The Synaptic Vesicle Protein Synaptophysin: Purification and Characterization of Its Channel Activity

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ABSTRACT The synaptic vesicle protein synaptophysin was solubilized from rat brain synaptosomes with a relatively low concentration of Triton X-100 (0.2%) and was highly purified (above 95%) using a rapid single chromatography step on hydroxyapatite/celite resin. Purified synaptophysin was reconstituted into a planar lipid bilayer and the channel activity of synaptophysin was characterized. In asymmetric KCl solutions (*cis* 300 mM/*trans* 100 mM), synaptophysin formed a fast-fluctuating channel with a conductance of 414 ± 13 pS at +60 mV. The open probability of synaptophysin channels was decreased upon depolarization, and channels were found to be cation-selective. Synaptophysin channels showed higher selectivity for K^+ over Cl^- ($P_{K^+}/P_{Cl^-} > 8$) and preferred K^+ over Li^+, Na^+, Rb^+, Cs^+, or choline$^+$. The synaptophysin channel is impermeable to Ca^{2+}, which has no effect on its channel activity. This study is the second demonstration of purified synaptophysin channel activity, but the first biophysical characterization of its channel properties. The availability of large amounts of purified synaptophysin and of its characteristic channel properties might help to establish the role of synaptophysin in synaptic transmission.

INTRODUCTION

Synaptic transmission involves the regulated release of transmitter molecules to the synaptic cleft, where they interact with postsynaptic receptors, which subsequently transduce the information. Synaptic vesicles accumulate neurotransmitters and release them during exocytosis to the synaptic cleft. In examining the exocytosis process many proteins from the synaptic vesicles have been studied, and several conserved family proteins in synaptic vesicles were shown (De Camilli and Jahn, 1990). One such family is the synaptophysin family, accounting for ~6–8% of the total synaptic vesicle protein (Jahn et al., 1985). Synaptophysin is a hexameric protein consisting of 38 kDa monomers and is a major integral membrane protein of transmitter-containing vesicles found in neurons and endocrine cells (Wiedenmann and Franke, 1985). Based on amino acid sequences deduced from rat and human cDNA and genomic clones, the predicted synaptophysin structure consists of four transmembrane domains, two intravesicular domains (Südhof et al., 1987; Buckley et al., 1987; Leube et al., 1987), and a cytoplasmic carboxyl tail containing a unique Ca^{2+}-binding repeat (Rehm et al., 1986). Based on the predicated structure, it was suggested that synaptophysin forms a channel in the synaptic vesicle membrane and acts as the major Ca^{2+}-binding protein in synaptic vesicles (Rehm et al., 1986). Indeed, it has been demonstrated that upon reconstitution into a planar lipid bilayer, purified synaptophysin displayed voltage-sensitive channel activity (Thomas et al., 1988). The function of synaptophysin is, however, as yet unknown. On the one hand, its location in the synaptic vesicle membrane and its interaction with VAMP (vesicle-associated membrane protein, also known as synaptobrevin), implicated in synaptic vesicle docking and fusion (Calakos and Scheller, 1994), suggests its involvement in exocytosis. However, the function of synaptophysin in neurotransmitter release has been questioned because mutant mice lacking synaptophysin I displayed normal synaptic transmission (McMahon et al., 1996). On the other hand, overexpression of synaptophysin enhanced neurotransmitter secretion at *Xenopus* neuromuscular synapses (Alder et al., 1995). The relationship between the channel activity of synaptophysin and its function is thus not clear. Furthermore, the function of synaptophysin remains unknown. Indeed, its involvement in regulation of exocytosis and/or endocytosis, rather than its direct participation in either process, has been postulated (Becher et al., 1999; Daly et al., 2000).

The purpose of this study was to purify synaptophysin and to characterize its channel properties. We have developed a novel and simple method for purification of large amounts of synaptophysin. The highly purified protein was reconstituted into planar lipid bilayer and single channel activity was recorded. The synaptophysin channel was found to be voltage-dependent, fast-fluctuating, and cation-selective, with high selectivity for potassium. The availability of high quantities of highly purified synaptophysin should help in the elucidation of its structure and to establish its function in exocytosis and/or endocytosis.

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MATERIALS AND METHODS

Materials

Potassium chloride, choline chloride, cesium chloride, Trizma base, Hepes, soybean asolectin, phenylmethylsulfonyl fluoride, leupeptin, n-decane, and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). Potassium gluconate was obtained from Fluka, and rubidium chloride,

calcium chloride, magnesium chloride, and lithium chloride from Merck. Alkaline phosphatase-conjugated goat anti-mouse IgG was obtained from Promega (Madison, WI). Synaptophysin monoclonal antibody (574780) was obtained from Cal Biochem (La Jolla, CA). Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad and celite was obtained from the British Drug Houses.

Synaptosome preparation

Synaptosomes were prepared from freshly dissected rat brain as described previously for sheep brain (Gincel et al., 2000). Synaptosomes were frozen in liquid nitrogen and stored at −80°C. Protein concentration was determined according to Lowry et al. (1951).

Purification of synaptophysin

Brain synaptosomal membranes (10 mg protein) were incubated for 30 min at 0°C (at 5 mg/ml) in a solution containing 5 mM NaH_2PO_4, pH 6.8, and 0.2% Triton X-100 (w/v). After centrifugation at 44,000 × *g* for 30 min, the Triton X-100 extract was applied to a dry hydroxyapatite/celite (2:1 w/w) (0.1 g/mg protein) and eluted with solubilization buffer (5 mM NaH_2PO_4, pH 6.8 and 0.2% Triton X-100). The resin bound proteins were eluted with 0.3M NaH_2PO_4, pH 6.8, and found to contain no synaptophysin. Synaptophysin-containing fractions were collected kept at −20°C and used within two weeks.

Gel electrophoresis and immunoblot analysis

Analysis of the protein profile was performed by SDS-PAGE with the discontinuous buffer system of Laemmli (1970) using 1.5-mm-thick slab gels of 10% and 3.5% acrylamide for separating and stacking gels, respectively. Gels were stained with Coomassie Brilliant blue. Molecular weight standards were from Bio-Rad (broad range). Western blot analysis was carried out by standard procedures (Towbin et al., 1979). The SDS-PAGE-separated proteins were electrophoretically transferred onto nitrocellulose membranes. For immunostaining, the membranes were blocked with 5% non-fat dry milk and 0.1% Tween-20 in Tris-buffered saline, incubated with monoclonal anti-synaptophysin antibodies (1:1,000) in Tris buffer-saline containing 1% non-fat dry milk. Antibody binding was visualized with alkaline-phosphatase conjugated anti-mouse IgG as a secondary antibody (1:10,000). The color was developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Single channel recording and analysis

Reconstitution of synaptophysin into planar lipid bilayers (PLB), single channel current recording, and data analysis were carried out as described previously (Gincel et al., 2001). Briefly, PLB were prepared from soybean asolectin dissolved in n-decane (50 mg/ml channel activity) in a chamber containing 10 mM Tris/Hepes pH 7.4 and KCl or other salt (100–300 mM). Only PLB with a resistance >100 GΩ were used. In some experiments, bilayers were prepared from mixture of purified phosphatidylethanolamine and phosphatidylserine (5:3) (Avanti Polar Lipids Inc., Alabaster, AL) and the obtained results were as with asolectin. Purified synaptophysin (1–2 ng) was added to one side of the bilayer (defined as the *cis* side). At KCl concentration gradient (300/100 mM, *cis/trans*), and at voltages between 0 and 100 mV, reconstituted synaptophysin channel activity is characterized by fast fluctuation in the outward currents. After one or a few channels inserted into the PLB, excess protein was removed by perfusion of the *cis* chamber with 20 volumes of a solution (with the same composition as before perfusion), to prevent further protein incorporation.

Currents were recorded under voltage-clamp using a Bilayer Clamp BC-525B amplifier (Warner Instrument Corp.). The currents were measured with respect to the *trans* side of the membrane (ground). The currents were low-pass filtered at 1 kHz (−3 dB), using a Bessel filter (Frequency Devices 902) and digitized on-line using a Digidata 1200 interface board and pCLAMP 6 software (Axon Instruments, Inc.). For analysis of single channel kinetic properties, we used custom-made programs.

Solutions

All the solutions used contained KCl or other salt as indicated (100–300 mM), and were adjusted to pH 7.4 using 10 mM Hepes/Tris. Experiments were performed at 23–25°C.

RESULTS

Synaptophysin purification

In previous studies, synaptophysin was solubilized from rat or frog brain with 1% Triton X-100 and purified using affinity chromatography column (anti-synaptophysin antibody coupled to Sepharose 4B) (Navone et al., 1986; Valtorta et al., 1988). This purification procedure is relatively complicated and yields small amounts of synaptophysin. In another study (Llona et al., 1992), synaptophysin was extracted with 2% Triton X-100 in the presence of 2 M KCl and was partially purified using carboxymethyl-Sepharose Fast Flow, and further purified by preparative SDS-polyacrylamide gel electrophoresis, resulting in denatured protein. Here, large quantities of synaptophysin were purified from rat brain synaptosomes using a low concentration of Triton X-100 (0.2% w/v) and rapid, single-step chromatography on hydroxyapatite/celite column (Fig. 1). We found that a major part of the synaptophysin was extracted from synaptosomes with 0.2% Triton X-100. This is noteworthy because higher concentrations of Triton X-100 extracted other proteins (such as the voltage-dependent anion channel, VDAC) that were found to co-purify with synaptophysin on a hydroxyapatite column (Gincel et al., 2000). Fig. 1 *A* shows that over 95% of the Triton-X-100 (0.2%)-extracted proteins applied to the hydroxyapatite column bound to the column, but not a 38 kDa protein. This protein was identified by specific monoclonal antibody as synaptophysin (Fig. 1*B*). The column-bound proteins were eluted from the resin by a high salt concentration (0.3 M NaH_2PO_4, pH 6.8) and contained no synaptophysin. Because the synaptophysin containing 0.2% Triton-X-100 extract may represent synaptic vesicle-associated synaptophysin, in some experiments the concentration of Triton-X-100 was increased to 2% before chromatography on the hydroxyapatite column. The same amount of synaptophysin was obtained when purified in the presence of the 0.2% or 2% Triton-X-100. Furthermore, after centrifugation of the 0.2% Triton-X-100 extract at 160,000 × *g*, most (over 80%) synaptophysin remained in the supernatant (data not shown). Similar synaptophysin purification was obtained using sheep brain synaptosomes (data not shown). Thus, using this purification

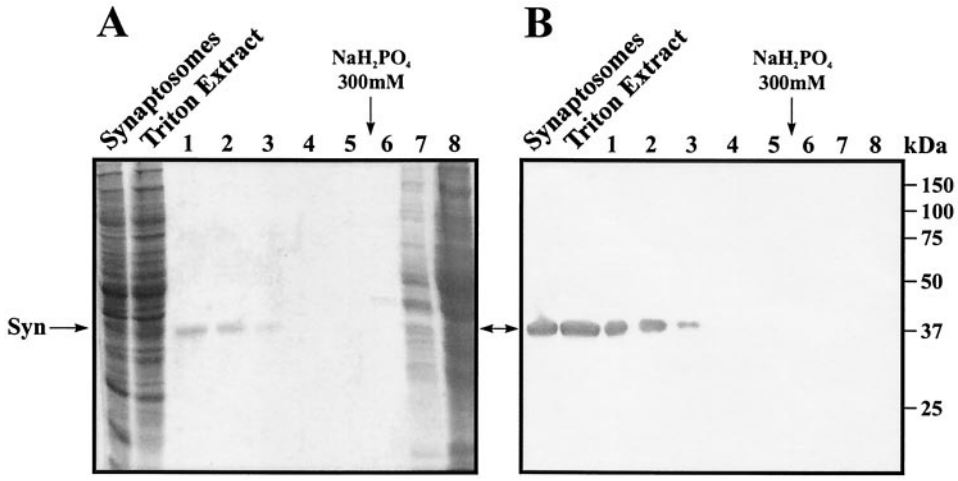

FIGURE 1 Purification of synaptophysin from brain synaptosomes. Synaptophysin was purified from rat brain synaptosomes using a simple one-step chromatography column of hydroxyapatite (HA). Synaptosomes were isolated and synaptophysin was purified as described under Materials and Methods. Synaptosomes, 0.2% Triton X-100 extract, flow-through fractions from HA (Fr 1–5), and 0.3 M NaH_2PO_4 eluted fraction (Fr 6–8) were subjected to SDS-PAGE (10% acrylamide) and Western blot analysis. The Coomassie stained gel is shown in *A* and the corresponding immunoblot in *B*. Synaptophysin (syn) and the positions of molecular weight standards (Bio-Rad) are indicated.

procedure, a relatively large amount of synaptophysin is obtained within 1 to 2 h.

Synaptophysin channel activity and permeability

Although synaptophysin was reported previously to act as an ion channel (Thomas et al., 1988), this activity was never thoroughly investigated. In the present study, synaptophysin was reconstituted into planar lipid bilayer (PLB) and current traces from a single synaptophysin molecule in response to voltage steps were recorded (Fig. 2). Fig. 2 *A* shows typical current traces recorded for synaptophysin channels. To determine whether the outward current is the result of an efflux of cations or an influx of anions, the *cis* side of the bilayer was successively exposed to 300 mM potassium chloride (*A*), choline chloride (*B*), or potassium gluconate (*C*), while the *trans* side of the bilayer in all cases was exposed to 100 mM KCl. A voltage change protocol was then used to generate families of currents under different ionic compositions. From an initial holding potential of 0 mV, the membrane potential (V_m) was stepped for 3.2 s to voltages ranging from −60 to +100 mV, in steps of +20 mV. It is apparent that when the 300 mM potassium chloride in the *cis* chamber was replaced by 300 mM choline chloride, the outward currents were eliminated (Fig. 2 *B*). Furthermore, replacement of choline chloride with potassium gluconate led to the recovery of outward currents at all voltages tested between 0 and +100 mV (Fig. 2 *C*). The current-voltage relationships, constructed from these current families, represent the changes in the current amplitude and E_{rev} (reversal potential) (Fig. 2 *D*). In contrast to choline chloride, substituting potassium gluconate for potassium chloride had relatively little effect on either channel kinetics (Fig. 2, *A* and *C*) or current-voltage (*I-V*) relationships (Fig. 2 *D*), suggesting that the cation, rather than the anion, carries the current. From this experiment, the permeability ratio of K^+ over Cl^- was calculated. The reversal potential point was found to be -19.6 ± 0.4 mV ($n = 6$) in 100 mM potassium chloride *trans* and 300 mM *cis*. Thus, a permeability ratio of $P_K/P_{Cl} > 8$ is estimated. Reconstitution experiments with fractions that contained no anti-synaptophysin antibodies cross-reacted protein (*fractions 5–6* in Fig. 1) showed no channel activity ($n = 3$).

Cation selectivity of the synaptophysin channel

The cation selectivity of the synaptophysin channels was determined in cation substitution experiments (Fig. 3). The 300 mM KCl (*A*) in the *cis* chamber was replaced by 300 mM of each of the following salts: RbCl (*B*), CsCl (*C*), NaCl (*D*), or LiCl (*E*), and families of current traces were obtained at voltages between −60 mV and +100 mV. The current-voltage relationships constructed from these current families show changes in the current amplitude and in E_{rev} (Fig. 3 *F*). The conductance of the channel decreased remarkably to zero level upon replacing the solution with any of the alkali salts. The reversal potential for the single unitary currents shifted to more positive values when Rb^+, Na^+, Li^+, or Cs^+ was substituted for K^+, indicating that these cations are less permeable than K^+ through the synaptophysin channel.

Kinetic properties of synaptophysin channel activity

The synaptophysin channel, as shown in Fig. 2, is completely closed at negative voltages. The kinetic properties of

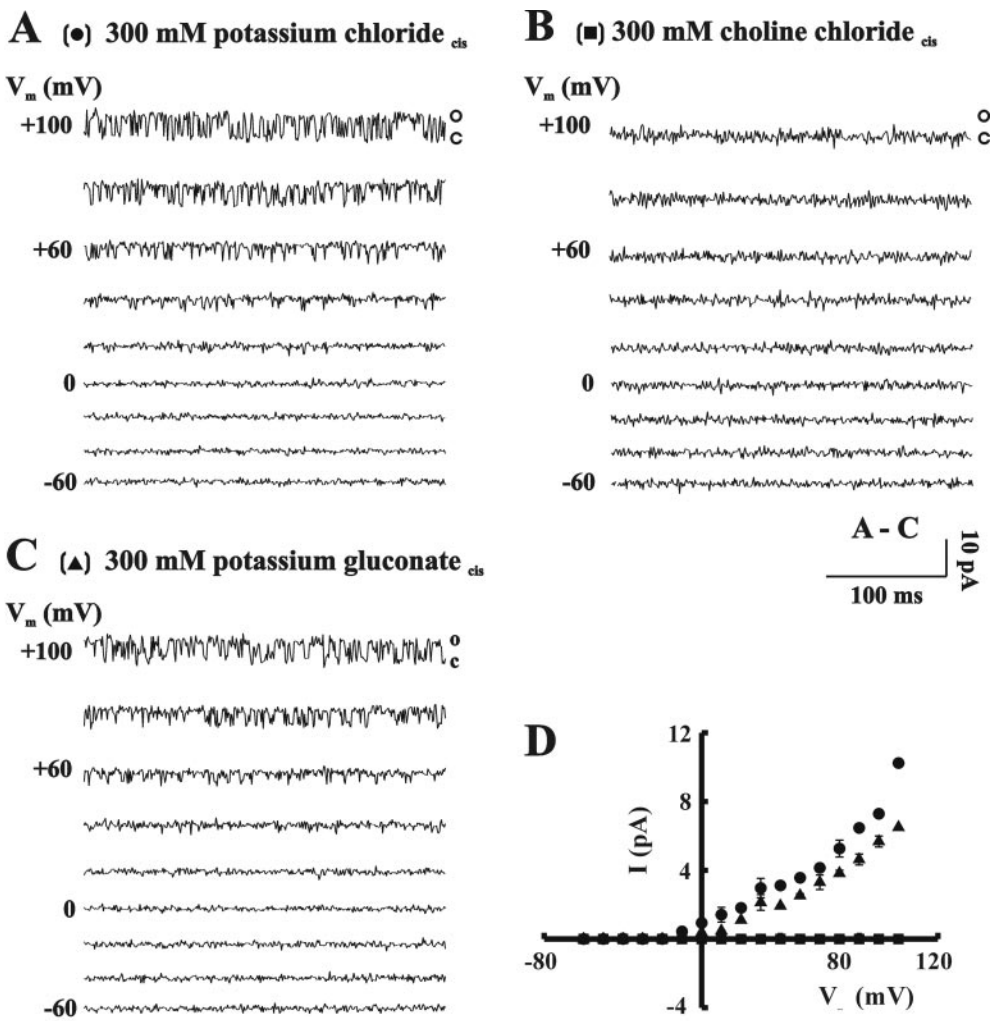

FIGURE 2 Synaptophysin forms fast cationic ion channels. Representative families of current traces illustrating the activity of synaptophysin channels recorded from voltage-clamped bilayer. The *cis* solution was 300 mM potassium chloride (*A*), 300 mM choline chloride (*B*), or 300 mM potassium gluconate (*C*). The *trans* solution was 100 mM potassium chloride in all experiments. Membrane voltages between −60 mV and +100 mV in 20 mV steps are shown in the left current traces *A–C*. Following insertion of a channel, the upward deflections denote activation of outward potassium current; potassium ions are moving from the *cis* chamber to the *trans* chamber. (*D*) Current-voltage relationships derived from the measurements of the open state current minus the closed state current in experiments *A–C*. For these experiments the values for E_{rev} were −19.6 ± 0.4 mV for potassium chloride (●), +52.3 ± 1.2 mV for choline chloride (■), and −17.5 ± 0.6 mV for potassium gluconate (▲). The data are the means ± standard error of three to four experiments.

synaptophysin channels were obtained by analyzing channel activity at positive voltages (Fig. 4). The probability of the channel being open (P_o) is voltage-dependent, with mean values between 0.87 and 0.49 at voltages between +40 and +120 mV, respectively (Fig. 4 *A*), suggesting decreasing channel activity by high voltages. The average open (*filled bars*) and closed (*open bars*) time histograms plotted against membrane voltage (Fig. 4 *B*) indicate that the mean values of open time decreased by ~72%, while those for the closed time increased only by ~40%. This suggests that the decrease of open probability at high positive voltages originated from the decrease in the open time of the channel, and that the channel voltage-sensor affects the channel open state rather then the closed state. Synaptophysin channel activity was not affected by Ca^{2+} (25 μM to 5 mM), EGTA (0.1 mM), La^{3+} (50 μM), Mg^{2+} (0.5 to 5 mM), a combination of Mg^{2+} and Ca^{2+}, or by tetraethylammonium (TEA) (data not shown).

DISCUSSION

Synaptophysin is among the most abundant and conserved synaptic vesicle proteins, but its function is still unknown (Südhof, 1995). Synaptophysin has been shown to interact with other proteins such as synaptobrevin (Calakos and Scheller, 1994; Prekeris and Terrian, 1997; Becher et al., 1999), to co-purify with other synaptic vesicle proteins such

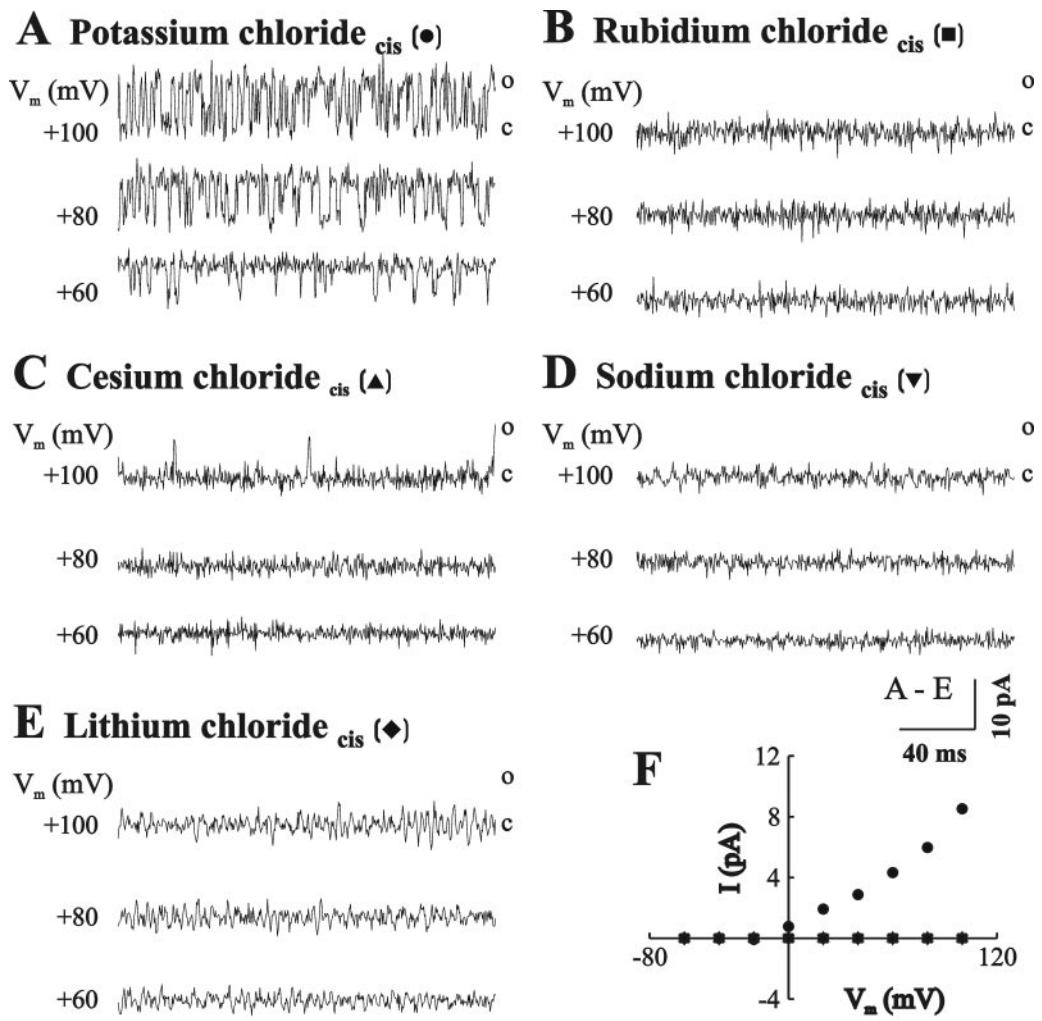

FIGURE 3 Monovalent cation selectivity of synaptophysin. Single channel currents of purified synaptophysin subjected to voltages between −60 mV and +100 mV in 20 mV voltages steps were recorded. For clarity, only representative traces are presented: (*A*) 300 mM $[KCl]_{cis}$; (*B*) 300 mM $[RbCl]_{cis}$; (*C*) 300 mM $[CsCl]_{cis}$; (*D*) 300 mM $[NaCl]_{cis}$; and (*E*) 300 mM $[LiCl]_{cis}$. (*F*) Current-voltage relationships derived from the measurements of the open state current minus the closed state current for the monovalent cations: K^+ (●), Rb^+ (■), Cs^+ (▲), Na^+ (▼), and Li^+ (◆). For Cs^+, although the open state is clearly seen at +100 mV it was not calculated as open state because it was only observed at +100 mV, which could be due to the large driving force and not to spontaneous channel opening. In each experiment, at the end of the experiment the *cis* solution was always exchanged back to KCl and control activity was recorded. The data in *F* are the mean ± standard error of two to three experiments.

as synapsin I (Llona et al., 1992), to possess a Ca^{2+}-binding site (Rehm et al., 1986), to play a role in synaptic plasticity by interacting with synaptogyrin I and preventing it from entering the SNARE complex (Janz et al., 1999), to inactivate the V-ATPase before exocytosis (Carrion-Vazquez et al., 1998), and to regulate clathrin-independent endocytosis (Daly et al., 2000), suggesting its involvement in synaptic vesicle recycling. Another synaptophysin family protein from the triad junction in skeletal muscle, mitsugumin29, has recently been described (Takeshima et al., 1998). The proposed function of mitsugumin29 is in the communication between the junctional sarcoplasmic reticulum and the T-tubular membrane by supporting the close association between the two membranes (Nishi et al., 1999). However, these synaptophysin activities are apparently not relevant to its activity as an ion channel. The rapid and simple method presented in this study for the purification of high quantities of highly purified synaptophysin would allow further study of synaptophysin function and of its interaction with associated proteins.

Despite the demonstration of synaptophysin channel activity ~15 years ago (Thomas et al., 1988), no further studies were carried out to confirm or to further characterize this activity. Furthermore, the role of synaptophysin channel activity remained murky. In the current study, the channel activity of synaptophysin purified from synaptosomes was characterized following its reconstitution into PLB. Synaptophysin exhibited the properties of a large and fast-fluctu-

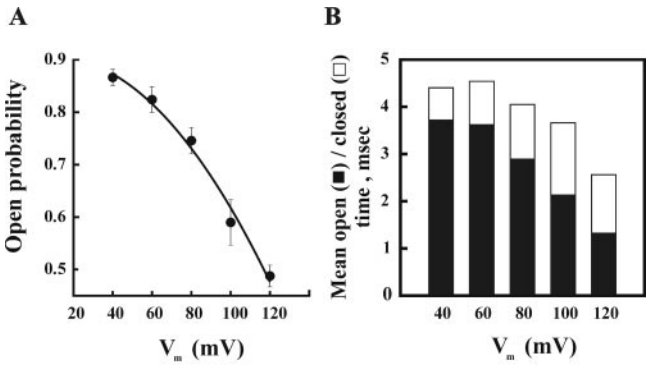

FIGURE 4 Voltage-dependence of kinetic parameters of the synaptophysin channel. (*A*) Open probability (P_o); (*B*) mean open time (*filled bars*) and mean closed time (*open bars*) of synaptophysin channel activity recorded in potassium chloride solutions. The threshold for channel detection was set at 50% of the current amplitude. The data are the mean of 10 to 15 channels. Only channel activities at positive voltages are presented because at negative voltages the channel was completely closed.

ating cation-selective channel with high specificity for potassium ions. This channel is distinguished from known K^+ channels not only in its specific location in synaptic vesicles and its structure, but also in its biophysical characteristics. Most K^+-selective channels are located in the plasma or ER membranes and are blocked by TEA^+, the general potassium channel blocker. TEA^+ had no effect on synaptophysin channel activity.

Synaptophysin is localized to synaptic vesicles, where its function there as a cation channel is still unclear. Synaptophysin was shown to be incorporated into the plasma membrane during exocytosis (Valtorta et al., 1988), yet the control mechanism(s) of synaptic vesicle membrane potential of synaptophysin channel activity is unknown. Furthermore, it is unknown whether a positive ion concentration gradient across the synaptic vesicles membrane does exist.

Regulation of synaptophysin channel activity by factors other then membrane potential cannot be ruled out. Since synaptophysin was shown to bind Ca^{2+} (Rehm et al., 1986), Ca^{2+} could be a good candidate for modulating synaptophysin activity. However, no effect of Ca^{2+} or EGTA on synaptophysin channel kinetic or biophysical parameters was observed. Ca^{2+} could, though, modulate synaptophysin activity indirectly, by interacting with synaptophysin-associated proteins. Several lines of evidence indicate that synaptophysin possesses several phosphorylation sites (Pang et al., 1988; Rubenstein et al., 1993). Regulation of synaptophysin channel activity or of its interaction with associated proteins that can modulate its activity by phosphorylation is a possible control mechanism, as shown for synapsins (Hosaka et al., 1999). Still, the role of synaptophysin as a channel and/or as a constituent of a protein complex of the vesicular machinery involved in synaptic transmission remains unclear. Of interest is a recent publication (Yin et al., 2002) demonstrating the presence of synaptophysin-like channel activity in neurosecretory granule membranes isolated from rat neurohypophysis. The inhibition by SY-38 anti-synaptophysin antibodies of both channel activity and the Ca^{2+}-triggering arginine vasopressin secretion may suggest that the protein channel activity is essential for neurosecretion.

Synaptophysin is a well-established synaptic vesicle protein. However, several channels considered as synaptic vesicle proteins have been biophysically characterized following reconstitution into PLB (Sato et al., 1992; Woodbury, 1995; Kelly and Woodbury, 1996). Their localization to synaptic vesicles could not, however, be confirmed because presynaptic plasma membrane contamination could not be ruled out. If these channels are in the synaptic vesicles their functions are unknown. One possibility is that they might be involved in volume regulation of the vesicles, and this could also be the role of synaptophysin channel activity.

To conclude, we have developed a rapid and simple method for the purification of synaptophysin. This purification enabled us to characterize the channel activity of the protein. The availability of large amounts of highly purified synaptophysin will allow the study of its association with other synaptosomal proteins and to establish its function in synaptic transmission.

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