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Ion channels in synaptic vesicles from *Torpedo* electric organ

(acetylcholine/transmitter release/K^+ channel/fusion)

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ABSTRACT **A simple method has been developed for fusing synaptic vesicles into spherical structures 20–50 μm in diameter. The method has been applied to purified cholinergic synaptic vesicles from *Torpedo* electric organ, and the membrane properties of these fused structures have been studied by the "cell"-attached version of the patch clamp technique. A large conductance potassium-preferring channel, termed the P channel, was consistently observed in preparations of fused synaptic vesicles. The selectivity of the channel for potassium over sodium was ≈2.8-fold. Two major conductance levels were observed during P-channel activity, and their relative proportion was dependent on the voltage applied to the membrane through the patch pipette. P channels were not seen in fused preparations of purified *Torpedo* lipids, nor was the frequency of their occurrence increased in preparations enriched with plasma membrane or nonvesicular membranes. We suggest, therefore, that the P channels are components of the synaptic vesicle membrane. Their function in synaptic transmission physiology is still unknown.**

Quantal transmitter release is a key step in synaptic transmission (1). Many lines of evidence suggest that the structural basis for transmitter quanta is the synaptic vesicles inside the presynaptic nerve terminal (2–4). In cholinergic synapses, these structures are known to concentrate the transmitter acetylcholine and, upon an appropriate signal, they fuse with the presynaptic surface membrane to discharge their contents. Although presumably very important in synaptic transmission and its regulation, these synaptic vesicles have not been amenable to direct physiological investigation because of their small size. We report here on a giant vesicle preparation, which was obtained by fusing presynaptic cholinergic vesicles from the *Torpedo* electric organ by using a simple chemical method. These giant vesicles reach a diameter of 50 μm and their properties can be studied by the patch clamp technique (5, 6). High resistance seals can routinely be obtained to allow the study of the ionic permeability of the vesicular membrane.

METHODS

Purification of Synaptic Vesicles. Synaptic vesicles were purified from electric organs of *Torpedo marmorata* by the method of Tashiro and Stadler (7). Briefly, vesicles were extracted from frozen and crushed electric organ in 0.4 M NaCl/3.5 mM EGTA/10 mM Tris·HCl, pH 7.4. They were prepurified on a step sucrose gradient (0.6 M sucrose/0.1 M NaCl/10 mM Tris·HCl, pH 7.4; 0.2 M sucrose/0.3 M NaCl/10 mM Tris·HCl, pH 7.4). The crude vesicle fraction at the interface (Vb) was collected, mixed with 1 M sucrose to a final sucrose concentration of 0.7–0.8 M, and further purified on a shallow continuous sucrose flotation gradient (sucrose concentrations: 0.7–0.8 M, 0.5 M, 0.45 M, 0.2 M) in either a zonal or swinging bucket rotor. The characterization of the vesicle fraction used in these experiments (Vf; band between 0.5 and 0.2 M sucrose) has been described in detail elsewhere (8). Neither Na/K-ATPase nor 5′ nucleotidase was detectable in the purified vesicle preparation.

Purification of *Torpedo* Lipids. Phospholipids were purified from electric organs of *T. marmorata* by a modification of the method of Bligh and Dyer (9). The frozen organ was thawed, minced, and then extracted by homogenization in chloroform/methanol. The samples were centrifuged (200 × *g* for 40 min), and the phospholipid-containing chloroform layer was removed and evaporated to dryness. Samples were redissolved in chloroform, loaded onto a column (4 × 80 cm^2) of silicic acid, and eluted with chloroform. Fractions were monitored by thin-layer chromatography (Merck; activated plate; 75:25:4, chloroform/methanol/water) for phospholipids; protein was determined by the method of Lowry *et al.* (10). A protein/lipid ratio of <1:1000 was obtained after one further cycle of chloroform/methanol extraction.

Preparation of Plasma Membranes. Plasma membranes were prepared by hypoosmotic lysis of synaptosomes prepared from *Torpedo* electric organ by the method of Michaelson and Sokolovsky (11). Synaptosomes were stirred for 30 min at 4°C in 20 vol of 5 mM potassium glutamate (pH 7.4). Plasma membranes were pelleted by centrifugation (20,000 × *g* for 20 min), resuspended in 0.4 M potassium glutamate (pH 7.4), and stored at −70°C.

Fusion. Fractions to be fused were pelleted by centrifugation for 3 hr at 100,000 × *g*. The pellet was suspended in 0.1 ml of fusion medium consisting of 20% dimethyl sulfoxide (Merck), 25% polyethylene glycol 1500 (PEG; Boehringer Mannheim), and an isosmotic buffer (see below). The suspension was incubated 2 min at 37°C, 0.2 ml of buffer was added, and the incubation continued at 37°C for another 10 min. After further dilution with 0.4–1.0 ml of buffer, the suspension was kept at room temperature. After 1–4 hr, "giant vesicles" were observed with sizes ranging from 1 to 50 μm in diameter. These became more abundant with time. The buffer used in the fusion varied depending on the experiment, but unless otherwise indicated, it was 0.4 M potassium glutamate (pH 7.4).

Electrophysiology. Conductance and single-channel measurements were made by using the "cell"-attached configuration of the patch clamp technique. Seals with resistances between 3 and 200 GΩ were routinely obtained ($n = 154$) by using thick-walled borosilicate glass electrodes with resistances of 2–10 MΩ. Ramp or step changes in pipette potential were applied through an EPC/7 amplifier (List Electronics) with a modified Rockwell AIM 65 microcomputer (Stühmer Electronics, Göttingen, F.R.G.). Data were collected on video tape and analyzed off-line with a PDP 11/73 computer.

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RESULTS

Physical and Biochemical Properties of Fused Vesicles. The formation of giant vesicles was easily observed under the phase-contrast microscope (Fig. 1). The giant vesicles usually appear nearly spherical. To obtain such a structure with a diameter of 20 μm from synaptic vesicles with diameters of 80 nm, it is necessary to fuse n unitary vesicles (in this case n = 62,500, assuming constant membrane properties). Thus, the fusion process increases the total entrapped volume by a factor of $n^{1/2}$; for a 20-μm diameter vesicle, this means that <0.5% of the volume is contributed from the original intravesicular content, while the rest comes, most likely, from the fusion medium.

Two lines of evidence support the notion that the intravesicular fluid composition is similar to that of the fusion medium. First, when the fluorescent dye Lucifer yellow is added to the fusion medium, it is trapped inside the giant vesicle. Dilution of the vesicles and a wash leaves the dye inside the vesicles and visible by fluorescence microscopy for several hours after washing. This suggests that the giant vesicles, once formed, are not leaky to substances of molecular mass in the order of 450 Da. The second line of evidence is from reversal potential measurements. When the fusion medium has a composition similar to that of the pipette solution and the extravesicular medium, the "zero current" measurements were obtained at patch pipette potentials very close to 0 mV.

In practice, the ion compositions of the extra- and intravesicular compartments were kept identical to avoid possible slow changes in the ionic content of the giant vesicle during the experiment.

The biochemical effects of fusion were assessed by measuring the Mg-ATPase activity in the presence of ouabain and oligomycin (12). The values obtained, 505 ± 33 mol of P_i per mg of protein per min at 30°C (n = 3) before and 430 ± 61 (n = 2) after fusion, suggest that this particular vesicular enzyme, and supposedly other vesicular proteins, may undergo the fusion reaction without gross alterations in their properties.

Ionic Conductances in the Giant Vesicle. Three main types of electrical activity were seen at the membrane of the giant vesicle with KCl solutions on both sides of the membrane patch. One type consisted of rapid currents (<1 ms duration on average), appearing in bursts. These currents could be abolished by replacing almost all the Cl^- ions in all three compartments (vesicle, bath, and pipette) with glutamate ions. The remaining two types of currents were cationic in nature; net outward currents occurring in discrete steps were observed under conditions in which the pipette potential was kept at 0 mV, but the intravesicular potassium glutamate concentration was higher than that in the pipette solution. In this article, we describe only one of the two types of currents: the P channel. For this channel, we have more data regarding its properties and we have the necessary control experiments.

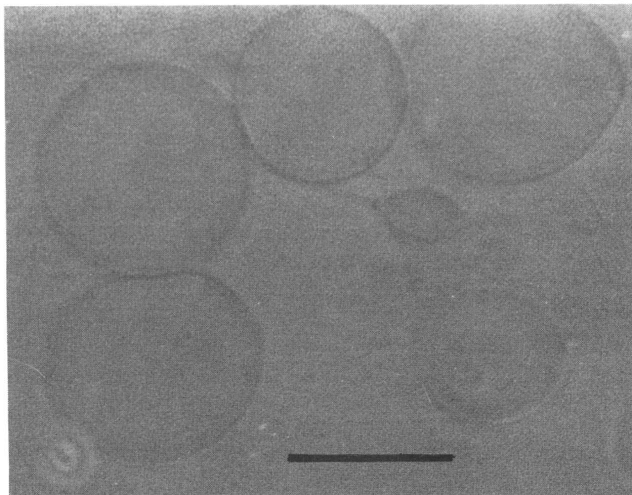

FIG. 1. Light micrograph of a giant vesicle. Phase-contrast microscopy. (Bar = 20 μm.)

The P Channel. Fig. 2 illustrates some of the properties of the P channel. A number of distinct characteristics were noted. One of these was that both short and long (many seconds) openings were observed (Fig. 2*A*). The long durations permitted examination of an entire current–voltage (*I–V*) relationship during a single opening of the channel (Fig. 2*B*). From these *I–V* curves, the single-channel conductance of 82 pS is obtained in the example shown in Fig. 2. The intercept with the voltage axis of the *I–V* relationship, obtained by subtracting the closed-channel *I–V* relationship from the open-channel *I–V* curve, measures reversal potential of the P channel with respect to the zero current potential of the leak current through the seal and patch membrane resistance (Fig. 2*C*). The reversal potential was dependent on the composition of the pipette solution and of the intravesicular compartment; with equal concentrations of potassium ions in these two compartments, the reversal potential was close to 0 mV [0.46 ± 1.1 mV (SD) in 11 determinations]. In one experiment with a transmembrane concentration gradient for potassium of 2-fold, with the pipette solution having a lower potassium concentration, the reversal potentials were shifted in the negative direction and gave a mean value of −14.2 ± 1.3 mV (SD). In five experiments under similar conditions, the mean values were between −12 and −15 mV. From the Nernst equation, a reversal potential of −17.7 mV (at 22°C) is expected. The small deviation from the expected value may be due either to the lower activity of ions in the concentrated salt solutions of *Torpedo* or to other conductances that exist in these patches during the measurements.

Discrimination Between Sodium and Potassium by the P Channel. Reversal potential measurements were also used to determine the relative permeability of the P channel to Na^+ and K^+ by using a simplified form of the Goldman–Hodgkin–Katz constant field equation (13–15). The reversal potential is given by

$$E_{rev} = (RT/F)\ \ln(P_K K_o + P_K Na_o)/(P_K K_{in} + P_{Na} Na_{in}), \quad [1]$$

where P denotes the permeabilities of the Na^+ and K^+ ions, and Na and K denote their respective concentrations in the vesicles (in) and in the pipette (o).

In the experiment, the intravesicular compartment contained 0.4 M potassium glutamate, while the pipette contained 0.4 M sodium glutamate. Therefore, Eq. **1** is reduced to

$$E_{rev} = (RT/F)\ \ln(P_{Na} Na_o)/(P_K K_{in}). \quad [2]$$

The concentrations of Na_o and K_{in} are the same; therefore, if the permeability of Na^+ and K^+ through the P channel is equal, the term inside the logarithm will be 1, and the reversal potential is expected to be 0 mV. In fact, in one set of recordings, the measured reversal potential was −25.9 ± 2.4 mV (n = 15), suggesting that this channel prefers potassium over sodium by a factor of 2.8. In five different experiments, the ratio of P_K/P_{Na} was between 2.6 and 4.0. We therefore named this channel the P channel (for potassium preferring). Inside the presynaptic nerve terminal, where the potassium concentration is much larger than that of sodium, this channel will presumably transport mainly K^+ ions because of both the high potassium concentration and the channel selectivity.

Amplitude Distribution of the P-Channel Currents. The conductance behavior of the P channels is far from simple.

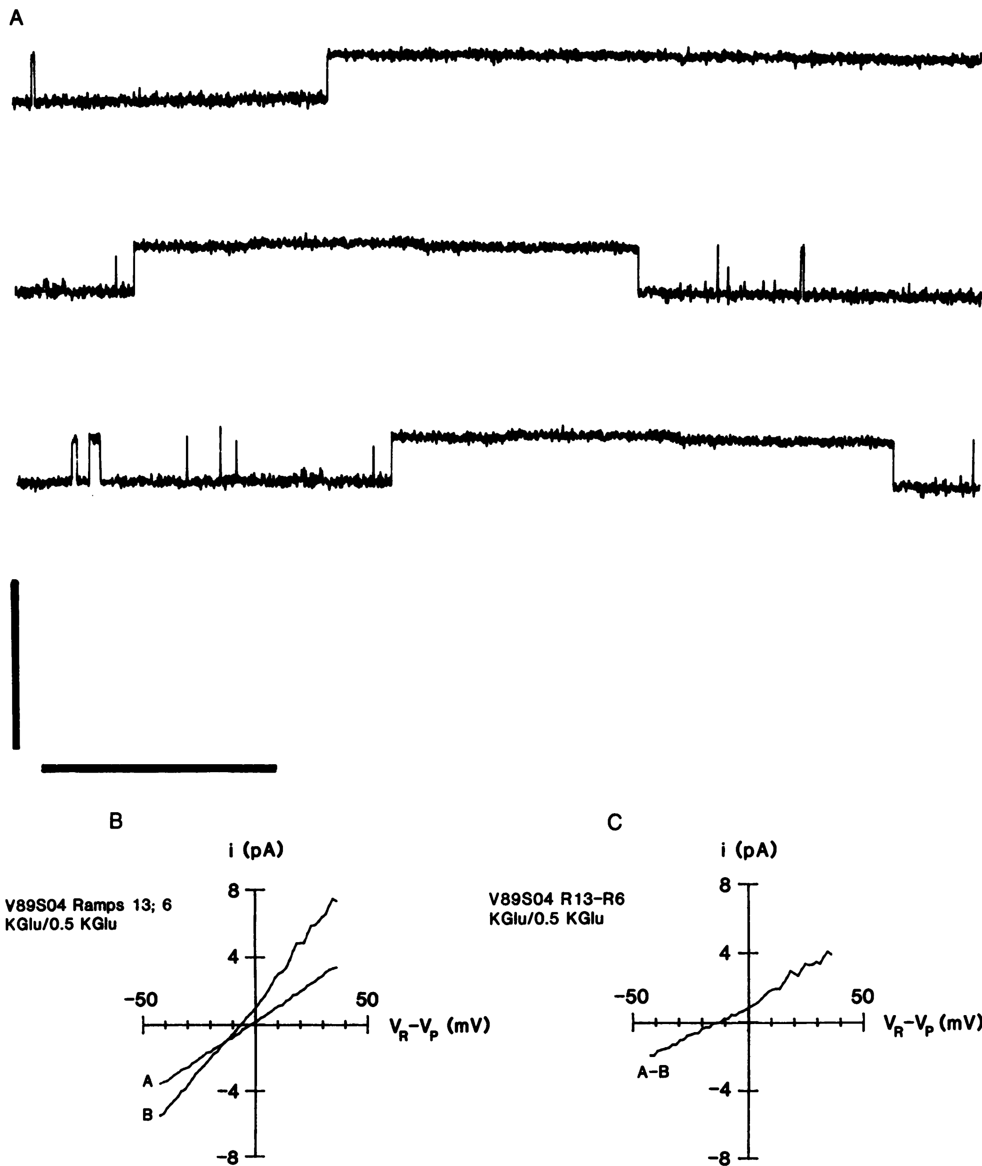

FIG. 2. (*A*) P channels in fused synaptic vesicles. The giant synaptic vesicles were prepared with dimethyl sulfoxide and PEG fusion medium, containing 400 mM potassium glutamate. After fusion, they were diluted in a medium containing 400 mM potassium glutamate and 2 mM KCl (KCl was added to reduce the polarization of the electrode and thus to help in balancing the preseal pipette potential). The vesicles were then put on a cover glass coated with 0.01% polylysine. The borosilicate patch pipette was filled with 100 mM potassium glutamate/2 mM KCl/3.2 μM $CaCl_2$. Pipette potential, 10 mV ($V_R - V_P = -10$ mV). Outward currents upward. Note short and long opening of channels. Vertical bar, 10 pA; horizontal bar, 2500 ms. (*B*) Current–voltage relationship. Pipette solution containing 200 mM potassium glutamate and 2 mM KCl, generating an ionic gradient of $\approx$2 for K^+ ions. Voltage ramps of 80 mV (from -42 mV) were applied to the patch pipette. Ramp duration of 1 s was digitized into 160 points. A, a ramp during closed state of the channel; B, a ramp when a P channel was open. (*C*) Same as *B*, showing the difference between A and B ramps. From 28 such *I–V* relationships on the same patch, the mean reversal potential was -14.23 ± 1.3 mV (SD). i, Current.

Fig. 3*A* shows amplitude distributions of currents obtained from a patch with 0.4 M potassium glutamate in the vesicle and bath and 0.1 M potassium glutamate in the pipette at zero pipette potential. Two peaks can be clearly seen; the mean value of the larger amplitudes (P2) being approximately double that of the smaller amplitudes (P1). In five experiments made under these conditions, the ratio P2/P1 varied between 1.78 and 2.07. Such a bimodal distribution of amplitudes could in principle originate from a number of different possibilities. For example, two similar channels that open and close simultaneously in a random fashion; two different types of channels; one type of channel with multiple conductance levels; or cooperative opening of two channels. The first possibility seems highly unlikely; the probability of two independent channels opening and closing simultaneously is very low. Although not excluded, it is unlikely that these two peaks originate from two completely different channels, since their reversal potentials are very similar.

A voltage-dependent transition between P2 and P1 seems to take place. In one example, there were 302 large current events and 1110 small current events (21.4% and 78.6%, respectively) at 0 mV pipette potential. When the same patch of membrane was depolarized by only 5 mV, the relative distribution changed dramatically to 786 large and 209 small current events (79.0% and 21.0%, respectively). We cannot distinguish at present whether several P channels open synchronously or whether this is a channel with multiple conductance levels (Fig. 3*B*).

In seven experiments with 0.4 M potassium glutamate in the bath and 0.1 M potassium glutamate in the pipette, the conductance of the P channel determined for the smaller of the two peaks (or for the only peak observed) varied between 80 and 109 pS.

Control Experiments. Three types of control experiments were performed to rule out the possibility that the fusogenic substances produce artifacts that resemble discrete openings and to strengthen the argument that P channels originate from the membrane of the synaptic vesicle. The first of these was to examine whether P channel-like activity exists in fused structures prepared from purified lipids of the electric organ. It was possible to use the same fusion technique used for the synaptic vesicles with a preparation of lipids in which the protein/lipid ratio was <1:1000. None of the 13 patches obtained exhibited any activity resembling that of the P channels.

The second control was to look at the level of P-channel activity in the crude vesicle fraction obtained from the initial discontinuous sucrose gradient (Vb). If the channels we observe are coming from a minor contaminant present in the pure vesicle fraction, but originating from a nonvesicular structure, then one might expect that the level of activity

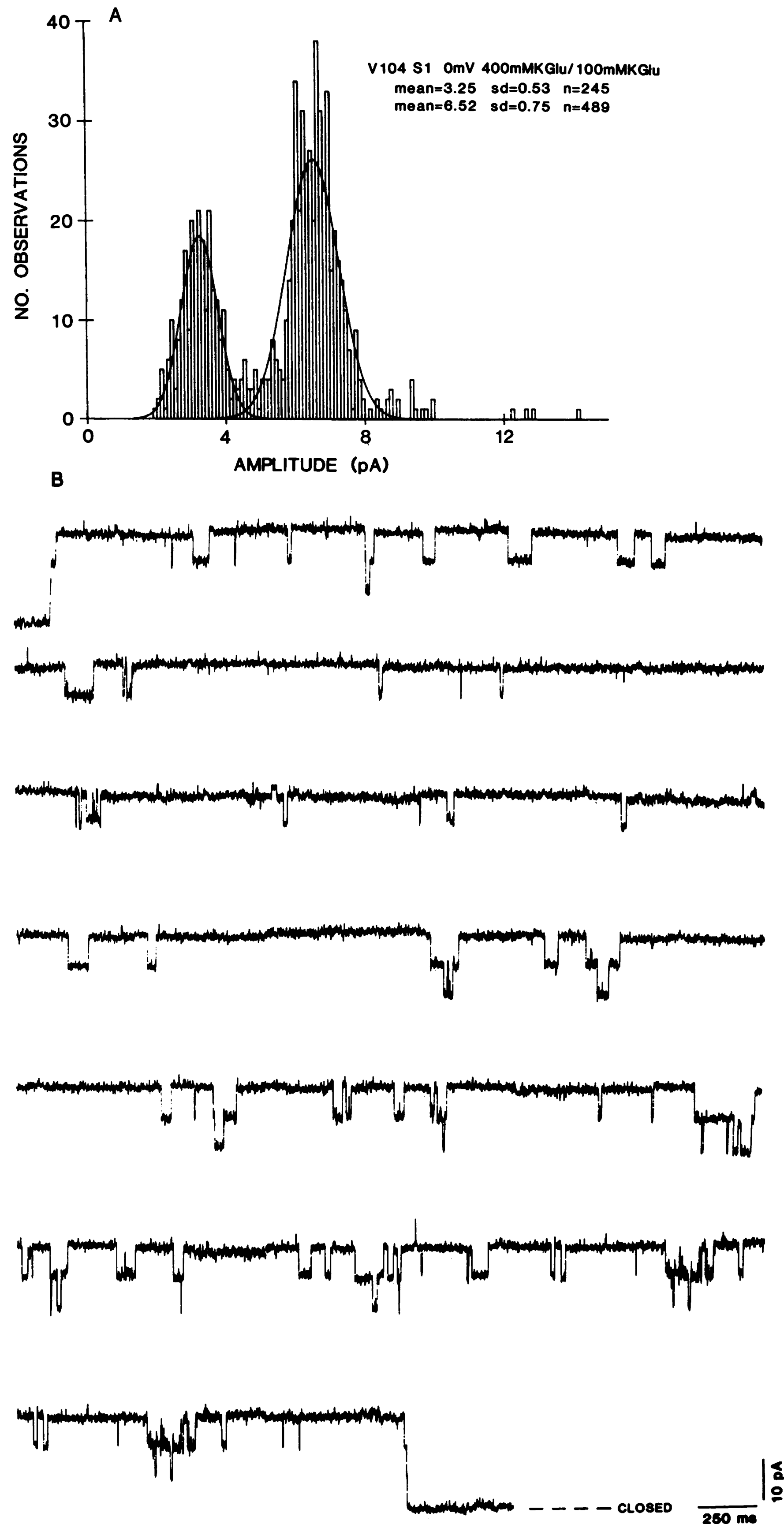

FIG. 3. Properties of the P channel. (*A*) Multiple levels of conductances at the same patch of membrane. Fusion in 400 mM potassium glutamate and 2 mM KCl bathing medium. Pipette composition, 100 mM potassium glutamate and 2 mM KCl. Pipette potential, 0 mV. Amplitude histogram of open-channel currents. Note the two peaks in the amplitude distribution: the first peak has a mean of 3.25 ± 0.53 pA (SD; n = 245) and the second peak has a mean of 6.52 ± 0.75 pA (SD; n = 489). The conductances were 102.5 and 205.7 pS, respectively. (*B*) An example of a period with a high rate of activity. Same patch of giant vesicle membrane as in *A*. These are consecutive recordings starting from the top. Data were recorded on a tape recorder with a 10-kHz filter and were digitized with a 1-kHz filter. Digitization rate, 5 kHz.

would be greater in the less purified fraction. Taking as a measure the maximal number of levels of opening (N) observed during an experiment, we saw no increase in N in four patches obtained from the fused Vb fraction (2.37 ± 0.41; mean ± SD) compared to five control recordings obtained from the same batch of vesicles (2.8 ± 1.08), carried through the final purification step.

One might expect that a major source of contaminating ion-channel proteins would be plasma membranes; therefore, we examined the effects of addition of purified synaptic plasma membranes on N. Addition of plasma membranes, giving ≈10% contamination, to the purified vesicle fraction prior to fusion produced no appreciable increase in N (2.4 ± 0.73 from five patches). These results support the conclusion that the P channels are presumably genuine components of the synaptic vesicle membrane.

DISCUSSION

The experiments presented in this article indicate that the synaptic vesicle possesses ionic channels. We do not know at present what roles these channels play in synaptic function. It is conceivable that channels may take part in any stage of vesicle function, such as assembly, transport, loading with neurotransmitter, fusion with the plasma membrane, recycling, or degradation. In the past several years, it has been suggested that the presence of channels in secretory and synaptic vesicles may be intimately related to the secretion process in a number of distinct ways (16–19). Stanley and Ehrenstein (19) proposed that the entry of calcium ions through the synaptic surface membranes causes an opening of calcium-activated K^+ channels, which in turn produce an increase in the number of osmotic particles in the vesicle, swelling, and facilitation of exocytosis. Although preliminary experiments (unpublished data) indicate that the P channels are calcium sensitive, we do not know whether this sensitivity is in the right concentration and time scale range to support such a hypothesis.

The long openings sometimes observed (many milliseconds or even seconds) would at first seem difficult to reconcile with a direct function in the synaptic transmission process, which occurs in a fraction of a millisecond (20). However, one must remember that we have examined these synaptic vesicle channels in conditions that are far from physiological. They are bathed in simplified solutions and their interior contains no more than 0.5% of their original components. Any modulatory processes, such as those controlling plasma membrane ion channels, are probably not functioning under these conditions in the same way as they might function within the intact cell.

In addition to simplifying the chemical environment, fusion may also alter the physical properties of the synaptic vesicle. For instance, the larger radius of the giant vesicle probably leads to a reduced surface tension in accordance with the Young–Laplace equation (21, 22).

The finding of ionic channels in the synaptic vesicle should probably be taken in the general context of channels in subcellular organelles. Recent reports show ion channels in mitochondrial (23) and plant vacuolar membranes (24); this, of course, is in addition to the better known channels in the sacroplasmic reticulum.

Particularly exciting is the recent finding of Breckenridge and Almers (25, 26), that during exocytosis of secretory vesicles in mast cells a "fusion pore" is formed between the vesicle and the surface membrane. The formation of this pore, which has the properties of an ionic channel, precedes the actual process of secretion. There is not enough information at present to speculate whether the P channels may serve as fusion pore analogs in the nervous system.

Whatever the properties of the channels are under physiological conditions, it seems that the synaptic vesicles are dynamic structures (27) with membrane potentials (28, 29), channels, and prospects for an interesting physiology.

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