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Large aqueous channels in membrane vesicles derived from the rough endoplasmic reticulum of canine pancreas or the plasma membrane of *Escherichia coli*

(planar lipid bilayer/fusion/protein translocation/GTP/guanosine 5′-[γ-thio]triphosphate)

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ABSTRACT **Voltage clamp conditions were used to study the membrane permeability properties of rough microsomes (RM) derived from the rough endoplasmic reticulum of canine pancreas and inverted vesicles (InV) derived from the plasma membrane of *Escherichia coli*. Membrane vesicles of RM or InV were fused to a planar lipid bilayer that was formed in a hole of a partition separating two chambers. Fusion of a single RM vesicle yielded a single-step conductance increase. Some preparations yielded unitary conductances of 20, 55, 80, and 115 pS in 45 mM potassium glutamate. These channels were largely open at negative membrane potential on the cytoplasmic side of the RM membrane, mostly closed at positive voltages, permeable to amino acids, and slightly more selective for anions than cations. There was a dramatic increase in the number of open channels when 100 μM GTP was added to the cytoplasmic side of the fused RM, whereas 100 μM guanosine 5′-[γ-thio]triphosphate caused closing of channels. ATP had no effect. A large channel of 115 pS at 45 mM potassium glutamate was also detected after the fusion of InV. As both RM and InV share the ability to translocate secretory proteins, it is possible that the 115-pS channel in both membranes represents a protein-conducting channel.**

A large number of specific cellular proteins can be translocated across or integrated into distinct cellular membranes. Targeting of these proteins is specified by membrane-specific signal sequences. The translocation process that is initiated by a signal sequence will go to completion unless it is interrupted by a stop-transfer sequence. The latter yields integration of the polypeptide into the lipid bilayer (1). It is not known how, after targeting, the polypeptide traverses the membrane. One hypothesis is that the polypeptide crosses the membrane directly through the lipid bilayer (2, 3). An alternative hypothesis is that the polypeptide traverses through an aqueous channel (1, 4, 5) in the membrane. More recently it was suggested (6) that such a protein-conducting channel might function like a ligand-gated channel and would open in response to signal sequence binding to a cytoplasmically exposed domain. Experimental evidence compatible with a protein-conducting channel has come from studies that showed that signal sequence insertion as well as subsequent translocation occurs in a space that is accessible to aqueous perturbants (7). It is generally accepted that the signal sequence inserts into the membrane in a loop configuration (8). Therefore, an open protein-conducting channel should be large enough to accommodate at least two antiparallel stretches of the polypeptide—i.e., it should be significantly larger and readily distinguishable from a typical ion-conducting channel.

To search for such a channel, we fused canine pancreas rough microsomes (RM) or inverted vesicles (InV) derived from the plasma membrane of *Escherichia coli* to a planar lipid bilayer (9–11). A voltage clamp was used to show the presence of ion-permeable transmembrane channels. Glutamate, which is impermeable to most ion channels, should easily permeate a protein-conducting channel. Thus, potassium glutamate was used as the probe for ion conductivity. A similar large conductance (115 pS at 45 mM potassium glutamate) channel was detected after fusion of single vesicles of either RM or InV.

METHODS

Preparation of Membrane Vesicles. RM from canine pancreas were prepared as described (12). RM were stored in 20-μl aliquots at −80°C in 250 mM sucrose/50 mM triethanolamine·HCl, pH 7.5/1 mM dithiothreitol (STD buffer) at a concentration of 50 A_{280} units/ml [determined in 1% sodium dodecyl sulfate (SDS)]. Plasma membrane InV from *E. coli* were prepared as described (13) and stored at −80°C in STD buffer at a concentration of 12.5 A_{280} units/ml (determined in 1% SDS).

Bilayer Formation. Planar phospholipid bilayers were formed in a 100-μm or 1-mm hole in a partition separating two chambers. Membrane vesicles were added to one side of the bilayer (see below) in what will be referred to as the cis chamber. The trans chamber held 3 ml of trans solution (45 mM potassium glutamate/50 mM Hepes·KOH, pH 7.5/1 mM dithiothreitol). The cis chamber was hyperosmotic and held 3 ml of cis solution (250 mM sucrose/45 mM potassium glutamate/50 mM Hepes·KOH, pH 7.5/1 mM dithiothreitol). When vesicles were microinjected into the cis chamber adjacent to the bilayer (see vesicle incorporation method 1 below) then both the cis and trans chambers additionally contained 10 mM $CaCl_2$. The two phospholipids used for these experiments were bacterial phosphatidylethanolamine (PtdEtn) (Avanti Polar Lipids) and bovine phosphatidylserine (Ptd Ser) (Avanti Polar Lipids). Two different types of bilayers were used.

Solvent-containing films. This method is essentially identical to the original method described by Mueller and Rudin for "black lipid membranes" (14, 15). The bilayer-forming solution was 1% (wt/vol) Ptd Etn and 1% (wt/vol) Ptd Ser dissolved in decane (Fluka). The thinning was monitored by observing both the visual interference patterns of light from the front and rear surfaces of the membrane (14, 15) and the

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Abbreviations: InV, inverted vesicles derived from the plasma membrane of *E. coli*; RM, rough microsomes of canine pancreas; V_m, membrane potential; GTP[γS], guanosine 5′-[γ-thio]triphosphate.

increase of capacitance of the membrane. Capacitance was measured by monitoring the transient current response to a step increase or decrease of voltage. Within a minute the membrane was black and the capacitance was $\approx 1\ \mu F/cm^2$.

Solvent-free films. "Solvent-free" bilayers were made as originally described by Takagi *et al.* (16) and modified by Montal and Mueller (17).

Incorporation of RM or InV into the Planar Lipid Bilayer. Two conditions are required to obtain fusion of membrane vesicles to the bilayer: raising the $CaCl_2$ on the cis side to a final concentration of 10 mM and establishing an osmotic gradient from the inside of the microsome to the trans side of the bilayer (11). The calcium is required for binding the vesicles to the bilayer (18), and the osmotic gradient provides the driving force for fusion (19). Two methods were used for adding the vesicles to the bilayer.

Method 1: Injecting vesicles onto the bilayer. This method was used essentially as described (18). For this technique the cis and trans solutions in the chambers were augmented with 10 mM $CaCl_2$. Vesicles were diluted to a concentration of 0.01–0.5 A_{280} unit/ml in cis solution without the added $CaCl_2$. The pipet was maneuvered within 20–50 μm of the cis face of the bilayer. At most 0.5 μl of vesicles were pressure-injected onto the bilayer (18). Increases of the bilayer conductance were observed within seconds of injecting the vesicles.

Method 2: Addition of vesicles to the bulk aqueous phase. The cis and trans chambers were filled with 3 ml of cis and trans solution, respectively. Approximately 3–10 μl of vesicles, diluted to 0.01–0.5 A_{280} unit/ml in cis solution, were added to the cis chamber. The solution was stirred for 10 min, 10 μl of 3 M $CaCl_2$ was added, and the solution was stirred for an additional 10 sec. Fusion usually started within 1 min. With this technique it was difficult to control the number of vesicles reaching the planar bilayer. The conductances observed were indistinguishable from those obtained with method 1 (injection adjacent to the bilayer). With both techniques the fusion could be halted by perfusing either the cis or trans chambers to eliminate the osmotic gradient.

All data were filtered at 500 Hz (Frequency Devices model 902, Haverhill, MA) and stored on a chart recorder (Kipp & Zonen, BD-41, Delft, Holland). The response time of the chart recorder eliminated detection of ion channels whose open or closed states are shorter than tens of milliseconds.

RESULTS

Fusion of Many RM Vesicles. The macroscopic permeability of RM was examined by fusing them to a planar lipid bilayer. This approach allows the use of electrodes to measure the conductivity of the RM membrane. The planar lipid bilayer was formed over a hole in a partition separating two aqueous chambers. Within seconds after injection of RM (Fig. 1*A*), there was a rapid increase in conductance across the lipid bilayer that continued for a period of 90 sec. The ongoing increase in conductance suggests that RM vesicles continued to fuse with the bilayer after the initial fusion of a single vesicle. A conductance increase was not observed if RM were not included in the micropipet or if $CaCl_2$ and sucrose were not added to the cis chamber. We conclude that fusion of RM with the planar bilayer occurred and that the observed conductance increase was due to a large number of channels.

The conductance of the membrane was studied as a function of voltage. A consistently greater membrane conductance was observed when the cis side of the chamber—i.e., the cytosolic side of the fused vesicles—was at negative potentials (Fig. 1*B*).

Detection of Single-Ion Channels. To examine the microscopic nature of the conductance properties of single RM vesicles, the concentration of RM was lowered. As soon as an increase in conductance was recorded, indicating RM

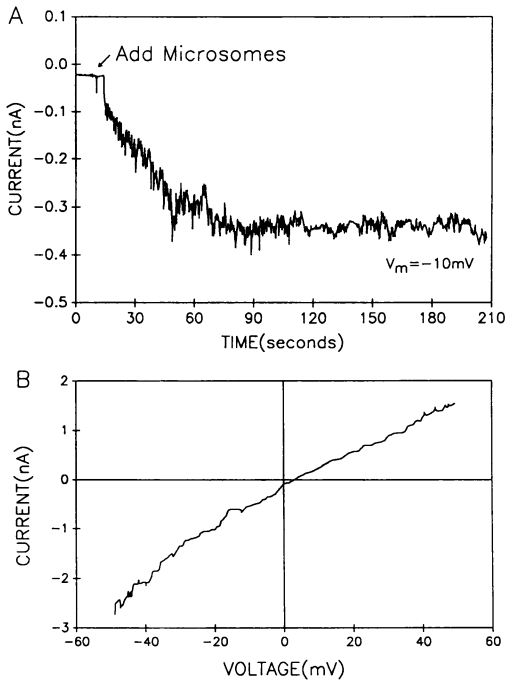

FIG. 1. Fusion of many RM vesicles to a planar lipid bilayer. (*A*) The current necessary to voltage-clamp the membrane at −10 mV (cis relative to trans) was recorded. Approximately 0.5 μl of RM (0.2 A_{280} unit/ml) was pressure injected (at the arrow) into the space adjacent to the bilayer. A few seconds later there was an increase of the current, indicating an increased conductance. The current stabilized at 350 pA, which corresponds to a total membrane conductance of 35 nS. (*B*) *I–V* plot after fusing many RM vesicles. The V_m was clamped to a triangular waveform of +/−70 mV.

fusion with the lipid bilayer, the osmotic gradient was eliminated by addition of sucrose to the trans chamber to prevent further fusion of additional RM vesicles.

Under these conditions (Fig. 2*A*), a single-step increase in conductance was observed (at the arrow) with no further conductance increase in time. This suggests that only a single RM vesicle fused with the bilayer. Quantitative analysis of the recording shown in Fig. 2*A* revealed conductances of 20, 50, 80, and 115 pS (Fig. 2*B*). All of the conductances rectified with ionic gradients as expected for passive pores increases in the concentration of KCl or potassium glutamate led to increases in the unitary conductances (data not shown). Presently, we cannot distinguish whether these different conductances represent distinct channels or subconductances of one or more channels that open to various degrees. When the voltage was varied from −70 to +70 mV, the channels opened more frequently at negative V_m (Fig. 2*C*).

Detection of a Single 115-pS Channel in RM. Some preparations showed only a single channel of 115 pS (Fig. 3*A Left* and *Middle*). In the record shown in Fig. 3*A Left*, the 115-pS channel stayed mostly closed at +50 mV and oscillated between the open and closed form at −50 mV. In another preparation (Fig. 3*A Middle*), this 115-pS channel was again mostly open when the voltage in the cis chamber was −80 mV. In a voltage sweep from −70 to +70 mV (Fig. 3*B Left* and *Middle*), the 115-pS channel in both preparations was predominantly open at negative V_m and largely closed at positive V_m. Increases in the KCl or potassium glutamate concentration led to an increase in conductance. At physiological salt concentration (i.e., at 150 mM), the conductance of this channel increased to 300 pS (data not shown).

We also obtained preparations that showed a single channel of 20 pS (Fig. 3*A Right*). This channel appears to be distinct from the 115-pS channel. It is open at both negative

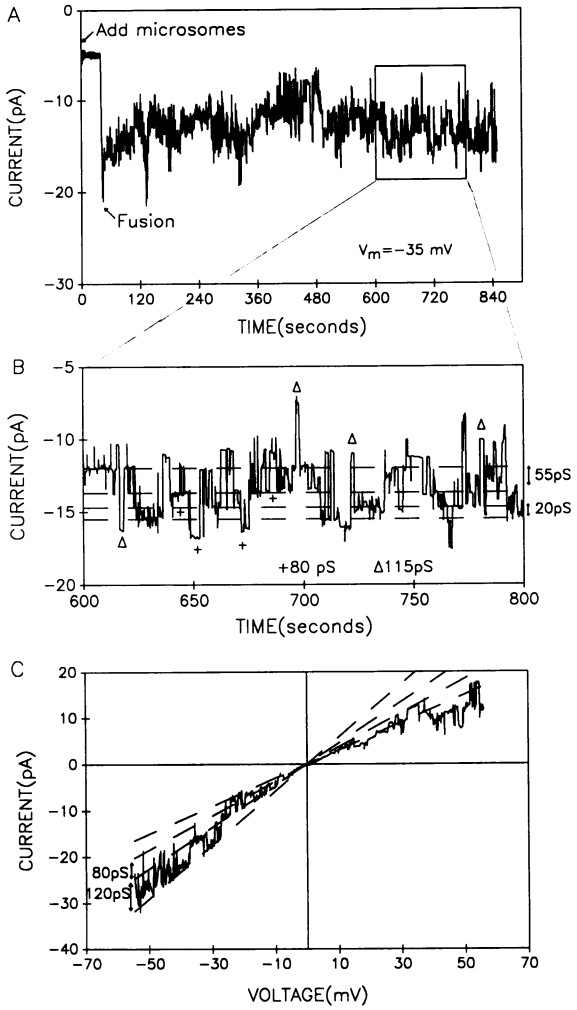

FIG. 2. Fusion of a single RM vesicle to the planar bilayer. (*A*) RM were added to planar lipid bilayer as in Fig. 1. However, the concentration of RM in the micropipet was reduced to 0.01 A_{280} unit/ml. After 45 sec there was a single 11-pA step increase in the current (300-pS conductance) of the membrane and a substantial increase in the fluctuations of the current. (*B*) A segment of the record of *A* from 600 to 800 sec is shown at an expanded time scale. Four distinct channel sizes can be distinguished: 20 pS, 50 pS, 80 pS (at the "+"), and 115 pS (at the "Δ"). (*C*) The membrane potential (V_m) was clamped to a triangular waveform of −/+50 mV. Individual channels can be distinguished opening and closing. More channels are open at more negative V_m.

and positive voltages in the cis chamber, although it has longer closed periods at positive voltages (Fig. 3*B Right*).

Ion Selectivity of the RM Channels. The ion selectivity of the channels was tested by increasing the concentration of either KCl or potassium glutamate on the trans side of the membrane and measuring the resulting reversal potential for the membrane—i.e., the voltage at which current flow is zero. If the channel is perfectly cation selective then the reversal potential would become more positive with each increase in ion concentration (Fig. 4, solid line). If the channel is anion selective, the reversal potential becomes more negative (Fig. 4, dashed line). At the end of each experiment, ≈10 mM valinomycin was added to the membrane to give the actual potassium equilibrium potential. The observed reversal potentials are plotted for the 115-pS channels in Fig. 4 for the addition of KCl and potassium glutamate. Both K^+ and Cl^- have very similar mobilities. Increasing the KCl concentration demonstrates that the channel is more permeable to Cl^-. When potassium glutamate was added to the trans compartment, there was little change in the reversal potential, indicating that the channel is roughly equally conductive to potassium ion and glutamate. The mobility of potassium (D_{K+} = 1.96 × 10^{-5} cm^2/sec) is ≈4 times the mobility of glutamate ($D_{gluatamate}$ ≈5 × 10^{-6} cm^2/sec). Therefore, the channel is substantially more selective for glutamate than potassium. To quantify the selectivity further, it is necessary to determine the activities of each of the ions on both sides of the membrane.

Effect of GTP and Guanosine 5′-[γ-thio]Triphosphate (GTP[γS]). The effect of GTP on channel activity was tested because GTP has been shown to be involved in protein translocation across RM (20). GTP (100 μM) was added to the cis chamber of a preparation that showed a single 55-pS channel in the 200-sec recording prior to the addition of GTP (Fig. 5*A*, top line). Within a minute after the addition of GTP, there was a substantial increase in conductance (Fig. 5*A*, middle and bottom lines). As there was no observable change in the size of the single-channel conductance, we conclude that the shift is the result of an increase in the number of open channels. The conductance increase ranged from 2- to 10-fold in five of six preparations. Addition of ATP or an ATP-regenerating system at the cis side of the chamber had no effect on the conductances (data not shown).

When GTP[γS] was added to the cis side of a preparation that contained channels of 20, 60, 80, and 115 pS (see Fig. 2*A*), there was a rapid decrease in the frequency of channel opening and a decrease in conductance (Fig. 5*B*).

A 115-pS Channel in InV of *E. coli*. Like RM, InV derived from *E. coli* plasma membrane are able to translocate secretory proteins in a cell-free system (13). If the 115 pS channel of RM were a protein-conducting channel, then InV should contain a similar channel. When a single InV was fused to the planar lipid bilayer, a 115-pS channel was detected. Like the 115-pS channel of RM, the 115 ps of InV closed completely without residual conductance. It was more selective for anions (Cl^-, glutamate$^-$) than cations (K^+), though it was permeable to both (data not shown). The 115-pS channel from InV was somewhat less affected by the voltage (Fig. 6*B*) across the membrane than the 115-pS channels from RM (Fig. 3*B*).

DISCUSSION

Large aqueous channels were detected here in canine pancreas RM and *E. coli* InV fused to planar lipid bilayers under voltage-clamp conditions. When only a single RM vesicle was fused to the bilayer, we sometimes observed several large channels (20, 55, 80, and 115 pS in 45 mM potassium glutamate) and sometimes only a single of these large channels. Strikingly, like single RM vesicles, single InV contained a similar 115-pS channel in 45 mM potassium glutamate. Since both RM and InV share the ability to translocate secretory proteins into their vesicle lumina, it is possible that this large channel represents the previously postulated protein-conducting channel (1, 4, 5).

The 20-pS channel of RM vesicles appears to differ from the larger channels in that its probability of opening is not dependent on the membrane voltage. The 55-, 80-, 115-pS channels are usually open only when the cis side of the membrane is negative. It is possible that each of these distinct conductances represents biochemically distinct channels. Alternatively, they may represent different subconductance states of the same channel, except perhaps for the 20-pS channel. Qualitatively similar subconductance states have been observed for the gap junction channel (21, 22).

Different subconductance states may be physiologically relevant for a protein-conducting channel that may have to open to various degrees to accommodate the different side chains or various secondary and tertiary structures of the polypeptide. The fact that we have, so far, not observed equivalent 55- and 80-pS channels in *E. coli* InV does not

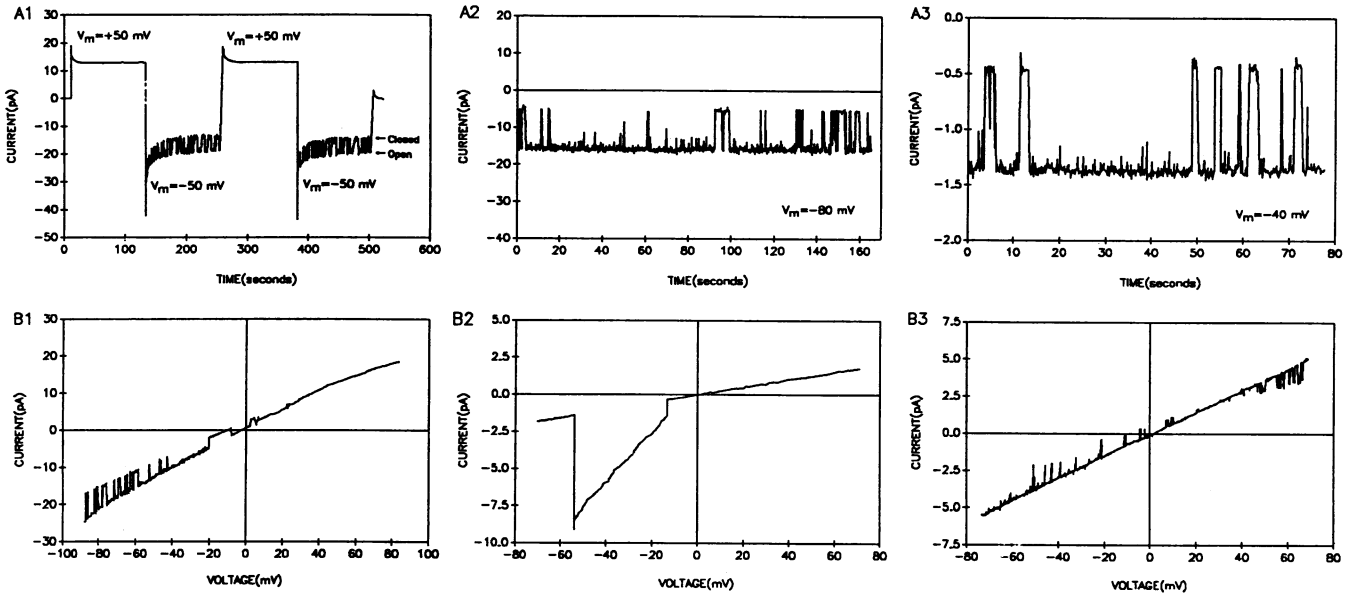

FIG. 3. Single-channel recordings from a single RM vesicle. Each record represents a different preparation of a single RM vesicle fused to a planar lipid bilayer. Two microliters of RM diluted to a concentration of 0.02 A_{280} unit/ml were added to the cis chamber by method 2. In each of these preparations, only single channels were observed. (*A Left* and *B Left*) A single 115-pS channel that opened only at negative V_m: the bilayer was alternatively clamped to +/−50 mV (*A Left*), and the V_m was changed from −87 to +87 mV (*B Left*). (*A Middle* and *B Middle*) A single 115-pS channel from a different preparation: the single-channel activity at a V_m of −80 mV. Note that the current is shown at an expanded time scale relative to *A Left*, and the voltage was varied +/−70 mV (*B Middle*). Similar to the 115-pS channel in *A* and *B Left*, this channel was primarily open when the voltage across the bilayer was negative (*B Middle*). (*A Right* and *B Right*) A 20-pS channel: at a V_m of −40 mV (*A Right*), and as the V_m was changed from −70 mV to +70 mV (*B Right*). This channel is open at most voltages. However, it closes more frequently at positive V_m.

necessarily argue against the one-channel-different subconductance-state idea. Differences in the preparation of InV or RM fractions might affect channel behavior.

Interestingly, 100 μm GTP, but not ATP, led to an increase in the numbers of channels open, whereas 100 μm GTP[γS] caused channel closing. It remains to be investigated whether and how these phenomena are related to the reported GTP requirement for the displacement of the signal sequence from the signal recognition particle (SRP) receptor (20). One of many possibilities is that GTP plays a role in a reversible association between SRP receptor and a signal sequence-gated channel. Such regulated association may be required, as the SRP receptor is present in less than stoichiometric amounts relative to a putative signal sequence-gated, protein-conducting channel.

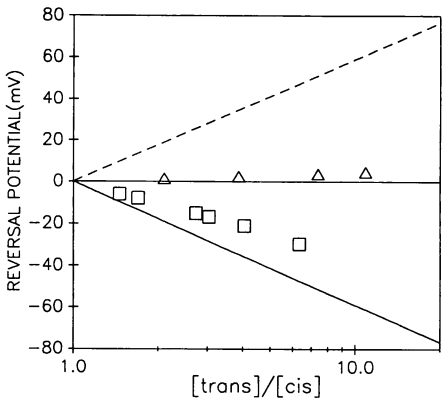

FIG. 4. Ion selectivity of the 115-pS channel. The concentration of KCl (□) or potassium glutamate (△) was increased on the trans side of the membrane to examine the relative permeability of the 115-pS channels to anions and cations. After each increase, the V_m was varied to find the equilibrium potential for the membrane (I_m = 0). The theoretically expected reversal potentials if the channel were only conductive to anions (—) or cations (---) are plotted.

It is not known if there is a V_m across the endoplasmic reticulum. Therefore, the observation that the channels open

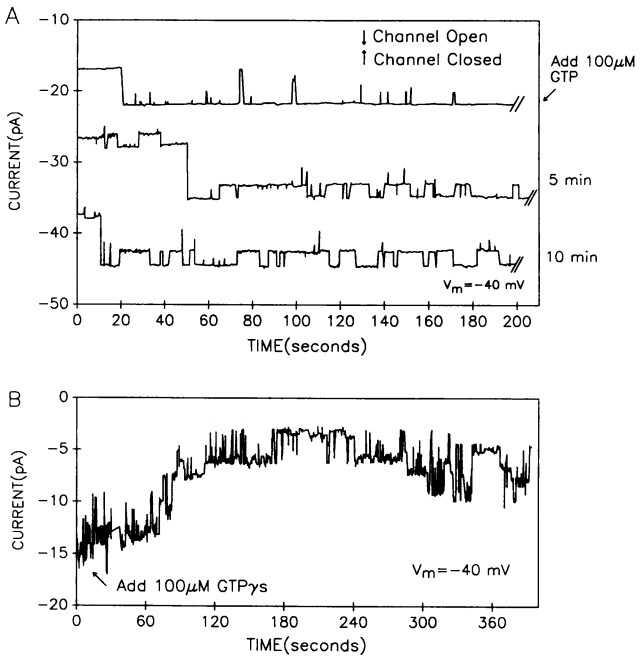

FIG. 5. Effect of GTP and GTP[γS] on single channels. (*A*) A single RM vesicle was fused with a planar lipid bilayer as described in Fig. 3. Ten microliters of 30 mM GTP was added to 3 ml of the cis solution. After addition of GTP, there was increase in the number of channels open. The three traces shown are consecutive recordings from the same preparation before addition of GTP (upper trace), immediately after addition of GTP (middle trace), and 7–10 min after addition of GTP (bottom trace). (*B*) Ten microliters of 30 mM GTP[γS] was added to the preparation shown in Fig. 2*A*. Within a few seconds there was a decrease in the number of open channels.

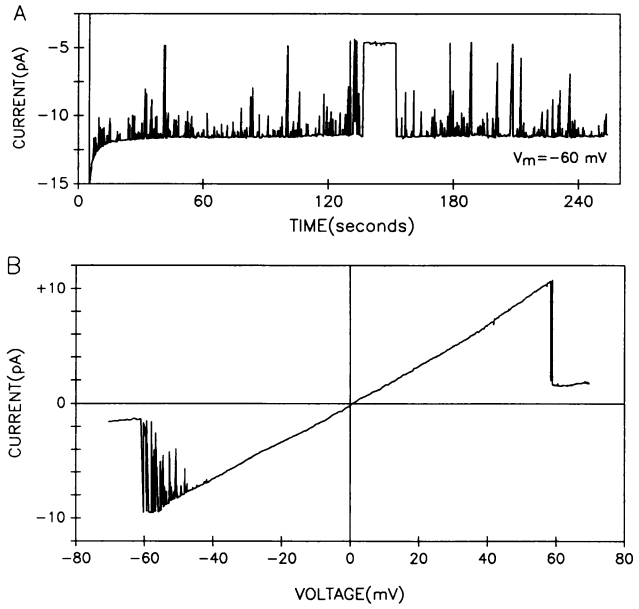

FIG. 6. Single-channel recordings from *E. coli* InV. A single InV was fused with a planar lipid bilayer as described in Fig. 3 at concentration of 0.125 A_{280} unit/ml. A single channel of 115-pS channel was observed. (*A*) The membrane was clamped to a constant voltage of −60 mV. (*B*) The V_m was voltage-clamped to a triangular waveform of +/−70 mV.

more frequently at negative voltage on their cytoplasmic side may not be of physiological relevance. It is not surprising that an electrical field of 50 mV across a 50-Å thick membrane (10^5 *V*/cm) can cause subtle conformational changes in protein structure. However, physiologically this channel may be opened by the binding of the ligand (i.e., signal sequence) to the channel. The effects of voltage are nonetheless useful in characterizing these channels and comparing them to previously described ion channels. Conductance measurements give misleading physical sizes for channels. However, the permeability of glutamate indicates that amino acids can easily pass through.

So far, of course, there is no evidence that the large channels described here are in fact, the long sought-after protein-conducting channel of RM or InV. The sensitivity of the methods used here is such that single channels can be detected. Even though we used highly purified preparations of RM or InV, the observed large channel might be indigenous to other cellular membranes. Potentially, it could be contributed by a small amount of contaminating vesicles derived from other cellular membranes. Alternatively, it could be a biosynthetic intermediate—i.e., a newly synthesized channel that was integrated into the rough endoplasmic reticulum or the *E. coli* plasma membranes but in fact is destined for other cellular membranes (in the case of the pancreatic cells) or to the outer membrane (in the case of *E. coli*) (23). However, the channels observed here are unique, relative to previously described channels, in their combination of features: large size, high conductance to glutamate, and ability to close completely. The large channels observed here are an order of magnitude larger than conventional ion channels (24). The only characterized channels of such large conductances in other cellular membranes are the gap junction channels or the voltage-dependent anion-conducting (VDAC) channels in the outer mitochondrial membrane of eukaryotes (25) and the porins in the outer membrane of *E. coli* (26). The channels described here are unlikely to represent the bacterial porins or mitochondrial VDAC. After porins open to allow ion flow, they close in three discrete steps of decreasing conductance (26). This stepwise closing has not been observed in the RM or InV channels. Further, when VDAC or porin channels "close" they never do so completely. Even in the closed state there is a large residual conductance (25, 26). When the RM or InV channels closed there was no residual conductance. The large RM or InV channels are most probably not gap junction channels, which appear to be slightly cation selective (21, 22). The observed RM and InV channels were clearly anion selective (see Fig. 4). InV, of course, do not contain gap junction proteins.

If, in fact, the large channels observed here in RM and InV represent protein-conducting channels then it is surprising that at times only single channels are observed. Indeed, some vesicles may have had no open channels but we would not have been able to observe their fusion. RM vesicles are large enough to contain on the order of 10–100 such channels. One of several possible explanations is that many of these channels might be occupied by polypeptides that are in the process of being conducted across the membrane. Such occupied channels might not be permeable to ions and therefore might be silent. If this were the case, one would expect an increase in the numbers of ion-conducting channels after release of polypeptides either naturally, after readout of the mRNA, or artificially after puromycin treatment.

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