Ion Channels from Synaptic Vesicle Membrane Fragments Reconstituted into Lipid Bilayers

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ABSTRACT Cholinergic synaptic vesicles were isolated from the electric organ of *Torpedo californica*. Vesicle membrane proteins were reconstituted into planar lipid bilayers by the nystatin/ergosterol fusion technique. After fusion, a variety of ion channels were observed. Here we identify four channels and describe two of them in detail. The two channels share a conductance of 13 pS. The first is anion selective and strongly voltage dependent, with a 50% open probability at membrane potentials of −15 mV. The second channel is slightly cation selective and voltage independent. It has a high open probability and a subconductance state. A third channel has a conductance of 4–7 pS, similar to the subconductance state of the second channel. This channel is fairly nonselective and has gating kinetics different from those of the cation channel. Finally, an ~10-pS, slightly cation selective channel was also observed. The data indicate that there are one or two copies of each of the above channels in every synaptic vesicle, for a total of six channels per vesicle. These observations confirm the existence of ion channels in synaptic vesicle membranes. It is hypothesized that these channels are involved in vesicle recycling and filling.

INTRODUCTION

The search for ion channels in synaptic vesicle (SV) membranes began 10 years ago with the idea that the activation of an ion channel could induce SVs to fuse with the presynaptic membrane (Stanley and Ehrenstein, 1985; Woodbury and Hall, 1988). This search has led to the identification of a variety of channels (for a review see Woodbury, 1995). Two techniques have been used to identify these ion channels from synaptic vesicles: patch clamping and planar lipid bilayers. Several channels have been characterized with the patch clamp technique (Rahamimoff et al., 1988, 1989; Yakir and Rahamimoff, 1995). The use of bilayers has also revealed the presence of SV channels (Thomas et al., 1988; Sato et al., 1992; Woodbury, 1993).

Originally it was predicted that SV channels would be Ca^{2+} activated. However, most of the SV channels found to date are not regulated by Ca^{2+}, and no connection between calcium, SV channels, and vesicle fusion has been found. It is not necessary for Ca^{2+} to have a direct effect on SV channels for SV channels to play a role in fusion. For example, the activation of a SV channel could be responsible for the formation of the fusion pore (the opening from the vesicle through the presynaptic membrane to the synaptic cleft), whereas Ca^{2+} may only be necessary for binding of a docking protein. Regardless of the possible involvement of channels in the formation of the fusion pore, another possibility is that SV channels aid in the refilling of recycled vesicles.

A review of the properties of SV may help in understanding the role of ion channels in the life cycle of a SV. Because an excellent review is provided by Whittaker (1992), a brief overview will suffice here. Cholinergic nerve terminals contain vesicles that deliver the neurotransmitter acetylcholine (ACh) into the synaptic cleft by fusing with the presynaptic membrane. Once neurotransmitter is released into the synaptic cleft, the vesicle retracts from the membrane via endocytosis and is recycled (Zimmermann et al., 1989). Recycling of these synaptic vesicles requires refilling the vesicle with its original contents, mainly ACh and ATP. The vesicle is replenished with acetylcholine through an ACh^+/H^+ exchanger (Parsons and Koenigsberger, 1980), and ATP is thought to be taken up through an ATP carrier known as component 11 (Lee and Witzemann, 1983).

The concentration of Na^+ in loaded synaptic vesicles is low compared to the cytosolic or extracellular fluids. When vesicles fuse, they dump their contents into the synaptic cleft. Therefore, it is reasonable to assume that vesicles are filled with Na^+ from synaptic cleft (extracellular) fluid after retraction from the presynaptic membrane. The problem then is how Na^+ and other ions are removed from vesicles after endocytosis. One possible solution is that the SV membrane becomes temporarily permeable to these ions, and the ions leave by moving down their electrochemical gradient. This is one possible function of ion channels in SV membranes.

To determine the true function(s) performed by SV membrane channels, it is necessary to search for and characterize SV channels. An excellent source for cholinergic SVs is the electric fish *Torpedo*. We have incorporated native synaptic vesicle membranes from *Torpedo* into artificial vesicles and fused these components into lipid bilayers. Here we report the initial characterization of four channels and focus on two of them. Most of the channels are permeable to both

Received for publication 7 September 1995 and in final form 28 February 1996.

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0006-3495/96/06/2593/07 $2.00

anion and cations, indicating that these channels are part of the recycling process. Our data imply that each vesicle contains one or two copies of each type of channel.

MATERIALS AND METHODS

Isolation of synaptic vesicles

Live *Torpedo californica* were acquired from Pacific Bio-Marine Laboratories (Venice, CA). The electric organ was excised, and SVs were isolated according to previously published methods (Woodbury and Kelly, 1994; Carlson et al., 1978). The final SV preparation contained 0.3–0.5 mg protein/ml and 2–3 mg lipid/ml. *Torpedo* cholinergic synaptic vesicles have a diameter of about 85 nm.

Preparation of fusigenic artificial vesicles

Artificial vesicles (AVs) were prepared in a manner similar to the original method (Woodbury and Miller, 1990). The modified procedure is as follows. Phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), and ergosterol (Erg) at a ratio of 2.1/0.9/1/1 (by weight) were dissolved in 10 mg/ml chloroform. Nystatin was added such that the final concentration was 50 μg/ml. The mixture was evaporated under nitrogen or argon until a dry plaque could be seen on the bottom of a 13 × 100 mm test tube. After evaporation, 125–250 ml of 150 mM NaCl, 8 mM HEPES (pH 7.2) was added to the test tube so that the final lipid concentration was 10 mg/ml. The mixture was vortexed for 5 min, and the cloudy mixture was sonicated in a water bath sonicator (at 22°C) for a total of 90 s. After the first 60 s of sonication, the mixture became translucent and was vortexed. Any solution on the sides was swirled down. After swirling, the mixture was sonicated for the remaining 30 s before quick freezing in liquid nitrogen or a dry ice/ethanol bath. We have found that the extent of sonication is critical for successful reconstitution; hence the procedure is described in detail below.

Sonication

A bath sonicator (G112SP1T; Laboratory Supplies Co., Hicksville, NY) was powered through a variable transformer set to about 85 V. The level of water in the bath was adjusted so that small droplets of water splashed from the center of the bath into the air. Aqueous solutions of vesicles (125 to 250 μl) were sonicated in a 13 × 100 mm test tube. The tube was placed several millimeters deep into the center of the bath. Sonication intensity and tube placement were adjusted so that the test tube vibrated just enough to give a tingling sensation to the fingers. If the test tube became warm, sonication was stopped until the test tube cooled down. Sonication was then continued for the desired time.

Preparation of joined vesicles

The preparation of joined vesicles (JVs) is summarized in Fig. 1. JVs were made by adding 20–30 μl of synaptic vesicles to 125 μl of fusigenic AVs. The SVs were then fused into the AVs by quick freezing, either in a dry ice/ethanol bath or in liquid nitrogen. Freezing was followed by a 5-min thaw at 4°C and a 15-s sonication. This freeze/thaw/sonicate cycle was repeated two more times. Except after the final thaw, the mixture was vortexed and sonicated for just 5 s or until the solution showed a slight pink hue when the test tube was held up next to a white light. All sonications were done using the protocol described above. It is crucial that this final sonication be neither too long nor too short. Oversonication will produce vesicles that are too small to fuse or to be observed. Undersonication will result in huge vesicles that deliver too much protein to the membrane at one time. Proper sonication produces vesicles that are about 250 nm in diameter (as estimated on an S-1000 sizing column).

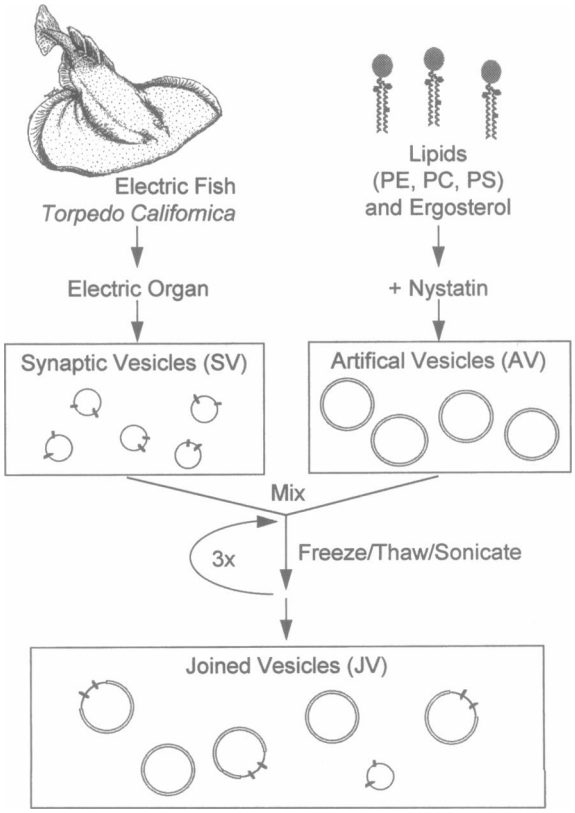

FIGURE 1 Outline of the procedure for forming joined vesicles (JVs). JVs are made from fusigenic artificial vesicles (AVs) and from synaptic vesicles (SVs) obtained from *Torpedo* electric organ. The two populations of vesicles are combined by three cycles of freezing, thawing, and sonicating.

Formation of bilayers

Planar bilayers were formed according to standard methods (Miller and White, 1984; Hanke and Schlue, 1993) as follows. Bilayers were formed from brain lipids (Avanti Polar Lipids, Alabaster, AL) and were composed of PE:PC (7:3 by weight), 10 mg/ml in chloroform. The organic solution was evaporated under argon or nitrogen until a plaque could be seen on the bottom of a 12 × 75 mm test tube. Lipids were redissolved in decane to give a final concentration of 20 mg/ml. Bilayers were formed in a small hole (~150 μm) made in 16-mm-diameter polystyrene cups. Holes were made in a manner similar to the method of Wonderlin et al. (1990), except that 0.047-inch piano wire mounted in a soldering iron heated to 350°C was used to melt a hole in the plastic cups. The cup hole was pretreated with lipid by placing a small amount (~1 μl) of the lipid-in-decane solution on the inside of the hole, and a tissue was applied to the outside to draw the solution through the hole. The pretreated cup was allowed to air dry for 5 min. The cup was placed in a standard plastic chamber, similar to Warner Instruments model BCH-13 (Hamden, CT), and filled with 1 ml of aqueous solution. A bilayer membrane was formed in the hole by using an air bubble on the end of a pipette to brush lipids over the outside of the hole. Membranes typically had capacitances of 60 pF and conductances of <5 pS. The head stage amplifiers transduce 1 pAmp into 100 mV. Current and voltage records were displayed on a computer screen, digitized, and saved on videotape for later analysis.

Incorporation of JVs into a lipid bilayer

The chamber to which vesicles were added is referred to as the "front chamber." The chamber that does not receive vesicles is electrically

grounded and is referred to as the "back chamber." JVs (5 μl) were added to the front chamber in the presence of a salt gradient. Normally the gradient was 650 or 860 mM NaCl front and 150 mM NaCl back (860 mM NaCl was sometimes used to increase the rate of fusion). To increase the rate of vesicle delivery to the bilayer membrane the front solution was stirred with a magnetic flea. Electrical noise due to stirring was minimized by operating the stir motor at a speed of 3 Hz. This was done under computer control using the Bilayer Control and Acquisition System from Biotech Products (Greenwood, IN). Fusion events were marked by a transient change in membrane conductance due to nystatin channels as previously described (Woodbury and Miller, 1990). After fusion of a channel-containing vesicle or five fusions (about 5 min), stirring was stopped and the channels incorporated into the membrane were analyzed. If no fusions were observed within 5 min, 5 μl more of JVs was added to the front chamber.

Channel nomenclature

The channels presented here are named on a scale from −10 to +10 according to their ion selectivity (determined from reversal potential). The sign (+ or −) refers to whether a channel is cation- or anion-selective, respectively. For example: with a 650/150 mM NaCl gradient, the Cl^- equilibrium potential is +37 mV. Therefore, a channel with a reversal potential of +8 mV would be classified as a type −2 channel, because the reversal potential is 20% of the Cl^- equilibrium potential. All channels of this type are referred to as "channel −2." In this paper, channel types with a reversal potential of less than 30% of the Cl^- or Na^+ equilibrium potential are considered nonselective.

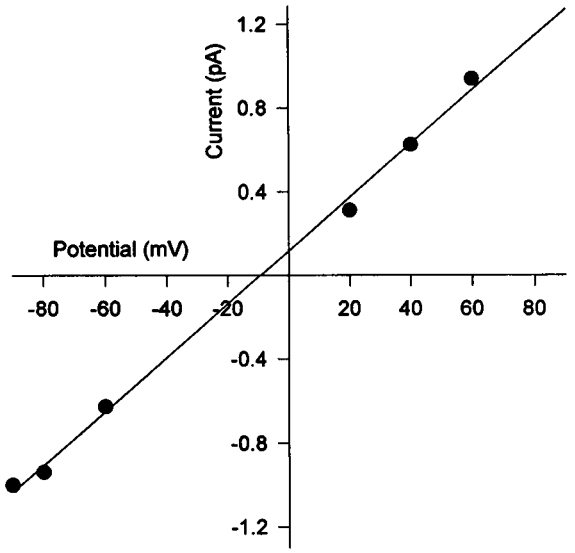

FIGURE 2 Current-voltage (*IV*) curve of channel type +3. The front solution was 650 mM NaCl and the back solution (cup) was 150 mM NaCl. Both solutions were buffered with 8 mM HEPES, pH 7.2. The curve crosses the voltage axis at −10 mV, which indicates that this channel is mildly cation selective. The slope of the *IV* curve indicates a conductance of 13 pS.

RESULTS

Membrane proteins from SVs were reconstituted into AVs to form JVs, as outlined in Fig. 1. This reconstitution was necessary as a means of decreasing the high channel density found in native SV. Joined vesicles were fused into planar lipid bilayers by the nystatin/ergosterol technique. We found that in 94% of our experiments (60 of 64), one or more channels were observed after about three vesicle fusion events. Usually, several different channels appeared together in one fusion event, which made it difficult to analyze channel properties. However, in 21 experiments three or fewer distinct channels were observed. In these cases, the channel(s) were characterized as to their conductance, ion selectivity, and gating kinetics. Two channels had distinctive gating properties that made them easier to identify and are described in detail.

Fig. 2 is a current-versus-voltage (IV) curve of a mildly cation-selective channel named +3. Channel +3, the most distinctive type of channel observed, has a conductance of 13 pS. Fig. 3 shows a current trace at constant voltage. The on/off times are relatively long (~2/s), and there is a high open probability. A subconductance state is also apparent in Fig. 3. This channel was usually observed to be "locked" in the open state, but there are occasional flickers between states before it inactivates (Fig. 3).

A second type of channel, channel −6, varies between 50% and 80% anion (Cl^-) selectivity. It is the most anionic channel that has been observed regularly. The channel was present in eight of the 21 experiments that could be analyzed for channel type. The IV curve of this channel shows a conductance of about 13 pS in a 850/150 mM NaCl solution (Fig. 4). This channel was usually observed in pairs. Fig. 5 shows current traces (at different voltages) of a membrane with two copies of channel −6. This channel is highly voltage dependent; the channel is mostly open at large negative voltages and mostly closed at positive voltages. The open probability (time spent in the open state divided by the total time) was measured at each voltage and is plotted in Fig. 6. The voltage dependence is steep, with a 50% open probability at about −15 mV. Both opened and closed time appeared to decrease at negative voltages but were not quantitated because of the pairing of channel type −6.

Several other types of channels were also observed, but proved difficult to characterize because they were rarely observed in the absence of other channels. A third type of channel, channel −2, has a conductance of 4–7 pS and fast

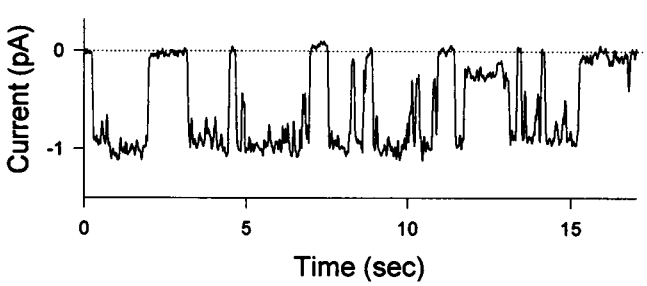

FIGURE 3 A current trace of channel type +3 with voltage held at −90 mV. In this trace a subconductance state of about 4 pS is visible at 12 s. Channel +3 has a long open and closed state. Other conditions were as in Fig. 2. Much of the noise (small fast flickering) visible in this trace is another channel.

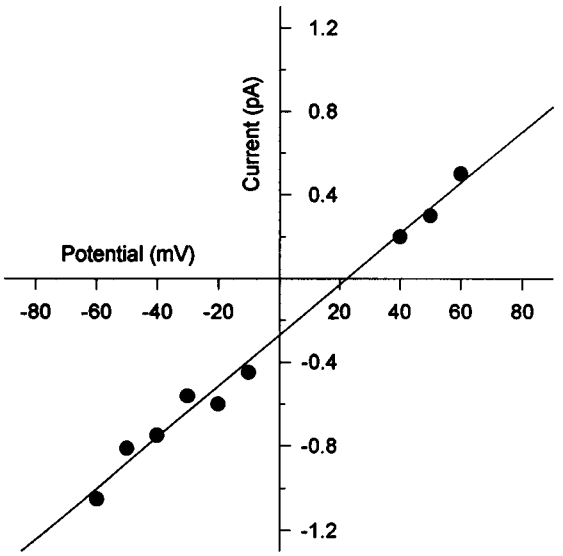

FIGURE 4 *IV* curve of channel type −6. Conditions are the same as Fig. 2. The curve crosses the voltage axis at +22 mV, which indicates that this channel is anion selective. As with channel +3, the slope of the *IV* curve indicates a conductance of 13 pS.

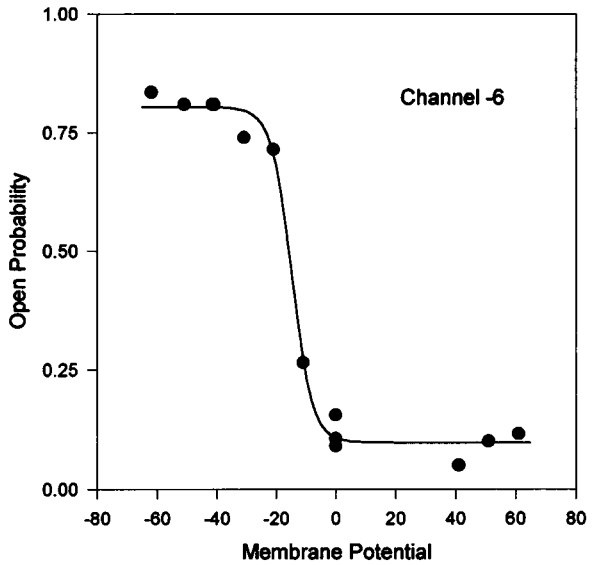

FIGURE 6 Open probability of channel type −6 as a function of voltage. The value at each voltage was obtained by integrating over the peak in the histogram of the current-time trace. The current traces used include those shown in Fig. 5. A binomial distribution of openings for the two channels was assumed. Under the conditions used (same as in Fig. 2), this channel has a reversal potential at +22 mV. Therefore, open states could not be clearly detected between 0 and 40 mV. The channel has a 50% open probability at a voltage of −15 mV. If it is assumed that this channel has the same orientation in SVs as JVs, then the 50% open probability corresponds to a voltage of +15 mV inside the synaptic vesicle.

kinetics that make it hard to resolve (Fig. 7). Channel −2 was usually observed in combination with other channel types. An example of two copies of this channel, together with channel +3, has been reported previously (figure 3 of Woodbury, 1995). In that work, two copies of fast gating channel −2 were mistaken as a single 13-pS channel. A fourth type, channel +4/+5, has a low open probability and a conductance of ~10 pS. This channel type was always observed in combination with other types and is shown in Fig. 8 together with channel −2. The variability in conductance of these channels suggests that they may really represent two types of channels, but are grouped together because of their similar kinetics and selectivity. The

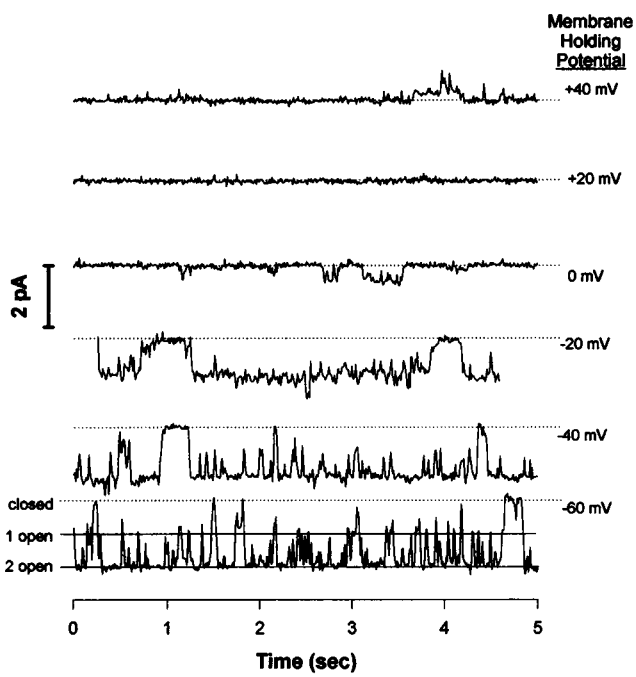

FIGURE 5 Current traces of channel type −6 at six different voltages. Conditions are the same as in Fig. 2. As shown here, these channels are usually observed in pairs. The dotted line represents the closed state. Open 1 and open 2 represent the current corresponding to one and two open channels, respectively. Notice that the open probability of the channels increases at more negative voltages.

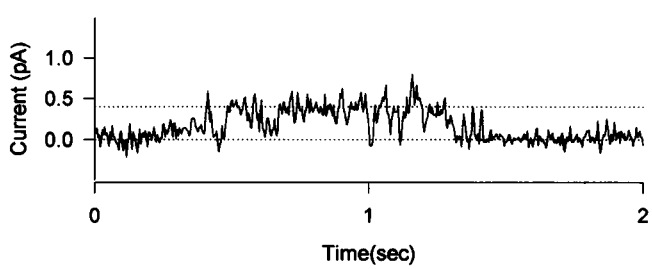

FIGURE 7 Current trace of channel type −2 at −92 mV. This channel has a conductance of 4–7 pS. The small size of this channel and its fast gating kinetics make it difficult to distinguish it from background noise without heavy filtering. The trace was smoothed by filtering at 40 Hz. This type of channel is most often found in the presence of other channels and prevents these other channel types from clearly being identified. Here a single copy of the channel is present in asymmetric solution (650/150 mM NaCl). The bottom dotted line represents the closed state.

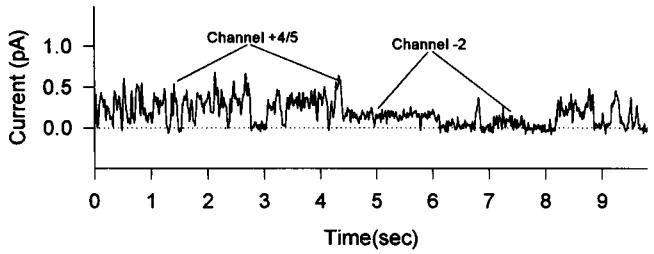

FIGURE 8 Current trace of channel type +4/+5, which has a conductance of 10 pS. A type −2 (4–7 pS) channel is also visible in this trace as the smaller current steps. The membrane holding potential was +40 mV; other conditions were as in Fig. 2. Channel +4/5 has never been observed in the absence of other channels.

properties of all four types of channels are summarized in Table 1.

Previously we have published data on an additional channel type with a conductance of 180 pS (Woodbury, 1995). In the present work, this type was rarely seen. This may be due, in part, to the high gain of our experimental system, which made it easier to characterize small conductance channels but prevented the clear observation of channels with conductances greater than 100 pS (because the large currents from these channels can saturate the amplifier and be mistaken for a broken membrane).

Two other channels were sporadically seen: a 75-pS nonselective channel and a 19-pS Cl^- channel. The Cl^- channel was identical as the double-barreled Cl^- channel reported by Miller and White (Miller and White, 1984; Woodbury and Miller, 1990), which is from the plasma membrane of the postsynaptic cell (electroplax membrane). Both of these channels were observed at a frequency that corresponds to less than one copy per synaptic vesicle (see below) and therefore are considered contaminants.

It should be noted that no rectification was observed with channel types −2, +3, and +4/+5. However, this is not surprising, because these channels are neither voltage dependent nor very ion selective.

DISCUSSION

We have observed at least four different types of channels after incorporation of synaptic vesicle membranes into bilayers. These channels are much smaller than most channels reported to be found in SV (reviewed in Woodbury, 1995) and may have been disregarded previously as noise because they were hard to resolve. Some of these channels may be contaminants from the presynaptic membrane, although they are unlike any channel previously observed in the plasma membrane. Even if they are, this does not preclude their existence in the SV membrane, because SVs are recycled from plasma membrane after fusion and a channel from the vesicle membrane may be incorporated into the plasma membrane after fusion, or vice versa.

Two additional facts support the hypothesis that most of these channels are from synaptic vesicle membrane. First, the channels were observed repeatedly in preparations of vesicles that are about 90% pure. This is significant because the nystatin/ergosterol technique makes all vesicles equally fusible; therefore, the observed frequency should correlate to natural abundance (Woodbury, 1993). Second, various channel types were frequently observed in clusters, indicating that most of the JVs contained one whole SV membrane with all of its channels. Thus, all of the membrane and protein from one SV becomes incorporated into the bilayer when a JV fuses. Identification of individual channels was possible only in those experiments where a JV containing SV membrane fragments with 1–3 channels fused into the bilayer.

Assuming that all of these channels are from the synaptic vesicle membrane, it is interesting to ask how many copies of each channel there are in one synaptic vesicle (assuming that all SVs have the same protein composition). To address this question, we first consider the simpler case that there is just one type of channel in each SV. The number of copies of this channel per synaptic vesicle can be calculated from the average number of channels reconstituted into the bilayer per fusion event. From this average and a calculation of the density of SVs per JV, the number of channels per SV can be determined. The information necessary to calculate the density of SVs per JV is given in Materials and Methods and is summarized in Table 2. From these numbers, the predicted surface density of one channel in a single SV is one per 0.0227 μm^2 or 44 channels/μm^2. As illustrated in Fig. 1, SVs (60 μg lipid) are joined with AVs (1250 μg

TABLE 2 Physical properties of vesicles shown in Fig. 1

Name	Diameter* (nm)	Surface Area (μm^2)	Volume added (μl)	[Lipid] (mg/ml)	Total Lipid (μg)
SV	~85	0.0227	20	3	60
AV	~250	~0.196	125	10	1250
JV	~250	~0.196	145	9	1310

*Diameter is defined as the distance across the vesicle from the middle of one membrane to the middle of the other. Diameters of AV and JV were estimated from an S-1000 sizing column (data not shown).

TABLE 1 Properties of four channels observed after reconstitution of synaptic vesicle membranes into bilayers

Channel name	No. of observations	Ion selectivity	Conductance (pS)	Voltage dependence	Mean on time (s)
+3	5	Slightly cationic	13	None	1
−6	8	Anionic	13	Strong	0.05
−2	9	Nonselective	4–7	None	<0.05
+4/+5	5	Slightly cationic	~10	None	~0.05

TABLE 3 Predicted and experimental values for the number of channels observed in the bilayer membrane after the fusion of three JVs

No. of channels in bilayer	Percentage of bilayers expected to have the indicated number of channels, assuming one SV contains 1, 4, 5, 6, 7, or 8 channels						Experimental observation (%)
	1	4	5	6	7	8	
0	50	6	3	**2**	0.8	0.4	**6**
1–3	49	64	52	**39**	28	20	**33**
>3	0.5	30	45	**59**	71	80	**61**

Note similarity between column labeled 6 and experimental observations.

lipid) to form JVs. The three freeze/thaw/sonicate cycles fuse about 58% of the SVs with AVs; the remaining 42% apparently remain intact (Woodbury and Kelly, 1994). Therefore, the surface density of one SV channel in the JVs is 1.2 channels/μm^2. Because JVs have a surface area of about 0.2 μm^2, this means that 20% of the JVs have at least one copy of this single channel (assuming a Poisson distribution). After fusion of three vesicles (the average number of fusions per experiment), there is a 50–50 chance that there is at least one channel in the membrane (see second row, first two columns of Table 3).

Similar calculations allow us to compare the experimental frequency of observed channels with predicted values. After about three fusions we observed that about 6% (4/64) of the bilayer membranes contained no channels and about 33% (21/64) contained 1–3 channels (Table 3, last column). The middle sections of Table 3 show the predicted probabilities of observing channels in the bilayer, for values of 1–8 channels per synaptic vesicle. These probabilities are based on the Poisson distribution, which assumes that all channels are distributed evenly. Notice that there is a very close match between our data and the assumption that there are six channels in the membrane of each SV.

Taking six as the total number of channels per vesicle, and the fact that channel types were observed at an approximate ratio of 1:2:2:1 (second column of Table 1), we conclude that each synaptic vesicle contains one copy each of channel types +3 and +4/+5, and two copies each of channel types −6 and −2. This is consistent with the observation that channel −6 was usually observed in pairs. This result demonstrates a novel approach to the determination of channel density in isolated vesicles.

The next question is, what is the purpose of these channels within the synaptic vesicle membrane? The simple answer is that we cannot be sure until further research has been done. However, we take this opportunity to speculate. Channel −2 has a small conductance with fast kinetics and may be the electrical manifestation of an altered pump or transporter from the vesicle membrane (Coady et al., 1995; DeFelice and Blakely, 1996). The anion channels (−6 and −2) may allow acidification of the vesicle when coupled to the H^+ pump (Wada and Anraku, 1994). However, because most of the channels are not very selective, they may be involved in equilibrating ionic composition. Synaptic vesicles require a mechanism to regulate the internal concentration of univalent ions, because it is different not only from the intracellular values, but also from the extracellular values to which the vesicles are exposed during exocytosis and endocytosis. Some of these channels may act to rapidly equilibrate internal vesicular concentration by using the natural electrical and chemical gradients of the ions. A voltage-dependent channel may be particularly well suited for this task because the channel would open or close as the membrane potential changed after exocytosis, endocytosis, and refilling. Channel −6 most closely fits this description.

The authors wish to thank Drs. J. Walter Woodbury, Dennis Przywara, and David Njus for their critical reading of the manuscript and Tom Woodbury for his drawing in Fig. 1.

This work was supported by National Institutes of Health grant R29 MH50003.

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