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Ca^{2+} Sensitivity of Synaptic Vesicle Dopamine, γ-Aminobutyric Acid, and Glutamate Transport Systems

Paula P. Gonçalves,[1,3] Sandra M. Meireles,[1] Paulo Neves,[1] and M. Graça P. Vale[2]

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The effect of Ca^{2+} on the uptake of neurotransmitters by synaptic vesicles was investigated in a synaptic vesicle enriched fraction isolated from sheep brain cortex. We observed that dopamine uptake, which is driven at expenses of the proton concentration gradient generated across the membrane by the H^+-ATPase activity, is strongly inhibited (70%) by 500 μM Ca^{2+}. Conversely, glutamate uptake, which essentially requires the electrical potential in the presence of low Cl^- concentrations, is not affected by Ca^{2+}, even when the proton concentration gradient greatly contributes for the proton electrochemical gradient. These observations were checked by adding Ca^{2+} to dopamine or glutamate loaded vesicles, which promoted dopamine release, whereas glutamate remained inside the vesicles. Furthermore, similar effects were obtained by adding 150 μM Zn^{2+} that, like Ca^{2+}, dissipates the proton concentration gradient by exchanging with H^+. With respect to γ-aminobutyric acid transport, which utilizes either the proton concentration gradient or the electrical potential as energy sources, we observed that Ca^{2+} or Zn^{2+} do not induce great alterations in the γ-aminobutyric acid accumulation by synaptic vesicles. These results clarify the nature of the energy source for accumulation of main neurotransmitters and suggest that stressing concentrations of Ca^{2+} or Zn^{2+} inhibit the proton concentration gradient-dependent neurotransmitter accumulation by inducing H^+ pump uncoupling rather than by interacting with the neurotransmitter transporter molecules.

KEY WORDS: Dopamine; GABA; glutamate; Ca^{2+}; neurotransmitter transport; synaptic vesicles.

INTRODUCTION

Synaptic vesicles are the primary organelles involved in storage and quantal release of neurotransmitters, which is a highly coordinated process at the nerve terminal. Alterations of neurotransmitter vesicular transport activities certainly modify the levels of neurotransmitter available for quantal release and provide a mechanism for the regulation of synaptic function in response to neural activity (1–3). Classical transmitters are accumulated in synaptic vesicles by distinct proton electrochemical gradient ($\Delta\mu H^+$)-dependent transport activities that differ in the extent to which they rely on the chemical (ΔpH) and electrical ($\Delta\varphi$) components of this gradient (1,4–10).

In previous investigations we found that sheep brain synaptic vesicles contain a Ca^{2+}/H^+ antiport sys-

[1] Centro de Biologia Celular, Departamento de Biologia, Universidade de Aveiro, 3810-193 Aveiro, Portugal.
[2] Centro de Neurociências, Departamento de Zoologia, Universidade de Coimbra, 3004-504 Coimbra, Portugal.
[3] Address reprint request to: Maria Paula Polónia Gonçalves. Tel: 351-234370776; Fax: 351-234426408; E-mail: pgoncalves@bio.ua.pt

Abbreviations: Acridine orange, bis[dimethylamino]acridine; 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; Oxonol VI, Bis-(3-propyl-5-oxoisoxazol-4-yl-)pentamethine oxonol; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; $\Delta\mu H^+$, proton electrochemical gradient; ΔpH, proton concentration gradient; $\Delta\varphi$, electrical potential.

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tem, which actively transport Ca^{2+} across the membrane, essentially at expenses of the ΔpH component of the $\Delta\mu H^+$ generated by the proton pumping ATPase (11–13). Consequently, Ca^{2+} dissipates the ΔpH under conditions (free Ca^{2+} concentration > 100 μM) that activates the exchanger (11). Furthermore, the antiporter has low affinity for Ca^{2+} and low selectivity, since various cations appear to be exchanged with protons according to their ionic radius in the dehydrated form: $Zn^{2+} > Cd^{2+} > Ca^{2+}$ (12).

In this work, we studied the impact of Ca^{2+}/H^+ antiport activation in the uptake of dopamine, glutamate and GABA by synaptic vesicles isolated from sheep brain cortex. The criteria for this neurotransmitter selection relays on their different transport characteristics, which depend differently on the ΔpH and Δφ components of the $\Delta\mu H^+$ (14–23). The implications of high intracellular Ca^{2+} or Zn^{2+} concentrations on the neurotransmission process are discussed.

EXPERIMENTAL PROCEDURE

Materials. All reagents were analytical grade. Carbonyl cyanide n-chlorophenylhydrozone (CCCP), ATP and bis[dimethylamino] acridine (acridine orange) were obtained from Sigma. Bis-(3-propyl-5-oxoisoxazol-4-yl-)pentamethine oxonol (oxonol VI) was supplied by Molecular Probes. [7,8-^{3}H]Dopamine (1,702 TBq/mol), 4-amino-*n*-[2,3-^{3}H]butyric acid (3,256 TBq/mol) and L-[G-^{3}H]glutamic acid (1,665 TBq/mol) were purchased from Amersham International, Bristol, UK.

Isolation of Synaptic Vesicles. Synaptic vesicles were isolated from sheep brain cortex according to the procedure described by Hell et al. (18,24,25). 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 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 obtained supernatant 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 highly pure synaptic vesicles fraction as revealed by assays of immunoblotting and marker enzymes activity (18,24). Analysis of protein was performed by the method of Gornall et al. (26).

Neurotransmitter Uptake Assays. Dopamine, glutamate and GABA accumulations by isolated synaptic vesicles were measured by rapid filtration and scintillation counting. In order to shift the $\Delta\mu H^+$ from ΔpH to Δφ, maintaining the total $\Delta\mu H^+$ unchanged (16,18), the membrane vesicles (1 mg protein/ml) were incubated at 30°C during 5 min, in the absence or in the presence of 500 μM $CaCl_2$, in the following incubation media: 1) to achieve maximal chemical gradient, we used a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and 10 mM Tris-HCl (pH 8.5) (*ΔpH medium*); 2) in contrast, maximal electrical gradient was achieved in the absence of permeant anions by using a medium containing 60 mM sucrose, 2 mM $MgSO_4$, 150 mM potassium gluconate, 50 μM EGTA and 10 mM Tris-H_2SO_4 (pH 8.5) (*Δφ medium*) or by using a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM potassium gluconate, 50 μM EGTA and 10 mM Tris-H_2SO_4 (pH 8.5) (*$\Delta\varphi + Cl^-$ medium*); 3) intermediary conditions were achieved in a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 15 mM KCl, 135 mM potassium gluconate, 50 μM EGTA and 10 mM Tris-HCl (pH 8.5) (*ΔpH/Δφ medium*), so that an proton electrochemical gradient with the two components (ΔpH and Δφ) was produced. The reactions were initiated by adding 0.2 μM [^{3}H]dopamine, 50 μM [^{3}H]glutamate or 450 μM [^{3}H]GABA after 50 s incubation in the presence of 210 μM ATP-Mg (except otherwise indicated). At designated reaction times, aliquots of 800 μl (800 μg protein) were rapidly filtered under vacuum through Millipore filters HAWP (Ø 0.45 μm), which were washed with 12 ml of the reaction medium without neurotransmitters, Ca^{2+} and ATP-Mg. The radioactivity of the filters was measured by liquid scintillation spectrometry and the amount of neurotransmitter accumulated in the vesicular space was calculated by subtracting the amount of radioactivity retained in the filters under conditions of H^+ pump uncoupling (presence of 10 μM CCCP) from the total amount retained in the presence of ATP-Mg and absence of CCCP. Filter blanks were determined by filtering aliquots of reaction medium with the respective radiolabeled neurotransmitter. The values for neurotransmitter uptake were expressed as pmol/mg protein.

Measurements of ΔpH and Δφ by Fluorescence Quenching. ATP-dependent proton transport was measured by following the fluorescence quenching of 2 μM acridine orange (25,27), while the Δφ across the synaptic vesicle membrane was monitored by measuring ATP-dependent fluorescence quenching of 3.3 μM oxonol VI (28). The membrane vesicles (600 or 900 μg protein/ml) were incubated at 30°C in different reaction media (2 ml), as indicated in the legends of the figures. Proton transport and Δφ generation across the synaptic vesicle membrane were initiated by adding ATP-Mg. Formation of ΔpH and Δφ 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 3.0 nm) and 612 nm (slit width 10.0 nm) using an excitation wavelength of 495 nm (slit width 2.5 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.

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

RESULTS

Since the transport of neurotransmitters into synaptic vesicles is energetically supported by the $\Delta\mu H^+$ across the membrane, we investigated how Ca^{2+} may affect the storage capacity of synaptic vesicles with respect to the neurotransmitters, dopamine, glutamate and GABA.

Fig. 1 shows that dopamine uptake by synaptic vesicles is essentially dependent on the ΔpH component of the $\Delta\mu H^+$. About 7 pmol of dopamine/mg protein are taken up when ΔpH is maximal and Δφ is

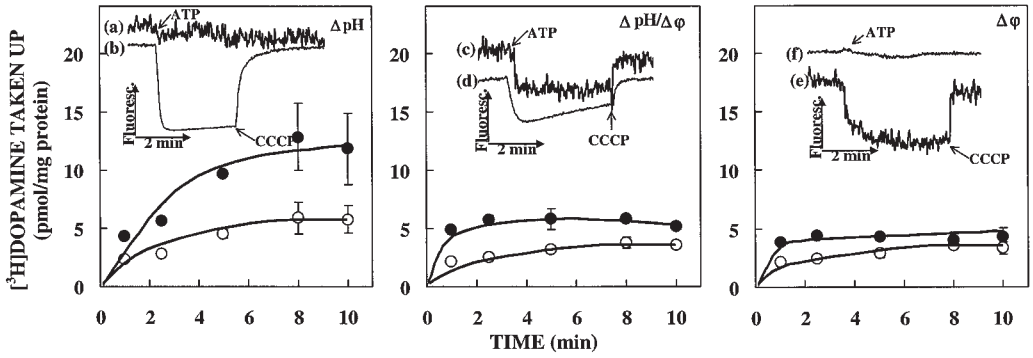

Fig. 1. $\Delta\mu H^+$-dependent dopamine uptake by isolated synaptic vesicles. The vesicles were incubated in *ΔpH medium* (*left panel*), *ΔpH/Δφ medium* (*central panel*) and *Δφ medium* (*right panel*) in the absence (*open symbols*) and in presence of 1 mM ATP-Mg (*close symbols*). Uptake assays were performed as described under "Experimental Procedure". Values represent the mean ± SD of three separate experiments. The inserted figures represent the magnitude of the Δφ (*a,c,e*) and the magnitude of the ΔpH (*b,d,f*) across the synaptic vesicle membrane under the experimental conditions used. The Δφ and ΔpH were visualized by quenching of 3.3 μM oxonol VI and 2 μM acridine orange fluorescence, respectively, as described in "Experimental Procedure".

negligible (presence of Cl^-) (Fig. 1, *left panel*). In contrast, when Δφ is predominant as compared to the ΔpH component (presence of impermeant anions), we observed that dopamine uptake is strongly reduced to ~0.9 pmol/mg protein (Fig. 1, *right panel*). Even when both components, ΔpH and Δφ, are simultaneously contributing to the $\Delta\mu H^+$ (Fig. 1, *central panel*), the dopamine uptake is significantly reduced (~2 pmol/mg protein), indicating that dopamine transport is essentially achieved at expenses of a large chemical gradient of protons. Under the various conditions studied, the ΔpH and Δφ magnitudes were evaluated by recording the fluorescence changes of acridine orange and Oxonol VI, respectively (Fig. 1, inserts). Association of dopamine uptake to the H^+ pump activity was checked by comparing these results with those obtained in control assays performed in the absence of ATP (Fig. 1).

The dopamine accumulation by synaptic vesicles was strongly reduced (~70%) by Ca^{2+} under the optimal conditions of maximal ΔpH, whereas no Ca^{2+} inhibition was observed under conditions of high Δφ where there is even a slight increase in the synaptic vesicle dopamine uptake (Fig. 2). When both ΔpH and Δφ components existed, the inhibitory effect of Ca^{2+} was reduced (~45%, suggesting that it was partially neutralized by the stimulatory effect observed under conditions of Δφ component predominance.

In contrast to dopamine uptake, the glutamate transport system of synaptic vesicles was low (~38 pmol/mg protein) under conditions of high ΔpH and low Δφ (Fig. 3, *left panel*). It appears that the main energy source for this neurotransmitter transport is Δφ, although negligible amounts of Cl^- (4 mM), which are not enough for ΔpH formation, were required to obtain substantial glutamate accumulation (~82 pmol/mg protein) (Fig. 3, *left panel*). Under conditions of higher concentrations of Cl^- (17 mM), at which ΔpH exists (Fig. 3, *central panel*), there was an increased glutamate accu-

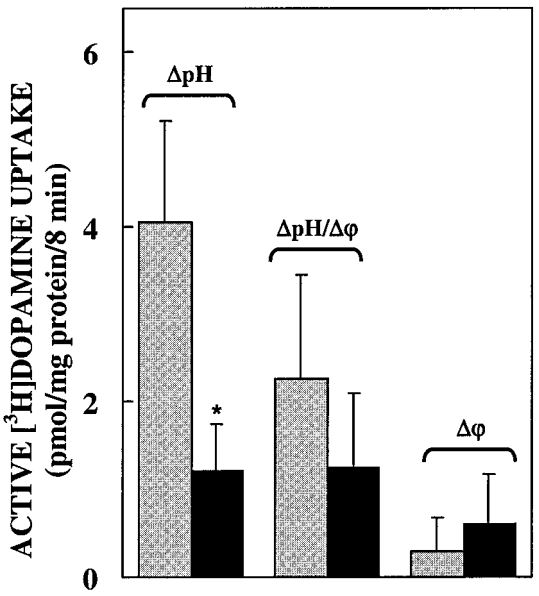

Fig. 2. Effect of Ca^{2+} on the active [^{3}H]dopamine uptake by isolated synaptic vesicles. The vesicles were incubated in *ΔpH*, *ΔpH/Δφ* and *Δφ* media in the absence (*grey bars*) and in presence of 500 μM Ca^{2+} (*black bars*). Uptake assays were performed as described in "Experimental Procedure", and the reactions were carried out during 8 min. Active [^{3}H]dopamine uptake activity was calculated as described in "Experimental Procedure". Values represent the mean ± SD of six separate experiments.
*$P < 0.001$.

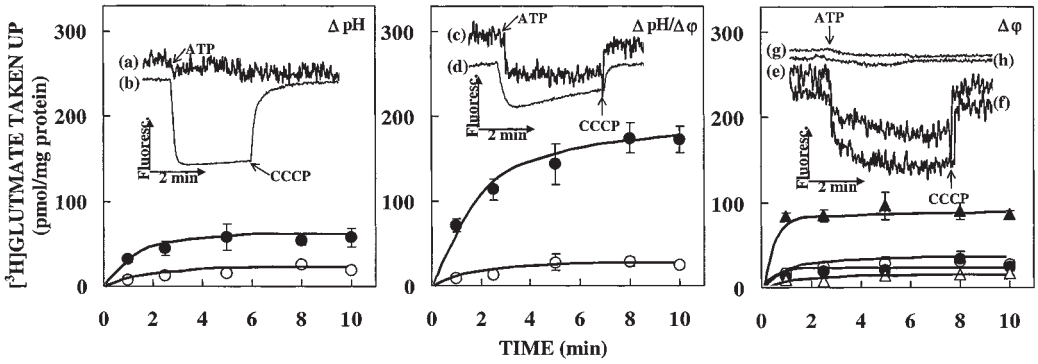

Fig. 3. $\Delta\mu H^+$-dependent glutamate uptake by isolated synaptic vesicles. The vesicles were incubated in *ΔpH medium* (*left panel*), *ΔpH/Δφ medium* (*central panel*) and *Δφ medium* (*circles*) or *Δφ + Cl⁻ medium* (*triangles*) (*right panel*) in the absence (*open symbols*) and in presence of 1 mM ATP-Mg (*close symbols*). Uptake assays were performed as described in "Experimental Procedure". Values represent the mean ± SD of three separate experiments. The inserted figures represent the magnitude of the Δφ (*a,c,e,f*) and the magnitude of the ΔpH (*b,d,g,h*) across the synaptic vesicle membrane under the experimental conditions used. The Δφ and ΔpH were visualized by quenching of 3.3 μM oxonol VI and 2 μM acridine orange fluorescence, respectively, as described in "Experimental Procedure".

mulation (~148 pmol/mg protein). However, we could not distinguish whether this is due to a Cl^--induced ΔpH enlargement (Fig. 3, *central panel*, insert) or to a concentration-dependent effect of Cl^- on the transporter, as previously suggested (21,22,29). This question was clarified by analysis of the Ca^{2+} on the glutamate uptake by synaptic vesicles (Fig. 4). We observed no Ca^{2+} effect either in the absence of Δφ or when the ΔpH component greatly contributed to the $\Delta\mu H^+$ (Fig. 4). Furthermore, a significant stimulatory effect of Ca^{2+} was observed when Δφ was the predominant component of the $\Delta\mu H^+$ in the presence of low concentrations of Cl^- (Fig. 4). These results indicate that the glutamate transport system prefers Δφ as energy source and it does not utilizes the ΔpH energy even in the presence of non-limiting Cl^- concentrations. Under these conditions, the ΔpH dissipative effect of Ca^{2+} did not decrease glutamate uptake, suggesting that high Cl^- permits optimal glutamate uptake by interaction with the transporter (30), rather than by generation of a large ΔpH.

Concerning the GABA uptake by synaptic vesicles, we observed that similar amounts (~186 and ~100 pmol/mg protein) are accumulated by using either the ΔpH or the Δφ components as energy source for the process (Fig. 5, *left and right panels*). Under intermediary conditions, when both components contributed for the $\Delta\mu H^+$, GABA update was slightly increased (292 pmol/mg protein) (Fig. 5, *central panel*), suggesting that an electrochemical gradient has not a great advantage relatively to the efficiency observed when the chemical or electrical gradient are operating in separate.

The GABA uptake by synaptic vesicles was slightly inhibited (~16%) by Ca^{2+} only when ΔpH contributed as main energy source, whereas there was no inhibitory effect when Δφ was the predominant component for the energetic support of the neurotransmitter transport (Fig. 6). Interestingly, Ca^{2+} did not alter GABA uptake when ΔpH and Δφ simultaneously exists

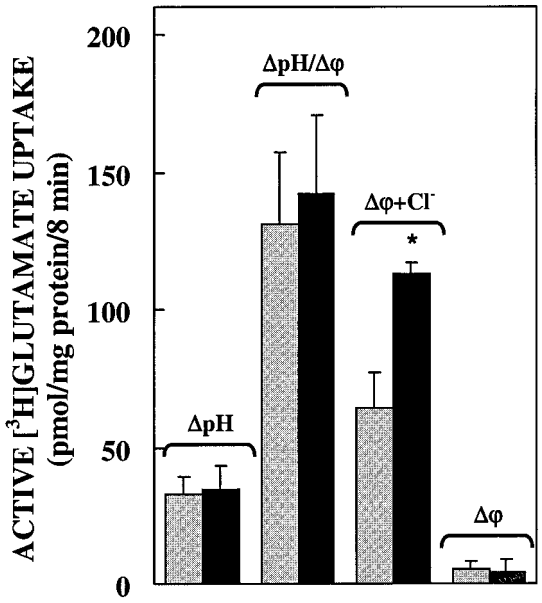

Fig. 4. Effect of Ca^{2+} on the active [^{3}H]glutamate uptake by isolated synaptic vesicles. The vesicles were incubated in *ΔpH, ΔpH/Δφ, Δφ + Cl⁻* and *Δφ media* in the absence (*grey bars*) and in presence of 500 μM Ca^{2+} (*black bars*). Uptake assays were performed as described in "Experimental Procedure", and the reactions were carried out during 8 min. Active [^{3}H]glutamate uptake activity was calculated as described in "Experimental Procedure". Values represent the mean ± SD of five separate experiments.
*$P < 0.01$.

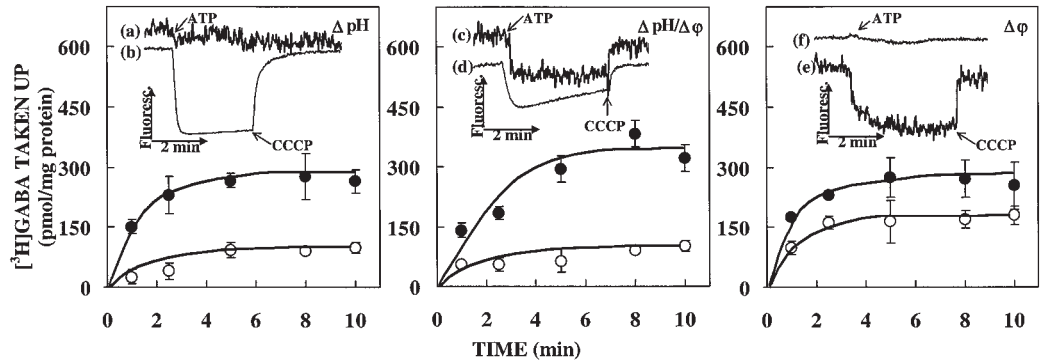

Fig. 5. $\Delta\mu H^+$-dependent GABA uptake by isolated synaptic vesicles. The vesicles were incubated in *ΔpH medium* (*left panel*), *ΔpH/Δφ medium* (*central panel*) and *Δφ medium* (*right panel*) in the absence (*open symbols*) and in presence of 1 mM ATP-Mg (*close symbols*). Uptake assays were performed as described under "Experimental Procedure". Values represent the mean ± SD of three separate experiments. The inserted figures represent the magnitude of the Δφ (*a,c,e*) and the magnitude of the ΔpH (*b,d,f*) across the synaptic vesicle membrane under the experimental conditions used. The Δφ and ΔpH were visualized by quenching of 3.3 μM oxonol VI and 2 μM acridine orange fluorescence, respectively as described in "Experimental Procedure".

across the membrane, suggesting that, under these conditions, Ca^{2+}-induced dissipation of ΔpH does not inhibit GABA uptake as it is similarly energized by Δφ.

Considering the effects of Ca^{2+} on the uptake of dopamine, GABA and glutamate by synaptic vesicles (Figs. 2, 4, 6), we investigated the action of Ca^{2+} when added to vesicles previously loaded with the various neurotransmitters (Fig. 7). In these conditions, we observed that Ca^{2+} rapidly induced complete release of dopamine from the vesicles (2 min), whereas GABA release was not greatly sensitive to Ca^{2+} and glutamate remained inside the vesicles. Interestingly, we observed that the various loaded vesicles responded to Zn^{2+} addition in a manner similar to that observed for Ca^{2+} (Fig. 7). It appears that Zn^{2+}, like Ca^{2+}, promoted liberation of the ΔH-dependent neurotransmitter (dopamine) by dissipation of ΔpH as they exchange with protons through a previous reported antiport system (12). The accumulation of the other neurotransmitters is not significantly affected by these cations since they are strictly or faculatively depedent on the Δφ, respectively.

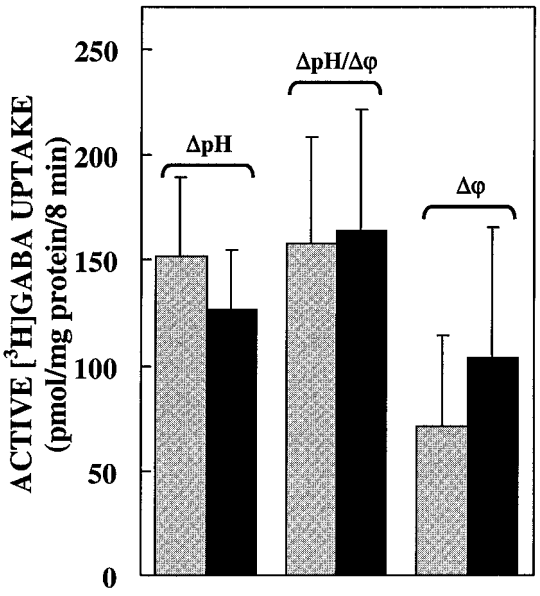

Fig. 6. Effect of Ca^{2+} on the active [^{3}H]GABA uptake by isolated synaptic vesicles. The vesicles were incubated in *ΔpH*, *ΔpH/Δφ* and *Δφ media* in the absence (*grey bars*) and in the presence of 500 μM Ca^{2+} (*black bars*). Uptake assays were performed as described in "Experimental Procedure", and the reactions were carried out during 8 min. Active [^{3}H]GABA uptake activity was calculated as described in "Experimental Procedure". Values represent the mean ± SD of six separate experiments.

DISCUSSION

Synaptic vesicles are important reservoirs to package the neurotransmitters for secretion after neuron stimulation. Therefore, alterations in their mechanisms of transport may have strong consequences at the physiological level.

Since Ca^{2+} (~500μM has revealed uncoupling properties with respect to synaptic vesicle H^+ pump activity (13), its effect on the neurotransmitter storage capacity of these vesicles appears to be bioenergetically relevant. We observed that Ca^{2+} strongly inhibits dopamine accumulation under conditions of high ΔpH, but it has a stimulatory effect when Δφ is predominant

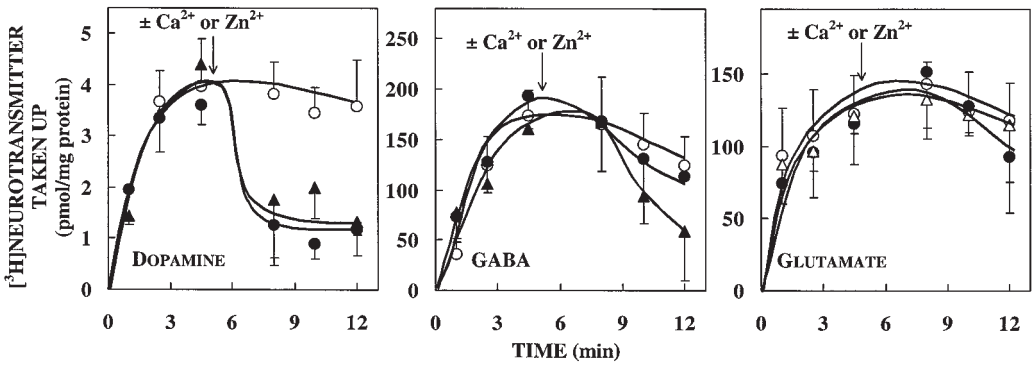

Fig. 7. Ca^{2+}- and Zn^{2+}-induced release of neurotransmitters from isolated synaptic vesicles. The neurotransmitter loading of the vesicles (*open circles*) was performed with [^{3}H]dopamine in *ΔpH medium* (*left panel*), [^{3}H]GABA in *ΔpH/Δφ medium* (*central panel*) and [^{3}H]glutamate in *Δφ + Cl^- medium* (right panel) as described in "Experimental Procedure". When indicated (at 5 min), Ca^{2+} (*close circles*) or Zn^{2+} (*close triangles*) were added to perform a final concentration of 500 or 150 μM, respectively. Active neurotransmitter uptake activities were calculated as describes in "Experimental Procedure". Values represent the mean ± SD of five separate experiments.

(Fig. 2). We also observed that, under conditions of non-limiting ATP concentration (1 mM), the inhibitory effect of Ca^{2+} is not visualized (results not shown). Under these conditions, the protons released in exchange with Ca^{2+} are rapidly taken up by H^+-ATPase activity and, therefore, no ΔpH dissipation occurs. These results indicate that Ca^{2+} inhibits dopamine uptake by dissipating the ΔpH rather than by interacting with the transport system. Indeed, dopamine loaded vesicles are rapidly discharged of dopamine by adding Ca^{2+} or Zn^{2+}, which appears to be also translocated by the cation/H^+ antiport activity previously observed in synaptic vesicles (12). The Ca^{2+} stimulatory effect observed under conditions of Δφ predominance is evident but dopamine uptake is greatly reduced as compared to that observed in a *ΔpH medium*. This evidence support the assumption that a coupled proton counter transport is required for dopamine accumulation and ΔpH provides both the energy source and the counter ions (*ΔpH medium*), as previously reported (31).

In contrast to dopamine transport, glutamate uptake strictly depends on Δφ, but it requires Cl^- anions (Fig. 3). By analysis of the Ca^{2+} effect, we observed that Ca^{2+} has a slight stimulatory effect when glutamate uptake is carried out in the *ΔpH/Δφ medium*, whereas a strong stimulation occurs in the *Δφ + Cl^- medium*. It appears that Ca^{2+}-induced membrane hyperpolarization may substitute (in a certain extent) the lack of optimal Cl^- concentration (17 mM). However, high Δφ does not support glutamate uptake in complete absence of Cl^- and there is no effect of Ca^{2+} (Fig. 4, *Δφ medium*). Furthermore, the lack of Ca^{2+} inhibition of glutamate uptake under conditions of high ΔpH (*ΔpH medium*) clearly shows that glutamate transporter utilizes exclusively the Δφ component of $\Delta\mu H^+$ as energy source for the transport process.

The transport system for GABA was operative either under conditions of high ΔpH or high Δφ (Fig. 5), and no additive amounts of GABA were taken up under conditions of significant contribution of both components (ΔpH and Δφ) (Fig. 5), which indicates that, essentially, only one component is required without preference for anyone. Ca^{2+} had a significant stimulatory effect on GABA uptake by the vesicles in a *Δφ medium*, whereas no effect was evident in *ΔpH/Δφ medium* and a slight inhibition was observed in a *ΔpH medium*. As Ca^{2+}-induced membrane hyperpolarization may neutralize the effect of ΔpH decrease in a *ΔpH/Δφ medium*, it appears plausible that no net Ca^{2+} effect is observed under these conditions.

The Ca^{2+} effects described above represent real evidence to demonstrate the type of energy source for accumulation of neurotransmitters by synaptic vesicles and they appears useful to distinguish the actual function of Cl^- in promote direct activation of the glutamate transport system. Furthermore, the results indicate that conditions of Ca^{2+} stress (> 100 μM) may alter neurosecretion by emptying synaptic vesicles of neurotransmitters whose accumulation is sensitive to ΔpH dissipation. In fact, various investigators observed that after prolonged stimulation, synaptic vesicles decrease their acetylcholine content in correlation with uptake of Ca^{2+} by the vesicles (32,33). In spite of the low affinity for Ca^{2+}, synaptic vesicles Ca^{2+}/H^+ antiport may be physiologically important as high Ca^{2+} concentrations are reached in specific microdomains near Ca^{2+} active zones (34). Under these conditions synaptic vesicle Ca^{2+} loading should allow forward Ca^{2+} extrusion

by exocytosis. At this level of neurosecretion reaction cascade, at which the cell recovers its ionic gradients, full activity of the Ca^{2+}/H^+ antiport should occur, since the cell may be ATP-deficient due to both increase ATP hydrolysis by activity of the ion pumps (Na^+/K^+-ATPase and Ca^{2+}-ATPase) and decreased ATP synthesis by mitochondria in consequence of their Ca^{2+}-induced depolarization. In conclusion, these results support the hypothesis that synaptic vesicle Ca^{2+}/H^+ antiport may participate in ruling cell recovering after a neurosecretory event.

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