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Ca^{2+}-H^+ antiport activity in synaptic vesicles isolated from sheep brain cortex

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Abstract

Synaptic vesicles isolated from sheep brain cortex exhibit an ATP-dependent Ca^{2+} accumulation that is inhibited by the protonophore uncoupler carbonyl cyanide *m*-chorophenylhydrazone (CCCP) and completely released by the Ca^{2+} ionophore ionomycin. This transport activity was sensitive to the V-type ATPase inhibitor, bafilomycin, but not to the P-type ATPase inhibitor, vanadate. We also observed that the proton gradient, established across the synaptic vesicle membranes in the presence of ATP, is partially dissipated by the addition of Ca^{2+} (100–860 μM) in correlation to an increase of ATP hydrolysis by the H^+-pumping ATPase. In contrast, the activity of the H^+-ATPase, measured under uncoupling conditions (presence of CCCP), appears to be unaltered by the calcium ion. The Ca^{2+}-induced H^+ release visualized by fluorescence quenching of acridine orange correlates well with the Ca^{2+} uptake determined isotopically. These results indicate that synaptic vesicles accumulate Ca^{2+}, via a low affinity Ca^{2+}–H^+ antiport system energized by the protonmotive force originated from the H^+-pumping ATPase activity. © 1998 Elsevier Science Ireland Ltd.

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The neuron Ca^{2+} homeostasis depends on the mechanisms of Ca^{2+} extrusion and on the capacity of Ca^{2+} sequestration by internal Ca^{2+} stores such as endoplasmic reticulum, mitochondria and Ca^{2+}-binding proteins [12]. Furthermore, there are evidences that synaptic vesicles may also play an active role in the regulation of cytoplasmic Ca^{2+} concentration [19]. They may function as internal Ca^{2+} stores to supply localized regions for initiation of neurotransmitter exocytosis [21], or as Ca^{2+} loaded bags useful for its exocytotic extrusion [20]. Indeed, it has been reported that synaptic vesicles are able to take up Ca^{2+} by an ATP-dependent process [11,16], but the mechanisms of Ca^{2+} accumulation are not still clarified. Although several types of ATPase systems have been observed [3,10,16,22,23], their activities were not well distinguished with respect to their specific biochemical characteristics.

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In this work, experiments were performed under conditions at which synaptic vesicles clearly show Ca^{2+} accumulation by a mechanism of Ca^{2+}–H^+ exchange that is energetically dependent on the activity of the H^+-pumping ATPase previously found [5,7,10,17] in synaptic vesicles.

Synaptic vesicles were isolated from sheep brain cortex according to the procedure described by Hell et al. [9]. The purified synaptic vesicles were frozen in liquid nitrogen and stored at −70°C. The analysis of protein was performed by the method of Gornall [8].

The Ca^{2+} uptake assays were performed by using 607.7 μM $CaCl_2$ supplemented with $^{45}CaCl_2$ (10 μCi/mmol). After designated incubation times, aliquots of 800 μl were rapidly filtered through Millipore HAWP filters ($\varnothing$ 0.45 μm), which were washed with 3 ml of the reaction medium without Ca^{2+}. The radioactivity of the filters was measured by liquid scintillation spectrometry and the amount of Ca^{2+} accumulated in the vesicular space was calculated. On the other hand, proton gradients across the membrane were measured by following the fluorescence quenching of 2

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μM acridine orange at excitation and emission wavelengths of 495 and 525 nm, respectively [6]. The ATPase activity was assayed by following the liberation of orthophosphate associated to ATP hydrolysis. The reaction was started by adding 504 μM ATP and 1 min later it was stopped by adding 10% trichloroacetic acid (w/v). After centrifuging for separation of precipitated protein, the supernatants were collected for analysis of P_i by the method of Taussky and Shorr [24]. All assays were carried out at 30ºC in continuously stirred reaction media containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and 10 mM Tris at pH 8.5. In some experiments, ionophores and inhibitors were added to the reaction media as detailed in the legends of the figures.

Statistical analysis was performed using the Student's *t*-test (two-tailed distribution; two-sample unequal variance). Statistical significance was accepted if $P < 0.02$.

The Ca^{2+}–H^+ antiport activity described in this work was revealed by both Ca^{2+}-induced H^+ release from isolated synaptic vesicles and proton gradient dependent Ca^{2+} transport into the vesicular space.

The first result indicative that a Ca^{2+}–H^+ antiport exists and can function at the level of synaptic vesicle membranes was obtained in studies of changes in the quenching of acridine orange fluorescence associated with proton accumulation inside the vesicles. Fig. 1 shows that the fluorescence signal was quenched about 35% by addition of ATP to the reaction mixture and it was restored to the original level by addition of 10 μM carbonyl cyanide *m*-chorophenylhydrazone (CCCP; Fig. 1). This reflects the ATP-dependent formation of a transmembrane pH gradient, interior acidic, which appears to be partially dissipated by addition of Ca^{2+} (Fig. 1). Indeed, $CaCl_2$ induces rapid H^+ release from the vesicles in a concentration dependent manner, which appears to be optimal at about 500 μM (Fig. 1). Under these conditions, diagram insert shows that about 20.1% of the ΔpH was decreased by Ca^{2+} addition as compared to the value (2.5%) obtained in the control experiment (absence of Ca^{2+}), where slight proton liberation is probably due to leakness of the membrane. The effect of Ca^{2+} on the H^+ release does not reflect exchange of membrane bound cations, since no change in the fluorescence quenching signal is observed when only passive Ca^{2+} uptake occurs (absence of ATP) and no proton gradient is formed (results not shown).

In order to eliminate the hypothesis that the Ca^{2+}-induced H^+ release could result from inhibition of the proton pump by Ca^{2+}, we performed experiments to investigate whether, under the conditions described above, Ca^{2+} was actually accumulated inside the vesicles in exchange with H^+. Fig. 2 shows that, indeed, Ca^{2+} transport occurs at expenses of ATP and that this active uptake is slightly decreased in the presence of vanadate (10 μM), whereas it is completely abolished by incubating the vesicles in a bafilomycin- or CCCP-containing medium. The amount of Ca^{2+} accumulated increased with time until it reached a plateau (2.7 nmol/mg protein) at about 3 min of reaction (Fig. 2). We also observed that addition of ionomycin to the reaction medium discharges all Ca^{2+} actively taken up by the vesicles (Fig. 2). This effect of the Ca^{2+} ionophore demonstrates that

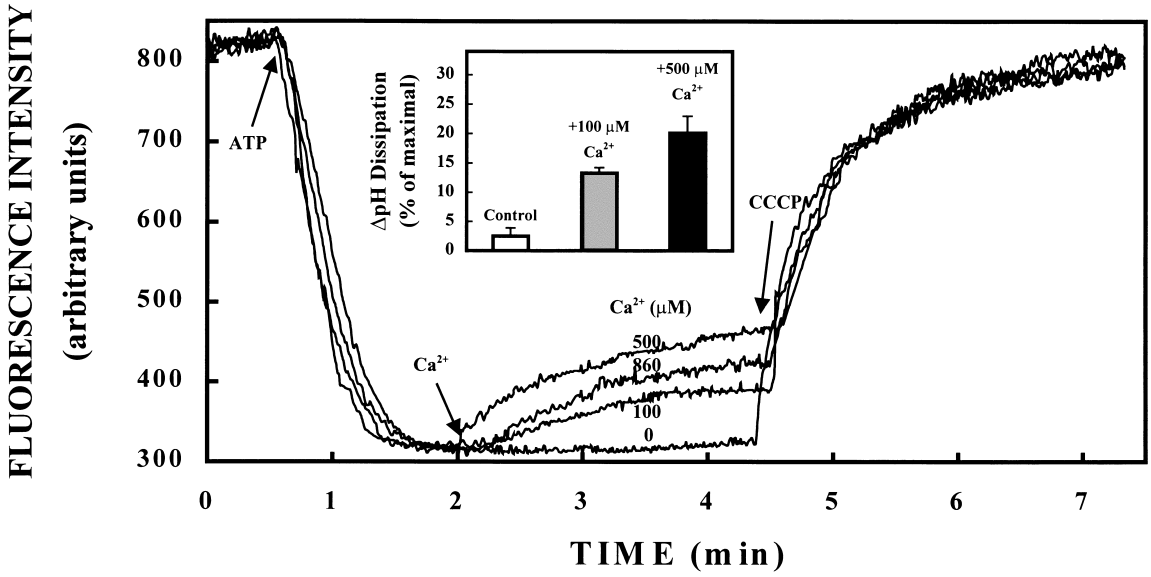

Fig. 1. Ca^{2+}-induced H^+ release in synaptic vesicles isolated from sheep brain cortex. Synaptic vesicles (1.2 mg protein) were incubated in 2 ml of a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and 10 mM Tris at pH 8.5. The reaction was started by adding 504 μM Mg-ATP and 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 100, 500 or 860 μM $CaCl_2$ (individual experiments). The protonophore CCCP (10 μM) was used to check that a pH gradient was formed at expenses of ATP. Diagram insert represents the percentage of ΔpH reduction by Ca^{2+} relatively to the control experiment performed in the absence of this cation. Data represent $\pm$ SD of three to six separate experiments.

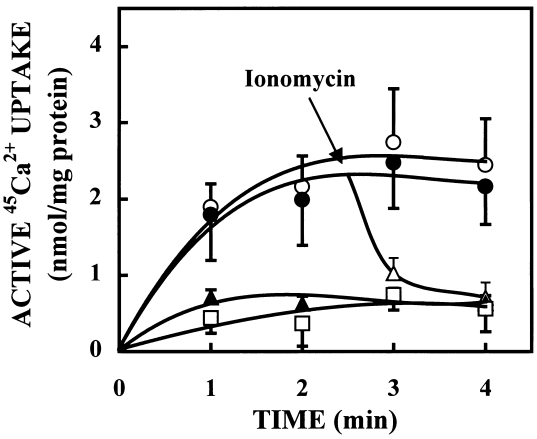

Fig. 2. Proton gradient-dependent Ca^{2+} uptake by synaptic vesicles isolated from sheep brain cortex. The vesicles (600 μg protein/ml) were incubated in a medium as described in Fig. 1, except that 500 μM free Ca^{2+} was used (607.7 μM $CaCl_2$ supplemented with 10 μCi/mmol $^{45}CaCl_2$). The reaction was started by adding 504 μM Mg-ATP and, at several time intervals, it was stopped by filtering 800 μl aliquots of reaction medium through Millipore filters ($\varnothing$ 0.45 μm). The radioactivity of the filters was measured as described in the text. Experiments were performed either in the absence of inhibitors (○), or in the presence of 10 μM vanadate (P-type ATPase inhibitor) (●), 10 μM vanadate plus 10 μM CCCP (protonophore) (▲), 10 μM vanadate plus 300 nM bafilomycin (V-type ATPase inhibitor) (□). The Ca^{2+}-ionophore, ionomycin (10 μM) was used to check that the ΔpH sensitive Ca^{2+} uptake was actually accumulated inside the vesicles (△). 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. Data represent ± SD of four to six separate experiments.

the Ca^{2+} ions in the vesicles were retained against a concentration gradient in a free, osmotically active form, rather than simply bound to the membranes.

Since the active Ca^{2+} uptake observed is strongly inhibited by the protonophore CCCP (10 μM) and by the H^+-ATPase inhibitor, bafilomycin (300 nM), it appears evident that it depends on the pH gradient formed across the membrane by the proton-pumping ATPase activity (Fig. 2). Moreover, the negligible effect of vanadate on this Ca^{2+} uptake indicates that, under the experimental conditions used, it is not supported by a P-type Ca^{2+}-ATPase. These studies of Ca^{2+} entry, performed under conditions at which the proton pump is either active or repressed, appeared to be useful to check the existence of a Ca^{2+}-H^+ antiport in synaptic vesicles.

On the other hand, studying the H^+-ATPase activity in its coupled form (absence of CCCP), we observed that Ca^{2+} (500 μM) promotes significant stimulation of the ATP hydrolysis (from 73.6 to about 99.2 nmol P_i/min per mg protein; Fig. 3). This 'uncoupling' effect of Ca^{2+} indicates that it has the ability to dissipate the proton gradient (Ca^{2+}-induced H^+ release) in agreement with the results depicted in Fig. 1. Conversely, the activity of uncoupled H^+-ATPase (presence of CCCP) is not significantly affected by Ca^{2+}, which indicates that the enzyme molecule, per se, is not altered by Ca^{2+}. These observations also support the conclusion that the Ca^{2+}-induced H^+ release observed in Fig. 1 is due to a Ca^{2+}-H^+ exchanger activity, rather than to a Ca^{2+} inhibition of the proton pump.

The results described here clearly demonstrate that a Ca^{2+}–H^+ antiport system exists in synaptic vesicles. It has been previously suggested [18], but direct observation of its operativity was not reported before. This Ca^{2+} transport system is not easily detected since it requires conditions of an accurate discernment between different types of ATPases, which have been studied by several investigators [3,10,16].

The Ca^{2+}–H^+ antiport constitutes a secondary active Ca^{2+} transport that is directly energized by the protonmotive force originated from the H^+-ATPase activity. It is indirectly dependent on ATP as the hydrolysis of this nucleotide is associated to the primary active transport of protons, forming a pH gradient across the synaptic vesicle membrane.

It is interesting to note that the Ca^{2+}–H^+ antiport is largely operative at about 500 μM (Fig. 1), which indicates that, essentially, it is a low affinity Ca^{2+} transport system. This observation readily explains why Ca^{2+} stimulates the V-type H^+-ATPase of synaptic vesicles (Fig. 3).

Ca^{2+} sequestering properties of synaptic vesicles were well-documented by Michaelson et al. [16]. However, they observed a Ca^{2+} translocation system with a $K_{m(Ca)}$ of 50 μM, whereas the ATPase activity exhibited a $K_{m(Ca)}$ of about 1 mM [15]. The 'uncoupling' between these processes may be due to the stimulatory effect of Ca^{2+} on the H^+-ATPase which, under the conditions reported by those authors, was not distinguished from the Ca^{2+}-ATPase activity. Indeed, a Ca^{2+}-ATPase activity of low K_m for Ca^{2+} was not yet identified in synaptic vesicles and its operativity was

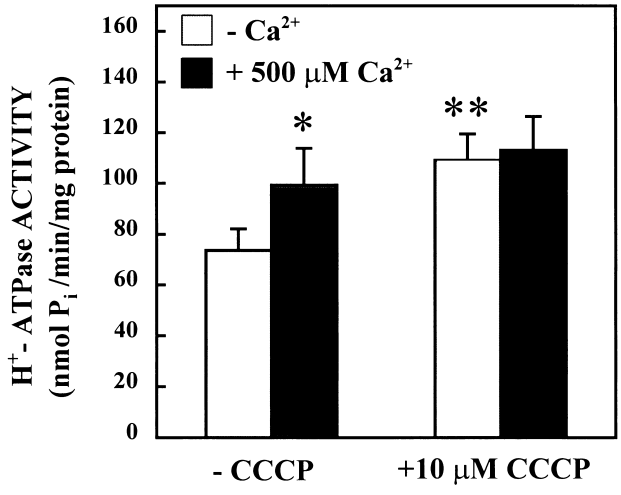

Fig. 3. Effect of Ca^{2+} on the H^+-ATPase activity of synaptic vesicles isolated from sheep brain cortex. The ATPase activity was assayed in a medium as described in Fig. 1. The reaction was started by adding 504 μM Mg-ATP and, 1 min later, P_i analysis was performed as described in the text. The experiments were performed under conditions of 'coupling' (absence of CCCP) or 'uncoupling' (presence of CCCP) of the proton pump. Data represent ±SD of six separate experiments. *$P < 0.02$ and **$P < 0.001$.

even questioned by the results of Stelzl et al. [22]. Preliminary results obtained in our laboratory show that high affinity Ca^{2+} transport is associated to the activity of a high affinity Ca^{2+} pump, which we are currently studying in detail. This system may be important for the physiological processes involved in the various steps of neurosecretion, whereas the low affinity Ca^{2+}–H^+ antiport described here appears to operate at the level of the high Ca^{2+} concentration microdomains previously suggested [13], or when a cytosolic Ca^{2+} excess is reached. Thus, synaptic vesicles may serve as a storage system of Ca^{2+} to provide a homeostatic reserve, or as a detoxification system to prevent the effects of excessive free Ca^{2+} in the cytoplasm.

If we keep in mind that the transport and retention of neurotransmitters by synaptic vesicles depend on the proton gradient across the membrane [14], its dissipation by high Ca^{2+} concentrations (100–860 μM; Fig. 1) is in agreement with the reported correlation between vesicular Ca^{2+} accumulation and acetylcholine content reduction [1], as well as with the inhibitory effects of Ca^{2+} on the neurotransmitter release [2,4]. This suggests that, under stress conditions (Ca^{2+} excess), synaptic vesicles behave as Ca^{2+}-sequestering compartments rather than as neurotransmitter reservoirs. Extensive studies are being elaborated in our laboratory to explore the biochemical properties and functions of both Ca^{2+} transport mechanisms (Ca^{2+}–H^+ antiport and Ca^{2+}-pump) across synaptic vesicle membranes.

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