Journal of Neurochemistry
Raven Press, New York

0022-3042/83/0901-670/$03.50/0

Effect of Monovalent Cations on Na^+/Ca^{2+} Exchange and ATP-Dependent Ca^{2+} Transport in Synaptic Plasma Membranes

O. P. Coutinho, A. P. Carvalho, and C. A. M. Carvalho

Center for Cell Biology, Department of Zoology, University of Coimbra, Coimbra, Portugal

Abstract: Two Ca^{2+} transport systems were investigated in plasma membrane vesicles isolated from sheep brain cortex synaptosomes by hypotonic lysis and partial purification. Synaptic plasma membrane vesicles loaded with Na^+ (Na^+_i) accumulate Ca^{2+} in exchange for Na^+, provided that a Na^+ gradient (in→out) is present. Agents that dissipate the Na^+ gradient (monensin) prevent the Na^+/Ca^{2+} exchange completely. Ca^{2+} accumulated by Na^+/Ca^{2+} exchange can be released by A 23187, indicating that Ca^{2+} is accumulated intravesicularly. In the absence of any Na^+ gradient (K^+_i-loaded vesicles), the membrane vesicles also accumulate Ca^{2+} owing to ATP hydrolysis. Monovalent cations stimulate Na^+/Ca^{2+} exchange as well as the ATP-dependent Ca^{2+} uptake activity. Taking the value for Na^+/Ca^{2+} exchange in the presence of choline chloride (external cation) as reference, other monovalent cations in the external media have the following effects: K^+ or NH_4^+ stimulates Na^+/Ca^{2+} exchange; Li^+ or Cs^+ inhibits Na^+/Ca^{2+} exchange. The ATP-dependent Ca^{2+} transport system is stimulated by increasing K^+ concentrations in the external medium (K_m for K^+ is 15 m*M*). Replacing K^+ by Na^+ in the external medium inhibits the ATP-dependent Ca^{2+} uptake, and this effect is due more to the reduction of K^+ than to the elevation of Na^+. The results suggest that synaptic membrane vesicles isolated from sheep brain cortex synaptosomes possess mechanisms for Na^+/Ca^{2+} exchange and ATP-dependent Ca^{2+} uptake, whose activity may be regulated by monovalent cations, specifically K^+, at physiological concentrations. **Key Words:** Synaptic membranes—Na^+/Ca^{2+} exchange—ATP-dependent Ca^{2+} transport—Monovalent cations. **Coutinho O. P. et al.** Effect of monovalent cations on Na^+/Ca^{2+} exchange and ATP-dependent Ca^{2+} transport in synaptic plasma membranes. *J. Neurochem.* **41**, 670–676 (1983).

The mechanisms in the plasma membranes of nerve cells that regulate the outward Ca^{2+} transport have been studied in great detail in the squid axon (Baker et al., 1969; DiPolo, 1977; Blaustein, 1977; DiPolo and Beaugé, 1980), since it is possible to control and to measure the intracellular ionic composition (Brinley and Mullins, 1967; DiPolo et al., 1976). It has been established that the extrusion of Ca^{2+} by the axonal membrane occurs by two mechanisms: (1) a Na^+/Ca^{2+} exchange in which the Na^+ gradient is utilized to drive the efflux of Ca^{2+} against its high electrochemical gradient (Blaustein and Hodgkin, 1969; Blaustein, 1977; Mullins and Brinley, 1975), and (2) a Ca^{2+}-ATPase which pumps Ca^{2+} out at the expense of ATP hydrolysis (DiPolo, 1977; 1978; DiPolo and Beaugé, 1979). These two mechanisms appear to be somewhat interlinked, since ATP affects the affinity of the Na^+/Ca^{2+} exchanger (Blaustein, 1977).

The approach used to study the regulation of Ca^{2+} in the squid axon cannot be utilized to study this phenomenon in mammalian nerve terminals *in situ*, but isolated synaptosomes have been widely used for this purpose (Blaustein and Oborn, 1975; Blaustein and Ector, 1976; Goddard and Robinson, 1976; Carvalho, 1978; 1979). More recently, several types of membrane preparations have been obtained from synaptosomes which form vesicles that contain both the Na^+/Ca^{2+} exchanger and the Ca^{2+}-ATPase mechanism for Ca^{2+} transport (Rahamimoff and Spanier, 1979; Michaelis and Michaelis, 1981; Gill et al., 1981). It is assumed that *in situ* these Ca^{2+} transport systems extrude Ca^{2+} from the cell; but it appears that a fraction of the vesicles formed are inside-out, so that the transport of Ca^{2+} is inward in these vesicles (Gill et al., 1981).

We have found that a similar type of vesicle obtained in our laboratory from synaptosomes iso-

Received November 29, 1982; accepted February 18, 1983.
Address correspondence and reprint requests to C. A. M. Carvalho, Department of Zoology, University of Coimbra, 3049 Coimbra Codex, Portugal.

lated from sheep brain cortex also displays both Ca^{2+} transport systems, and that these transport systems are present in the same population of vesicles (Carvalho and Coutinho, 1982). The internal monovalent cationic content of these vesicles can be easily manipulated by equilibration in media of different composition and with the aid of ionophores (Rahamimoff and Spanier, 1979; Gill et al., 1981; Carvalho and Coutinho, 1982). In this paper we report the influence of various ionic gradients and of ATP on the Ca^{2+} transport by these synaptic plasma membrane vesicles. It appears that K^+ specifically stimulates the Na^+/Ca^{2+} exchange and the ATP-dependent Ca^{2+} transport.

MATERIALS AND METHODS

Isolation of synaptic plasma membrane vesicles

Synaptic plasma membrane vesicles were obtained after osmotic lysis of sheep brain cortex synaptosomes according to the method described by Michaelis and Michaelis (1981), with some modifications. The synaptosomal pellets, obtained by the method of Hajós (1975), were washed by resuspension in 0.32 *M* sucrose buffered with 10 m*M* HEPES-Tris, pH 7.4, and centrifugation at 20,000 × *g* for 30 min, as described previously (Carvalho and Carvalho, 1979). The washed pellets were resuspended in 10 volumes of hypotonic medium (5 m*M* HEPES-Tris, pH 8.6) and were stirred at 0–4°C for 15 min. The suspension was centrifuged at 8000 × *g* for 5 min, and the pellets, containing mostly mitochondria and intact synaptosomes, were kept for future analysis. The supernatants were centrifuged at 20,000 × *g* for 30 min to sediment the synaptic plasma membrane preparation. The pellets used for enzymatic characterization were resuspended in 0.32 *M* buffered sucrose, whereas the pellets to be used for Ca^{2+} uptake (Na^+/Ca^{2+} exchange or ATP-dependent Ca^{2+} uptake) were washed and resuspended in ionic medium, usually containing 150 m*M* NaCl or KCl, 1 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* HEPES-Tris, pH 7.4, at a protein concentration of about 10 mg/ml as determined by the biuret method described by Layne (1957), with bovine serum albumin as standard.

This method of preparation of synaptic plasma membrane vesicles yields about 30 mg of membrane protein per 50 g of brain cortex, and has the advantage over others previously described (Michaelis and Michaelis, 1981; Gill et al., 1981) that most of the contamination by mitochondria and intact synaptosomes is eliminated by a short (5 min) centrifugation at low speed (8,000 × *g*) of the lysed synaptosomal suspension.

Calcium uptake assays

Na^+/Ca^{2+} exchange. The synaptic plasma membrane vesicles were prepared for Na^+/Ca^{2+} exchange assays by incubation overnight at 0–4°C in medium containing 150 m*M* NaCl, 1 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* HEPES-Tris, pH 7.4, in order to load the membrane vesicles with Na^+. Other membrane samples were loaded as indicated above, except that the medium contained 150 m*M* KCl, substituted for Na^+; these were utilized as a control for Na^+/Ca^{2+} exchange experiments. Na^+/Ca^{2+} exchange was normally assayed in media containing 150 m*M* KCl or NaCl and 20 μ*M* $CaCl_2$ supplemented with $^{45}CaCl_2$ (0.0025 μCi/nmol). The reaction was initiated by 20-fold dilution of the synaptic membrane suspension (0.5 mg protein/ml final concentration) into the reaction medium at 30°C. At the desired times, the reaction was stopped by filtration of 0.5-ml samples (0.25 mg protein) through glass microfiber filters (Whatman GF/B) prewashed with 10 ml 0.32 *M* sucrose buffered with 10 m*M* Tris-Cl, pH 7.4. After filtration, the samples on the filters were washed with 10 ml of buffered sucrose. The presence of 1 m*M* $LaCl_3$ in this solution gives essentially the same results. Blanks were always determined by filtering the same volumes of reaction media containing $^{45}CaCl_2$, without protein, and were treated the same way as the samples.

The dried filters were placed in vials containing 8 ml of scintillation fluid [composition per liter of toluene: 7.3 g 2,5-diphenyloxazole (PPO), 176 mg *p*-bis -[2-(5-phenyloxazolyl)]benzene (POPOP), and 250 ml Triton X-100], and the radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer, model 460-CD. The quenching of radioactivity in the samples was corrected by using the external standard technique described by the manufacturer in the operation manual.

ATP-dependent Ca^{2+} uptake. ATP-dependent Ca^{2+} uptake was normally assayed in medium containing 150 m*M* KCl or NaCl (or as indicated in the figure legends), 1 m*M* $MgCl_2$, 10 m*M* glucose, 10 m*M* HEPES-Tris, pH 7.4, 20 μ*M* $CaCl_2$ supplemented with $^{45}CaCl_2$ (0.0025 μCi/nmol), 0.1 m*M* dinitrophenol, 0.1 m*M* azide, and 1.0 μg/ml oligomycin. Synaptic plasma membrane vesicles were diluted 20-fold into the reaction medium (final protein concentration, 0.5 mg/ml), preequilibrated at 30°C. The reaction was initiated by adding 1 m*M* ATP-Mg and was terminated by filtration as described above.

The radioisotope method used here to follow Ca^{2+} uptake by synaptic plasma membrane vesicles was checked by using a Ca^{2+}-selective electrode coupled to a pH meter (Madeira, 1975), and the data obtained by both methods agree within ±5%. The Ca^{2+} uptake values reported for this and the previous experiments (Figs. 1–6) are representative of at least four independent experiments.

Other assays

$(Na^+ + K^+)$-ATPase activity was taken as the rate of ATP hydrolysis which was inhibited by 1.0 m*M* ouabain at 30°C in media containing 100 m*M* NaCl, 30 m*M* KCl, 3 m*M* $MgCl_2$, 5 m*M* Tris-Cl, pH 7.4, and 2 m*M* ATP-Mg (in the presence or absence of ouabain). ATP hydrolysis was monitored continuously by following the production of H^+ during ATP hydrolysis, by means of a Radiometer combined electrode, type GK 2321 C, connected to a Radiometer pH meter, model PHM 64, and a Perkin-Elmer recorder, model 56. The system was calibrated with freshly titrated NaOH at the end of each determination. The ratio H^+ produced/ATP hydrolyzed is 1.15.

RNA content was determined in the synaptic membrane fractions by the method of Schneider (1945).

Reagents

The ionophores A 23187 and monensin were kindly supplied by Dr. Robert L. Hamill, the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN, U.S.A. Vanadium-free ATP (disodium salt), PPO, POPOP, oligomycin, dinitrophenol, and azide were purchased from Sigma Chemical Co. All other reagents were of analytical grade. $^{45}CaCl_2$ (20 mCi/mg) was obtained from Amer-

sham, England. Filters used for the separation of vesicles were obtained from Whatman (type GF/B).

RESULTS

Properties of synaptosomal membrane fractions

The synaptic plasma membrane fraction isolated by osmotic lysis and differential centrifugation of sheep brain cortex synaptosomes, as described in Materials and Methods, is rich in $(Na^+ + K^+)$-ATPase activity, a marker for plasma membranes (Jones and Matus, 1974), and has a low RNA content, as shown in Table 1, suggesting that it is predominantly of plasma membrane origin.

The presence in synaptic membrane fractions of membranes derived from endoplasmic reticulum was determined by measuring RNA content (Gurd et al., 1974). On the basis of RNA, synaptic membranes do not contain more than 20% of microsomal contamination (Table 1). The table also shows the distribution of the Na^+/Ca^{2+} exchange activity in the synaptosomal membrane fractions. It is observed that this Ca^{2+} transport activity is low in lysed synaptosomes (3.0 nmol Ca^{2+}/mg protein) and in the pellet obtained by centrifugation at 8000 × *g* of the lysed synaptosomes (2.5 nmol Ca^{2+}/mg protein), whereas there is an enrichment of activity in the synaptic membrane fraction (5 nmol Ca^{2+}/mg protein), as well as in the microsomal fraction (8.5 nmol Ca^{2+}/mg protein). The presence of Na^+/Ca^{2+} exchange activity in brain microsomes is in agreement with previous observations in our laboratory (not published) and with results published recently (Schellenberg and Swanson, 1981). This alters the previous notion that the Na^+/Ca^{2+} exchanger carrier in nerve tissue is exclusively located in the plasma membrane (Blaustein and Hodgkin, 1969; Mullins and Brinley, 1975; Blaustein and Ector, 1976; Goddard and Robinson, 1976) and mitochondria (Crompton et al., 1978).

The data in Fig. 1 show the effect of a combination of mitochondrial inhibitors (azide, dinitrophenol, and oligomycin) on the ATP-dependent Ca^{2+} uptake by the fractions. It is observed that about 50% of the Ca^{2+} uptake (from 13.5 to 7.0 nmol Ca^{2+}/mg protein) is inhibited in the fraction designated pellet 8000 × *g*, which is removed before sedimenting the synaptic membrane fraction, indicating the presence of mitochondria in this pellet. In contrast, the ATP-dependent Ca^{2+} uptake is reduced only 13% by mitochondrial inhibitors (from 10.5 to 9.15 nmol Ca^{2+}/mg protein) in the synaptic membrane fraction, indicating a low contamination with mitochondria. Nevertheless, all the experiments on ATP-dependent Ca^{2+} uptake were performed in the presence of mitochondrial inhibitors.

Stimulation by K^+ of Na^+/Ca^{2+} exchange

Ca^{2+} uptake by synaptic membrane vesicles was studied in the presence or absence of a Na^+ gradient, as shown in Fig. 2. The membrane vesicles were loaded with NaCl medium (Na_i) overnight, and were diluted in Ca^{2+} uptake media containing 150 m*M* KCl (K_o) or 150 m*M* NaCl (Na_o) at 30°C. It is found that, in the presence of a Na^+ gradient (Na_i/K_o), Ca^{2+} is taken up in a time-dependent manner, reaching a maximal value of about 10 nmol/mg protein within 3–5 min. This value of Ca^{2+} uptake varies from preparation to preparation and depends greatly on the degree of Na^+ loading. The results in Fig. 2 also show that, in the absence of a Na^+ gradient (Na_i/Na_o), there is only a small Ca^{2+} retention, which probably corresponds to Ca^{2+} binding to the surface of the membrane vesicles. On the other hand, if the Na^+gradient is abolished by adding to the reaction medium the ionophore monensin, which catalyzes the electroneutral exchange of Na^+ for H^+ (Gómez-Puyou and Gómez-Lojero, 1977), no Ca^{2+} accumulation is obtained, confirming the need for a Na^+ gradient to drive the uptake of Ca^{2+} into the membrane vesicles, as reported by others (Rahamimoff and Spanier, 1979; Michaelis and Michaelis, 1981; Gill et al., 1981).

Figure 2 also shows that A 23187, a Ca^{2+} ion-

TABLE 1. *Characterization of synaptic plasma membrane fractions isolated from sheep brain by differential centrifugation*

Fraction	$(Na^+ + K^+)$-ATPase (nmol · min^{-1} · mg^{-1})	RNA (μg RNA · mg^{-1} protein)	Na^+/Ca^{2+} exchange (nmol Ca^{2+} · mg^{-1} protein)
Lysed synaptosomes	105	8	3.0
Pellet (8000 × *g*)	85	—	2.5
Synaptic membranes	173	9	5.0
Microsomes	34	43	8.5

The synaptosomal membrane fractions were obtained as described in Materials and Methods. Microsomal fraction was prepared by centrifugation of the supernatant of the crude mitochondrial fraction at 100,000 × *g*, for 30 min, and the pellet was washed and resuspended in 0.32 *M* sucrose buffered with 10 m*M* HEPES-Tris, pH 7.4 (samples used for enzymatic and RNA assays) or in ionic medium (150 m*M* NaCl, 1 m*M* $MgCl_2$, 10 m*M* HEPES-Tris, pH 7.4) for Na^+/Ca^{2+} exchange assays. The Na^+/Ca^{2+} exchange activity was assayed at 30°C by diluting the membrane fractions in ionic medium containing either 150 m*M* NaCl or KCl and 20 μ*M* $^{45}CaCl_2$. After 3 min of incubation, the uptake was terminated by filtration. The difference between Ca^{2+} uptake in KCl and NaCl media was taken as the Na^+/Ca^{2+} exchange activity. The results reported are means of two different experiments which agreed within 10%.

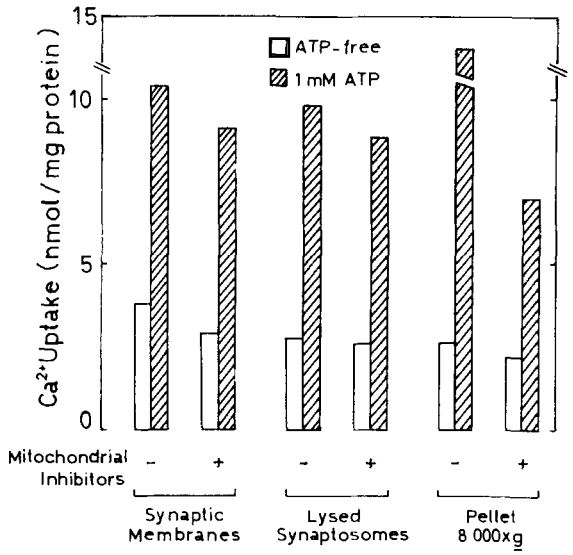

FIG. 1. Effect of mitochondrial inhibitors of ATP-dependent Ca^{2+} uptake in brain membrane fractions. The brain fractions were washed and suspended in media containing 130 m*M* choline chloride, 20 m*M* KCl, 1 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* HEPES-Tris, pH 7.4. Ca^{2+} uptake was assayed at 30°C by diluting the fractions in the same medium, containing in addition 20 μ*M* $CaCl_2$ + $^{45}CaCl_2$ and mitochondrial inhibitors (0.1 m*M* DNP, 0.1 m*M* NaN_3, and 1 μg/ml oligomycin), at a final protein concentration of 0.5 mg/ml. Reactions were initiated by addition of 1 m*M* ATP-Mg. After 5 min, duplicate samples (0.5 ml) were filtered as described in Materials and Methods.

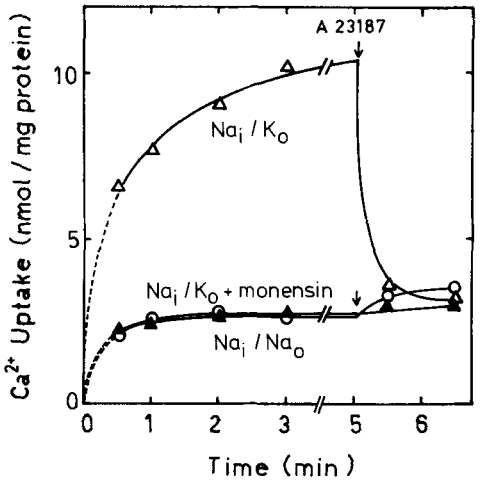

FIG. 2. Time course of sodium-dependent calcium uptake by synaptic membrane vesicles: effect of monensin. Membrane vesicles were preloaded with 150 m*M* NaCl medium and diluted 20-fold in Ca^{2+} uptake media containing 150 m*M* NaCl (○) or KCl (△) and 20 μ*M* $CaCl_2$ + $^{45}CaCl_2$, as described in Materials and Methods. The arrows indicate the time at which the Ca^{2+} ionophore A 23187 was added, at a concentration of 40 μ*M*. In the presence of 40 μ*M* monensin (▲) a Na^+ ionophore, the Ca^{2+} uptake due to the outward sodium gradient was abolished.

ophore that catalyzes the electroneutral exchange of Ca^{2+} for $2H^+$ without disturbing the monovalent ion gradients (Gómez-Puyon and Gómez-Lojero, 1977), releases most of the Ca^{2+} that was taken up by the membrane vesicles in the presence of a Na^+ gradient, indicating that this Ca^{2+} is accumulated intravesicularly rather than being bound to the membranes.

The following experiments were devised to determine the influence of parameters other than the Na^+ gradient on the Na^+/Ca^{2+} exchange activity in synaptic membrane vesicles. Therefore, membrane vesicles loaded with NaCl (Na_i) were diluted 20-fold in Ca^{2+} uptake media without Na^+, thus maintaining a fixed Na^+ gradient, but with variable external ionic composition. The results reported in Fog. 3 show the time course of Na^+/Ca^{2+} exchange by membrane vesicles in the presence of external medium containing 150 m*M* choline chloride, 75 m*M* choline chloride plus 75 m*M* KCl, or 150 m*M* KCl. It is seen that the presence of 150 m*M* KCl in the external medium approximately doubles both the rate and the extent of Ca^{2+} uptake by synaptic membrane vesicles as compared with the values obtained in the presence of choline. Thus, the total Ca^{2+} taken up after 5 min of reaction is about 5 nmol/mg protein in choline chloride (Ch_o), but it is 10 nmol/mg of protein in the presence of KCl (K_o). The value for Ca^{2+} uptake is intermediate between these two values (about 7.5 nmol/mg protein) when half of the choline chloride is substituted by K^+ (Ch_o + K_o). The data suggest, therefore, that K^+ activates, directly or indirectly, the Na^+/Ca^{2+} exchange. A similar stimulation of the Na^+/Ca^{2+} carrier has previously been reported for cardiac mitochondria (Crompton et al., 1980).

We further explored this aspect by determining

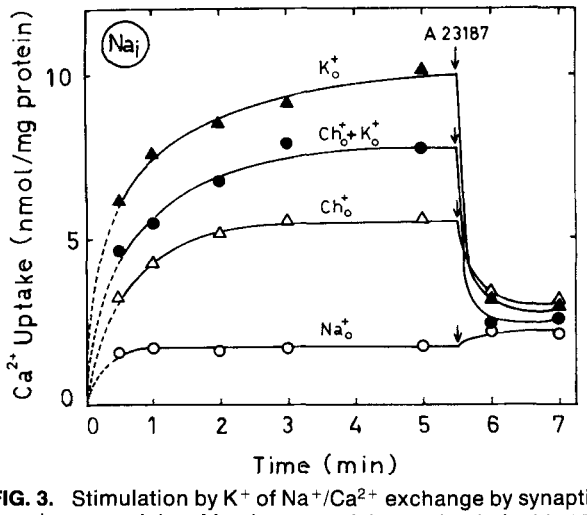

FIG. 3. Stimulation by K^+ of Na^+/Ca^{2+} exchange by synaptic membrane vesicles. Membrane vesicles preloaded with 150 m*M* NaCl medium (Na_i) were diluted 20-fold in Ca^{2+} uptake media containing either 150 m*M* NaCl (Na^+_o), 150 m*M* choline chloride (Ch^+_o), 75 m*M* choline chloride plus 75 m*M* KCl (Ch^+_o + K^+_o), or 150 m*M* KCl (K^+_o) and 20 μ*M* $CaCl_2$ + $^{45}CaCl_2$, as described in the text. Ca^{2+} uptake was followed as a function of time. At the time indicated by the arrows, 40 μ*M* A 23187 was added to the reaction media.

the effect of other monovalent cations on the Na^+/Ca^{2+} exchange (Fig. 4), and the results show that, taking the value for Ca^{2+} uptake (Na^+/Ca^{2+} exchange) obtained in the presence of choline as reference, the other monovalent cations in the external medium have the following effects: K^+ or NH_4^+ stimulates the Na^+/Ca^{2+} exchange, whereas Li^+ or Cs^+ inhibits Na^+/Ca^{2+} exchange. Figure 4 also shows the level of Ca^{2+} retention by the membrane vesicles obtained in the presence of the various monovalent cations in the external media containing, additionally, the Na^+ ionophore monensin. The level of Ca^{2+} bound to the membrane vesicles in the absence of a Na^+ gradient is about the same for the various external media utilized, except for choline and Cs, for which it is slightly higher (Fig. 4). This observation excludes the possibility that the stimulatory effect of K^+ on Ca^{2+} uptake might be due to an increase in Ca^{2+} binding rather than to a stimulatory effect on Na^+/Ca^{2+} exchange activity.

Stimulation by K^+ of ATP-dependent Ca^{2+} transport by synaptic membrane vesicles

The results shown in Fig. 1, as well as previous observations by other authors (Rahamimoff and Spanier, 1979; Michaelis and Michaelis, 1981; Gill et al., 1981), indicate that Ca^{2+} uptake by synaptic membrane vesicles is stimulated by ATP. Furthermore, it is known that, as with Na^+/Ca^{2+} exchange, the ionic composition of the external medium influences the ATP-dependent Ca^{2+} uptake (Gill et al., 1981). Thus, we devised experiments to test whether the Na^+ in the external medium would antagonize the ATP-dependent Ca^{2+} accumulation, since the Na^+/Ca^{2+} exchange, working in the opposite direction, would induce the efflux of Ca^{2+} from the vesicles. The results shown in Fig. 5 refer to the ATP-dependent Ca^{2+} uptake by membrane vesicles studied in incubation media in which K^+ was replaced by increasing concentrations of Na^+, up to 100 m*M*. It is observed that both the rate and the total extent of Ca^{2+} retained are decreased by increasing the external NaCl concentration, and Ca^{2+} uptake is half- maximal with approximately 50 m*M* NaCl. In the absence of ATP, the maximal NaCl concentration utilized has no significant effect on the passive Ca^{2+} retention by the membranes (Fig. 5).

These results can be explained partially in terms of the effect of the increased external Na^+ on the efflux of Ca^{2+} through the Na^+/Ca^{2+} exchanger, which would cause release of the Ca^{2+} transported through the Ca^{2+}-ATPase. However, in these experiments with elevated NaCl (Fig. 5), the KCl concentration in the medium was proportionally reduced to maintain the isoosmolarity. Therefore, it is important to determine whether the decrease in Ca^{2+} retained was specifically caused by the elevated Na^+ or by the reduced K^+ utilized. Figure 6 shows the results of an experiment designed to study separately the effects of K^+ and Na^+ on the ATP-dependent Ca^{2+} uptake by the membrane vesicles. Furthermore, the effect of increasing concentrations of Li^+ was also tested (Fig. 6). The data show that an increase in K^+ concentration from 0 to 50

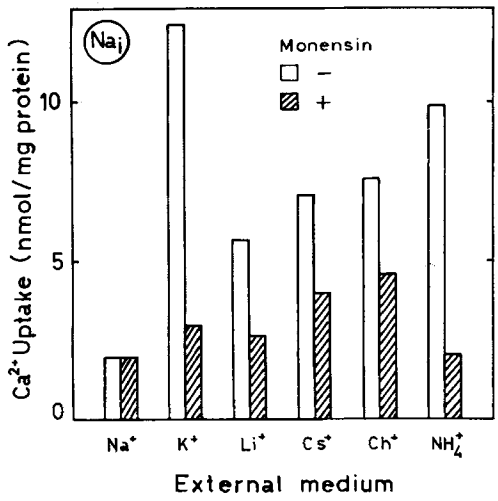

FIG. 4. Effect of various monovalent cations on Na^+/Ca^{2+} exchange by synaptic membrane vesicles; effect of monensin. Membrane vesicles preloaded with 150 m*M* NaCl medium (Na_i) were diluted 20-fold in media containing 150 m*M* of NaCl, KCl, LiCl, choline chloride, or NH_4Cl and 20 μ*M* $CaCl_2$ + $^{45}CaCl_2$, as indicated in Materials and Methods. Ca^{2+} uptake was conducted for 5 min at 30°C, and the reaction was terminated by filtration. When present, 40 μ*M* monensin was added to the reaction medium.

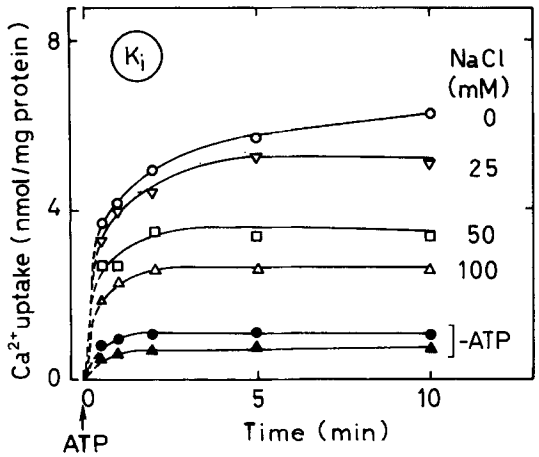

FIG. 5. Effect of increasing NaCl concentrations on ATP-dependent Ca^{2+} uptake by synaptic membrane vesicles. Synaptic membrane vesicles preloaded with 150 m*M* KCl (K_i) were diluted 20-fold in media containing increasing concentrations of NaCl (0 to 100 m*M*) isoosmotically compensated by KCl to a total concentration of 150 m*M*. In addition, the medium included 20 μ*M* $CaCl_2$ + $^{45}CaCl_2$. Ca^{2+} uptake was initiated by addition of 1 m*M* ATP-Mg, and the time course of the reaction (at 30°C) was followed by filtering samples (0.5 ml, containing 0.25 mg protein) of the reaction mixture at indicated time intervals. Controls were made for the Ca^{2+} retained in the absence of ATP for the 0 and 100 m*M* NaCl concentrations (filled symbols).

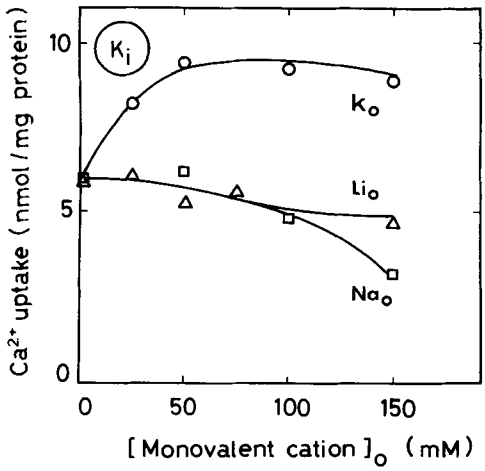

FIG. 6. Effect of monovalent cations on ATP-dependent Ca^{2+} uptake by synaptic plasma membrane vesicles. Synaptic membrane vesicles preloaded with 150 m*M* KCl (K_i) were diluted 20-fold in media containing increasing concentrations of KCl, LiCl, or NaCl isoosmotically compensated with choline chloride to a total concentration of 150 m*M*, so that the effects of Na^+, K^+, and Li^+ on Ca^{2+} uptake were studied separately. Because of this dilution procedure, there was always a minimal K^+ concentration of 7.5 m*M* in the Ca^{2+} uptake media. Reaction media also contained 20 μM $CaCl_2$ + $^{45}CaCl_2$; Ca^{2+} uptake was measured 5 min after the addition of 1 m*M* ATP-Mg.

m*M* in the external medium (maintaining osmolarity with choline chloride) stimulates the ATP-dependent Ca^{2+} uptake by 50% over the uptake observed in the presence of 150 m*M* choline chloride, and the effect is maintained constant for K^+ concentrations up to 150 m*M*. On the other hand, Na^+ or Li^+ failed to affect Ca^{2+} uptake significantly, except that Na^+ for concentrations of 100–150 m*M* reduces the Ca^{2+} transport activity, probably owing to an efflux of Ca^{2+} through the Na^+/Ca^{2+} exchange mechanism. Therefore, K^+ appears to stimulate the ATP-dependent Ca^{2+} transport process in synaptic plasma membrane vesicles. Note that a similar effect of K^+ is observed for the Na^+/Ca^{2+} exchange (Fig. 3). Thus, it appears that the two mechanisms for Ca^{2+} extrusion from the nerve cell are stimulated by K^+.

DISCUSSION

In this work we studied the properties of the two Ca^{2+} transport systems present in synaptic plasma membrane vesicles isolated from sheep brain cortex synaptosomes by hypotonic lysis and partial purification. The two mechanisms of Ca^{2+} transport are (1) a mediated antiport system, the Na^+/Ca^{2+} exchange mechanism, whereby Ca^{2+} transport is coupled to the Na^+ gradient; and (2) an ATP-dependent Ca^{2+} transport.

The preparation of synaptic membrane vesicles utilized in these studies is predominantly derived from the plasma membrane of synaptosomes, since it is rich in (Na^+ + K^+)-ATPase and has a low RNA content (Table 1). Furthermore, the method of preparation of the membrane vesicles permits the elimination of most of the mitochondrial contamination (Fig. 1), and thus represents in improvement over other methods used previously (Rahamimoff and Spanier, 1979; Michaelis and Michaelis, 1981; Gill et al., 1981).

The ionic composition of the external medium greatly affects the Ca^{2+} uptake activity. Thus, the Na^+/Ca^{2+} exchange system is strongly activated by K^+ and NH_4^+ in the external medium, although the mechanism of activation is not understood (Figs. 3 and 4). From the results shown in Fig. 4 one can conclude that only the more permeant cations (K^+ and NH_4^+) can stimulate the Na^+/Ca^{2+} exchange mechanism, which may indicate the involvement of charge transfer in Na^+/Ca^{2+} exchange. However, the stoichiometry of Na^+/Ca^{2+} exchange activity is not well established in synaptic plasma membranes vesicles, although in squid axons and isolated synaptosomes there is some evidence that three Na^+ exchange for one Ca^{2+} (Blaustein, 1977; Blaustein and Oborn, 1975). Another explanation for the stimulatory effect of K^+ (Figs. 3 and 4) is that K^+ combines with the carrier and modifies its Na^+/Ca^{2+} exchange activity, as was previously suggested for the activation by K^+ of the Na^+/Ca^{2+} carrier of cardiac mitochondria (Crompton et al., 1980).

The results reported for the effect of Na^+, K^+, and Li^+ on the Ca^{2+} transport due to ATP hydrolysis in synaptic membranes vesicles indicate that K^+ specifically stimulates Ca^{2+} transport, whereas Li^+ has no effect and Na^+ slightly inhibits Ca^{2+} uptake for concentrations higher than 100 m*M*. It is suggested that the stimulatory effect of K^+ reflects stimulation of the Ca^{2+} transport system. Similar stimulation has been shown for the Ca^{2+} transport system of cardiac membrane vesicles (Jones et al., 1977) and for the Ca^{2+}-ATPases of sarcolemma (Morcos and Drummond, 1980) and brain (Robinson, 1981).

In summary, the effects observed in this study for K^+ stimulation of both Na^+/Ca^{2+} exchange and ATP-dependent Ca^{2+} uptake by synaptic membrane vesicles suggest that K^+ may normally influence the extrusion of Ca^{2+} from the nerve cell through both Ca^{2+} transport systems, thus regulating the intracellular Ca^{2+} concentration. It should be noted that the vesicles that accumulate Ca^{2+} actively in the presence of ATP are probably inside-out, so that in our studies we saw the effect of K^+ on the inner membrane surface. The Na^+/Ca^{2+} exchanger appears to be reversible (Rahamimoff and Spanier, 1979; Gill et al., 1981); thus, a fraction of the vesicles that accumulate Ca^{2+} by Na^+/Ca^{2+} exchange is inside-out and another fraction may be right-side-out. It would be of interest to differentiate between these two vesicle populations and to determine whether the effect of K^+ on the stimulation of the

Na^+/Ca^{2+} exchange is selective for one side of the membrane.

Acknowledgment: The present work was supported by I.N.I.C., The Portuguese National Institute for Scientific Research, and by NATO Research Grant no. 1513.

REFERENCES

Baker P. F., Blaustein M. P., Hodgkin A. L., and Steinhart R. A. (1969) The influence of calcium on sodium efflux in squid axons. *J. Physiol. (Lond.)* **200**, 431–458.

Blaustein M. P. and Ector A. C. (1976) Carrier-mediated sodium-dependent and calcium-dependent calcium efflux from pinched-off presynaptic nerve terminals (synaptosomes) *in vitro*. *Biochim. Biophys. Acta* **419**, 295–308.

Blaustein M. P. and Hodgkin A. L. (1969) The effect of cyanide on the efflux of calcium from squid axons. *J. Physiol. (Lond.)* **200**, 497–527.

Blaustein M. P. and Oborn C. J. (1975) The influence of sodium on calcium fluxes in pinched-off nerve terminals *in vitro*. *J. Physiol. (Lond.)* **247**, 657–686.

Blaustein M. P. (1977) Effects of internal and external cations and of ATP on sodium-calcium and calcium-calcium exchange in squid axons. *Biophys. J.* **20**, 79–111.

Brinley F. J., Jr. and Mullins L. J. (1967) Sodium extrusion by internally dialyzed squid axons. *J. Gen. Physiol.* **50**, 2303–2331.

Carvalho C. A. M. (1978) Effects of monovalent cations and of temperature on the release of Ca^{2+} from synaptosomes. *Ciênc. Biol. (Portugal)* **3**, 57–78.

Carvalho C. A. M. (1979) Fluxes of Ca^{2+}, Sr^{2+} and Mg^{2+} in synaptosomes. *Life Sci.* **25**, 73–82.

Carvalho C. A. M. and Carvalho A. P. (1979) Effect of temperature and ionophores on the permeability of synaptosomes. *J. Neurochem.* **33**, 309–317.

Carvalho C. A. M. and Coutinho O. P. (1982) Ca^{2+} translocation in synaptic membranes by Na^+/Ca^{2+} exchange and Ca^{2+}-ATPase. Special FEBS Meeting, April 25–29, Athens, Greece (Abst.).

Crompton M., Moser R., Lüdi H., and Carafoli E. (1978) The interrelations between the transport of sodium and calcium in mitochondria of various mammalian tissues. *Eur. J. Biochem.* **82**, 25–31.

Crompton M., Heid I., and Carafoli E. (1980) The activation by potassium of the sodium-calcium carrier of cardiac mitochondria. *FEBS Lett.* **115**, 257–259.

DiPolo R. (1977). Characterization of the ATP-dependent calcium efflux in dialyzed squid axons. *J. Gen. Physiol.* **69**, 795–814.

DiPolo R. (1978) Ca pump driven by ATP in squid axons. *Nature* **274**, 390–392.

DiPolo R. and Beaugé L. (1979) Physiological role of ATP-driven calcium pump in squid axon. *Nature* **278**, 271–273.

DiPolo R. and Beaugé L. (1980) Mechanisms of calcium transport in the giant axon of the squid and their physiological role. *Cell Calcium* **1**, 147–169.

DiPolo R., Requena R., Brinley F. J., Jr., Mullins L. J., Scarpa A., and Tiffert T. (1976) Ionized calcium concentrations in squid axons. *J. Gen. Physiol.* **67**, 433–467.

Gill D. L., Grollman E. F., and Kohn L. D. (1981) Calcium transport mechanisms in membrane vesicles from guinea pig brain synaptosomes. *J. Biol. Chem.* **256**, 184–192.

Goddard G. A. and Robinson J. D. (1976) Uptake and release of calcium by rat brain synaptosomes. *Brain Res.* **110**, 331–350.

Gómez-Puyou A. and Gómez-Lojero C. (1977) The use of ionophores and channel formers in the study of the function of biological membranes. *Curr. Topics Bioenerg.* **6**, 221–257.

Gurd J. W., Jones L. R., Mahler H. R., and Moore W. J. (1974) Isolation and partial characterization of rat brain synaptic plasma membranes. *J. Neurochem.* **22**, 281–290.

Hajós F. (1975) An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res.* **93**, 485–489.

Jones D. H. and Matus A. I. (1974) Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. *Biochim. Biophys. Acta* **356**, 276–287.

Jones L. R., Besch H. R., and Watanabe A. M. (1977) Monovalent cations stimulation of Ca^{2+} uptake by cardiac membrane vesicles. *J. Biol. Chem.* **252**, 3315–3323.

Layne E. (1957) Spectrophotometric and turbidimetric method for measuring proteins, in *Methods in Enzymology, Vol. 3* (Colowick S. P. and Kaplan N. O., eds), pp. 447–451. Academic Press, New York.

Madeira V. M. C. (1975) A rapid and ultrasensitive method to measure Ca^{2+} movements across biological membranes. *Biochem. Biophys. Res. Commun.* **64**, 870–876.

Michaelis M. L. and Michaelis E. K. (1981) Ca^{2+} fluxes in resealed synaptic plasma membrane vesicles. *Life Sci.* **28**, 37–45.

Morcos N. C. and Drummond G. I. (1980) (Ca^{2+} + Mg^{2+})-ATPase in enriched sarcolemma from dog heart. *Biochim. Biophys. Acta* **598**, 27–39.

Mullins L. J. and Brinley F. J., Jr. (1975) Sensitivity of calcium efflux from squid axons to changes in membrane potential. *J. Gen. Physiol.* **65**, 135–152.

Rahamimoff H. and Spanier R. (1979) Sodium-dependent calcium uptake in membrane vesicles derived from rat brain synaptosomes *FEBS Lett.* **104**, 111–114.

Robinson J. D. (1981) Effects of cations on (Ca^{2+} + Mg^{2+})-activated ATPase from rat brain. *J. Neurochem.* **37**, 140–146.

Schellenberg G. D. and Swanson P. D. (1981) Sodium-dependent and calcium-dependent calcium transport by rat brain microsomes. *Biochim. Biophys. Acta* **648**, 13–27.

Schneider W. C. (1945) Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and pentose nucleic acid. *J. Biol. Chem.* **161**, 293–303.