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Brain Research Protocols 5 (2000) 102–108

BRAIN RESEARCH PROTOCOLS

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Protocol

Methods for analysis of Ca^{2+}/H^{+} antiport activity in synaptic vesicles isolated from sheep brain cortex

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Accepted 15 October 1999

Abstract

The involvement of Ca^{2+}-storage organelles in the modulation of synaptic transmission is well-established [M.K. Bennett, Ca^{2+} and the regulation of neurotransmitter secretion, Curr. Opin. Neurobiol. 7 (1997) 316–322 [1]; M.J. Berridge, Neuronal calcium signaling, Neuron 21 (1998) 13–26 [2]; Ph. Fossier, L. Tauc, G. Baux, Calcium transients and neurotransmitter release at an identified synapse, Trends Neurosci. 22 (1999) 161–166 [7]]. Various Ca^{2+} sequestering reservoirs (mitochondria, endoplasmic reticulum and synaptic vesicles) have been reported at the level of brain nerve terminals [P. Kostyuk, A. Verkhratsky, Calcium stores in neurons and glia, Neuroscience 63 (1994) 381–404 [18]; V. Mizuhira, H. Hasegawa, Microwave fixation and localization of calcium in synaptic terminals using X-ray microanalysis and electron energy loss spectroscopy imaging, Brain Res. Bull. 43 (1997) 53–58 [21]; A. Parducz, Y. Dunant, Transient increase of calcium in synaptic vesicles after stimulation, Neuroscience 52 (1993) 27–33 [23]; O.H. Petersen, Can Ca^{2+} be released from secretory granules or synaptic vesicles?, Trends Neurosci. 19 (1996) 411–413 [24]]. However, the knowledge of the specific contribution of each compartment for spatial and temporal control of the cytoplasmic Ca^{2+} concentration requires detailed characterization of the Ca^{2+} uptake and Ca^{2+} release mechanisms by the distinct intracellular stores. In this work, we described rapid and simple experimental procedures for analysis of a Ca^{2+}/H^{+} antiport system that transport Ca^{2+} into synaptic vesicles at expenses of the energy of a ΔpH generated either by activity of the proton pump or by a pH jumping of the vesicles passively loaded with protons. This secondary active Ca^{2+} transport system requires high Ca^{2+} concentrations (>100 μM) for activation, it is dependent on the chemical component (ΔpH) of the proton electrochemical gradient across the synaptic vesicle membrane and its selectivity is essentially determined by the size of the transported cation [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 [10]; P.P. Gonçalves, S.M. Meireles, P. Neves, M.G.P. Vale, Ionic selectivity of the Ca^{2+}/H^{+} antiport in synaptic vesicles of sheep brain cortex, Mol. Brain Res. 67 (1999) 283–291 [11]; P.P. Gonçalves, S.M. Meireles, P. Neves, M.G.P. Vale, Synaptic vesicle Ca^{2+}/H^{+} antiport: dependence on the proton electrochemical gradient, Mol. Brain Res. 71 (1999) 178–184 [12]]. The protocols described here allow to ascertain the characteristics of the Ca^{2+}/H^{+} antiport in synaptic vesicles and, therefore, may be useful for clarification of the physiological role of synaptic vesicles in fast buffering of Ca^{2+} at various sites of the neurotransmission machinery.Theme: Excitable membranes and synaptic transmission.Topic: Presynaptic mechanisms. © 2000 Elsevier Science B.V. All rights reserved.

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

1. Type of research

(A) Isolation of a synaptic vesicle enriched fraction [14,15].
(B) Generation of a proton electrochemical gradient across synaptic vesicle membrane [10–12].
(C) Measurement of Ca^{2+} uptake by synaptic vesicles [10–12].
(D). Measurement of proton fluxes across synaptic vesicle membrane [10–12].

2. Time required

The time required to run the total protocol is 9 h subdivided in four distinct periods:
(A) 4 h is the time required to isolate the synaptic vesicle enriched fraction.
(B) 1 min is the time required to generate a proton electrochemical gradient across synaptic vesicle mem-

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PII: S1385-299X(99)00061-6

brane by activation of the proton pump. However, 4 h are required when the gradient is artificially imposed after transferring passively proton loading vesicles to a basic reaction medium.

(C) 10 min plus the time spent in counting radioactivity is the time required to run the Ca^{2+} uptake assay.

(D) 10 min is the time required to measure proton fluxes across synaptic vesicle membrane either by the fluorescence quenching of acridine orange or by potentiometry.

3. Materials

(A) Sheep brains were removed, immediately after death, from young male sheep examined by the veterinarian in the public slaughterhouse.

(B) All reagents were of analytical grade. The $^{45}CaCl_2$, with a specific activity of 2 mCi/ml, was obtained from Amersham, England. Bafilomycin A_1, carbonyl cyanide *n*-chlorophenylhydrozone (CCCP), pepstatin, leupeptin, ATP and bis[dimethylamino]acridine (acridine orange) were obtained from Sigma. The scintillation cocktail ''Universol™ ES'' and the mixed cellulose esters (nitrate and acetate) filters HAWP 0.25.00, type HA (∅ 0.45 μm) were purchased from Biomedicals and Millipore, respectively.

(C) The equipment utilized includes:

- Teflon-glass homogenizer ''Potter-Elvejhem'';
- electric motor ''Franz Morat, model R25'';
- centrifuges ''Beckman Optima LE-80 K (50.2 Ti rotor)'' and ''Beckman Avanti J-25 I (JA-25.50 and JA 25-15 rotors)'';
- hydrogen electrode ''Ingold U402-M6-S7/100'' associated to an electrometer ''Crison pH mV-meter, model 2002'' connected to the recorder ''Zipp&Zonen, model BD 111'';
- diaphragm vacuum pump and compressor ''KF Neuberger, No. 35.1.2.18'';
- computer-controlled spectrofluorometer ''Perkin-Elmer, model LS-50'';
- liquid scintillation spectrometer ''Packard Tri-Carb 460 CD''.

4. Detailed procedure

4.1. Isolation of synaptic vesicle enriched fraction

Synaptic vesicles were isolated from sheep brain cortex according to the procedure described by Hell et al. [14,15]. After removing the cortex from the sheep brain (*Ovis aries*, breed merino), it is cut in small pieces and frozen in liquid nitrogen to facilitate its crushing with a mortar and pestle. The fine powder obtained is homogenized in 6 volumes of homogenization buffer (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) by a motor driven Teflon-glass homogenizer at 900 rpm. Then, the homogenate is centrifuged at $47{,}000 \times g$ for 10 min and the supernatant is collected for new centrifugation at $120{,}000 \times g$ during 40 min. The obtained supernatant (21 ml aliquots) is layered onto 5 ml cushions of 0.65 mM sucrose and 10 mM HEPES–K (pH 7.3) and centrifugation is run for 2 h at $260{,}000 \times g$. The resulting pellets are resuspended in 0.32 M sucrose and 10 mM HEPES–K (pH 7.4) and centrifuged for 10 min at $27{,}000 \times g$. At this stage, the collected supernatant is essentially enriched in synaptic vesicles. Indeed, contamination with plasma membranes was not observed as judged by absence of ouabain-sensitive (Na^+/K^+)ATPase activity, but a little contamination with microsomes (~ 15%) was detected by measuring the activity of the marker enzyme glucose-6-phosphatase. All steps of the purification procedure are performed at 4°C. Finally, the protein is determined (for example, by the method described by Gornall et al. [13], using bovine serum albumin as a standard), and fraction is divided into various aliquots that must be frozen in liquid nitrogen to store at −70°C. They are thawed at room temperature immediately before use in the experimental assays.

4.2. Generation of a proton electrochemical gradient across synaptic vesicle membrane

The synaptic vesicle Ca^{2+}/H^+ antiport accumulates Ca^{2+} into the vesicles by a process that depends on the energy of a proton electrochemical gradient across the membranes. This gradient may be achieved by passive proton loading of the vesicles or by activity of the proton pump. To obtain the artificially imposed pH gradient, synaptic vesicles (15 mg protein/ml) are incubated in loading medium (300 mM sorbitol, 1 mM DL-dithiothreitol (DTT), 10 mM potassium gluconate and 20 mM Mes–Tris, pH 5.6) during 4 h at 4°C. In contrast, for pump-mediated proton loading, synaptic vesicles (600 μg protein/ml) are 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 210–504 μM ATP–Mg. Under these conditions, the ATP-dependent proton transport may be visualized by following the fluorescence quenching of 2 μM acridine orange [5] at 525 nm (slit width 3.5 nm), using an excitation wavelength of 495 nm (slit width 3.0 nm). The formation of the proton gradient (ΔpH) can be checked by inducing its dissipation or by preventing its formation when a protonophore (10 μM CCCP) is added after or before the proton uptake reaction, respectively.

4.3. Measurement of Ca^{2+} uptake by synaptic vesicles

The use of the radioisotope $^{45}Ca^{2+}$ is suitable for measuring Ca^{2+} accumulation by isolated synaptic vesicles when combined with rapid filtration under vacuum and subsequent scintillation counting. The rapid filtration under vacuum permits separation of the synaptic vesicles

from the incubation media, so that they can be analyzed with respect to their radioactive content. In standard assay conditions for measuring Ca^{2+} uptake driven by an artificially imposed ΔpH (ATP-independent), the membrane vesicles (600 μg protein/ml) are incubated at 30°C in media containing 300 mM sorbitol, 10 mM potassium gluconate and 1 mM Tris (pH 8.5), whereas for measuring Ca^{2+} uptake driven by an ATP-dependent ΔpH, the membrane vesicles (600 μg protein/ml) are incubated 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 210 μM ATP–Mg. After 50 s, the reaction starts by adding $CaCl_2$ supplemented with $^{45}CaCl_2$ (10 μCi/mmol) and, at designated times, the reaction is stopped by rapid filtration of 800 μl aliquots through mixed cellulose esters (nitrate and acetate) filters (Millipore HAWP). These filters, with a maximal thickness of 180 μm and a pore size of 0.45 μm, have a protein retention capacity of 150 $\mu g/cm^2$. The cycle ''pre-washing, filtration, washing and drying'' should last no more than 30 s. The pre-washing and the washing after filtration with 1.5 and 3 ml of the reaction medium without Ca^{2+} and ATP–Mg, respectively, decrease the amount of the radioactivity in the filters which improves the ratio noise/signal, so that the noise is less than 2% of the signal. This value should be calculated in each experience and subtracted from the total counts. After drying, the filters are immersed in 3 ml of the scintillation cocktail ''Universol™ ES'', where they stay for at least 6 h before counting the radioactivity in the liquid scintillation spectrometer. In the counting programs of the apparatus, we introduced a statistic precision (2σ) of 1% for all the channels, as well as the curves of the correlation efficiency to different levels of muddiness, in order to assure that the reading counts are not lower than the real emissions. The Ca^{2+} accumulated in the vesicular space through the Ca^{2+}/H^+ antiport is expressed as nmol/mg protein and it can be checked by its discharging with 10 μM ionomycin, a selective Ca^{2+} ionophore.

4.4. Measurement of proton fluxes across synaptic vesicle membrane

Synaptic vesicles take up Ca^{2+} in exchange with H^+ [10–12]. Therefore, Ca^{2+}/H^+ antiport activity can be followed by recording the H^+ efflux from synaptic vesicles. A direct estimation of the Ca^{2+}-induced dissipation of the ΔpH artificially imposed to synaptic vesicles is achieved by using a ''Ingold U402-M6-S7/100'' hydrogen electrode associated to a ''Crison micro pH-meter 2002'' connected to a ''Zipp&Zonen BD 111'' recorder. In standard assay conditions, inside-acid synaptic vesicles (600 μg protein/ml) are incubated in a medium containing 300 mM sorbitol, 10 mM potassium gluconate and 1 mM Tris (pH 8.5). The reaction is started by adding 500 μM $CaCl_2$ and proceeds until a steady state is reached in a constantly stirred reaction mixture thermostated at 30°C. Ca^{2+}-induced medium acidification is quantified with successive additions of freshly titrated NaOH at the end of each experiment. The Ca^{2+}/H^+ antiport activity can be expressed as nmol H^+ released/mg protein. The registration of fluorescence quenching of 2 μM acridine orange is appropriated to visualize the efflux of H^+ from synaptic vesicles in exchange with Ca^{2+}, when proton loading is mediated by activation of the proton pump, as described above.

5. Results

5.1. Ca^{2+} uptake driven by the proton electrochemical gradient generated by the proton pump activity

The Ca^{2+}/H^+ antiport activity assures Ca^{2+} accumulation by synaptic vesicles and requires a proton electrochemical gradient. In physiological conditions, ΔpH represents the main component of the proton electrochemical gradient across synaptic vesicle membrane, which is essen-

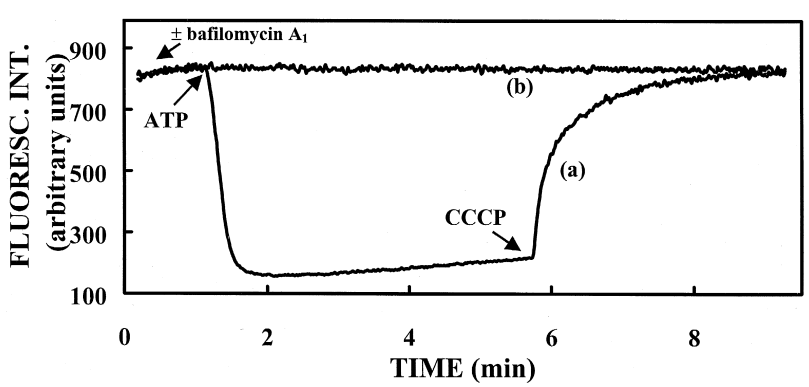

Fig. 1. Time-course of active H^+ loading of synaptic vesicles. Synaptic vesicles (1.2 mg protein) were incubated in 2 ml of a medium containing 2 μM acridine orange, 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and 10 mM Tris at pH 8.5 in the absence (curve a) and in the presence (curve b) of 300 nM bafilomycin A_1 (selective V-type H^+–ATPase inhibitor). The reaction was started by adding 210 μM 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.

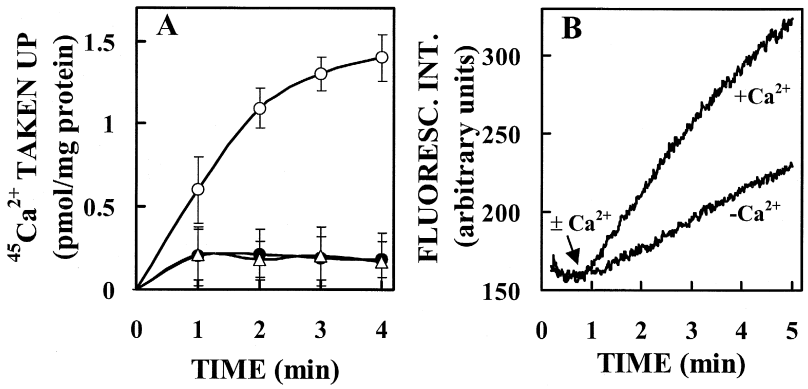

Fig. 2. Ca^{2+}/H^{+} antiport activity driven by ATP-dependent formation of ΔpH. The time-courses of Ca^{2+} uptake and H^{+} release by synaptic vesicles are represented in Panel A and Panel B, respectively. The vesicles (600 μg protein/ml) were incubated in a medium containing 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA and 10 mM Tris (pH 8.5) in the presence of 210 μM ATP–Mg. After 50 s, the reactions were started by adding 607.7 μM $CaCl_2$ to obtain a free Ca^{2+} concentration of 500 μM. The measurements of active Ca^{2+} uptake (Panel A) were performed in the absence (○-○) and in the presence of 10 μM CCCP (●-●) or 300 nM bafilomycin A_1 (△-△), as well as 10 μCi/mmol $^{45}CaCl_2$ (typical data ranged from 2600 to 3800 cpm total bound and 200 to 400 cpm background bound ^{45}Ca). The reactions were stopped at designated times by filtering 800 μl aliquots of reaction medium through Millipore filters (∅ 0.45 μm). The radioactivity of the filters was quantified as described in the text. The measurements of H^{+} release (Panel B) was performed in the presence of 2 μM acridine orange as described in the legend of Fig. 1.

tially generated and maintained by the V-type H^{+}–ATPase activity. The procedure for isolation of synaptic vesicles from sheep brain cortex allows to obtain a population of well sealing vesicles that are able to form a transmembrane pH gradient in response to ATP supply. Fig. 1 represents the time-course of H^{+} loading of synaptic vesicles in response to activation of the V-type H^{+}–ATPase. In the presence of saturating ATP concentrations (210 μM), the accumulation of H^{+} is linear over a 30 s incubation period. After that the intensity of acridine orange fluorescence signal remains unchanged during at least 4 min, which corresponds to the maximal ΔpH maintained by the H^{+}–ATPase activity at steady-state (Fig. 1, curve a). Under these experimental conditions, ATP-dependent ΔpH formation across synaptic vesicle membrane involves the proton pump activity as shows the total prevention of ΔpH formation by bafilomycin A_1, a specific inhibitor of the V-type H^{+}–ATPase (Fig. 1, curve b).

After formation of the ΔpH across the membrane, synaptic vesicles actively accumulate Ca^{2+} in exchange with protons, in a time-dependent way (Fig. 2). Moreover, the uptake of Ca^{2+} was found to be concentration-dependent and saturable (data not shown). The ΔpH-dependent Ca^{2+} uptake by synaptic vesicles is easily demonstrated in experiments whose results are depicted in Fig. 2A. Indeed, the activity of Ca^{2+} uptake at 500 μM Ca^{2+} is almost completely abolished by the protonophore CCCP and by the V-type ATPase inhibitor bafilomycin A_1. This indi-

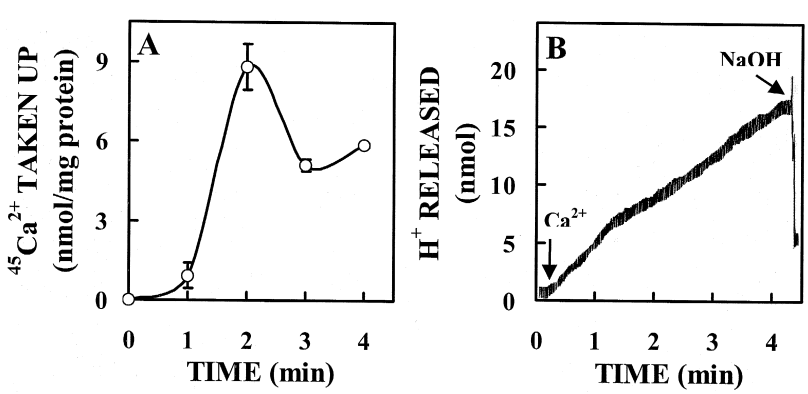

Fig. 3. Ca^{2+}/H^{+} antiport activity driven by artificially imposed ΔpH (ATP-independent). The time-courses of Ca^{2+} uptake and H^{+} release by synaptic vesicles are represented in Panel A and Panel B, respectively. The vesicles (600 μg protein/ml) previously incubated in 300 mM sorbitol, 1 mM 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 $CaCl_2$ (Panel B) or 500 μM $CaCl_2$ supplemented with 10 μCi/mmol $^{45}CaCl_2$ (Panel A). Ca^{2+} uptake was measured as described in Fig. 2 and 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.

cates a 100% contribution of the H^+–ATPase for the Ca^{2+} uptake process since, under these conditions, it is the only system responsible for the proton gradient formation.

5.2. Ca^{2+} uptake driven by an artificially imposed proton electrochemical gradient

The efflux of protons in exchange with Ca^{2+} influx is accompanied by the permanent re-uptake of protons by the H^+–ATPase into the synaptic vesicles. This implies that net Ca^{2+} induced proton efflux is reduced. Thus, a further characterization of the synaptic vesicle Ca^{2+}/H^+ antiport activity can be achieved by using synaptic vesicles passively loaded with protons. The radiometric and potentiometric approaches described in this work are suitable for direct estimation of the cation fluxes across synaptic vesicle membrane. Fig. 3 shows the time-courses of Ca^{2+} uptake and H^+ release associated with Ca^{2+}/H^+ exchange in synaptic vesicles made acidic in an ATP-independent way. The synaptic vesicles (internal pH of 5.6), when incubated at pH 8.5 in the presence of 500 μM Ca^{2+}, sequester radioactive Ca^{2+} in exchange with equivalent H^+ release from the vesicles. In these experimental conditions, steady-state levels of Ca^{2+} uptake and H^+ release are reached after ~3.5 min and correspond to 5.5 nmol Ca^{2+}/mg protein and 15 nmol H^+/mg protein, respectively.

6. Discussion

6.1. Trouble shooting

The protocol described here has been used in our laboratory to demonstrate and to characterize the Ca^{2+}/H^+ antiport activity in isolated synaptic vesicles [10–12]. Its visualization essentially requires preservation of membrane integrity to form well sealing vesicles. In order to obtain a suitable fraction of synaptic vesicles isolated from sheep brain cortex, we used the procedure developed by Hell et al. [14,15], where two experimental conditions, maintenance of the tissue in liquid nitrogen until homogenization and formation of a fine powder during tissue crush, appear to be important for the good quality of the preparation.

The Ca^{2+}/H^+ antiport represents a secondary active transport system for Ca^{2+} sequestration inside various types of cellular compartments [3,27]. Recently, we observed its existence in synaptic vesicle membranes [10], where, under physiological conditions, its capacity and translocation rate appear to depend on the activity of the V-type H^+–ATPase, which is stimulated by Ca^{2+}. Thus, Ca^{2+} influx to the vesicles is accompanied by H^+ efflux through the antiport, which implies an increased activity of the H^+ pumping into the vesicles and a consequent stimulation of ATP hydrolysis by the H^+–ATPase. The main encountered problem during the proposed procedure was to correlate the Ca^{2+} influx with H^+ efflux mediated by the Ca^{2+}/H^+ antiport system. In this work, we present two distinct approaches to obviate this problem. The first one was by adjusting the ATP concentration in the incubation medium to a level that is sufficient for maintenance of a high ΔpH across the membrane, but it is not high enough to support a significant stimulation of the H^+ pump in response to Ca^{2+} addition (Fig. 2). The second alternative was the creation of an artificially pH jump across the synaptic vesicle membrane (Fig. 3), which is lost in the presence of Ca^{2+} without H^+ reloading by the ATPase. It appears that the workability of the assays to follow H^+ release from the synaptic vesicles is limited by the methodology for acidification of the synaptic vesicles and, therefore, the proton electrochemical gradient generation across synaptic vesicle membrane must be taken into account, according to the purpose of the experiments.

6.2. Alternative and support protocols

The Ca^{2+}/H^+ antiport activity in synaptic vesicles was recently described [10–12] and, consequently, there are no reports of alternative protocols in the literature. However, the basic techniques used can be performed in any biochemical laboratory and have been currently employed by several investigators [20,30].

The synaptic vesicle fraction can be obtained from frozen and fresh tissue [6,9,16,17,29] by more consuming time procedures. Immuno-isolation of synaptic vesicles with Dynabeads [4,19] probably represents a real alternative protocol, but requires the characterization of the ionic transport behavior of the purified vesicles.

In the absence of ATP, Ca^{2+}/H^+ antiport activity requires the acidification of isolated synaptic vesicles, in order to generate a suitable ΔpH gradient to drive Ca^{2+} transport into the vesicles. When necessary, higher proton electrochemical gradients across the membrane can be achieved by centrifugation of the vesicles after passive H^+-loading, which reduces the total volume of synaptic vesicles and, consequently, increase the magnitude of the generated ΔpH gradient. Synaptic vesicles can be also refill with protons by combined use of ionophores and ionic manipulation of the intra- and extravesicular media compositions [22,25].

The indirect coupling of the Ca^{2+}/H^+ antiport activity with the V-type H^+–ATPase implies that, under physiological conditions, proton release through the antiporter can not be evaluated by following the variation of pH in the incubation medium (potentiometric method). As already mentioned above, in this case, it is recommended to use fluorescent dyes as intravesicular pH indicators. Nowadays, there are a variety of pH fluorescent probes that can be used instead of acridine orange, and its selection must be determined according to the experimental purposes. The establishment of the adequate ATP concentration (210–504 μM) represents a crucial step for successful visualization

of Ca^{2+}-induced H^+ release from the synaptic vesicles in the presence of ATP. On this basis, it is recommendable a preliminary study to investigate whether Ca^{2+} has a stimulatory effect on the hydrolytic activity of the H^+–ATPase under the same experimental conditions. The ATPase activity of the synaptic vesicles is easily determined by measuring the Pi liberation associated with the hydrolysis of ATP. The simultaneous performance of assays in the absence and in the presence of bafilomycin A_1 (a selective inhibitor of the V-type H^+–ATPase) allows identification of the V-type H^+–ATPase activity among other Pi sources [10]. Pi analysis [26] can be performed at the end of the ATPase assay after removing the precipitated protein by centrifugation with ice-cold trichloroacetic acid. By continuous spectrophotometric monitoring of the increase in absorbance at 360 nm, it is possible to follow the kinetics of ATPase activity. This method requires the additional presence of purine nucleoside phosphorylase (PNP) and 2-amino-6-mercapto-7-methylpurine ribose (MESG) and is based on the proportionality between Pi consumption by MESG/PNP reaction and the production of 2-amino-6-mercapto-7 methylpurine [28]. Similarly, the pair phosphoenolpyruvate lactate dehydrogenase and pyruvate kinase can be use for continuous sprectrophotometric monitoring of ATPase activity [8].

Although the Ca^{2+}-induced proton efflux strongly indicates the existence of a Ca^{2+}/H^+ antiport system in synaptic vesicles, its full demonstration must be accomplished by measuring the Ca^{2+} accumulation into the vesicles in correlation with the magnitude of the pH gradient across the membranes. The possibility that other Ca^{2+} transporters such as Ca^{2+} pumps could contribute for the Ca^{2+} accumulation observed was ruled out since the process is vanadate insensitive (results not shown).

In conclusion, the protocols depicted in the present work have been employed to study the Ca^{2+}/H^+ antiport of synaptic vesicles and represent putative tools for further characterization of the synaptic vesicle participation on the Ca^{2+} homeostasis at the level of nerve terminals. The protocols can be also applied to other types of vesicles originated from various cellular compartments (plant cell plasma membranes, vacuoles, chromaffin granules, etc.) that contain a proton pump to form a ΔpH responsible for energize the Ca^{2+}/H^+ antiport system.

7. Quick procedure

7.1. Isolation of synaptic vesicle enriched fraction

- Dissection of the brain and separation of little pieces of gray matter to freeze in liquid nitrogen.
- Pulverization and homogenization of the frozen tissue.
- Centrifugation of the homogenate (H) at $47{,}000 \times g$ for 10 min and subsequent centrifugation of the supernatant (S_1) for 40 min at $120{,}000 \times g$.
- Recovery of the supernatant (S_2) and ultracentrifugation through 0.65 mM sucrose and 10 mM HEPES–K (pH 7.3) for 2 h at $260{,}000 \times g$.
- Resuspension of the pellet (P_3) and centrifugation for 10 min at $27{,}000 \times g$.
- Recovery of the synaptic vesicle enriched fraction (S_4).
- Storage of the synaptic vesicles at -70°C after freezing in liquid nitrogen.
- Determination of the protein content of the synaptic vesicle preparation.

7.2. Proton electrochemical gradient generation across synaptic vesicle membrane

- Passive proton loading of synaptic vesicles: incubation of synaptic vesicles (15 mg protein/ml) in loading medium (300 mM sorbitol, 1 mM DL-dithiothreitol (DTT), 10 mM potassium gluconate and 20 mM Mes–Tris, pH 5.6) during 4 h at 4°C.
- Active proton loading of synaptic vesicles: incubation of synaptic vesicles (600 μg protein/ml) 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 210-504 μM ATP–Mg during 50 s at 30°C.

7.3. Measurement of Ca^{2+} transport by synaptic vesicles

- Incubation of synaptic vesicles (600 μg protein/ml) at 30°C in media containing 300 mM sorbitol, 10 mM potassium gluconate and 1 mM Tris (pH 8.5) (Ca^{2+} uptake driven by ΔpH gradient artificially imposed) or 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 (Ca^{2+} uptake driven by ΔpH gradient generated by the proton pump).
- Start the reaction by the addition of $CaCl_2$ supplemented with $^{45}CaCl_2$ (10 μCi/mmol).
- Separation of the synaptic vesicles from the incubation media by rapid filtration under vacuum.
- Counting of the radioactivity retained in the filters by liquid scintillation spectrometry.

7.4. Measurement of proton fluxes across synaptic vesicle membrane

- Potentiometric assay: Addition of inside-acid synaptic vesicles (600 μg protein/ml) to a medium (1 ml) containing 300 mM sorbitol, 10 mM potassium gluconate and 1 mM Tris (pH 8.5) in a thermostatized cuvette with magnetic stirring. Start the reaction by adding $CaCl_2$. Continuous measurement of the medium acidification. Calibration of the response with freshly titrated NaOH at the end of each experiment.
- Fluorimetric assay: Addition of synaptic vesicles (600 μg protein/ml) to a medium (2 ml) containing 2 μM acridine orange, 60 mM sucrose, 2 mM $MgCl_2$, 150 mM KCl, 50 μM EGTA, 10 mm Tris–HCl (pH 8.5) and 210

μM ATP–Mg. Start the reaction by adding $CaCl_2$. Continuous registration of the acridine orange fluorescence quenching at 525 nm (slit width 3.5 nm), using an excitation wavelength of 495 nm (slit width 3.0 nm). For evaluation of the net Ca^{2+}-induced proton efflux from synaptic vesicles, add 10 μM CCCP at the end of each experiment.

8. Essential references

Essential references: Refs. [10–12,14,15].

Acknowledgements

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

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