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Research report

Ionic selectivity of the Ca^{2+}/H^{+} antiport in synaptic vesicles of sheep brain cortex

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Abstract

As we previously reported, synaptic vesicles isolated from sheep brain cortex contain a Ca^{2+}/H^{+} antiport that permits Ca^{2+} accumulation inside the vesicles ($\sim$5 nmol/mg protein) at expenses of the pH gradient generated by the H^{+}-pumping ATPase. We observed that the system associates Ca^{2+} influx to H^{+} release and operates with low affinity for Ca^{2+}. In the present work, we found that Ca^{2+}/H^{+} antiport mediates exchange of protons with other cations such as Zn^{2+} and Cd^{2+}, suggesting that these cations and Ca^{2+} share the same transporter molecules to enter the intravesicular space. Zn^{2+} and Cd^{2+} induce H^{+} release in a concentration-dependent manner (fluorimetrically evaluated) and they inhibit the antiport-mediated Ca^{2+} uptake by the vesicles (isotopically measured). In contrast, large cations such as Ba^{2+} and Cs^{+} do not alter Ca^{2+} influx and they are unable to induce proton release from the vesicles. With respect to Sr^{2+}, which has an intermediary size relatively to the other groups of cations, we found that it does not induce H^{+} liberation from the vesicles, but it has a concentration-dependent inhibitory effect on the Ca^{2+}-induced H^{+} release and Ca^{2+} uptake by the vesicles. These results indicate that the cation selectivity of the synaptic vesicles Ca^{2+}/H^{+} antiport is essentially determined by the size of the cation transported into the vesicles.

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

1. Introduction

In neurons, synaptic vesicles function as reservoirs of neurotransmitters that are ready to deliver them by exocytosis after a stimulus-linked signal. However, there are evidences that synaptic vesicles also sequester Ca^{2+} [36–38], suggesting that they may function as internal Ca^{2+} stores required in localized regions of the exocytotic machinery [3–5,16,40,44,45] or as Ca^{2+} loaded bags useful for its exocytotic extrusion [16,37,40]. An ATP-dependent Ca^{2+} accumulation has been reported by several investigators [24,32] and, in previous experiments, we found a ΔpH-dependent, Ca^{2+}/H^{+} antiport-mediated Ca^{2+} uptake by synaptic vesicles [18], which was recently distinguished from a Ca^{2+} transport system mediated by a Ca^{2+}-ATPase.

On the other hand, Zn^{2+} accumulation inside synaptic vesicles has been visualized with fluorescent probes, particularly in brain neurons [9,17] where release of Zn^{2+} to the synaptic cleft appears to modulate the activity of various receptors including ionotropic glutamate receptors and GABA receptors [1,11,14,30,51,52]. The mechanism of Zn^{2+} transport into synaptic vesicles is not known, but a putative ZnT-3 transporter has been suggested by Palmiter et al. [34] and Wenzel et al. [50]. They cloned ZnT-3 cDNA and, by in situ hybridization, they detected predominance of ZnT-3 mRNA in the hippocampus and cerebral cortex. Furthermore, they observed that, in brain, the antibody-staining pattern against ZnT-3 protein is very similar to the histochemical localization of zinc by the Timm's reaction [50].

In this work, we investigated the cation selectivity of the ΔpH-dependent Ca^{2+}/H^{+} antiport of synaptic vesicles and we found that it is has poor specificity for Ca^{2+}, since

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EGTA, ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol

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it is very efficient to exchange H^+ with small ionic radius cations such as Zn^{2+} and Cd^{2+}.

2. Materials and methods

2.1. Materials

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

2.2. Isolation of synaptic vesicles

Synaptic vesicles were isolated from sheep brain cortex (obtained from a local slaughterhouse) according to the procedure described previously [20,21]. Briefly, the frozen and crushed sheep brain cortical tissue 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 $47\,000 \times g$, 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 containing 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 plus 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 evaluated by assays of immunoblotting and marker enzymes activity [20,21]. Analysis of protein was performed by the method of Gornall et al. [19].

2.3. Assay of Ca^{2+} uptake

Ca^{2+} accumulation by isolated synaptic vesicles was measured by rapid filtration and scintillation counting as described previously [18]. Briefly, 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, 10 mM Tris–HCl, pH 8.5 and 504 μM ATP-Mg. The reactions were initiated by adding 607.7 μM $CaCl_2$ supplemented with $^{45}CaCl_2$ (10 μCi/mmol) and they were carried out in the absence and in the presence of different cation specimens as indicated in the legends of the figures. At designated reaction times, the reactions were stopped by filtering 800 μl aliquots under vacuum. After washing the filters (Millipore HAWP ∅ 0.45 μm) with 3 ml of the reaction medium without Ca^{2+} and ATP-Mg, the retained radioactivity was measured by liquid scintillation spectrometry.

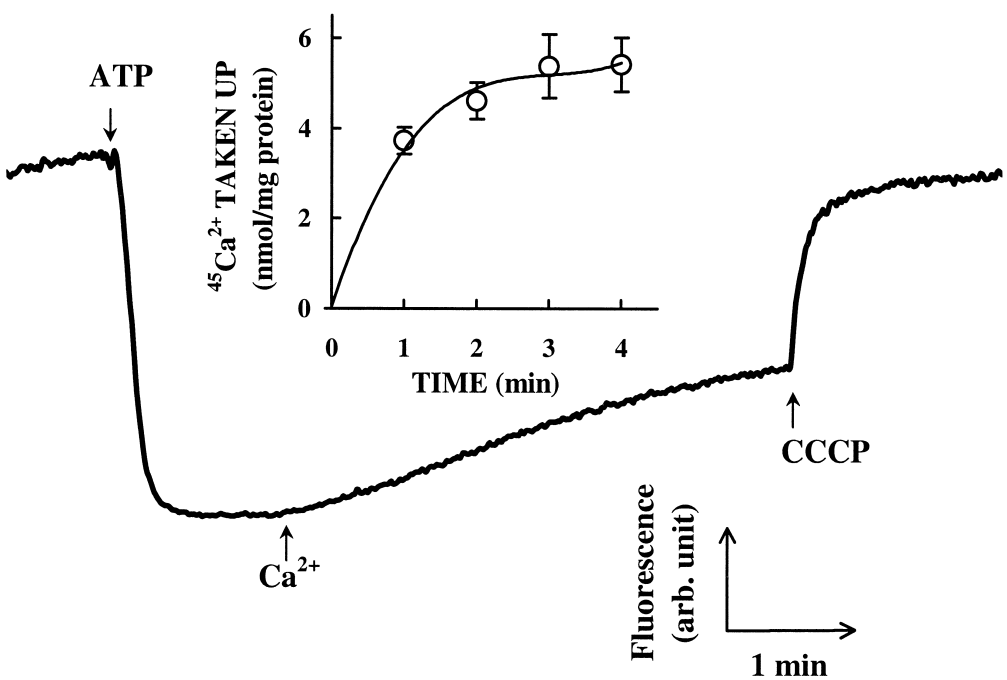

Fig. 1. Ca^{2+}/H^+ antiport activity of synaptic vesicles isolated from sheep brain cortex. Synaptic vesicles (1.2 mg protein) were incubated, at 30°C, in 2 ml of a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and 10 mM Tris–HCl at pH 8.5. The reaction was started by adding 504 μM Mg-ATP and the proton transport was visualized by quenching of acridine orange fluorescence as described in the text. At 1 min of reaction, the Ca^{2+}/H^+ antiport was induced by adding 607.7 μM $CaCl_2$ (500 μM Ca^{2+} free concentration) and its activity was evaluated by recording the fluorescence changes associated to the proton efflux from the vesicles. The ATP-dependent ΔpH was checked by using the protonophore CCCP (10 μM). The time-course of the Ca^{2+} uptake associated to H^+ release (plot insert) was measured under the same experimental conditions, except that radioactive calcium (^{45}Ca) was used as described in the text. Values represent ±S.D. of four separate experiments.

2.4. Assays of proton transport and cation-induced H^+ release

ATP-dependent proton transport was measured by following the fluorescence quenching of 2 μM acridine orange [15] as previously described [18]. 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 504 μM ATP-Mg and the pH gradient formed was checked by inducing H^+ release with the protonophore CCCP (10 μM). In some experiments, H^+-release was induced by adding $CaCl_2$ (final free concentration of 500 μM) or by adding the various cations as described in the legends of the figures. Proton efflux was evaluated by the fluorescence increase after addition of the uncoupling agents. Controls in media without synaptic vesicles showed no alterations in the fluorescence signal.

The fluorescence changes associated to proton 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. Analysis of the data

Statistical differences were performed by the Student's *t*-test (two-tailed distribution; two-sample unequal vari-

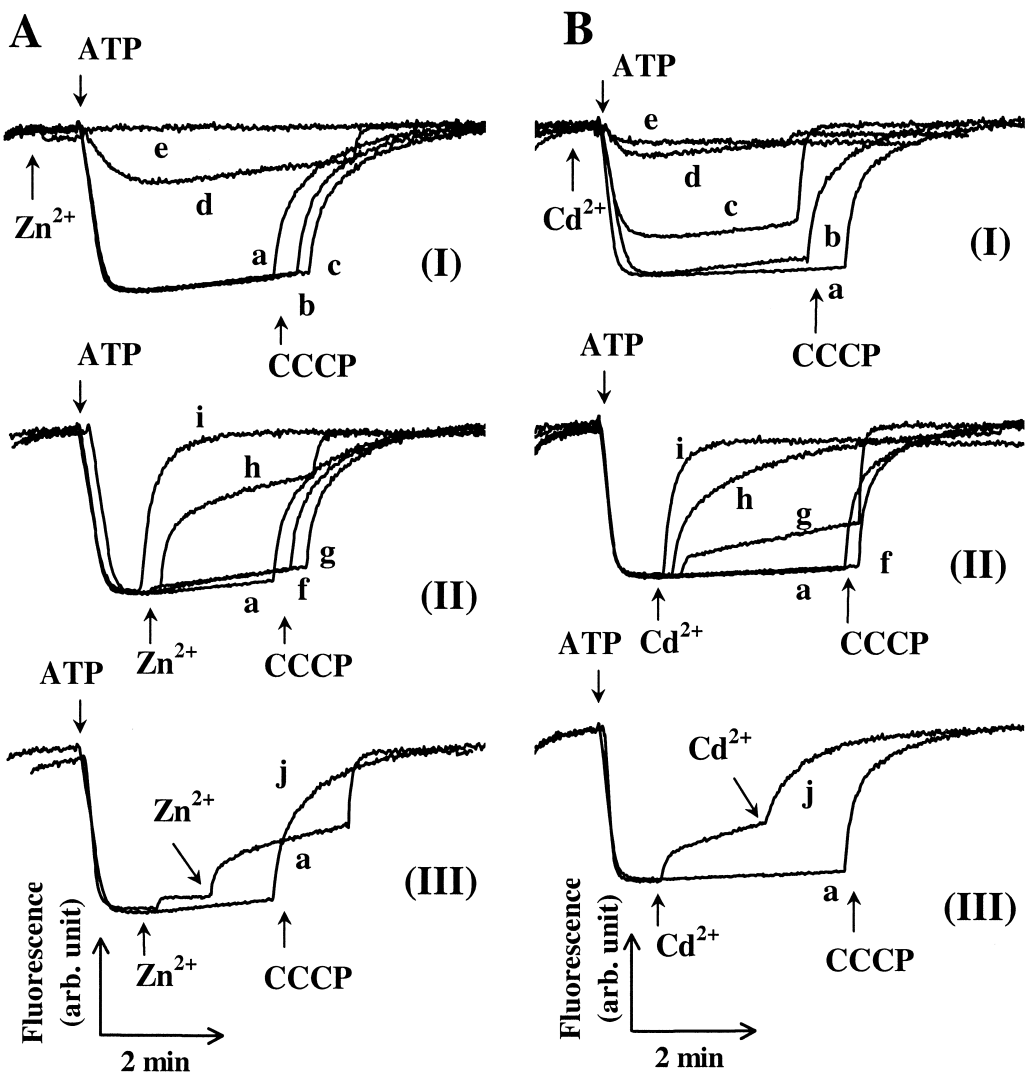

Fig. 2. Zn^{2+} (A) and Cd^{2+} (B) -induced H^+ release from synaptic vesicle: decrease of the ΔpH magnitude. The experiments were performed as described in the legend of Fig. 1. Panel A, Group I: control (a), and presence of 1 μM (b), 10 μM (c), 100 μM (d) or 250 μM (e) of $ZnCl_2$, before starting the reaction with 504 μM ATP-Mg; Group II: control (a) and addition of 1 μM (f), 10 μM (g), 100 μM (h) or 250 μM (i) of $ZnCl_2$, after reaching maximal ΔpH; Group III: control (a) and effect of successive additions of 50 μM $ZnCl_2$ to proton-loaded vesicles (j). Panel B, Group I: control (a) and presence of 20 μM (b), 100 μM (c), 250 μM (d) or 500 μM (e) of $CdCl_2$, before starting the reaction with 504 μM ATP-Mg; Group II: control (a) and addition of 20 μM (f), 100 μM (g), 250 μM (h) or 500 μM (i) of $CdCl_2$, after reaching maximal ΔpH; Group III: control (a) and effect of successive additions of 150 μM $CdCl_2$ (j) to proton-loaded vesicles. The protonophore CCCP (10 μM) was used to check ΔpH generation at expenses of ATP.

ance) and the *p*-values are indicated in the legends of the figures.

3. Results

As reported before [18], we observed that, after formation of a pH gradient across synaptic vesicle membranes, addition of Ca^{2+} (500 μM free concentration) to the reaction medium induces H^+ release from the vesicles (Fig. 1). Indeed, the fluorescence quenching of acridine orange, observed during the proton accumulation by synaptic vesicles, was reversed by Ca^{2+} addition to the medium. We also observed that the Ca^{2+}-induced H^+ release from the vesicles was accompanied by a Ca^{2+} movement into the vesicles, as judged by measurements of the Ca^{2+} taken up along the time under the same experimental conditions (Fig. 1, insert). Ca^{2+} uptake increased with time, reaching a plateau (~5 nmol/mg protein) at 2 min of reaction, in good correlation with the time course of the H^+ release induced by Ca^{2+} (Fig. 1).

The ΔpH dissipation by Ca^{2+} is partial, since further addition of the protonophore CCCP promotes complete discharge of the ATP-dependent proton accumulated (Fig. 1).

Interestingly, we observed that Zn^{2+} and Cd^{2+} also promotes dissipation of the pH gradient across the synaptic vesicle membranes (Fig. 2A and B). The magnitude of this effect is Zn^{2+} or Cd^{2+} concentration-dependent, being maximal at about 250 μM Zn^{2+} or 500 μM Cd^{2+}, respectively (Fig. 2A and B, group II, curves 'i'). We also observed that, after partial H^+ release induced by 100 μM Zn^{2+} or 150 μM Cd^{2+}, a new fraction of H^+ was liberated by further addition of Ca^{2+} (500 μM free concentration) (Fig. 3, curves 'b' and 'c') or of a new aliquot of Zn^{2+} or Cd^{2+} (Fig. 2A and B, group III, curves 'j'). It

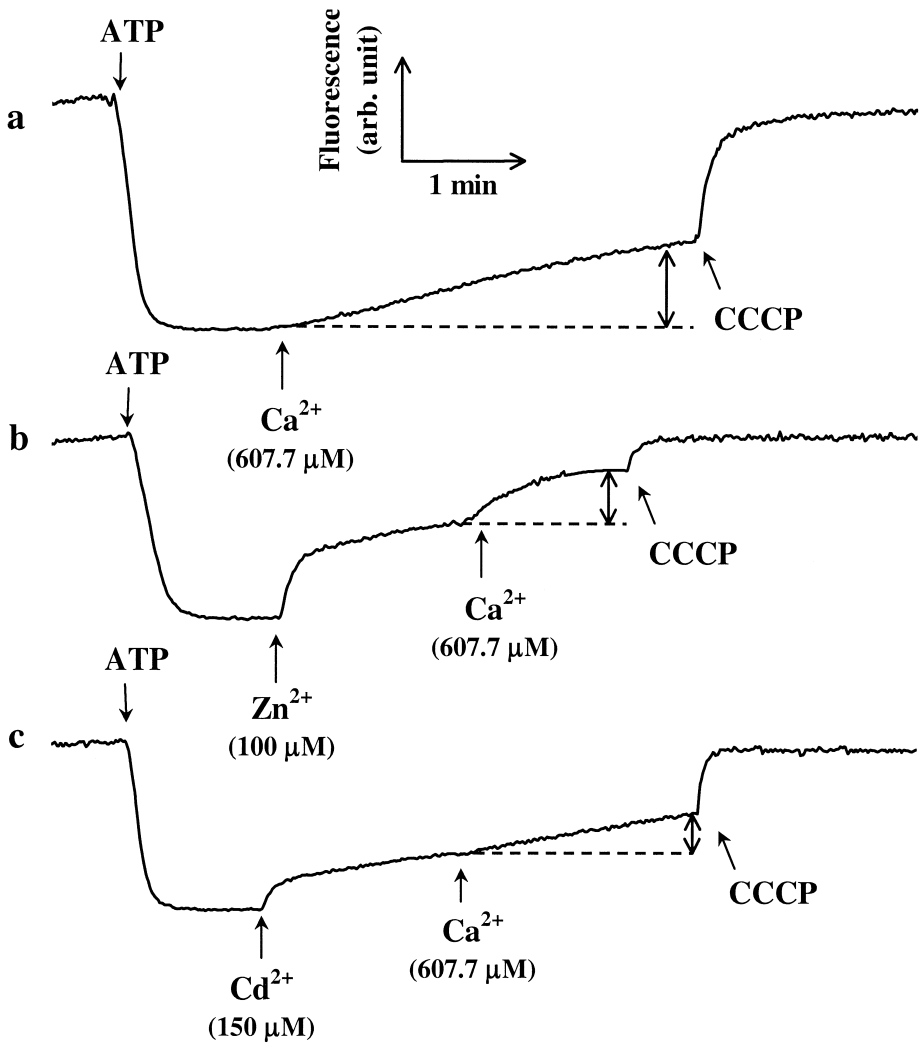

Fig. 3. Zn^{2+} and Cd^{2+} inhibition of the Ca^{2+}-induced H^+ release from synaptic vesicle. The experiments were performed as described in the legend of Fig. 1. (a), control; (b), effect of successive additions of 100 μM $ZnCl_2$ and 607.7 μM $CaCl_2$ (500 μM free concentration) to proton-loaded vesicles; (c), effect of successive additions of 150 μM $CdCl_2$ and 607.7 μM $CaCl_2$ (500 μM free concentration) to proton-loaded vesicles. The protonophore CCCP (10 μM) was used to check ΔpH generation at expenses of ATP.

appears that, like it happens with Ca^{2+}, Zn^{2+} and Cd^{2+} enter the vesicles in exchange with protons. However, the antiport activity reaches a steady-state that is maintained by the activity of the proton-pump until new addition of cations reactivates the antiport by increasing the cation concentration in the medium.

The uncoupler properties of Zn^{2+} and Cd^{2+}, demonstrated by the cation-induced H^+ release, were checked in experiments where the presence of these cations before ATP addition to the medium reduced the magnitude of the ΔpH or, at critical concentrations (250 μM Zn^{2+} or 500 μM Cd^{2+}), prevented its formation across the vesicle membranes (Fig. 2A and B, group I, curves 'e').

Studying the antiport with respect to the Ca^{2+} transport under conditions where Ca^{2+} was added to a Zn^{2+} or Cd^{2+}-containing medium, we observed that it was reduced as the concentration of those cations increased in the extravesicular space (Fig. 4A and B). This suggests that, indeed, these cations enter the vesicles at expenses of the ΔpH, and promote its dissipation by inducing H^+ release.

In contrast to the small cations Zn^{2+} and Cd^{2+} (0.74 and 0.97 Å dehydrated ionic radius, respectively), we observed that cations with large ionic radius, such as Ba^{2+} and Cs^+ (1.35 and 1.69 Å dehydrated ionic radius, respectively) do not show uncoupling effects on the proton pump activity (Fig. 5) and do not inhibit significantly the Ca^{2+}/H^+ antiport-mediated Ca^{2+} accumulation by the vesicles (Fig. 6). In fact, no H^+ release was induced by Ba^{2+} or Cs^+, even at the high concentration of 500 μM (Fig. 5A and B, group II, curves 'c'). Similarly, they do not prevent ΔpH formation across the synaptic vesicle membranes as revealed by the identical magnitude of the acridine orange fluorescence quenching obtained when those cations are absent (control) or present in the reaction medium before starting H^+ transport with addition of ATP (Fig. 5A and B, group I, curves 'b'). On the other hand, Ba^{2+} and Cs^+ did not reduce significantly the secondary active Ca^{2+} transport (Ca^{2+}/H^+ antiport-mediated) by the vesicles (Fig. 5, group III) as it happens with the other cations (Fig. 4).

Taking in consideration that large cations such as Ba^{2+} and Cs^+ do not induce antiport activity like Ca^{2+} (0.99 Å dehydrated ionic radius) or the group of small cations (Zn^{2+} and Cd^{2+}) does, we investigated whether Sr^{2+}, which has an intermediary size (1.13 Å dehydrated ionic radius), is able to induce exchange reaction. Interestingly, we observed that high concentrations of Sr^{2+} (500 μM) do not induce H^+ release from proton loaded vesicles (Fig. 6, group II, curve 'c'), but it has a significant inhibitory effect (24%) on the Ca^{2+} uptake by synaptic vesicles (Fig. 7A) and on the Ca^{2+}-induced H^+ release from the vesicles (Fig. 7B, curve 'a') when both cations, at similar concentrations (500 μM), are present in the reaction medium. It appears, therefore, that Sr^{2+} blocks the cation/H^+ antiport, preventing proton efflux in exchange with Ca^{2+} influx. However, when Ca^{2+} is further added to a Sr^{2+}-containing medium, a slight Ca^{2+}/H^+ exchange occurs. These results indicate that Sr^{2+} inhibits synaptic vesicles Ca^{2+} uptake, by blocking the antiport with consequent decrease in the number of its available molecules.

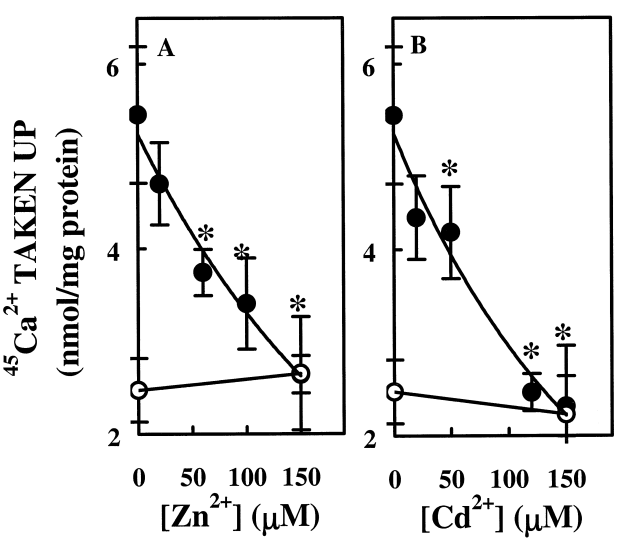

Fig. 4. Zn^{2+} (A) and Cd^{2+} (B) inhibition of the Ca^{2+} transport mediated by the synaptic vesicles Ca^{2+}/H^+ antiport. The vesicles (600 μg protein/ml) were incubated during 50 s in a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, various concentrations of $ZnCl_2$ or $CdCl_2$ (if present), 50 μM EGTA and 10 mM Tris–HCl (pH 8.5) in the absence (○-○) and in the presence (●-●) of 504 μM Mg-ATP. Ca^{2+} uptake reaction was started by adding 500 μM free Ca^{2+} (607.7 μM $CaCl_2$ supplemented with 10 μCi/mmol $^{45}CaCl_2$) and carried out for 3 min. The Ca^{2+} taken up was determined as described in the text. Values represent $\pm$S.D. of four separate experiments. Asterisk indicates significant inhibition (* $p < 0.01$) of Ca^{2+} uptake.

4. Discussion

In previous investigations, we observed that Ca^{2+} (100–500 μM) partially dissipates the pH gradient generated by synaptic vesicles H^+-ATPase and, simultaneously, it is accumulated inside the vesicles at expenses of the ΔpH energy [18]. Recently, we have observed that synaptic vesicles also accumulate Ca^{2+} by a process which requires low Ca^{2+} concentrations (< 100 μM) and it is ΔpH-independent and vanadate-inhibited. It appears, therefore, that synaptic vesicles can take up Ca^{2+} by two distinct mechanisms: a low Ca^{2+} affinity, Ca^{2+}/H^+ antiport-mediated system and a high Ca^{2+} affinity, Ca^{2+}-ATPase-mediated system.

Interestingly, we observed in this work that the Ca^{2+}/H^+ antiport system has poor specificity for Ca^{2+}, since it exchanges protons with various types of smaller ionic radius cations. We found that small cations such as Zn^{2+} and Cd^{2+} easily dissipate the pH gradient in a concentration-dependent manner as it happens with Ca^{2+}.

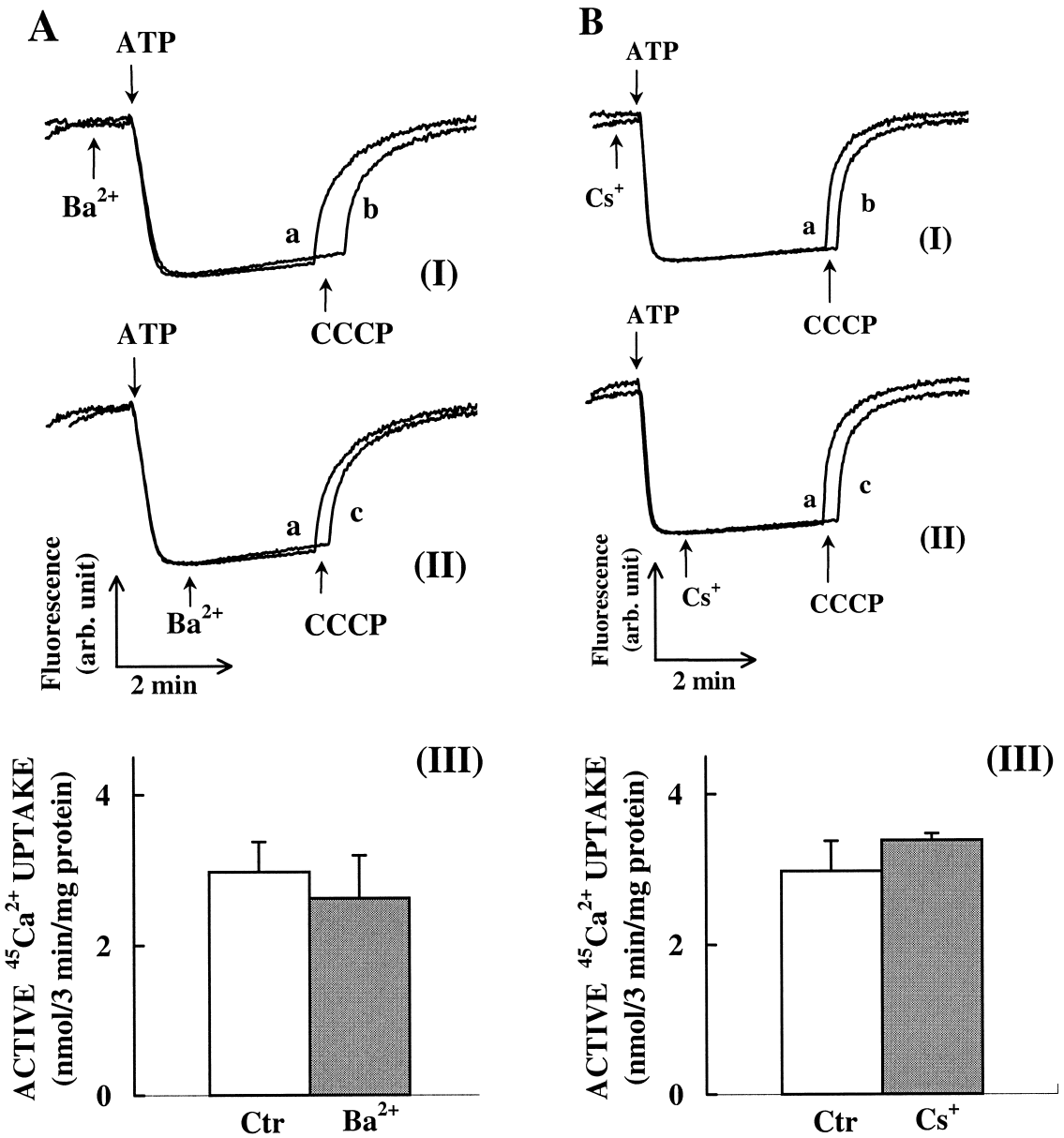

Fig. 5. Inability of Ba^{2+} (A) and Cs^{+} (B) to alter the ΔpH and the antiport-mediated Ca^{2+} uptake by synaptic vesicle membranes. The experiments were performed as described in the legend of Fig. 1. The protonophore CCCP (10 μM) was used to check ΔpH generation at expenses of ATP. Panel A, Group I: control (a) and addition of 500 μM $BaCl_2$ before starting the reaction with 504 μM ATP-Mg (b); Group II: control (a) and addition of 500 μM $BaCl_2$ after reaching maximal ΔpH (c). Panel B, Group I: control (a) and addition of 500 μM CsCl before starting the reaction with 504 μM ATP-Mg (b); Group II: control (a) and addition of 500 μM CsCl after reaching maximal ΔpH (c). The Ca^{2+} uptake was measured as described in Fig. 4, except that 500 μM $BaCl_2$ or CsCl were used instead of $ZnCl_2$ and $CdCl_2$ (Group III). Values represent ±S.D. of four separate experiments.

However, the cation concentrations required for the effect decreased with the ionic radius of cations. About 500 μM $CaCl_2$ is efficient to induce H^+ release (Fig. 1), whereas only 250 μM $CdCl_2$ and about 100 μM $ZnCl_2$ are high enough to induce strong liberation of protons (Fig. 2A and B). It appears that the affinity of the antiport is determined by the size of cations and, probably, competition exists between them, when they are simultaneously present in the reaction medium. Indeed, Ca^{2+}-induced H^+ release was inhibited when 607.7 μM $CaCl_2$ (500 μM free concentration) was added in the presence of 150 μM $CdCl_2$ (Fig. 3, curve 'c') or 100 μM $ZnCl_2$ (Fig. 3, curve 'b') as compared to the release observed when exclusively $CaCl_2$ existed in the reaction medium (Fig. 3, curve 'a'). This conclusion appears plausible if we take in consideration that, in contrast to Ca^{2+}, a second addition of $ZnCl_2$ or $CdCl_2$ (Fig. 2A and B, group III) does not decrease H^+ liberation from the vesicles but, instead, it promotes H^+ release stimulation due to an increased concentration of these cations in the reaction medium (steep dose response).

On the other hand, Sr^{2+}, which is larger than Ca^{2+}, does not induce H^+ release from the vesicles (Fig. 6) and

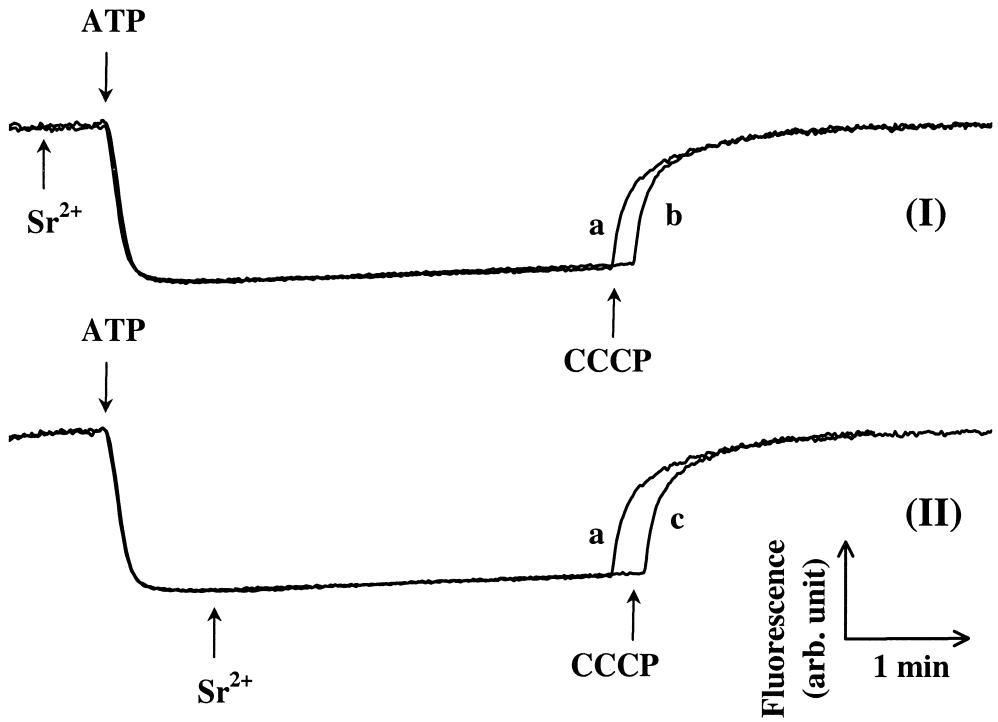

Fig. 6. Effect of Sr^{2+} on the ATP-dependent ΔpH formation across synaptic vesicle membranes. The experiments were performed as described in the legend of the Fig. 1. The protonophore CCCP (10 μM) was used to check ΔpH generation at expenses of ATP. Group I: control (a) and addition of 500 μM $SrCl_2$ before starting the reaction with 504 μM ATP-Mg (b); Group II: control (a) and addition of 500 μM $SrCl_2$ after reaching maximal ΔpH (c).

it has a strong inhibitory effect on both Ca^{2+} uptake (Fig. 7A) and Ca^{2+}-induced H^+ release (Fig. 7B) mediated by the antiporter. This indicates that Sr^{2+} is not small enough to enter the vesicles in exchange with protons, but it blocks

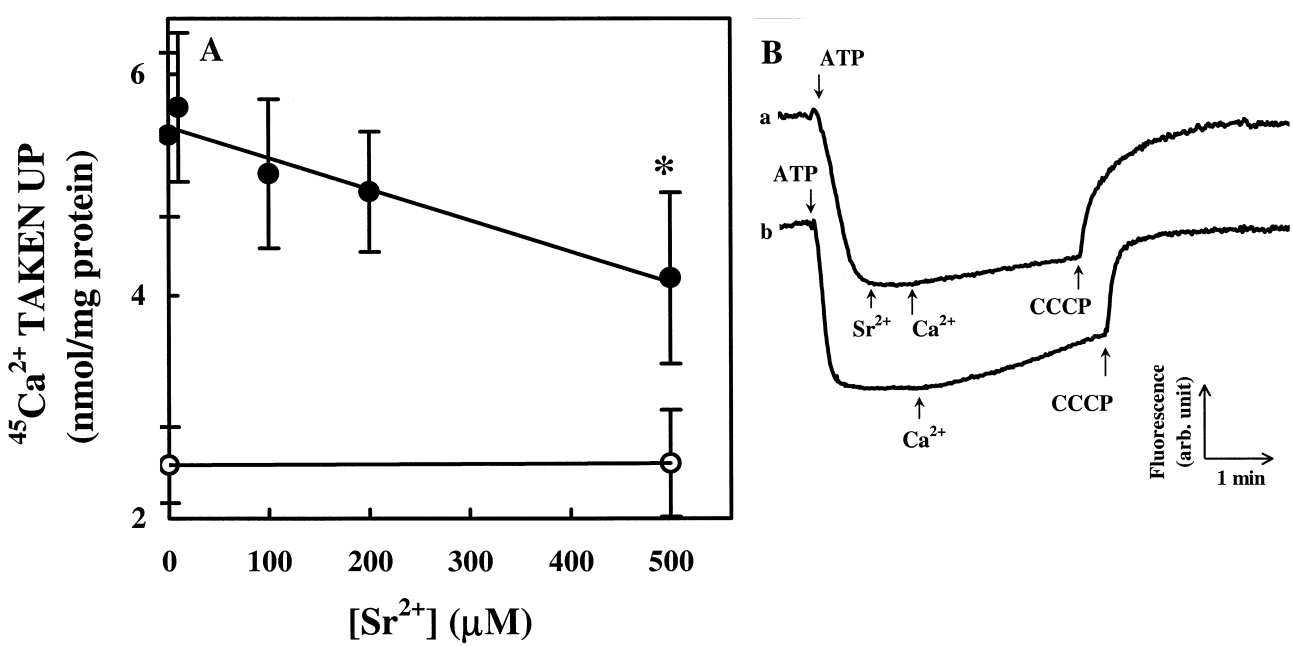

Fig. 7. Effect of Sr^{2+} on the Ca^{2+} uptake (A) and on the Ca^{2+}-induced H^+ release (B) mediated by the synaptic vesicle Ca^{2+}/H^+ antiport. Panel A: the vesicles (600 μg protein/ml) were incubated during 50 s in a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA, various concentrations of $SrCl_2$ (if present) and 10 mM Tris–HCl (pH 8.5) in the absence (○-○) or in the presence (●-●) of 504 μM Mg-ATP. The reactions were started by adding 500 μM free Ca^{2+} (607.7 μM $CaCl_2$ supplemented with 10 μCi/mmol $^{45}CaCl_2$) and carried out for 3 min. The Ca^{2+} taken up was measured as described in the text. Values represent ±S.D. of four separate experiments. Asterisk indicates significant inhibition ($^*p < 0.01$) of Ca^{2+} uptake. Panel B: the experiments were performed as described in the legend of Fig. 1. (a), effect of successive additions of 500 μM $SrCl_2$ and 607.7 μM $CaCl_2$ (500 μM free concentration) to proton-loaded vesicles; (b), addition of 607.7 μM $CaCl_2$ (500 μM free concentration) to proton-loaded vesicles.

the antiporter by interacting with it, causing consequent loss of its availability for other cations (Ca^{2+}).

In contrast, larger cations, such as Ba^{2+} and Cs^{+}, do not induce H^{+} release from the vesicles and do not inhibit Ca^{2+} uptake either (Fig. 5), suggesting that they are too large, not only to enter the vesicles through the antiport, but also to interact with it at the level of its cation binding site. It appears, therefore, that Ba^{2+} and Cs^{+} are neither blockers, nor competitive inhibitors of the cation/proton exchanger, as it happens with Sr^{2+} and with smaller cations (Zn^{2+} and Cd^{2+}), respectively. It is interesting to note that all reactions were carried out in the presence of $\sim$2 mM Mg^{2+} (0.65 Å dehydrated ionic radius) which, under these conditions, functions as a coupling factor of the H^{+} pump activity necessary to generate the motive force for the antiport operativity. We do not know whether Mg^{2+} interferes with the antiport activity, however, it should not affect its response specificity to the other cations studied, as all reactions were performed under the same conditions (Mg^{2+} present).

An important question about the cation/proton exchanger in synaptic vesicles concerns its physiological function. As we report here, it is poorly selective for small divalent cations, so that its specificity is essentially determined by the ionic radius of the cations.

Although no important role has been reported for Cd^{2+} in the central nervous system, Zn^{2+} and Ca^{2+} appear to be involved in important neuronal functions [2–4,6,22, 23,44,45,49,53]. Ca^{2+} acts at various levels of the exocytotic machinery [5,28,46,48,54] and Zn^{2+} has been described as an efficient modulator of both excitatory and inhibitory synaptic transmission [11,14,30,51–53]. Both cations have been found in synaptic vesicles [9,17,24, 31,32,36,38,39,43,50] which, essentially, serve as neurotransmitter reservoirs [25,29,33,41].

The Ca^{2+} sequestration properties of synaptic vesicles may be important to regulate cytoplasm Ca^{2+} concentration [7,16,36,40], particularly at the level of the high Ca^{2+} concentration microdomains previously suggested [27,47]. On the other hand, simultaneous exocytosis of Zn^{2+} and neurotransmitters may permit receptor modulation by this cation.

In spite of these important functions, both cations become toxic when their intracellular concentration exceed levels of tolerance [8,12,13,26,42], unless protective mechanisms of sequestration are induced in the cell. Our results show that Ca^{2+} and Zn^{2+} may be taken up by a low affinity cation/proton exchange system and, under conditions of cation concentration stress, they have a strong dissipater effect on the synaptic vesicle ΔpH, due to accumulation of cations in exchange with protons release. Indeed, Ca^{2+} inhibition of neurotransmitter exocytosis has been reported [10] probably because the proton motive force responsible for transport of neurotransmitters into the vesicles is abolished by high Ca^{2+} concentrations. A similar inhibitory effect on neurotransmitter release was reported for Zn^{2+} [35], which is not surprising since Zn^{2+} (>100 μM), like Ca^{2+} (>300 μM), uncouples the proton pump by inducing H^{+} release through the antiport reported here.

Our results indicate that Zn^{2+}, Ca^{2+} and other small cations (Cd^{2+}) share the same antiport system to enter the vesicles in exchange with protons. However, we do not know whether the antiport described here corresponds to the same transporter molecule (ZnT-3) previously suggested by Palmiter et al. in cloning experiments [34] and ultrastructural observations [50].

The results reported here suggest that moderate concentrations of Zn^{2+} and Ca^{2+} permit their limited storing into the vesicles concomitantly with neurotransmitters, contributing altogether for the neurotransmission process, whereas high concentrations of those cations permit a great cation loading which may be of physiological importance as a mean of their exocytotic discharging under stress conditions.

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References

[1] Ph. Ascher, Zinc, Src and NMDA receptors—a transmembrane connection, Nature Neurosci. 1 (1998) 173–175.
[2] S.Y. Assaf, Sh.-H. Chung, Release of endogenous Zn^{2+} from brain tissue during activity, Nature 308 (1984) 734–736.
[3] G.J. Augustine, E.M. Adler, M.P. Charlton, The calcium signal for transmitter secretion from presynaptic nerve terminals, Ann. N.Y. Acad. Sci. 563 (1992) 365–381.
[4] G.J. Augustine, M.P. Charlton, S.J. Smith, Calcium action in synaptic transmitter release, Annu. Rev. Neurosci. 10 (1987) 633–693.
[5] M.K. Bennett, Ca^{2+} and the regulation of neurotransmitter secretion, Curr. Opin. Neurobiol. 7 (1997) 316–322.
[6] M.J. Berridge, Neuronal calcium signaling, Neuron 21 (1998) 13–26.
[7] M.P. Blaustein, Calcium transport and buffering in neurons, Trends Neurosci. 11 (1988) 438–443.
[8] J. Borovansky, P.A. Riley, Cytotoxicity of zinc in vitro, Chem. Biol. Interact. 69 (1989) 279–291.
[9] T. Budde, A. Minta, J.A. White, A.R. Kay, Imaging free zinc in synaptic terminals in live hippocampal slices, Neuroscience 79 (1997) 347–358.
[10] 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.
[11] N. Chen, A. Moshaver, L.A. Raymond, Differential sensitivity of recombinant *N*-methyl-D-aspartate receptor subtypes to zinc inhibition, Mol. Pharmacol. 51 (1997) 1015–1023.
[12] D.W. Choi, Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage, Trends Neurosci. 11 (1988) 465–469.
[13] D.W. Choi, J.Y. Koh, Zinc and brain injury, Annu. Rev. Neurosci. 21 (1998) 347–375.
[14] Ch.W. Christine, D.W. Choi, Effect of zinc on NMDA receptor-

mediated channel currents in cortical neurons, J. Neurosci. 10 (1990) 108–116.
[15] 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.
[16] Y. Dunant, Hormones and neurotransmitters release: four mechanisms of secretion, Cell Biol. Int. 18 (1994) 327–336.
[17] C.J. Frederickson, D.W. Moncrieff, Zinc-containing neurons, Biol. Signals 3 (1994) 127–139.
[18] 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.
[19] 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.
[20] 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.
[21] 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.
[22] G.A. Howell, M.G. Welch, Ch.J. Frederickson, Stimulation-induced uptake and release of zinc in hippocampal slices, Nature 308 (1984) 736–738.
[23] E.P. Huang, Metal ions and synaptic transmission: think zinc, Proc. Natl. Acad. Sci. USA 94 (1997) 13386–13387.
[24] 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.
[25] R.B. Kelly, Storage and release of neurotransmitters, Neuron 10 (1993) 43–53, Cell 72.
[26] H. Komulainen, S. Bondy, Increased free intracellular Ca^{2+} by toxic agents: index of potential neurotoxicity?, Trends Pharmacol. Sci. 9 (1988) 154–156.
[27] R. Llinás, M. Sugimori, R.B. Silver, Microdomains of high calcium concentration in a presynaptic terminal, Science 256 (1992) 677–679.
[28] Th.F.J. Martin, Stages of regulated exocytosis, Trends Cell Biol. 7 (1997) 271–276.
[29] P.R. Maycox, J.W. Hell, R. Jahn, Amino acid neurotransmission: spotlight on synaptic vesicles, Trends Neurosci. 13 (1990) 83–87.
[30] M.L. Mayer, L. Vyklicky Jr., G.L. Westbrook, Modulation of excitatory amino acid receptors by group IIB metal cations in cultured mouse hippocampal neurons, J. Physiol. (London) 415 (1989) 329–350.
[31] R.J. McMahon, R.J. Cousins, Mammalian zinc transporters, J. Nutr. 128 (1998) 667–670.
[32] D.M. Michaelson, I. Ophir, I. Angel, ATP-stimulated Ca^{2+} transport into cholinergic *Torpedo* synaptic vesicles, J. Neurochem. 35 (1980) 116–124.
[33] 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.
[34] R.D. Palmiter, T.B. Cole, C.J. Quaife, S.D. Findley, ZnT-3, a putative transporter of zinc into synaptic vesicles, Proc. Natl. Acad. Sci. USA 93 (1996) 14934–14939.
[35] A. Parducz, P. Correges, P. Sors, Y. Dunant, Zinc blocks acetylcholine release but not vesicle fusion at the *Torpedo* nerve-electroplate junction, Eur. J. Neurosci. 9 (1997) 732–738.
[36] A. Parducz, Y. Dunant, Transient increase of calcium in synaptic vesicles after stimulation, Neuroscience 52 (1993) 27–33.
[37] 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.
[38] 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.
[39] J. Perez-Clausell, G. Danscher, Intravesicular localization of zinc in rat telencephalic boutons: a histochemical study, Brain Res. 337 (1985) 91–98.
[40] O.H. Petersen, Can Ca^{2+} be released from secretory granules or synaptic vesicles?, Trends Neurosci. 19 (1996) 411–413.
[41] R.J. Reimer, E.A. Fon, R.H. Edwards, Vesicular neurotransmitter transport and the presynaptic regulation of quantal size, Curr. Opin. Neurobiol. 8 (1998) 405–412.
[42] H.E. Scharfman, P.A. Schwartzkroin, Protection of dentate hilar cells from prolonged stimulation by intracellular calcium chelation, Science 246 (1989) 257–260.
[43] R. Schmidt, H. Zimmermann, V.P. Whittaker, Metal ion content of cholinergic synaptic vesicles isolated from the electric organ of *Torpedo*: effect of stimulation-induced transmitter release, Neuroscience 5 (1980) 625–638.
[44] 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.
[45] S.J. Smith, G.J. Augustine, Calcium ions, active zones and synaptic transmitter release, Trends Neurosci. 11 (1988) 458–464.
[46] Th.C. Sudhof, The synaptic vesicle cycle: a cascade of protein–protein interactions, Nature 375 (1995) 645–653.
[47] 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.
[48] P. Thomas, W. Almers, Exocytosis and its control at the synapse, Curr. Opin. Neurobiol. 2 (1992) 308–311.
[49] J.C. Wallwork, Zinc and the central nervous system, Prog. Food Nutr. Sci. 11 (1987) 203–247.
[50] H.J. Wenzel, T.B. Cole, D.E. Born, P.A. Schwartkroin, R.D. Palmiter, Ultrastructural localization of zinc transporter-3 (ZnT-3) to synaptic vesicle membranes within mossy fiber boutons in the hippocampus of mouse and monkey, Proc. Natl. Acad. Sci. USA 94 (1997) 12676–12681.
[51] G.L. Westbrook, M.L. Mayer, Micromolar concentrations of Zn^{2+} antagonize NMDA and GABA responses of hippocampal neurons, Nature 328 (1987) 640–643.
[52] X. Xie, R.C. Hider, T.G. Smart, Modulation of GABA-mediated synaptic transmission by endogenous zinc in the immature rat hippocampus in vitro, J. Physiol. (London) 478 (1994) 75–86.
[53] X.M. Xie, T.G. Smart, A physiological role for endogenous zinc in rat hippocampal synaptic neurotransmission, Nature 349 (1991) 521–524.
[54] H. Zimmermann, Neurotransmitter release, FEBS Lett. 268 (1990) 394–399.