7. Temperature dependence of the $^{45}Ca^{2+}$ uptake by secretory vesicles isolated from bovine adrenal medulla. The vesicles were incubated with 100 μM free Ca^{2+} in K^+ medium (18 mM). An osmolality of 420 mosmol/kg was maintained by addition of sucrose. Incubations were carried out for 5 (●———●) or 10 min (○———○).

catecholamine-nucleotide-protein complex (cf. Ref. 10). The Ca^{2+} uptake by the secretory vesicles from medium containing 100 μM free Ca^{2+} is temperature dependent (Fig. 7). The curve obtained after 5 min is similar to the one obtained after 10 min of incubation. From the uptake of Ca^{2+} between 3°C and 30°C we have calculated an energy of activation of about 16 kcal/mol.

The concentration of Ca^{2+} (100 μM) used in the experiments described so far may be compatible with the concentration of free Ca^{2+} in stimulated secretory cells. In further experiments we tried to find out whether secretory vesicles are able to take up Ca^{2+} also from solutions containing free Ca^{2+} in concentrations found in resting cells. In media containing K^+, Ca^{2+} uptake occurs already with 0.1 μM free Ca^{2+} (Fig. 8). As shown after 10 min of incubation, Ca^{2+} uptake by the secretory vesicles increased roughly by a factor of 2 between 0.1 μM and 100 μM free Ca^{2+} (Fig. 8). If the Ca^{2+} uptake data shown in Fig. 8 are plotted as a function of the Ca^{2+} concentration on a linear scale it is evident that only a partial saturation of the process takes place.

The uptake of Ca^{2+} by secretory vesicles in media varying pH values is shown in Fig. 9. The uptake increased slightly with increasing pH. It is known that isolated secretory vesicles from adrenal medulla posses a transmembrane electrochemical gradient for protons (cf. Ref. 10). The relative insensitivity of Ca^{2+} uptake to large changes in proton concentration seen in Fig. 9 argues against a major importance of thos proton gradient for Ca^{2+} accumulation. In further experiments, we have focussed our attention on the Mg^{2+}-ATPase present within the chromaffin secretory vesicle membrane, which transports H^+ into the secretory vesicles (cf. Ref. 10).

Additional proton accumulation within the secretory vesicles brought about by 2 mM Mg^{2+}-

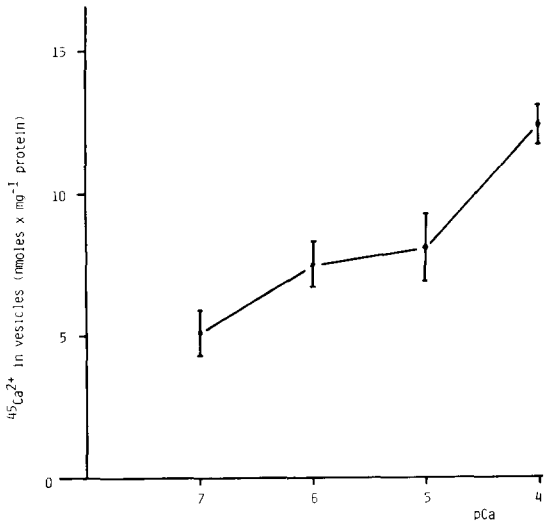

Fig. 8. Uptake of $^{45}Ca^{2+}$ by secretory vesicles isolated from bovine adrenal medulla as a function of the free concentration of Ca^{2+}. The vesicles were incubated at 37°C in a medium containing 18 mM K^+, 0.5 mM EGTA and 20 mM Mops (pH 7.3). An osmolality of 420 mosmol/kg was maintained by addition of sucrose. Incubations were carried out for 10 min (mean of three preparations investigated, S.D.).

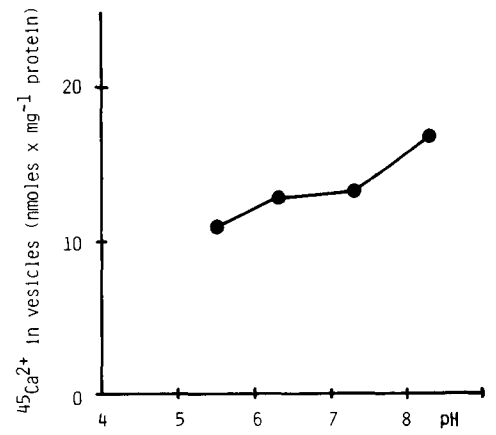

Fig. 9. pH dependence of the $^{45}Ca^{2+}$ uptake by secretory vesicles isolated from bovine adrenal medulla. The vesicles were incubated at 37°C with 100 μM free Ca^{2+}. Constant pH during incubation was maintained with 20 mM Mes (pH<7) with 20 mM Mops (pH 7.3) and or with 20 mM Hepes (pH>7.3). All incubation media contained 18 mM K^+ and sucrose to give a final osmolality of 420 mosmol/kg. Incubations were carried out for 10 min.

ATP does not affect the uptake of Ca^{2+} (Table I). Ca^{2+} uptake by the secretory vesicles was similarly independent of Mg^{2+}-ATP at concentrations of free Ca^{2+} between 10^{-7} M and 10^{-4} M (not shown). Also, 2 mM Mg^{2+} does not influence the Ca^{2+} transport but 2 mM Sr^{2+} was strongly inhibitory (Table I). Chromaffin secretory vesicles contain high concentrations of ATP (cf. Ref. 10). To exclude the possibility that Ca^{2+} uptake is supported by endogenous (leaked out) ATP, we have used known inhibitors of the adrenal medullary secretory vesicles Mg^{2+}-ATPase (F_1), *N*-ethylmaleimide (11), and of the F_0-protein (the proton translocating part), dicyclohexylcarbodiimide [12]. Neither of the two substances was effective in inhibiting the uptake of Ca^{2+} (Table I). As expected (see e.g. Fig. 1) Ca^{2+} uptake by secretory vesicles was insensitive to the inhibitors of mitochondrial Ca^{2+} uptake, oligomycin A and ruthenium red (Table I). It can be concluded from the experiments described in Table I that Ca^{2+} uptake by intact secretory vesicles isolated from adrenal medulla is not directly energized by the proton translocating Mg^{2+}-ATPase and that the process is different from the uptake of Ca^{2+} by mitochondria driven by respiration and/or ATP hydrolysis.

Discussion

Recently, we have observed for the first time that secretory vesicles isolated from adrenal medulla take up Ca^{2+} ($^{45}Ca^{2+}$ uptake as well as net Ca^{2+} uptake) in the presence of K^+ but not in the presence of Na^+ [1]. The results presented in this contribution indicate that the Ca^{2+} uptake into intact secretory vesicles does not derive its energy directly from ATP but from the gradient of Na^+ across the membrane. The observations that Ca^{2+} can be released from the vesicles by Na^+ and that Ca^{2+} uptake is inhibited by extravesicular Na^+ or by addition of the Na^+ ionophore monensin implies that the Ca^{2+} transport (Na^+-Ca^{2+} exchange) may operate in either direction, depending on the difference between the electrochemical gradients for Na^+ and Ca^{2+} across the secretory vesicle membrane.

Several attempts to investigate Ca^{2+} uptake by chromaffin secretory vesicle in vitro have been

made in the past by various groups. It was reported that Ca^{2+} is not taken up by the vesicles unless the ionophore A23187 is added [9]. Probably, the method used in their investigation (measurement of the Ca^{2+} concentration in the extravesicular space using Arsenazo III) was not sensitive enough to detect Ca^{2+} accumulation with no ionophore added. Both, ionophore-dependent and -independent (but Na^+-inhibited) Ca^{2+} uptake by the vesicles was recognized in the present investigation (Fig. 6).

Using secretory vesicles of varying degrees of mitochondrial contamination, it was concluded from a use of inhibitors, that an ATP-dependent uptake for Ca^{2+} exists within the vesicles [13,14]. Using highly purified secretory vesicles [4] we could not confirm an ATP-effect (or of inhibitors such as dicyclohexylcarbodiimide, *N*-ethylmaleimide or Ruthenium red) on Ca^{2+} uptake.

The properties of the Ca^{2+} uptake found with the highly purified secretory vesicles are in some respects consistent with uptake experiments performed with crude secretory vesicles (i.e. containing mitochondria) in which the secretory vesicles were separated from mitochondria on sucrose gradients after Ca^{2+} uptake had taken place [15]; i.e. there is an agreement on the inhibition by Sr^{2+} as well as on the lack of an influence of *N*-ethylmaleimide, Mg^{2+} or Mg^{2+}-ATP on the Ca^{2+} uptake and of its linearity for 10 min. However, the two studies differ in the dependence of the Ca^{2+} uptake on the concentration of Ca^{2+} (we have found uptake already with 0.1 μM free Ca^{2+}) as well as in the absolute amounts of Ca^{2+} taken up (our values are higher). These differences cannot be resolved easily since the values given earlier [15] comprise total Ca^{2+} whilst the values given in this report give the actual free Ca^{2+} concentration present during incubation. Furthermore Ca^{2+} uptake was stopped by addition of a medium containing Na^+ [15] which has been shown in this study to release Ca^{2+} from the vesicles.

Ca^{2+} uptake by the vesicles exhibits only partial saturation with increasing Ca^{2+} concentrations which is in accordance with earlier findings [15]. One reason for this fact might be that components of the secretory vesicle contents (cf. Ref. 10) participate in the accumulation of Ca^{2+}. This is also supported by recent studies, in which we observed that Ca^{2+} uptake by secretory vesicle ghosts (which are devoid of the intravesicular components) saturates at 1 μM free Ca^{2+} (Krieger-Brauer, H. and Gratzl, M., unpublished data).

As opposed to the present investigation, in which stable vesicles prepared according to a new procedure [4] were used, studies on the uptake of Ca^{2+} by secretory vesicles from adrenal medulla have hitherto been hampered by the fragility and concomitant leakage of Ca^{2+} from the isolated vesicles. Similar problems have arisen previously when secretory vesicle ghosts were used to investigate Ca^{2+} transport across the vesicle membrane [16]. Addition of EGTA to this preparation resulted in an instantaneous leakage of Ca^{2+} from the vesicles, which was only slightly modified by Na^+ or K^+. Despite it has been described that roughly two Na^+ may enter the ghosts during efflux of one Ca^{2+} [16]. In this context it is interesting to note that the data concerning the effect of Na^+ on the inhibition of Ca^{2+} uptake by intact secretory vesicles (see Results) are compatible with an exchange of two Na^+ for one Ca^{2+} across the vesicular membrane. It has been reported that Ca^{2+}-Ca^{2+} exchange across the vesicular membrane is inhibited by Mg^{2+} and Ruthenium red [16]. Since we did not find an effect of both substances on Ca^{2+} uptake, Ca^{2+}-Ca^{2+} exchange does not contribute significently to the uptake of Ca^{2+} by intact secretory vesicles which results in a net increase of intravesicular Ca^{2+} [1].

The apparent concentration of Na^+ present within the secretory vesicles is around 3-times higher than that found in the cytosol of mammalian cells. On mechanism of Ca^{2+} extrusion from cells has been identified as Na^+-Ca^{2+} exchange across the cell membrane [17,18] a process that we have shown here to work also in secretory vesicles from adrenal medulla. This means that the Ca^{2+} influx system into secretory vesicles as well as the Ca^{2+} efflux from cells can use Na^+ gradients and transport Ca^{2+} with similar mechanisms.

The Ca^{2+} sequestration within membrane bounded intracellular systems (e.g. mitochondria or endoplasmic reticulum), is limited in extent by the relatively small internal volume of the structures involved unless they can also release Ca^{2+} again. For secretory vesicles this limitation could

be circumvented since Ca^{2+} taken up can subsequently leave the cell during exocytosis.

An inherent puzzle, which is not yet solved, is that secretory vesicles fuse upon addition of Ca^{2+} [2] and that the same organelles are, in addition, able to take up Ca^{2+}. That means the same membrane, within the sequence of events between stimulus and secretion by exocytosis, acts as an acceptor of the signal as well as a 'scavenger' of the signal.

In earlier work [2] high affinity-binding sites for Ca^{2+} have been identified on the secretory vesicle membrane. Occupation of these sites has been found to parallel the fusion of secretory vesicles. The Ca^{2+} binding was studied with secretory vesicle ghosts, i.e. under conditions where no Na^{+} gradient exists across the membrane and under which no uptake of Ca^{2+} takes place (Krieger-Brauer, H. and Gratzl, M., unpublished data). It remains to be established, whether the binding sites found comprise the Na^{+}-Ca^{2+} exchange system or the Ca^{2+} receptor which must be occupied for fusion, or both.

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