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Distinction between Ca^{2+} pump and Ca^{2+}/H^{+} antiport activities in synaptic vesicles of sheep brain cortex

Paula P. Gonçalves[a,*], Sandra M. Meireles[a], Paulo Neves[a], M. Graça P. Vale[b]

[a]*Centro de Biologia Celular, Departamento de Biologia, Universidade de Aveiro, 3810-193 Aveiro, Portugal*
[b]*Centro de Neurociências, Departamento de Zoologia, Faculdade de Ciências, Universidade de Coimbra, 3004-517 Coimbra, Portugal*

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Abstract

Synaptic vesicles, isolated from a sheep brain cortex, accumulate Ca^{2+} in a manner that depends on the pH and pCa values. In the presence of 100 μM $CaCl_2$, most of the Ca^{2+} taken up by the vesicles was vanadate-inhibited (86%) at pH 7.4, whereas at pH 8.5, part of the Ca^{2+} accumulated (36%) was ΔpH-dependent (bafilomycin and CCCP inhibited) and part was insensitive to those drugs (31%). We also observed that both vanadate-sensitive and bafilomycin-sensitive Ca^{2+} accumulations were completely released by the Ca^{2+} ionophore, ionomycin, and that these processes work with high ($K_{0.5} = 0.6$ μM) and low ($K_{0.5} = 217$ μM) affinity for Ca^{2+}, respectively. The ΔpH-dependent Ca^{2+} transport appears to be largely operative at Ca^{2+} concentrations (>100 μM) which completely inhibited the vanadate-sensitive Ca^{2+} uptake. These Ca^{2+} effects on the Ca^{2+} accumulation were well correlated with those observed on the vanadate-inhibited Ca^{2+}-ATPase and bafilomycin-inhibited H^{+}-ATPase, respectively. The Ca^{2+}-ATPase activity reached a maximum at about 25 μM (pH 7.4) and sharply declined at higher Ca^{2+} concentrations. In contrast, Ca^{2+} had a significant stimulatory effect on the H^{+}-ATPase between 250 and 500 μM Ca^{2+} concentration. Furthermore, we found that ΔpH-sensitive Ca^{2+} transport was associated with proton release from the vesicles. About 21% of the maximal proton gradient was dissipated by addition of 607.7 μM $CaCl_2$ to the reaction medium and, if $CaCl_2$ was present before the proton accumulation, lower pH gradients were reached. Both vanadate-inhibited and bafilomycin-inhibited systems transported Ca^{2+} into the same vesicle pool of our preparation, suggesting that they belong to the same cellular compartment. These results indicate that synaptic vesicles of the sheep brain cortex contain two distinct mechanisms of Ca^{2+} transport: a high Ca^{2+} affinity, proton gradient-independent Ca^{2+} pump that has an optimal activity at pH 7.4, and a low Ca^{2+} affinity, proton gradient-dependent Ca^{2+}/H^{+} antiport that works maximally at pH 8.5.

1. Introduction

The main mechanism for exocytotic release of neurotransmitters depends on the presence of Ca^{2+} ions that enter the nerve cells by a stimulus-induced depolarizing wave (Sihra and Nichols, 1993). However, the concentration of free Ca^{2+} is raised momentarily in restricted spots (microdomains) located under the plasma membrane surrounding the nerve terminals (Llinás et al., 1992). This implies that the trigger signal is essentially associated to synchronized periodic Ca^{2+} spikes rather than to a large rise in cytoplasmic Ca^{2+} concentration. It appears, therefore, that the cellular Ca^{2+} oscillations reflect the action of efficient and rapid Ca^{2+} sequestering systems. Although the endoplasmic reticulum, mitochondria and Ca^{2+}-binding proteins greatly contribute for the intracellular Ca^{2+} homeostasis (Kostyuk and Verkhratsky, 1994), there are evidences that synaptic vesicles may also play an active role as internal Ca^{2+} stores which may be important either for localized Ca^{2+} signals (Parducz and Dunant, 1993; Petersen, 1996) or for

Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; Mes, 2[*N*-morpholino] ethanesulfonic acid.

* Corresponding author. Tel.: +351-34-370-776; fax: +351-34-426-408.

E-mail address: pgoncalves@bio.ua.pt (P.P. Gonçalves).

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extrusion of excessive intracellular Ca^{2+} (Parducz et al., 1994).

It has been reported that synaptic vesicles are able to take up Ca^{2+} by an ATP-dependent process (Israël et al., 1980; Michaelson and Ophir, 1980) and that they have Ca^{2+}-binding properties (Kostyuk and Verkhratsky, 1994). Furthermore, after prolonged stimulation, synaptic vesicles accumulate Ca^{2+} in correlation with a decrease in the vesicular acetilcholine content (Parducz et al., 1994). Recently, evidences that IP_3 (inositol triphosphate) and cyclic ADP-ribose induce release of Ca^{2+} from isolated granules (Gerasimenko et al., 1996; Nguyen et al., 1998) also support the idea that synaptic vesicles and secretory granules can play an active role as internal Ca^{2+} stores.

In spite of these observations, the mechanisms of Ca^{2+} transport by synaptic vesicles remain poorly understood since, at present, the several ATPase systems found at the level of the synaptic vesicle membranes (Stelzl et al., 1987; Cidon and Sihra, 1989; Floor et al., 1990; Moriyama and Futai, 1990; Hicks and Parsons, 1992) were not perfectly distinguished and identified. On this basis, we recently reported the presence of a Ca^{2+}/H^+ antiport system that required suitable experiments for detection, although it has specific characteristics of operativity in synaptic vesicles (Gonçalves et al., 1998, 1999a,b).

In this work, experiments were performed to distinguish between Ca^{2+} pump and Ca^{2+}/H^+ antiporter activities in synaptic vesicles of sheep brain cortex. The method was based on the ability to separate the activities of the two types of transport by a study of pH dependency, pCa dependency and sensitivity to specific inhibitors.

2. Experimental procedures

2.1. Isolation of synaptic vesicles and microsomes

Synaptic vesicles were isolated from sheep brain cortex according to the procedure described by Hell et al. (1988, 1990). The brains were cut into small pieces, frozen in liquid nitrogen and crushed to form a fine powder that was homogenized in a solution containing 0.32 M sucrose, 10 mM HEPES-K (pH 7.3), 0.2 mM EGTA, 0.5 μg/ml pepstatin and 1 μg/ml leupeptin. After centrifugation during 10 min at 47,000 *g*, the supernatant was collected and centrifuged again for 40 min at 120,000 *g*. The supernatant obtained was layered onto a cushion of 0.65 mM sucrose and 10 mM HEPES-K (pH 7.3) and centrifuged for 2 h at 260,000 *g*. The resulting pellet was resuspended in 0.32 M sucrose and 10 mM HEPES-K (pH 7.4) and, after centrifuging for 10 min at 27,000 *g*, the supernatant containing the purified synaptic vesicles was collected, frozen in liquid nitrogen and stored at −70°C. This method allows preparation of a fraction highly enriched in synaptic vesicles, as revealed by assays of immunoblotting and marker enzymes activity (Hell et al., 1988, 1990). Usually, our preparations were not contaminated with plasma membrane as judged by absence of ouabain-sensitive (Na^+/K^+)ATPase activity. However, we observed a little (∼15%) contamination with microsomes, as was demonstrated by analysis of the glucose-6-phosphatase activity according to the method of Swanson (1955) and Huang and Veech (1986). For control experiments, we also isolated a microsomal fraction (endoplasmic reticulum enriched) from the axonal region of cortical neurons where the synaptic vesicles are absent. The procedure involved a differential centrifugation method as described by Racay et al. (1998).

Both types of membranes were isolated at 4°C and the protein was determined by the method of Gornall et al. (1949).

2.2. Ca^{2+} uptake assays

Ca^{2+} accumulation by isolated synaptic vesicles was measured by rapid filtration and scintillation counting. The membrane vesicles (600 μg protein/ml) were incubated at 30°C in a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA buffered at different pH values (10 mM Mes, HEPES and Tris to pH 5.6, 7.4 and 8.5, respectively) and 504 μM ATP–Mg (except otherwise indicated). The reaction was initiated by adding various concentrations of $CaCl_2$ supplemented with $^{45}CaCl_2$ (10 μCi/mmol) and, at designated reaction times, aliquots of 800 μl (480 μg protein) were rapidly filtered under vacuum through Millipore filters HAWP (0.45 μm), which were washed with 3 ml of the reaction medium without Ca^{2+} and ATP–Mg. The radioactivity of the filters was measured by liquid scintillation spectrometry and the amount of Ca^{2+} accumulated in the vesicular space was calculated.

The Ca^{2+} uptake assays were performed at various pH values (5.6, 7.4 and 8.5) in the presence of vanadate (10 μM) or bafilomycin (300 nM), to conform, we were testing the activity of the Ca^{2+}/H^+ antiport or of the Ca^{2+} pump, respectively. The active Ca^{2+} uptake was determined by subtracting passive Ca^{2+} bound (absence of ATP) from the total amount of Ca^{2+} taken up under the various experimental conditions. The actual Ca^{2+} accumulation inside the vesicles was demonstrated by using the Ca^{2+} ionophore, ionomycin, whose presence in the medium prevents formation of Ca^{2+} gradients across the membranes. In some experiments, the pH gradient sensitive Ca^{2+} uptake was evaluated by measuring the Ca^{2+} accumu-

lation either in the absence or in the presence of the protonophore, CCCP.

A set of experiments was performed under conditions where the vesicles were firstly incubated at 25 μM Ca^{2+} concentration and pH 7.4. After 2.5 min, Ca^{2+} and Tris were added to perform a 500 μM concentration and pH 8.5, respectively. For control, we evaluated the time course (6 min) for Ca^{2+} accumulation under conditions where only Tris was added at 2.5 min or under conditions where synaptic vesicles, in all time (6 min) took up Ca^{2+} in the presence of 25 μM Ca^{2+} (pH 7.4) or of 500 μM (pH 8.5), respectively. The isotopic method was also applied to determine the Ca^{2+} uptake by the microsomal fraction at various Ca^{2+} concentrations (0.03–25μM).

2.3. ATPase assays

The ATPase activity of the synaptic vesicles was determined by measuring the P_i liberation associated with the hydrolysis of ATP. Membrane vesicles (600 μg protein/ml) were incubated in 1.1 ml of 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA buffered at different pH values (10 mM HEPES and Tris to pH 7.4 and 8.5, respectively) and various concentrations of $CaCl_2$. The reaction was started by adding 504 μM ATP–Mg and, after 1 min at 30°C, it was stopped by adding 50 μl of ice-cold 20% (v/v) trichloroacetic acid. The precipitated protein was discarded by centrifugation and the supernatant was collected for P_i analysis by the method of Taussky and Shorr (1953).

The assays of the Ca^{2+}-ATPase were performed in the presence of bafilomycin (V-type ATPase inhibitor), whereas the H^+-ATPase was assayed in the presence of vanadate (P-type ATPase inhibitor).

2.4. Proton transport and Ca^{2+}-induced H^+ release assays

ATP-dependent proton transport was measured by following the fluorescence quenching of 2 μM acridine orange (Deamer et al., 1972). The membrane vesicles (600 μg protein/ml) were incubated at 30°C in a medium (2 ml) containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and 10 mM Tris–HCl (pH 8.5). Proton accumulation inside the vesicles was initiated by adding 210 μM ATP–Mg and it was allowed to proceed for about 1 min. Then, H^+ was released by adding $CaCl_2$ (final free concentration of 500 μM) as revealed by the fluorescence increase during about 2 min.

In some experiments, the H^+ transport reaction was started by adding 22.4 μM ATP–Mg in the presence of various Ca^{2+} concentrations (100, 250, 500 and 860 μM).

The proton accumulation inside the vesicles was checked by inducing H^+ release with the protonophore CCCP (10 μM).

The fluorescence changes due to H^+ movements were followed by using a Perkin–Elmer computer-controlled spectrofluorometer, Model LS-50. The fluorescence emission was measured at 525 nm (slit width 3.5 nm) using an excitation wavelength of 495 nm (slit width 3.0 nm).

2.5. Materials

All reagents were analytical grade. Bafilomycin A_1, CCCP, vanadate, ATP and acridine orange were obtained from Sigma. $^{45}CaCl_2$ (2 mCi/ml) was purchased from Amersham.

2.6. Statistical analysis

Statistical analysis was performed using the Student's *t*-test (two-tailed distribution; two-sample unequal variance) and *P* values are presented in the legends of the figures.

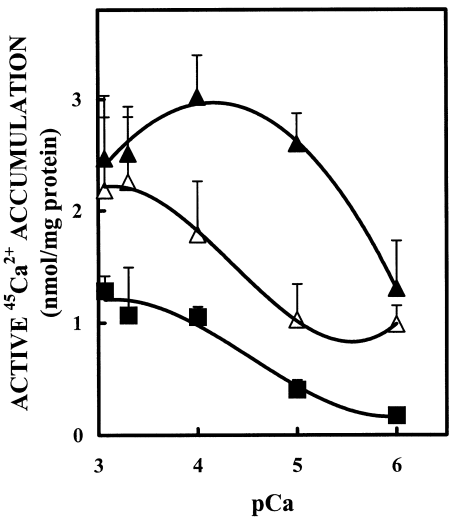

Fig. 1. Ca^{2+} uptake by synaptic vesicles as a function of pCa and pH. The vesicles (600 μg protein/ml) were incubated in a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and buffered at different pH values (10 mM Tris, HEPES and Mes to pH 8.5 (△–△), 7.4 (▲–▲) and 5.6 (■–■), respectively) in the presence of 504 μM ATP–Mg. The reactions were started by adding 10 μCi/mmol $^{45}CaCl_2$ supplemented with $CaCl_2$ to obtain free Ca^{2+} concentration in the range of 1–860 μM. After 3 min, the reactions were stopped as described in the text. Values represent the mean ± SD of at least three separate experiments.

3. Results

3.1. Active Ca^{2+} uptake by synaptic vesicles

As previously reported (Gonçalves et al., 1998), we observed that synaptic vesicles take up Ca^{2+} in the presence of ATP. However, by studying the process under various conditions of pCa and pH, we found a characteristic behavior that could not be explained by a simple mechanism of Ca^{2+} transport.

Fig. 1 shows that, in the range of neutral pH (~7.4), Ca^{2+} was actively accumulated by synaptic vesicles in a pCa-dependent manner. As the Ca^{2+} concentration increased in the medium, higher amounts of Ca^{2+} were taken up by the vesicles until they reached a maximum (3.02 nmol/mg protein) at about pCa 4.0. Then, the Ca^{2+} uptake declined, although it remained efficient at high Ca^{2+} concentrations (pCa = 3.1). On the other hand, at pH 8.5, the Ca^{2+} transport was low at pCa values up to 5.0, but it increased substantially at higher Ca^{2+} concentrations (pCa = 3.3), reaching about the same levels (2.25 nmol/mg protein) observed at pH 7.4. At acidic pH values (pH 5.6), Ca^{2+} accumulation was reduced, although significant amounts (1.28 nmol/mg protein) were taken up at pCa values above 4.0.

It appears, therefore, that the inhibitory effect of high Ca^{2+} concentrations observed at neutral pH does not exist in the other ranges of pH studied. In contrast, high Ca^{2+} concentrations (pCa = 3.3) maximize the active Ca^{2+} uptake either at basic or acidic pH values, which suggests that, under these conditions, other mechanisms support Ca^{2+} uptake by synaptic vesicles.

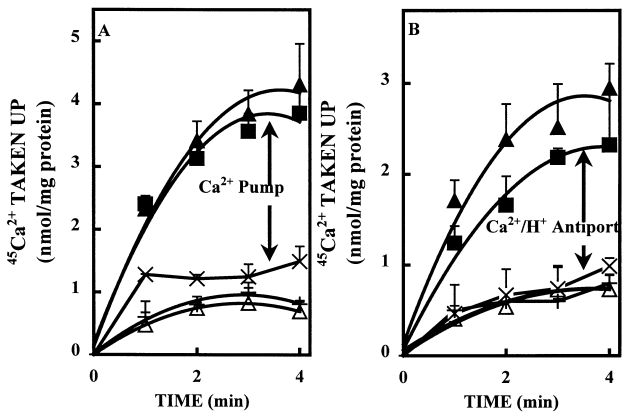

Fig. 2. Differentiation between the ATP-dependent Ca^{2+} uptake processes by synaptic vesicles. The vesicles (600 μg protein/ml) were incubated in a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and 10 mM HEPES-K pH 7.4 or 10 mM HEPES–Tris pH 8.5 in the absence (△–△) and in the presence (▲–▲) of 504 μM Mg–ATP. The reactions were started by adding 100 μM free Ca^{2+} (162.8 μM $CaCl_2$ supplemented with 10 μCi/mmol $^{45}CaCl_2$) and, at several time intervals, stopped as described in Fig. 1. The reactions were carried out at pH 7.4 in the presence of 300 nM bafilomycin (A) or at pH 8.5 in the presence of 10 μM vanadate (B) (■–■). The Ca^{2+} accumulation was checked by adding 10 μM ionomycin (X–X) or by adding vanadate plus bafilomycin (−+). The arrows in the plots represent the Ca^{2+} pump and the Ca^{2+}/H^+ antiport activities. Values represent the mean ± SD of at least four separate experiments.

3.2. Distinction between different Ca^{2+} transport systems in synaptic vesicles: Ca^{2+} pump and Ca^{2+}/H^+ antiport activities

In order to identify the different Ca^{2+} transport systems suggested in the experiments described above, we measured the Ca^{2+} accumulation by synaptic vesicles under various experimental conditions in a medium (pH 7.4) containing a V-type ATPase inhibitor (bafilomycin) or in a medium (pH 8.5) containing a P-type ATPase inhibitor (vanadate). Fig. 2 shows that, indeed, Ca^{2+} can be accumulated into the vesicles by both, a bafilomycin-resistant, vanadate-sensitive process (Fig. 2A) and a vanadate-resistant, bafilomycin-sensitive process (Fig. 2B). Both Ca^{2+} transport systems have similar time-courses, reaching a maximum level at about 3 min of reaction. However at the Ca^{2+} concentration of 100 μM, higher amounts of Ca^{2+} (~3 nmol/mg protein) are taken up at pH 7.4 as compared to those (~1 nmol/mg protein) taken up at the pH 8.5. These fractions of Ca^{2+} uptake are actually accumulated inside the vesicles against a Ca^{2+} concentration gradient, since they are abolished in the presence of the Ca^{2+} ionophore, ionomycin (Fig. 2). We also observed that the bafilomycin-sensitive Ca^{2+} accumulation was ΔpH dependent since it is abolished in the presence of CCCP (results not shown).

These results indicate that, essentially, two distinct mechanisms of ATP-dependent Ca^{2+} transport exist in synaptic vesicles: one appears to be mediated by a P-type ATPase (vanadate-inhibited) which behaves as a Ca^{2+} pump and another appears to be associated to a V-type ATPase activity (bafilomycin-inhibited) which behaves as a Ca^{2+}/H^+ antiport. Although at 100 μM $CaCl_2$, the antiport is significantly operative (Fig. 2B), we reported before that it is a low-affinity Ca^{2+} transport system with high activity at about 500 μM (Gonçalves et al., 1998). In this work, we studied the sensitivity to Ca^{2+} of both, the Ca^{2+} pump and the Ca^{2+}/H^+ antiport (Fig. 3). We observed that, at pH 8.5, the activity of the pump is low whereas, at pH 7.4 it is much higher, reaching a maximum (1.5 nmol/mg protein) at about 25 μM Ca^{2+}. Above this concentration the pump-mediated Ca^{2+} uptake sharply declined until complete abolishment at about 250 μM Ca^{2+} (Fig. 3A). In contrast, Fig. 3B shows that, at both pH values (pH 7.4 and 8.5) the antiport-mediated Ca^{2+} transport is enhanced as the Ca^{2+} con-

centration increases in the medium up to about 500 μM. Higher Ca^{2+} concentrations appear to have a gradual inhibitory effect. Although the Ca^{2+}/H^{+} antiport is maximally operative at the pH 8.5 (Fig. 3B), it also works significantly at pH 7.4 (Fig. 3B), which is optimal for operativity of the Ca^{2+} pump (Fig. 3A). It is interesting to note that, at pH 7.4, the Ca^{2+}/H^{+} antiport begins to work at Ca^{2+} concentrations (>100 μM) which partially inhibit the Ca^{2+} pump activity and it is maximally operative at concentrations of Ca^{2+} (~500 μM) which completely abolish the activity of the pump. By calculating kinetic parameters of both processes at the two pH values studied, we verified that, indeed, at pH 7.4 the values for $K_{0.5(Ca)}$ and J_{max} of the Ca^{2+} pump are 0.6 μM and 1.9 nmol/min/mg protein, whereas those for the Ca^{2+}/H^{+} antiport are 258 μM and 1.5 nmol/min/mg protein, respectively (Fig. 3). Substitution of the pump activity by the antiport activity was also observed when, at pH 8.5, the Ca^{2+} pump operativity appears to be reduced, whereas the Ca^{2+}/H^{+} antiport is maximal, exhibiting values of $K_{0.5(Ca)}$ and J_{max} of about 217 μM and 1.9 nmol/min/mg protein, respectively (Fig. 3). Since the vanadate-sensitive Ca^{2+} uptake represents the operativity of a P-type ATPase (Ca^{2+} pump), we measured the Ca^{2+}-stimulated ATP hydrolysis (Ca^{2+}-ATPase) under conditions identical to those of the Ca^{2+} uptake (Fig. 4A). The Ca^{2+}-ATPase activity (bafilomycin insensitive) is very low at pH 8.5, whereas it is largely active at pH 7.4. Furthermore, at this pH, the Ca^{2+}-ATPase varies in a Ca^{2+}-dependent manner. It increases with Ca^{2+} concentration in the medium, reaching a maximum at about 25 μM. At higher Ca^{2+} concentrations, the ATPase is inhibited and completely abolished at about 500 μM $CaCl_2$ (Fig. 4A). At pH 7.4, the Ca^{2+}-dependent ATP hydrolysis proceeds with a $K_{0.5(Ca)}$ of 1.4 μM and a V_{max} of about 161 nmolP_i/min/mg protein.

On the other hand, the bafilomycin-sensitive Ca^{2+} uptake appears to be well correlated with the activity of the V-type H^{+}-ATPase (Fig. 4B) found in synaptic vesicles (Tanaka et al., 1976; Breer et al., 1977; Cidon and Sihra, 1989; Floor et al., 1990; Moriyama and Futai, 1990; Hicks and Parsons, 1992). This enzyme appears to be largely operative at about pH 8.5, although at pH 7.4, it also exhibits great activity (Fig. 4B). We also observed that the H^{+}-ATPase is Ca^{2+}-sensitive since it is stimulated at concentrations up to about 350 μM whereas high Ca^{2+} concentrations (>500 μM) appears to have a slight inhibitory effect. These results are in good correlation with the values obtained for the Ca^{2+} uptake under the same conditions (Fig. 3B). This is not surprising if we take in consideration that Ca^{2+}/H^{+} exchange by the antiport dissipates the pH gradient across the membrane and consequently stimulates the H^{+}-ATPase. This indirect action of Ca^{2+} on the H^{+}-ATPase activity was supported by the observation that Ca^{2+} has no

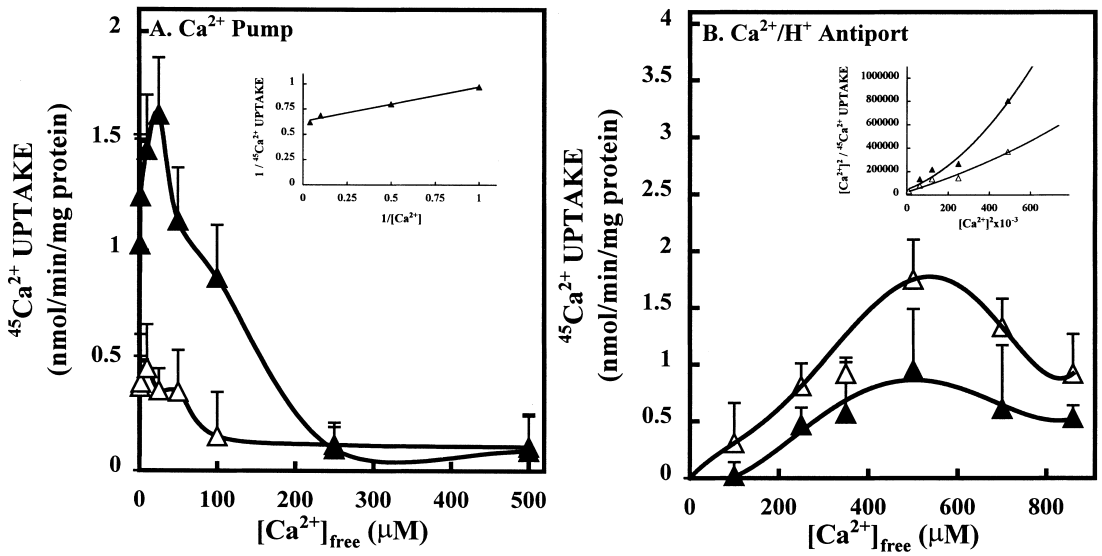

Fig. 3. Ca^{2+} pump (A) and ΔpH-dependent Ca^{2+} uptake (B) dependency on the Ca^{2+} concentration. The vesicles (600 μg protein/ml) were incubated in a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and buffered at different pH values (10 mM Tris and HEPES to pH 8.5 (△–△) and 7.4 (▲–▲), respectively) in the presence of 504 μM ATP–Mg. The reactions were started by adding 10 μCi/mmol $^{45}CaCl_2$ supplemented with $CaCl_2$ to obtain free Ca^{2+} concentration in the range of 1–860 μM. The reactions were stopped after 1 min as described in the text. Ca^{2+} uptake mediated by the Ca^{2+} pump (A) and by Ca^{2+}/H^{+} antiport (B) were respectively determined from the amount of Ca^{2+} taken up in the presence of 300 nM bafilomycin or 10 μM vanadate by subtracting the amount of Ca^{2+} taken up in the presence of both; bafilomycin and vanadate, under the various experimental conditions. Values represent the mean ± SD of at least five separate experiments. Plots of $1/Ca^{2+}$ uptake vs $1/[Ca^{2+}]$ (A) and of $[Ca^{2+}]^2/Ca^{2+}$ uptake vs $[Ca^{2+}]^2$ (B) are presented as inserts.

significant effect on the enzyme previously uncoupled by CCCP (Fig. 4B, table insert).

3.3. Effect of calcium on the proton accumulation by synaptic vesicles

In order to demonstrate that, indeed, the low affinity Ca^{2+} influx reported above is associated to the H^+ efflux, we elaborated experiments to visualize Ca^{2+}-induced changes in the proton accumulation mediated by the synaptic vesicle H^+-ATPase system. The results were obtained by recording the variation in the quenching of acridine orange fluorescence associated to the H^+ movements across the membranes.

Fig. 5 shows that after formation of the proton gradient in the presence of 210 μM ATP, addition of 500 μM Ca^{2+} accelerates proton liberation with consequent dissipation of the ΔpH. This reflects activity of the Ca^{2+}/H^+ antiport in agreement with the results of the ΔpH dependent Ca^{2+} accumulation (Fig. 5 insert). The ATP-dependent H^+ accumulation was checked by using the protonophore CCCP. It prevented formation of the ΔpH when present in the H^+ uptake reaction medium and it completely dissipated the proton gradient when added after its formation (Fig. 5).

The uncoupling effect of Ca^{2+} on the synaptic vesicles proton pump is also evidenced by the results depicted in Fig. 6. In fact, the magnitude of the ATP dependent proton gradient decreases as the Ca^{2+} concentration increases in the H^+ uptake reaction medium. The operativity of the Ca^{2+}/H^+ antiport system is well evidenced under non saturating conditions of ATP (22.4 μM) since it is masked when the proton-pump is highly efficient to reload the H^+ released in exchange for Ca^{2+}.

All these experiments showed the operativity of two distinct Ca^{2+} transport systems. However, demonstration that they belong to the same pool (synaptic vesicles) is necessary since contaminant microsomes in our preparation could be responsible for the Ca^{2+} pump activity observed.

For this purpose, we compared the amount of Ca^{2+} taken up by our synaptic vesicle preparation under various experimental conditions (Fig. 7). We observed that, after reaching a steady-state value of Ca^{2+} uptake (~2 nmol/mg protein) under conditions of maximal activity of the Ca^{2+} pump (25 μM Ca^{2+}, pH 7.4), a fraction of about 1.3 nmol Ca^{2+}/mg protein could be further taken up by increasing the Ca^{2+} concentration and the pH of the medium to the values (500 μM Ca^{2+} and pH 8.5) required for optimal operativity of the Ca^{2+}/H^+ antiport (curve a). On the other hand, when synaptic vesicles were loaded with Ca^{2+} exclusively by operativity of the Ca^{2+}/H^+ antiport, higher amounts of Ca^{2+} (3.5 nmol/mg protein) were accumulated inside the vesicles (curve b).

Since the previous pump-mediated Ca^{2+} loading prevented further maximal Ca^{2+} accumulation by the Ca^{2+}/H^+ antiport (Fig. 7), it appears plausible to assume that the same pool (synaptic vesicles) takes up

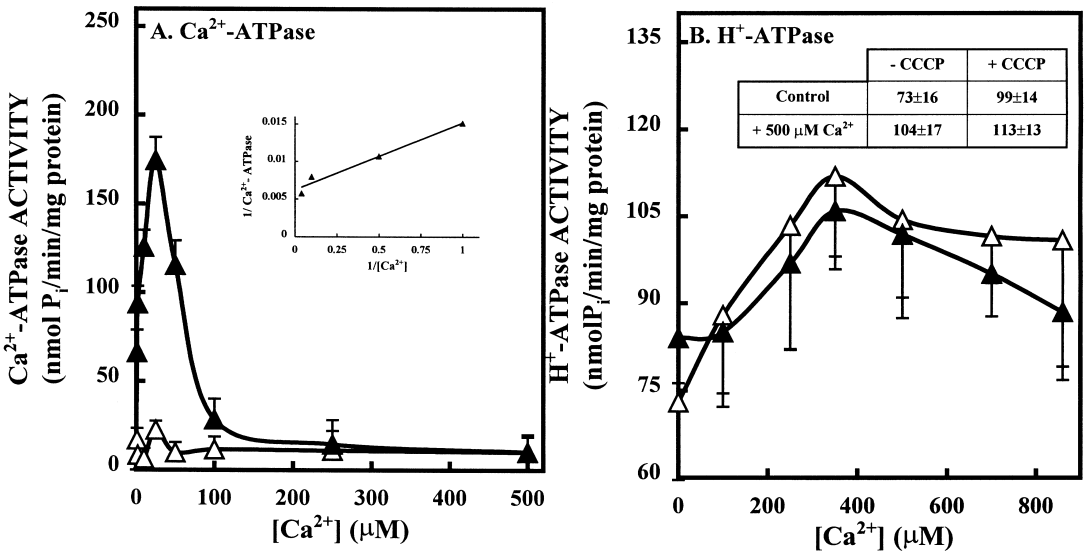

Fig. 4. Ca^{2+}-ATPase (A) and H^+-ATPase (B) dependency on the Ca^{2+} concentration. The vesicles (600 μg protein/ml) were incubated in a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and buffered at different pH values (10 mM Tris and HEPES to pH 8.5 (△–△) and 7.4 (▲–▲), respectively). The ATPase activities were assayed as described in Fig. 3. The reactions were started by adding 504 μM Mg–ATP and, 1 min later, P_i analysis was performed as described in the text. Values represent the mean ± SD of at least five separate experiments. The plot of $1/Ca^{2+}$-ATPase activity vs $1/[Ca^{2+}]$ (A) and the effect of 500 μM Ca^{2+} on the H^+-ATPase activity under conditions of 'coupling' (absence of CCCP) or 'uncoupling' (presence of 10 μM CCCP) of the proton pump (B) are presented as inserts.

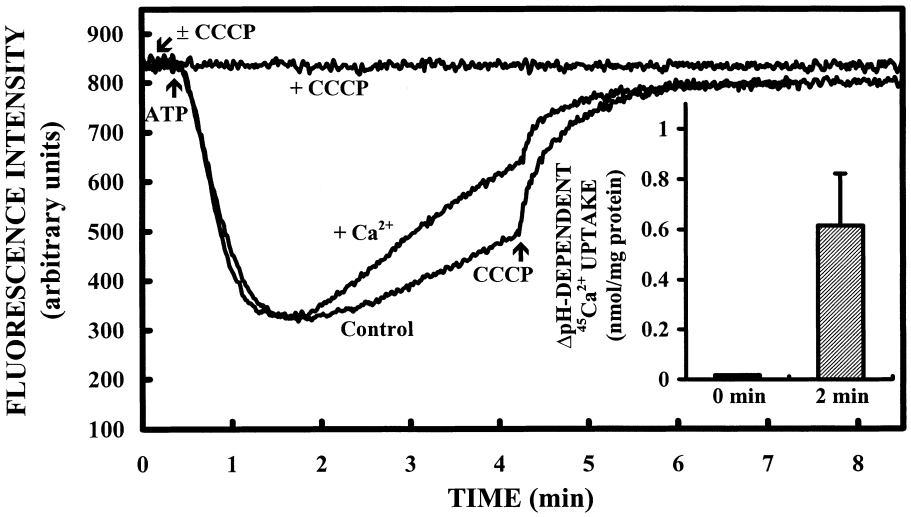

Fig. 5. Ca^{2+}-induced H^+ release in synaptic vesicles. Synaptic vesicles (1.2 mg protein) were incubated in 2 ml of a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and 10 mM Tris at pH 8.5. The reaction was started by adding 210 μM Mg–ATP and the proton transport was visualized by quenching of acridine orange fluorescence as described in the text. At 2 min of reaction, H^+ liberation was induced by adding 500 μM $CaCl_2$ (representative experiment). The protonophore CCCP (10 μM) was used to check that a pH gradient was formed at expenses of ATP. Diagram insert represents the ΔpH-dependent Ca^{2+} uptake by synaptic vesicles after 2 min of reaction. Values represent the mean ± SD of four separate experiments.

Ca^{2+} by the two distinct mechanisms. Furthermore, activity of the microsomal Ca^{2+} pump does not occur under the conditions reported above. Indeed, isolated microsomes exhibited a Ca^{2+} pump system with higher affinity for Ca^{2+} ($K_{0.5} = 0.017$ μM) as compared to that of synaptic vesicles ($K_{0.5} = 0.6$ μM), and it was inhibited by Ca^{2+} concentrations (25 μM) that were optimal for activity of the synaptic vesicle Ca^{2+} pump system (Fig. 8).

4. Discussion

By using specific inhibitors in reaction media buffered for suitable values of pH and pCa, we could distinguish between two types of Ca^{2+} transport in synaptic vesicles of sheep brain cortex. One of them is mediated by Ca^{2+}-ATPase and the other is mediated by Ca^{2+}/H^+ antiporter.

The first indication that two mechanisms of Ca^{2+}

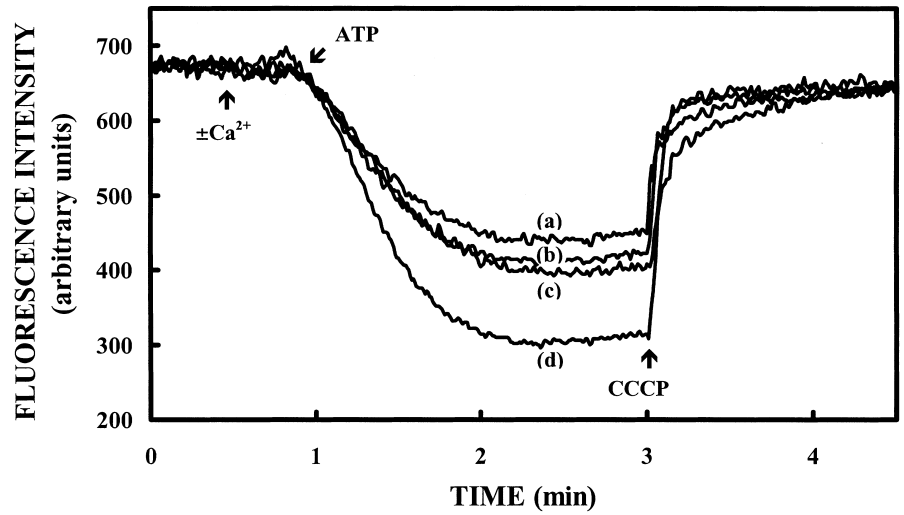

Fig. 6. Ca^{2+} effect on the proton gradient magnitude across synaptic vesicle membrane. Synaptic vesicles (1.2 mg protein) were incubated in 2 ml of a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and 10 mM Tris at pH 8.5. The reaction was started by adding 22.4 μM Mg–ATP and the proton transport was visualized by quenching of acridine orange fluorescence as described in the text. The reactions were carried out in the absence (d) and in the presence of 100 (b), 500 (a) or 860 (c) μM $CaCl_2$ (representative experiments). The protonophore CCCP (10 μM) was used to check that a pH gradient was formed at expenses of ATP.

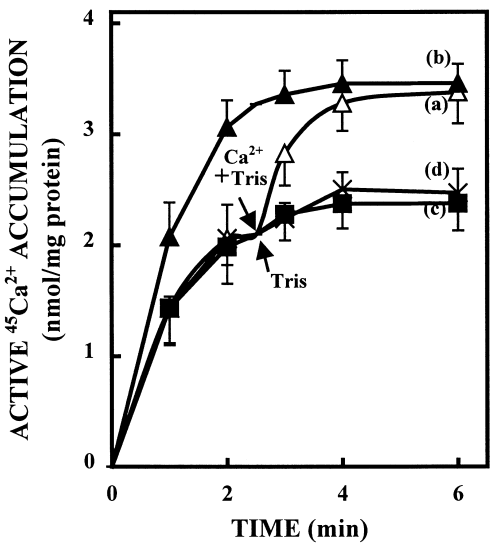

Fig. 7. Synaptic vesicles Ca^{2+} uptake successively mediated by the Ca^{2+} pump and by the Ca^{2+}/H^{+} antiport. The vesicles (0.6 mg protein/ml) were firstly incubated in the reaction medium containing 25 μM Ca^{2+} (pH 7.4) and, after 2.5 min, Tris and Ca^{2+} were added to perform pH 8.5 and 500 μM Ca^{2+}, respectively (curve a, △–△). Ca^{2+} uptake was measured as described in the text. Controls of Ca^{2+} uptake exclusively mediated by Ca^{2+}/H^{+} antiport (curve b, ▲–▲) or by the Ca^{2+} pump (curve c, ■–■) were performed as indicated in the graph. The effect of increasing pH to 8.5, remaining Ca^{2+} concentration at 25 μM was also tested (curve d, X–X). Values represent the mean ± SD of four separate experiments.

transport exist in synaptic vesicles resulted from the observation that low Ca^{2+} concentrations (pCa > 4) support efficient Ca^{2+} uptake at pH 7.4, whereas at pH 8.5, the process was highly operative only at high Ca^{2+} concentrations (pCa = 3.3) (Fig. 1).

These mechanisms of high and low affinity for Ca^{2+} were also distinguished by their sensitivity to vanadate and bafilomycin, respectively (Fig. 2). Thus, these drugs appeared to be useful as tools to analyze, in separate, the activity of both Ca^{2+} transport systems.

The vanadate-inhibited Ca^{2+} uptake was revealed to be associated to a P-type Ca^{2+}-ATPase that works by pumping Ca^{2+} into the vesicles. Indeed, we observed good correlation between this pump-mediated Ca^{2+} uptake and the Ca^{2+}-dependent ATP hydrolysis with respect to their sensitivity to Ca^{2+}. They reached a maximal activity at about 25 μM Ca^{2+}, whereas they were completely abolished at 250 μM Ca^{2+} in the reaction medium (Fig. 3A). These characteristics appeared to be different from those of the microsomal Ca^{2+} pump (Fig. 8) which indicates that the Ca^{2+} pump activity observed in our synaptic vesicle preparation does not correspond to the Ca^{2+} pump activity of the contaminant microsomes. Furthermore, we observed that the pool which accumulated Ca^{2+} by previous activity of the Ca^{2+} pump, also accumulated Ca^{2+} by activity of the Ca^{2+}/H^{+} antiport, whose dependence on the proton ATPase (Gonçalves et al., 1999b) assures synaptic vesicles identification. Indeed, a V-type ATPase responsible for generation of a proton gradient across the membrane has been reported in synaptic vesicles by several investigators (Tanaka et al., 1976; Breer et al., 1977, Stelzl et al., 1987; Cidon and Sihra, 1989; Floor et al., 1990; Moriyama and Futai, 1990; Hicks and Parsons, 1992).

The sensitivity to Ca^{2+}, together with that to vanadate, indicates that good correlation exists between Ca^{2+} transport and Ca^{2+}-ATPase activities. However, the values of J_{max} (1.9 nmol Ca/min/mg protein) for Ca^{2+} uptake and V_{max} (161 nmol P_i/min/mg protein) for the Ca^{2+}-ATPase (Figs. 3 and 4) do not reflect an actual coupling ratio for the Ca^{2+} pump, revealing an excessive ATP hydrolysis. It is possible that a residual H^{+}-ATPase activity that is not completely abolished in the presence of bafilomycin also contributes for the high P_i liberation observed in the assays of Ca^{2+}-ATPase at pH 7.4.

The presence of a Ca^{2+}-ATPase in synaptic vesicles has been previously suggested (Michaelson and Ophir, 1980; Michaelson et al., 1980). However, no correspondence was observed between the Ca^{2+} transport [$K_{0.5(Ca)} = 50$ μM] and the Ca^{2+}-stimulated ATPase [$K_{0.5(Ca)} = 1$ mM]. Breer et al. (1977) also reported an ATPase activity in *Torpedo marmorata* synaptic vesicles which was maximally stimulated by 5 mM $CaCl_2$, and identical observations by Stelzl et al. (1987) led to the conclusion that ATP-dependent vesicular Ca^{2+} uptake is not due to the presence of a (Ca^{2+}–Mg^{2+})-ATPase stimulated by micromolar Ca^{2+} concen-

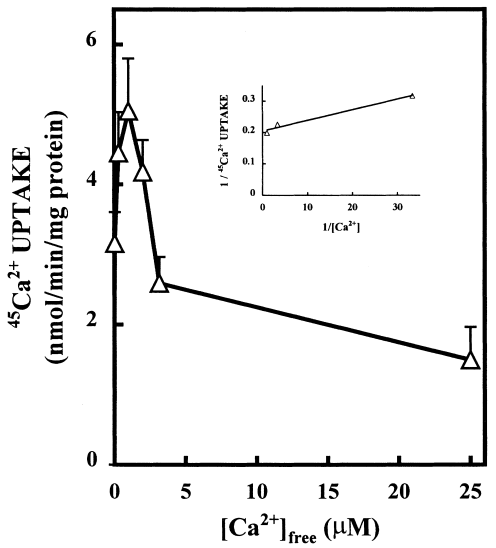

Fig. 8. Ca^{2+} sensitivity of the brain microsomal Ca^{2+} pump. Microsomal vesicles were incubated as described in Fig. 3A, except that the Ca^{2+} concentrations changed from 0.03 μM to 25 μM. Values represent the mean ± SD of four separate experiments. The plot of 1/ Ca^{2+} uptake vs 1/$[Ca^{2+}]$ is presented as insert.

trations. Hicks and Parsons (1992) also reported the presence of a P-type ATPase activity (vanadate-sensitive) in synaptic vesicles, but they did not observed Ca^{2+} stimulation. Instead, the activity of that ATPase was low in the presence of Ca^{2+} (2 mM). These controversial conclusions may be explained by the fact that the activity of the Ca^{2+}-ATPase was not perfectly distinguished from the H^+-ATPase activity, which appears to be indirectly stimulated by high Ca^{2+} concentrations (~500 μM) (Fig. 4B, insert). Moreover, under the conditions reported by those investigators (millimolar Ca^{2+} concentrations), the Ca^{2+}-ATPase is completely inhibited (Fig. 3A), which justifies the low activity of the P-type ATPase observed by Hicks and Parsons (1992) in the presence of 2 mM $CaCl_2$.

Besides the Ca^{2+} pump activity that functions in the micromolar range of Ca^{2+} concentration, we observed that Ca^{2+} can be also accumulated inside the vesicles by a process that depends on both high Ca^{2+} concentrations and ΔpH gradient across the membranes. Indeed, this low affinity Ca^{2+} transport system is abolished in the presence of the protonophore, CCCP, or of the V-type ATPase inhibitor, bafilomycin (Fig. 2), suggesting requirement of the H^+-ATPase activity. It appears that the proton gradient is the driving force to support Ca^{2+} accumulation through a Ca^{2+}/H^+ antiporter which works at high Ca^{2+} concentrations as recently reported (Gonçalves et al., 1998, 1999b).

In this work we show maximal operativity of this system at pH 8.5 (Fig. 3B) in agreement with the optimal pH (8.5) observed for activity of the H^+-ATPase (Fig. 4B) that is responsible for the pH gradient maintenance across the membrane.

These findings indicate that a Ca^{2+}/H^+ antiport, indirectly associated to the H^+-ATPase activity, acts as a secondary active Ca^{2+} transport, whereas the Ca^{2+} pump acts as a primary active Ca^{2+} transport directly associated to the Ca^{2+}-ATPase activity. In both cases, the Ca^{2+} taken up by the vesicles is completely discharged by the Ca^{2+} ionophore, ionomycin, which demonstrates that, indeed, the Ca^{2+} was taken up against its concentration gradient (Fig. 2).

The presence of a Ca^{2+}/H^+ antiport system in synaptic vesicles was checked by the observation that Ca^{2+} addition promotes H^+ release from proton loaded vesicles or decreases the magnitude of the pH gradient formed in its presence (Figs. 5 and 6).

The observation of Ca^{2+}/H^+ antiport activity in synaptic vesicles provides a ready explanation for stimulation of the H^+-ATPase by high Ca^{2+} concentrations (~500 μM), as was previously observed (Gonçalves et al., 1998) and checked in this work.

As the well-known P-type Ca^{2+} pumps require a Ca^{2+}/H^+ exchange for activity (Carafoli, 1987), distinction of an antiport system from direct pumping should be difficult. However, the results presented here suggest that the Ca^{2+} pump and the Ca^{2+}/H^+ antiport are different mechanisms for Ca^{2+} transport across synaptic vesicle membranes.

Since the antiport activity is maximal at Ca^{2+} concentrations (>100 μM) greater than those observed in either resting cell or those experiencing a Ca^{2+} transient (10 μM), it is plausible to assume that it plays a significant role in reducing cytoplasmic Ca^{2+} under extreme conditions of Ca^{2+} stress. On the other hand, the function of the high affinity Ca^{2+} pump is likely predominant under physiological conditions. It appears that, under Ca^{2+} excess the Ca^{2+}/H^+ antiport substitutes the Ca^{2+} pump in removing cytoplasmic Ca^{2+}, since it works efficiently under conditions which completely inhibit the Ca^{2+} pump (Fig. 3A and B).

In spite of these assumptions, we cannot rule out the hypothesis that, even under non-stressing Ca^{2+} conditions, Ca^{2+}/H^+ antiport has also physiological importance. Indeed, as suggested by several investigators (Augustine et al., 1992; Llinás et al., 1992; Neher, 1998), submembrane microdomains may reach high Ca^{2+} concentrations (>100 μM), so that synaptic vesicles may function physiologically as intracellular Ca^{2+} stores (Parducz and Dunant, 1993) or exocytotic bags for extrusion of Ca^{2+} (Petersen, 1996). These aspects may be important if we consider that a rise in the cytosolic Ca^{2+} concentration induces brief cellular responses (classic Ca^{2+} signal), whereas changes in the microdomains Ca^{2+} concentration appear to be responsible for sustained responses (Ca^{2+} cycling signal) as suggested by Rasmussen (1989).

If we keep in mind that Ca^{2+} entry in exchange for H^+ may induce dissipation of the synaptic vesicle proton gradient (Fig. 5), it is expected that synaptic vesicle neurotransmitters uptake is reduced. In fact, various investigators observed that after prolonged stimulation, synaptic vesicles accumulate Ca^{2+} in correlation with decrease in the vesicular acetylcholine content (Babel-Guérin et al., 1977; Parducz et al., 1994) and, under certain conditions, Ca^{2+} inhibits exocytotic release of neurotransmitters (Carvalho et al., 1991; Duarte et al., 1993). We are currently exploring these implications in isolated synaptic vesicle preparations.

The results reported here not only demonstrate the presence of two distinct mechanisms of Ca^{2+} transport in synaptic vesicles of brain cortex, but permit clarification of some controversial conclusions previously reported by other investigators (Breer et al., 1977; Michaelson et al., 1980; Stelzl et al., 1987).

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