ELSEVIER

Molecular Brain Research 71 (1999) 178–184

MOLECULAR BRAIN RESEARCH

www.elsevier.com/locate/bres

Synaptic vesicle Ca^{2+}/H^{+} antiport: dependence on the proton electrochemical gradient

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 Aveiro, Portugal*

[b] *Centro de Neurociências, Departamento de Zoologia, Faculdade de Ciências, Universidade de Coimbra, 3049 Coimbra, Portugal*

Accepted 25 May 1999

Abstract

Synaptic vesicles isolated from sheep brain cortex accumulate Ca^{2+} by a mechanism of secondary active transport associated to the H^{+}-pump activity. The process can be visualized either by measuring Ca^{2+}-induced H^{+} release or ΔpH-dependent Ca^{2+} accumulation. We observed that the amount of Ca^{2+} taken up by the vesicles increases with the magnitude of the ΔpH across the membrane, particularly at Ca^{2+} concentrations ($\sim$ 500 μM) found optimal for the antiporter activity. Similarly, H^{+} release induced by Ca^{2+} increased with the magnitude of ΔpH. However, above 60% ΔpH (high H^{+}-pump activity), the net H^{+} release from the vesicles decreased as the pump-mediated H^{+} influx exceeded the Ca^{2+}-induced H^{+} efflux. We also observed that the Ca^{2+}/H^{+} antiport activity depends, essentially, on the ΔpH component of the electrochemical gradient ($\sim$ 3 nmol Ca^{2+} taken up/mg protein), although the $\Delta\varphi$ component may also support some Ca^{2+} accumulation by the vesicles ($\sim$ 1 nmol/mg protein) in the absence of ΔpH. Both Ca^{2+}-induced H^{+} release and ΔpH-dependent Ca^{2+} uptake could be driven by an artificially imposed proton motive force. Under normal conditions (H^{+} pump-induced ΔpH), the electrochemical gradient dependence of Ca^{2+} uptake by the vesicles was checked by inhibition of the process with specific inhibitors (bafilomycin A_1, ergocryptin, folymicin, DCCD) of the H^{+}-pump activity. These results indicate that synaptic vesicles Ca^{2+}/H^{+} antiport is indirectly linked to ATP hydrolysis and it is essentially dependent on the chemical component (ΔpH) of the electrochemical gradient generated by the H^{+}-pump activity.

Keywords: Ca^{2+}/H^{+} antiport; Synaptic vesicle; Brain cortex

1. Introduction

The exocytotic release of neurotransmitters depends on the presence of calcium ions that enter the nerve cells by stimulus-induced depolarization wave [25]. Therefore, the maintenance of the cell Ca^{2+} homeostasis by intracellular Ca^{2+} stores appears to be important for the neurotransmission regulation. Not only mitochondria and endoplasmic reticulum accumulate Ca^{2+} [15], but also synaptic vesicles appear to have Ca^{2+} sequestering properties well visualized in previous cytochemical studies [20–22].

In previous investigations we found that sheep brain synaptic vesicles contain a Ca^{2+}/H^{+} antiport system, which actively transport Ca^{2+} across the membrane at expenses of the ΔpH energy generated by the proton pumping ATPase [7]. The antiporter has low affinity for Ca^{2+} and, recently, we observed that it has low selectivity for this cation [8]. Indeed, various cations appear to be exchanged with protons according to their ionic radius in the dehydrated form: $Zn^{2+} > Cd^{2+} > Ca^{2+}$. Therefore, synaptic vesicles appear to serve as neurotransmitter reservoirs used in the neurosecretion process or as cation bags to be exocytotically discharged under stress conditions. Ca^{2+} accumulation inside the vesicles was previously observed by several authors [13,18], although its actual mechanism of transport was not well established.

Abbreviations: Acridine orange, bis[dimethylamino]acridine; CCCP, carbonyl cyanide *n*-chlorophenylhydrazone; DCCD, *N,N′*-dicyclohexylcarboiimide; DTT, DL-dithiothreitol; EGTA, ethyleneglycol-bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; Ergocryptine, 2-bromo-α-ergocryptine; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethane-sulfonic acid; Mes, 2[*N*-morpholino]-ethanesulfonic acid; Oxonol VI, Bis-(3-propyl-5-oxoisoxazol-4-yl)-pentamethine oxonol; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol

* Corresponding author. Fax: +351-34-26408; E-mail: pgoncalves@bio.ua.pt

0169-328X/99/$ - see front matter
PII: S0169-328X(99)00183-7

In this work, we observed that a proton gradient across synaptic vesicle membranes, either naturally formed by the H^+-pump activity or artificially imposed by a pH jump, constitutes the direct energy source for Ca^{2+} transport and that the process is essentially dependent on the ΔpH component of the proton electrochemical gradient ($\Delta\mu H^+$) across the membrane.

2. Materials and methods

2.1. Materials

All reagents were analytical grade. Bafilomycin A_1, ergocryptine (2-bromo-α-ergocryptine), DCCD (*N,N′*-dicyclohexylcarboiimide), CCCP (carbonyl cyanide *n*-chlorophenylhydrozone), ATP and acridine orange (bis[dimethylamino]acridine) were obtained from Sigma. Oxonol VI (Bis-(3-propyl-5-oxoisoxazol-4-yl)-pentamethine oxonol) and folimycin were supplied by Molecular Probes and Calbiochem, respectively. $^{45}CaCl_2$ (2 mCi/ml) was purchased from Amersham.

2.2. Isolation of synaptic vesicles

Synaptic vesicles were isolated from sheep brain cortex according to the procedure described by Hell et al. [10,11]. The brains were cut in 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 *N*-2-hydroxyethylpiperazine-*N′*-2-ethane-sulfonic acid (HEPES)-K (pH 7.3), 0.2 mM EGTA (ethyleneglycol-bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid), 0.5 μg/ml pepstatin and 1 μg/ml leupeptin. After centrifugation during 10 min at $47{,}000 \times g$, the supernatant was collected and centrifuged again for 40 min at $120{,}000 \times g$. 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 $260{,}000 \times g$. 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 $27{,}000 \times g$, the supernatant containing the purified synaptic vesicles was collected, frozen in liquid nitrogen and stored at −70°C. This method allows preparation of a highly pure synaptic vesicles fraction as revealed by assays of immunoblotting and marker enzymes activity [10,11].

Artificial pH gradient across the synaptic vesicle membrane was imposed by incubation of synaptic vesicles (15 mg protein/ml) in loading medium (300 mM sorbitol, 1 mM DTT (DL-dithiothreitol), 10 mM potassium gluconate and 20 mM 2[*N*-morpholino]-ethanesulfonic acid (Mes)–2-amino-2-hydroxymethylpropane-1,3-diol (Tris), pH 5.6) during 4 h at 4°C.

Analysis of protein was performed by the method of Gornall et al. [9].

2.3. Ca^{2+} uptake assays

Ca^{2+} accumulation by isolated synaptic vesicles was measured by rapid filtration and scintillation counting. In standard uptake assay conditions, 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, Tris–HCl (pH 8.5) and 504 μM ATP-Mg (except otherwise indicated). After 50 s, the reaction was initiated by adding $CaCl_2$ 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 inhibitors folimycin, DCCD, bafilomycin A_1 and ergocryptine were added at the beginning of the Ca^{2+} uptake reaction.

ATP-dependent Ca^{2+} uptake activities were calculated by subtracting the amount of Ca^{2+} taken up in the absence of ATP-Mg from the total amount taken up in the presence of ATP-Mg and the values were expressed as nmol/mg protein.

2.4. Measurements of $\Delta\varphi$ and ΔpH by fluorescence quenching

ATP-dependent proton transport was measured by following the fluorescence quenching of 2 μM acridine orange [4], while the electrical potential ($\Delta\varphi$) across the synaptic vesicle membrane was monitored by measuring ATP-dependent fluorescence quenching of 3.3 μM oxonol VI [27]. The membrane vesicles (600 or 900 μg protein/ml) were incubated at 30°C in different reaction media (2 ml), as indicated in the legend of the figures. Proton transport and $\Delta\varphi$ generation across the synaptic vesicle membrane were initiated by adding ATP-Mg. Formation of ΔpH and $\Delta\varphi$ (electrochemical gradient components) were checked by promoting their elimination with the protonophore CCCP (10 μM).

The fluorescence emissions were measured in the presence of 2 μM acridine orange and 3.3 μM oxonol VI at 525 nm (slit width 3.5 nm) and 612 nm (slit width 10.0 nm) using an excitation wavelength of 495 nm (slit width 3.0 nm) and 591 nm (slit width 5.0 nm), respectively. The fluorescence changes due to H^+ movements were followed by using a Perkin-Elmer computer-controlled spectrofluorometer, Model LS-50. Fluorescence changes were quantified using the following equation:

$$\%\ \text{fractional quench} = [F_t - F_{ATP}]/F_o \times 100$$

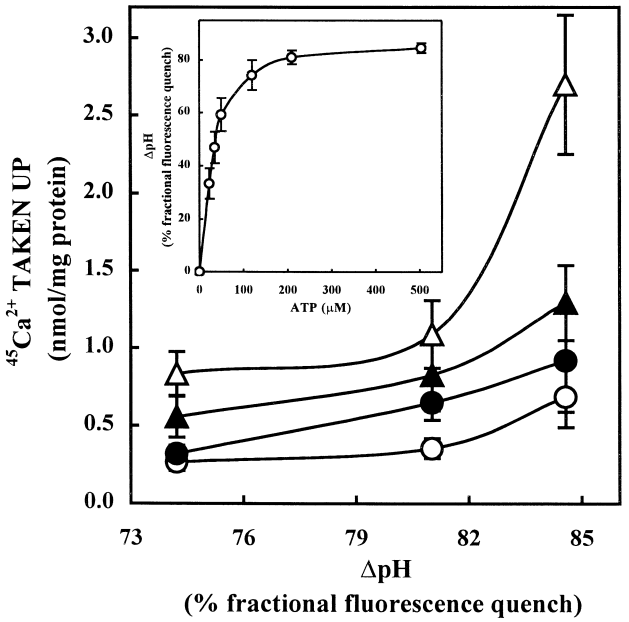

Fig. 1. ΔpH-dependent Ca^{2+} uptake by isolated synaptic vesicles. The vesicles (600 μg protein/ml) were incubated in a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and 10 mM Tris (pH 8.5) in the presence of increasing concentrations (120–504 μM) of ATP-Mg. After 50 s, the reactions were started by adding 10 μCi/mmol $^{45}CaCl_2$ supplemented with $CaCl_2$ to obtain free Ca^{2+} concentration of 100 μM (○-○), 350 μM (●-●), 500 μM (△-△) and 700 μM (▲-▲). The reactions were stopped after 2 min by filtering 800 μl aliquots of reaction medium through Millipore filters (∅ 0.45 μm). The radioactivity of the filters was measured as described in the text. The figure insert represents the magnitude of the ΔpH as a function of ATP concentration. Synaptic vesicles (1.2 mg protein) were incubated in 2 ml of a medium containing 2 μM acridine orange, 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and 10 mM Tris at pH 8.5, and the reaction was started by adding increasing concentrations (22.4–504 μM) of Mg-ATP. The proton transport was visualized by quenching of acridine orange fluorescence, and the fractional fluorescence quench at steady-state was calculated as described in the text. The protonophore CCCP (10 μM) was used to check ATP-dependent generation of ΔpH whose quantification was performed by using the equation expressed in Section 2. Values represent the mean ± S.D. of 10 separate experiments.

where F_t is the initial fluorescence intensity, and F_{ATP} and F_o are the fluorescence intensities after addition of ATP-Mg and CCCP, respectively.

2.5. Measurements of Ca^{2+}-induced H^+ release by potentiometry

The Ca^{2+}-induced dissipation of the ΔpH artificially imposed to synaptic vesicles was assayed by following the release of H^+ with a ''Ingold U402-M6-S7/100'' hydrogen electrode associated to a ''Crison micro pH meter 2002'' which was connected to a ''Zipp&Zonen BD 111'' recorder. Inside-acid synaptic vesicles (600 μg protein/ml) were incubated in a medium containing 300 mM sorbitol, 10 mM potassium gluconate and 1 mM Tris (pH 8.5). The reaction was started by adding 500 μM $CaCl_2$ and it proceeded in a constantly stirred reaction mixture thermostated at 30°C. The Ca^{2+}-induced medium acidification was checked by calibrating the system with freshly titrated NaOH at the end of each experiment. The Ca^{2+}/H^+ antiport activity was expressed as nmol H^+ released/mg protein.

2.6. Treatment of the data

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

3. Results

3.1. Influence of the ΔpH magnitude on the synaptic vesicle Ca^{2+}/H^+ antiport activity

The Ca^{2+}/H^+ antiport activity described here was studied by measuring either the Ca^{2+}-induced H^+ release or the ΔpH-dependent Ca^{2+} uptake by synaptic vesicles.

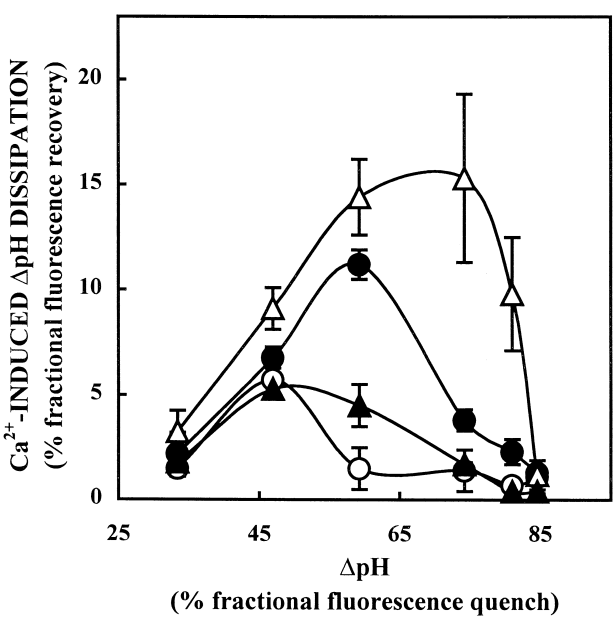

Fig. 2. Ca^{2+}-induced H^+ release from synaptic vesicles pre-loaded with protons in the presence of increasing concentrations of ATP-Mg. The vesicles (600 μg protein/ml) were incubated in a medium containing 2 μM acridine orange, 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and 10 mM Tris (pH 8.5) in the presence of increasing concentrations (120–504 μM) of ATP-Mg. After 50 s, the reactions were started by adding $CaCl_2$ to obtain free Ca^{2+} concentration of 100 μM (○-○), 350 μM (●-●), 500 μM (△-△) and 700 μM (▲-▲). The total amount of H^+ released during 2 min in response of Ca^{2+} addition was evaluated as described in the text and it was expressed as fractional acridine fluorescence recovery. The ATP-dependent ΔpH across the synaptic vesicle membrane was measured as described in the legend of Fig. 1 and it was expressed as fractional acridine orange fluorescence quench. Values represent the mean ± S.D. of 3–11 separate experiments.

Fig. 1 shows that Ca^{2+} uptake increases as the magnitude of ΔpH (evaluated in percentage of acridine orange fluorescence quenching) increases above 81%. This observation is particularly evident (Ca^{2+} uptake increasing from about 0.8 to 2.7 nmol/mg protein), when the uptake reaction was performed at 500 μM Ca^{2+} concentration which has been previously revealed as the optimal Ca^{2+} concentration for the Ca^{2+}/H^+ antiporter activity [7]. Various values of ΔpH magnitude were obtained by using different concentrations of ATP. It increased up to about 200 μM ATP, reaching a plateau (84.6 ± 1.8%) at high concentrations of the nucleotide (Fig. 1, insert). The $K_{0.5(ATP)}$ obtained for H^+ transport (42 μM) agrees with that for H^+-ATPase activity (26 μM) reported by other investigators [12]. The non-limiting concentrations of ATP permit a delayed steady state (results not shown) as the proton release by some leaking of the membrane may be recovered by the vesicles at the expense of ATP.

It is interesting to note that ΔpH was well correlated not only with the magnitude of Ca^{2+} influx (Fig. 1), but also with the amount of Ca^{2+}-induced H^+ efflux (Fig. 2), suggesting that, indeed both ion movements are associated. However, above 60% ΔpH, where high ATP concentration maintains high H^+-pump activity, the capacity of Ca^{2+} to dissipate the proton gradient decreased. This apparent discrepancy appears to result from a high pump-mediated H^+

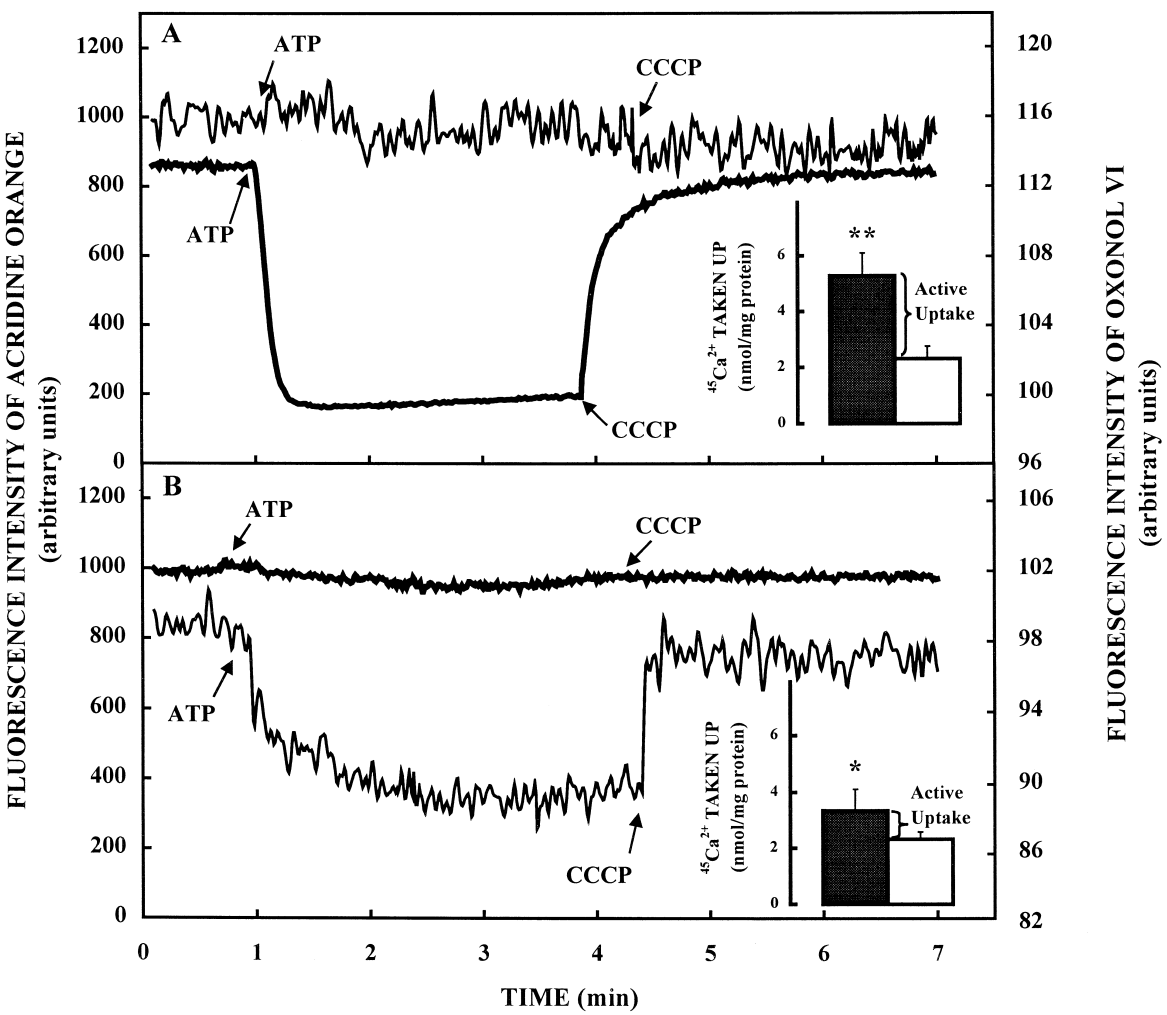

Fig. 3. Ca^{2+}/H^+ antiport activity as a function of the ΔpH and Δφ formed across synaptic vesicle membranes. The synaptic vesicles were incubated in 2 ml of media containing 60 mM sucrose, 50 μM EGTA, 10 mM Tris (pH 8.5), supplemented with 2 mM $MgCl_2$ and 150 mM KCl (A) or 2 mM $MgSO_4$ and 150 mM potassium gluconate (B), respectively. The reactions were started by adding 504 μM Mg-ATP and the ΔpH and the Δφ were evaluated by measuring the fluorescence quenching of acridine orange (dark record) and oxonol VI (light record), respectively. The protonophore CCCP (10 μM) was used to check that a ΔpH and Δφ were formed at expenses of ATP (representative experiments). The $^{45}Ca^{2+}$ taken up under the same experimental conditions are presented as an insert. The assays were initiated by addition of 607.7 μM $CaCl_2$ supplemented with 10 μCi/mmol $^{45}CaCl_2$ and allowed to proceed during 2 min in the absence (rectangle) and in the presence (filled rectangle) of 504 μM Mg-ATP. Values represent ± S.D. of 4 separate experiments (** $p < 0.001$ and * $p < 0.5$).

influx that, exceeding the Ca^{2+}-induced H^+ efflux, does not permit observation of ''net'' proton gradient dissipation by Ca^{2+} under conditions of high ΔpH (Fig. 2).

3.2. Influence of the proton electrochemical gradient components on the Ca^{2+}/H^+ antiport activity of synaptic vesicle

The synaptic vesicle proton ATPase generates an electrochemical gradient ($\Delta\mu H^+$) across the membrane which is composed of two inter-convertible components, a chemical gradient (ΔpH) and an electrical gradient ($\Delta\varphi$), that, at steady state, are related by the expression: $\Delta\mu H^+/F = \Delta\varphi - 59\ \Delta$pH.

In order to investigate the contribution of both chemical component and electrical component for the antiport activity, we measured the Ca^{2+} uptake by the vesicles under conditions where ΔpH was preponderant and $\Delta\varphi$ negligible (Fig. 3A) or, conversely, $\Delta\varphi$ was preponderant and ΔpH negligible (B).

The first condition was obtained by using a reaction medium with high concentration (150 mM) of the permeant anion (Cl^-), whereas the second condition was obtained in a medium containing the impermeant anion (gluconate) in the absence of Cl^-. These conditions were checked by measuring ΔpH and $\Delta\varphi$ through the analysis of the fluorescence quenching of acridine orange and oxonol VI, respectively (Fig. 3).

As shown in Fig. 3A, a chloride-containing medium permits large ΔpH formation by the H^+-ATPase activity, since Cl^- serves as a charge compensatory ion which, preventing membrane hyperpolarization due to H^+ entry, favors ΔpH generation. Conversely, in a gluconate-containing medium (Fig. 3B), ΔpH was not formed, but an ATP-dependent electrical potential across the membrane was evident. This is not surprising as gluconate does not permeate the membranes and, therefore, it is not able to reduce the electrical component of the electrochemical gradient [3].

In both situations, ΔpH and $\Delta\varphi$ magnitude were precisely evaluated by measuring the total amount of CCCP-reversible quenching after a steady state be obtained in the H^+ pumping reaction (Fig. 3A and B).

It is interesting to note that the amount of Ca^{2+} actively accumulated inside the vesicles under conditions of maximal ΔpH (Fig. 3A, insert) is higher ($\sim$3 nmol/mg protein) than that ($\sim$1 nmol/mg protein) accumulated when ΔpH is zero and $\Delta\varphi$ maximal (Fig. 3B, insert), suggesting that the ΔpH component of the proton electrochemical gradient constitutes the main driving force for the Ca^{2+}/H^+ antiport activity. Intermediary values of Ca^{2+} accumulation were obtained when both components (ΔpH and $\Delta\varphi$) existed across the synaptic membranes (results not shown).

On the other hand, we observed that under the conditions reported here, the active Ca^{2+} accumulation is directly dependent on the proton gradient and not on the presence of ATP. Indeed, by using synaptic vesicles, which were made acidic inside under pH jump conditions, we observed that Ca^{2+} was able to dissipate the proton gradient across the vesicle membranes with consequent decrease of pH in the reaction medium (Fig. 4). Moreover, as Ca^{2+}-induced proton release from the vesicles acidified the reaction medium (potentiometrically evaluated), Ca^{2+} was taken up by the vesicles, reaching a maximum level ($\sim$8.8 nmol/mg protein) at about 2 min of Ca^{2+}-induced reaction (Fig. 4, insert). This suggests that, even in the absence of ATP, a proton gradient artificially imposed is able to promote Ca^{2+} accumulation by the vesicles, which confirms the existence of a ΔpH-dependent Ca^{2+}/H^+ antiport in synaptic vesicles of sheep brain cortex.

Correlation between the Ca^{2+} transport into the vesicles and ΔpH formation across the membrane could be also demonstrated by studying the effect of specific H^+-ATPase inhibitors on the amount of Ca^{2+} accumulated by the vesicles (Fig. 5). We observed that bafilomycin A_1 and folimycin, which strongly inhibited (100%) the ΔpH formation (Fig. 5, insert), also greatly inhibited the Ca^{2+} uptake by the vesicles ($>$86% inhibition), whereas the weaker inhibitors of the H^+-pump, ergocryptin and DCCD, promoted about 60–80% inhibition on both types of pa-

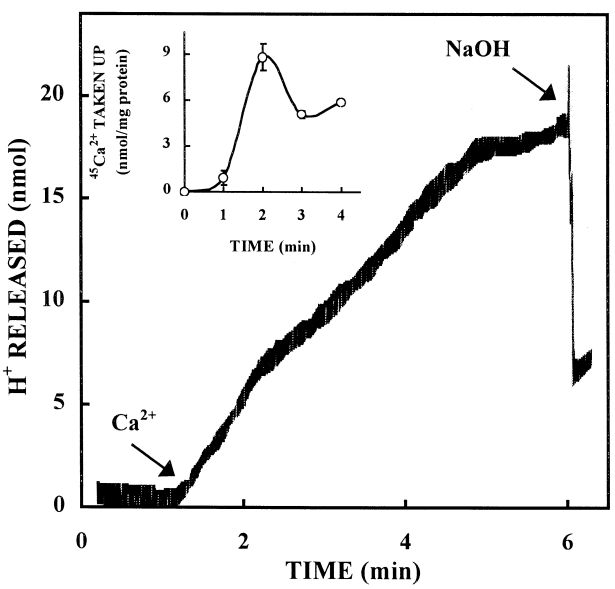

Fig. 4. Time-course of synaptic vesicle Ca^{2+}/H^+ antiport activity driven by an artificially imposed proton motive force. The vesicles (600 μg protein/ml) previously incubated in 300 mM sorbitol, 1 mM DTT, 10 mM potassium gluconate and 20 mM Mes–Tris, pH 5.6 were added to a medium containing 300 mM sorbitol, 10 mM potassium gluconate and 1 mM Tris (pH 8.5). The reactions were started by adding 500 μM $CaCl_2$ (potentiometric assay for detection of H^+ release) or 500 μM $CaCl_2$ supplemented with 10 μCi/mmol $^{45}CaCl_2$ (isotopic assay for detection of Ca^{2+} uptake). The Ca^{2+}-induced acidification of the medium was checked by reverting the signal with NaOH addition (12.5 nmol). The measurements were performed as described in the text. Values represent $\pm$S.D. of 4 separate experiments.

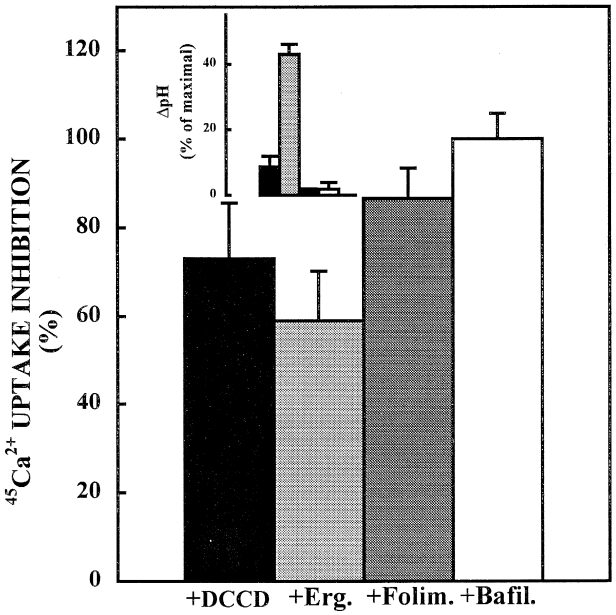

Fig. 5. Effect of H^+-ATPase inhibitors on the Ca^{2+}/H^+ antiport activity of synaptic vesicles. The vesicles (600 μg protein/ml) were incubated in a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA, 10 mM Tris (pH 8.5) and 504 μM ATP-Mg in the absence and in the presence of various H^+-ATPase inhibitors used at concentrations which correspond to the maximal inhibitory effect on the proton-pump: 120 μM DCCD (black rectangle), 50 μM ergocryptine (light grey rectangle), 0.04 μM folimycin (dark grey rectangle) and 0.3 μM bafilomycin A_1 (white rectangle). The reactions were started by adding 607.7 μM $CaCl_2$ supplemented with 10 μCi/mmol $^{45}CaCl_2$ and were stopped after 3 min by filtering 800 μl aliquots of reaction medium, as described in Fig. 1. Ca^{2+} uptake inhibition was calculated as a percentage of maximal uptake (absence of inhibitors) after subtracting the amount of Ca^{2+} taken up in the presence of 10 μM CCCP. The histogram insert represents the inhibitory effect of the drugs on the H^+-pump activity. Values represent ±S.D. of 4–8 separate experiments.

rameters evaluated (ΔpH formation and Ca^{2+} accumulation).

It appears therefore, that the Ca^{2+}/H^+ antiport activity of sheep brain synaptic vesicles is indeed a ΔpH-energized process for Ca^{2+} accumulation into the vesicles.

4. Discussion

A Ca^{2+}/H^+ antiport activity in sheep brain synaptic vesicles has been observed in our laboratory [7]. The antiporter appears to have low affinity for Ca^{2+}, works optimally at alkaline pH (~8.5) and appears to transport other cations (Zn^{2+} and Cd^{2+}) in exchange with protons [8]. The process constitutes a secondary active Ca^{2+} transport that is dependent on the energy of the ΔpH generated by the H^+-pump activity [7].

In this work, we observed that, indeed, Ca^{2+}/H^+ antiport depends on the ΔpH magnitude, as is evidenced by measuring the Ca^{2+} uptake by the vesicles under conditions of optimal operativity of the antiporter (Fig. 1). In correlation, we observed that the proton gradient across the vesicle membranes can be dissipated by Ca^{2+} addition to the medium, although this property can be visualized only when the H^+-pump activity is not high enough to retrieve into the vesicles the protons released by Ca^{2+} (Fig. 2).

Since the proton ATPase generates an electrochemical gradient ($\Delta\mu H^+$) across the vesicle membranes [3,19], we studied the influence of both, the chemical component (ΔpH) and the electrical component ($\Delta\varphi$) on the Ca^{2+}/H^+ antiport activity. Our results show that Ca^{2+} transport is essentially determined by ΔpH (Fig. 3A), whereas $\Delta\varphi$ has low contribution for the antiporter activity (Fig. 3B). The ΔpH dependence of the Ca^{2+}/H^+ antiport activity was also supported by experiments with H^+-pump inhibitors. Indeed, both Ca^{2+} uptake (Fig. 5) and ΔpH formation (insert) were inhibited in similar extent by bafilomycin A_1, ergocryptin, folymicin and DCCD. None of these inhibitors was specific for the synaptic vesicle antiporter as it was reported for the Ca^{2+}/H^+ antiport system of out roots that was strongly inhibited by concentrations of DCCD (IC_{50} = 3 μM) not sufficient to inhibit significantly the ΔpH formation [24]. Similarly, ruthenium red has no effect on the Ca^{2+}/H^+ antiport of synaptic vesicles (results not shown), whereas it inhibited specifically (IC_{50} = 40 μM) that of oat roots [24].

The main evidence that the Ca^{2+}/H^+ antiport system of synaptic vesicles is actually a secondary active transport, which is directly dependent on the membrane ΔpH and not on the presence of ATP, was obtained from experiments where a ΔpH artificially imposed by pH jump was sufficient to drive Ca^{2+} accumulation into the vesicles (Fig. 4). Therefore, the Ca^{2+}/H^+ antiport of synaptic vesicles appears to constitute a ΔpH-dependent Ca^{2+} transport mechanism, which is distinct from other ATP-dependent mechanisms previously suggested by other investigators [13,18] and currently studied in our laboratory.

If we bear in mind that neurotransmitter transport and their maintenance inside the vesicles also depends on the proton electrochemical gradient across the membrane [1,6,14a,14b,15,19,29], it is plausible to assume that Ca^{2+}, through its dissipater effect on the proton gradient, may regulate neurosecretion by decreasing exocytosis particularly under stress conditions of cytoplasmic Ca^{2+} excess. Indeed, Carvalho et al. [2] and Duarte et al. [5] observed that influx of Ca^{2+} into synaptosomes mediated either by the Na^+/Ca^{2+} exchanger or by glutamate receptors activation does not induce GABA release, in spite of cytoplasmic Ca^{2+} concentration exceeds the values usually required for exocytosis when it is triggered by K^+ depolarization of the membrane. Probably, cytoplasmic Ca^{2+}, entering the vesicles in exchange with protons, makes synaptic vesicles emptied of neurotransmitters due to abolishment of the proton motive force required for their accumulation in the vesicles. Conversely, Ca^{2+} entry induced by K^+ depolarization of the membrane has been interpreted on the basis that Ca^{2+} channels exist in ''active

zones'', forming Ca^{2+} concentration microdomains particularly efficient for exocytosis induced by membrane depolarization [16,26,28].

Since Ca^{2+} appears to discharge synaptic vesicle ΔpH with maintenance of $\Delta\varphi$ [7], we presume that it decreases strongly the ΔpH-dependent neurotransmitter storage (dopamine) by the vesicles [17,29], whereas the $\Delta\varphi$-dependent neurotransmitter accumulation (glutamate) [6,17] should not be greatly affected. We are currently exploring these effects of Ca^{2+} on the transport of various neurotransmitters by synaptic vesicles.

The results reported here suggest that Ca^{2+}/H^{+} antiport activity may regulate the synaptic vesicle neurotransmitter storage, so that Ca^{2+} exocytosis may occur instead neurotransmitter exocytosis, particularly under stress conditions. This may be physiologically important in agreement with previous suggestions of several investigators [20,21,23].

Acknowledgements

This research was supported by PRAXIS XXI and JNICT (Junta Nacional de Investigação Científica e Tecnológica).

References

[1] P.M. Burger, J. Hell, E. Mehl, C. Krasel, F. Lottspeich, R. Jahn, GABA and glycine in synaptic vesicles: storage and transport characteristics, Neuron 7 (1991) 287–293.
[2] C.M. Carvalho, C. Bandeira-Duarte, I.L. Ferreira, A.P. Carvalho, Regulation of carrier-mediated and exocytotic release of [^{3}H]GABA in rat brain synaptosomes, Neurochem. Res. 16 (1991) 763–772.
[3] S. Cidon, T.S. Sihra, Characterization of a H^{+}-ATPase in rat brain synaptic vesicles. Coupling to L-glutamate transport, J. Biol. Chem. 264 (1990) 8281–8288.
[4] D.W. Deamer, R.C. Prince, A.R. Crofts, The response of fluorescent amines to pH gradients across liposome membranes, Biochim. Biophys. Acta 274 (1972) 323–335.
[5] C.B. Duarte, I.L. Fereira, P.F. Santos, C.R. Oliveira, A.P. Carvalho, Glutamate increases the $[Ca^{2+}]_i$ but inhibits Ca^{2+}-independent release of ^{3}H-GABA in cultured chick retina cells, Brain Res. 611 (1993) 130–138.
[6] E.M. Fykse, F. Fonnum, Transport of the g-aminobutyrate and L-glutamate into synaptic vesicles, Biochem. J. 276 (1991) 363–367.
[7] P.P. Gonçalves, S.M. Meireles, C. Gravato, M.G.P. Vale, Ca^{2+}–H^{+}-Antiport activity in synaptic vesicles isolated from sheep brain cortex, Neurosci. Lett. 247 (1998) 87–90.
[8] P.P. Gonçalves, S.M. Meireles, C. Gravato, M.G.P. Vale, Ionic selectivity of the Ca^{2+}/H^{+}-antiporter in synaptic vesicles of sheep brain cortex, Molec. Brain Res. 67 (1999) 283–291.
[9] A.G. Gornall, Ch.J. Bardawill, M.M. David, Determination of serum proteins by means of the biuret reaction, J. Biol. Chem. 177 (1949) 751–766.
[10] J.W. Hell, P.R. Maycox, R. Jahn, Energy dependence and functional reconstitution of the γ-aminobutyric acid carrier from synaptic vesicles, J. Biol. Chem. 265 (1990) 2111–2117.
[11] J.W. Hell, P.R. Maycox, H. Stadler, R. Jahn, Uptake of GABA by rat brain synaptic vesicles isolated by a new procedure, EMBO J. 7 (1988) 3023–3029.
[12] B.W. Hicks, S.M. Parsons, Characterization of the P-type and V-type ATPases of cholinergic synaptic vesicles and coupling of nucleotide hydrolysis to acetylcholine transport, J. Neurochem. 58 (1992) 1211–1220.
[13] M. Israël, R. Manaranche, J. Marsal, F.M. Meunier, N. Morel, P. Franchon, B. Lesbats, ATP-dependent calcium uptake by cholinergic synaptic vesicles isolated from *Torpedo* electric organ, J. Membr. Biol. 54 (1980) 115–126.
[14a] R.B. Kelly, Storage and release of neurotransmitters, Cell 72 (1993) .
[14b] R.B. Kelly, Storage and release of neurotransmitters, Neuron 10 (1993) 43–53.
[15] P. Kostyuk, A. Verkhratsky, Calcium stores in neurons and glia, Neuroscience 63 (1994) 381–404.
[16] R. Llinás, M. Sugimori, R.B. Silver, Microdomains of high calcium concentration in a presynaptic terminal, Science 256 (1992) 677–679.
[17] P.R. Maycox, J.W. Hell, R. Jahn, Amino acid neurotransmission: spotlight on synaptic vesicles, TINS 13 (1990) 83–87.
[18] D.M. Michaelson, I. Ophir, I. Angel, ATP-stimulated Ca^{2+} transport into cholinergic *Torpedo* synaptic vesicles, J. Neurochem. 35 (1980) 116–124.
[19] Y. Moriyama, M. Futai, H^{+}-ATPase, a primary pump for accumulation of neurotransmitters, is a major constituent of brain synaptic vesicles, Biochem. Biophys. Res. Commun. 173 (1990) 443–448.
[20] A. Parducz, Y. Dunant, Transient increase of calcium in synaptic vesicles after stimulation, Neuroscience 52 (1993) 27–33.
[21] A. Parducz, F. Loctin, E. Babel-Guérin, Y. Dunant, Exo–Endocytotic activity during recovery from a brief tetanic stimulation: a role in calcium extrusion? Neuroscience 62 (1994) 93–103.
[22] Á. Párducz, J. Toldi, F. Joó, L. Siklós, J.R. Wolff, Transient increase of calcium in pre- and postsynaptic organelles of rat superior cervical ganglion after tetanizing stimulation, Neuroscience 23 (1987) 1057–1061.
[23] O.H. Petersen, Can Ca^{2+} be released from secretory granules or synaptic vesicles? Trends Neurosci. 19 (1996) 411–413.
[24] K.S. Schumaker, H. Sze, Calcium transport into vacuole of oat roots. Characterization of H^{+}/Ca^{2+} exchange activity, J. Biol. Chem. 261 (1986) 12172–12178.
[25] T.S. Sihra, R.A. Nichols, Mechanisms in the regulation of neurotransmitter release from brain nerve terminals: current hypotheses, Neurochem. Res. 18 (1993) 47–58.
[26] S.J. Smith, G.J. Augustine, Calcium ions, active zones and synaptic transmitter release, TINS 11 (1988) 458–464.
[27] J.C. Smith, B. Chance, Kinetics of the potential-sensitive extrinsic probe Oxonol VI in beef heart submitochondrial particles, J. Membr. Biol. 46 (1979) 255–282.
[28] M. Sugimori, E.J. Lang, R.B. Silver, R. Llinas, High-resolution measurement of the time course of calcium-concentration microdomains at squid presynaptic terminals, Biol. Bull. 187 (1994) 300–303.
[29] L. Toll, B.D. Howard, Role of Mg^{2+}-ATPase and pH gradient in the storage of cathecolamines in synaptic vesicles, Biochemistry 17 (1978) 2517–2523.