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Gating of a Voltage-Dependent Channel (Colicin E1) in Planar Lipid Bilayers: The Role of Protein Translocation

Stephen L. Slatin, Lynn Raymond, and Alan Finkelstein
Departments of Physiology & Biophysics and Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461

Summary. The voltage-dependent channel formed in planar lipid bilayers by colicin E1, or its channel-forming C-terminal fragments, is susceptible to destruction by the nonspecific protease pepsin under well-defined conditions. In particular, pepsin acts only from the *cis* side (the side to which colicin has been added) and only upon channels in the closed state. Channels in the open state are refractory to destruction by *cis* pepsin, and neither open nor closed channels are destroyed by *trans* pepsin. Colicin E1 channels are normally turned on by *cis* positive voltages and turned off by *cis* negative voltages. For large (>80 mV) positive voltages, however, channels inactivate subsequent to opening. Associated with the inactivated state, some channels become capable of being turned on by *cis* negative voltages and turned off by *cis* positive voltages, as if the channel-forming region of the molecule has been translocated across the membrane. Consistent with this interpretation is the ability now of *trans* pepsin to destroy these "reversed" channels when they are closed, but not when they are open, whereas *cis* pepsin has no effect on them in either the open or closed state. Our results indicate that voltage gating of the E1 channel involves translocation of parts of the protein across the membrane, exposing different domains to the *cis* and *trans* solutions in the different channel states.

Key Words colicins · lipid bilayers · channels · voltage gating · inactivation · protein translocation

Introduction

Aside from the recently sequenced sodium channel from electroplax (Noda et al., 1984), the only voltage-dependent channels formed by proteins for which the primary structures are known are diphtheria toxin (Greenfield et al., 1983) and colicins E1, A, and Ib—a related group of bacteriocins produced by *E. coli* (Yamada et al., 1982; Morlon et al., 1983; Varley & Boulnois, 1984). These latter, water-soluble molecules bind to the outer membrane of target bacteria, which they then kill by forming ion-permeable channels in the inner membrane; they also form these channels in artificial lipid bilayers, making them excellent candidates for model voltage-dependent channels (Schein, Kagan & Finkelstein, 1978). The channel-forming ability of the 60 kD colicin E1 protein resides in the 152-amino acid C-terminal portion produced by cyanogen bromide cleavage of the molecule (Cleveland, Slatin, Finkelstein & Levinthal, 1983). The remaining 43-kD N-terminal region is involved in binding to the outer membrane receptor and in subsequent translocation to the inner membrane (for review *see* Cramer, Dankert & Uratani, 1983). The C-terminal fragment includes a 35-amino acid hydrophobic stretch near its C-terminal end, which undoubtedly inserts in the membrane; a similar hydrophobic region is found in colicins A and Ib. The rest of the sequence appears to be very hydrophilic, with many positive and negative charges. The pore formed is at least 8 Å in diameter, although the single channel conductance (in 1 M KCl, pH 6) is only about 24 pS (Raymond, Slatin & Finkelstein, 1985). The ionic selectivity, opening and closing rates, and total channel activity are all highly pH dependent over a pH range of 3.5 to 7. Most of the experiments to be described below were performed at pH 3.5, where the conductance can reach a steady-state value. In the steady state at this pH, all the channels are turned on for voltages greater than +30 mV and turned off for voltages less than −30 mV; in the narrow voltage range −30 to +30 mV, the rates are too slow for the conductance to reach a steady state.

This paper describes qualitatively several different states of the E1 channel and reports results of experiments in which the channels are subjected to the nonspecific protease pepsin from either side of the bilayer. The results imply that voltage-driven movements of protein into and across the membrane play a role in gating. These findings apply equally to channels formed from the intact colicin E1 molecule and to those formed from its C-terminal fragments [produced by either enzymatic or cyanogen bromide cleavage, as described by Cleveland et al. (1983)]. A preliminary report of some of

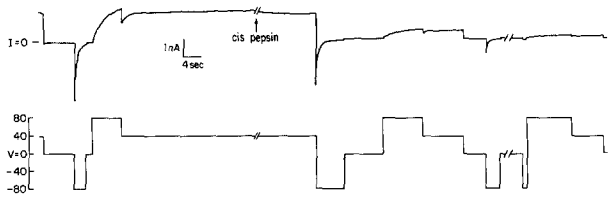

Fig. 1. The effect of *cis* pepsin on colicin channels. A membrane formed from a 4:1 mixture of diphytanoyl phosphatidylcholine/phosphatidylinositol separated symmetric buffer solutions of 1 M KCl, 20 mM DMG (dimethylglutaric acid), 5 mM $CaCl_2$, pH 3.5. 120 ng of ThCF was added to the *cis* compartment prior to the section of record shown here, and a steady-state level of conductance was obtained. Near the beginning of this record the channels were turned off with a −80 mV pulse and then turned on again to the same steady-state level seen initially. At the arrow, 5 mg of pepsin dissolved in a small volume of buffer was stirred into the *cis* compartment (compartment volume ca. 0.7 ml). Approximately 90 sec later the channels were turned off with a −80 mV pulse. Subsequent attempts to turn the channels on with +80 mV pulses met with diminishing success. (The second break in the record is 50 sec)

these results appeared earlier (Slatin, Raymond & Finkelstein, 1985).

Materials and Methods

For most experiments, membranes were formed at room temperature from two lipid monolayers folded together across a 50 to 150 μm hole in a Teflon partition (Montal, 1974) that had been pretreated with squalene; the partition separated two compartments (1–5 ml) of a Teflon chamber containing buffered salt solutions, which were stirred independently by magnetic fleas. Monolayers were spread from 1% lipid solutions in hexane, and the solvent was allowed to evaporate before membrane formation. In some experiments, membranes (ca. 1 mm^2 area) were formed by the brush technique of Mueller, Rudin, Tien and Wescott (1963) from a solution of lipid in decane. The lipid used was generally asolectin (soybean lecithin type IIS, Sigma Chemical Co., St. Louis, Mo.) from which neutral lipids had been extracted with acetone (Kagawa & Racker, 1971). Other lipids used to form membranes were from Avanti Polar Lipids, Birmingham, Ala. After membrane formation, colicin E1 or one of its C-terminal fragments was added from stock aqueous solutions to one of the compartments, defined as the *cis* compartment, to a final concentration of 0.005 to 1.0 μg/ml. pH changes in the solutions bathing the membrane were made by stirring in small aliquots of concentrated buffer solutions. Electrical measurements were made under voltage-clamp conditions using a single pair of Ag/AgCl electrodes, contacting the solutions via 3 M KCl agar bridges; current was monitored on an oscilloscope and a Physiograph chart recorder. The *trans* compartment was held at virtual ground; all voltages, therefore, refer to that of the *cis* compartment.

Colicin E1 and its channel-forming fragments were prepared in the laboratory of Cyrus Levinthal, Columbia University, as previously described (Cleveland et al., 1983). Stock aqueous solutions of 1 mg/ml protein [E1; its C-terminal thermolysin fragment (ThCF); its trypsin fragment (TrCF)] in 10 mM Tris, pH 7, were stored at 4°C, and under these conditions they were generally stable for more than a year. Any dilution of the stock solutions was usually done just prior to addition to the bilayer, as dilute colicin solutions tended to lose activity over time. Since the cyanogen bromide fragment (CNBr) is sparingly soluble in aqueous solutions, it was added (after membrane formation) from stock solutions (1 mg/ml in 10 mM Tris, pH 7) containing 1% octyl glucoside. After several days at 4°C, these solutions often contained some precipitate which could be easily redissolved with agitation; the solutions usually remained active for several months. Colicin A was a gift of S.E. Luria of M.I.T.; colicins Ia and Ib were gifts of J. Konisky of the University of Illinois. Porcine pepsin was from Sigma, as was pronase (Sigma protease type XIV).

Results

Effect of Aqueous Proteases on Colicin Gating

To probe the orientation of colicin E1 channels in the membrane, we used the proteolytic enzyme pepsin, which is active at acidic pH (pH optimum 2.5). Membranes containing, in the steady state, 10^3–10^5 channels formed by colicin E1 (or one of its C-terminal fragments) were treated with pepsin from either the *cis* or *trans* side. Our basic finding, illustrated in Fig. 1, is that *cis* pepsin has no discernible effect on channels that are in the open state, but destroys those that are turned off. Essentially, membrane conductance is unaffected by *cis* pepsin so long as the channels are held open (even up to 20 min) by positive voltages, and the channels turn off at negative voltages with the same kinetics seen in the absence of pepsin. When the voltage is again made positive (>+30 mV), however, the conductance does not return to the original level (as it does in the absence of enzyme), and, as found in numerous experiments, the deficit in conductance increases with the off time. After several minutes in the off state, essentially none of the conductance returns upon switching back to positive voltages. In contrast, pepsin added to the *trans* side has no noticeable effect on conductance or gating under these conditions of symmetric pH 3.5 [*see*, however, the following paper (Raymond et al., 1986)]. Similar results were obtained at pH 6.1 with colicins A, Ia and Ib using pronase (a nonspecific protease that is active at neutral pH); that is, *cis* pronase destroys colicin A, Ia and Ib channels only when they are closed, and *trans* pronase is ineffective on channels in either the open or the closed state. These data suggest that colicin reorients in the membrane in going from the closed to the open state. In the open state it is protected from pepsin (from either side), but upon turning off it exposes enough of itself to the *cis* solution to have its chan-

nel-forming capability destroyed by the enzyme. We shall argue below that it is the protein's relationship to the membrane that accounts for the differing susceptibility to enzymatic destruction.

Gating in Decane-Containing vs. "Hydrocarbon-Free" Membranes

Colicin E1 is several orders of magnitude more active in "hydrocarbon-free" membranes than in so-called black lipid membranes (BLM's) containing decane. Figure 2*A* is a record showing two individual TrCF channels in a BLM at pH 3.5 (under conditions in which a hydrocarbon-free membrane of 1% the area would have approximately 10^3 channels). The channels flicker on a time scale of tens of milliseconds.

It is possible to convert a hydrocarbon-free membrane into a BLM by stirring into the bathing solutions a suspension of decane sonicated in buffer. The membrane takes up the decane and becomes thicker, as manifested by the decrease in its capacitance. This maneuver, if performed while the colicin E1 channels are held in the open state by positive voltage, has very little effect on total conductance (Fig. 2*B*). After the channels are turned off by negative voltage, however, they fail to turn on again in response to positive voltages (Fig. 2*B*). If the turn-off process involves part of the protein deinserting from the bilayer, as suggested by the previously described pepsin experiments, then the failure to turn on again may reflect the inability of part of the protein to reinsert into the thicker BLM. Notice, though, that individual channels in a BLM turn off and on rapidly (Fig. 2*A*), demonstrating that there must be more than one closed state. A channel in the "shallow" closed state of Fig. 2*A* turns on again in a few tens or hundreds of milliseconds. This state presumably represents a more subtle rearrangement of the protein than the "deep" closed state seen at negative voltage in Fig. 2*B*, from which most channels do not turn on again during the lifetime of the membrane.

Inactivation

At very low pH (<4) colicin channels exhibit several interesting properties not seen at higher pH. All of the voltage dependence of the steady-state conductance occurs between +30 mV and −30 mV; outside this range all the channels are either on (>+30 mV) or off (<−30 mV). [In fact, it is only at such low pH's that the macroscopic conductance ever reaches a steady-state value; at higher pH's the conductance increases indefinitively at positive voltages.] However, when the voltage is clamped at values above about +80 mV, channels begin to turn off into a different closed state—that is, they inactivate (Fig. 3). Inactivated channels turn on again with kinetics different from those of "off" channels, and do so in a way that depends upon the particular conditions of the experiment; they may never reappear (during the lifetime of the membrane), or they may reappear at a slow rate (but *see below*). The rate of inactivation from the open state is faster at larger voltages (*compare* Fig. 3*A* to *C*). It appears that to pass into the inactivated state, the channel must first enter the open state (*see* Fig. 3*B* and *C*). In particular, whereas a 3-msec pulse of +320 mV will inactivate about half of the channels from the open state, it has no discernible inactivating effect on channels in the closed state (Fig. 3*C*).[1] Inactivation occurs at the single channel level (Fig.

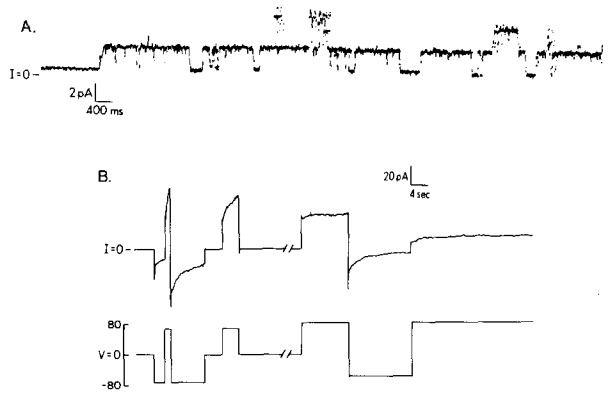

Fig. 2. The gating of colicin channels in bilayers containing decane. (*A*) This is a current record from a decane-containing black lipid membrane clamped at +60 mV. The membrane was made of asolectin and separated symmetric solutions of 1 M KCl, 5 mM DMG, 5 mM $CaCl_2$, pH 3.5. Prior to the section shown here, 0.2 μg of TrCF was added to the *cis* compartment. The record is unfiltered, but the recorder pen limits the response to about 100 Hz. (*B*) This membrane was made from asolectin monolayers and separated symmetric solutions of 100 mM KCl, 5 mM DMG, 5 mM $CaCl_2$, pH 4.5. ThCF was added to the *cis* compartment prior to the record shown here. The capacitance was continuously monitored on an oscilloscope with the concurrent application of a 1-kHz symmetric square wave (0.5 mV peak to peak). At the break in the record 200 μl of decane (30% in buffer, sonicated immediately before use) was added to the *cis* compartment, and a significant reduction in capacitance was observed. Note that the turn-off of conductance with a negative voltage pulse was comparable to that seen prior to the addition of decane, but the subsequent turn-on of conductance with a positive voltage pulse was meager in comparison

[1] This short, sharp shock was chosen because it was too short to turn closed channels on, but large enough to inactivate a significant percentage of open channels.

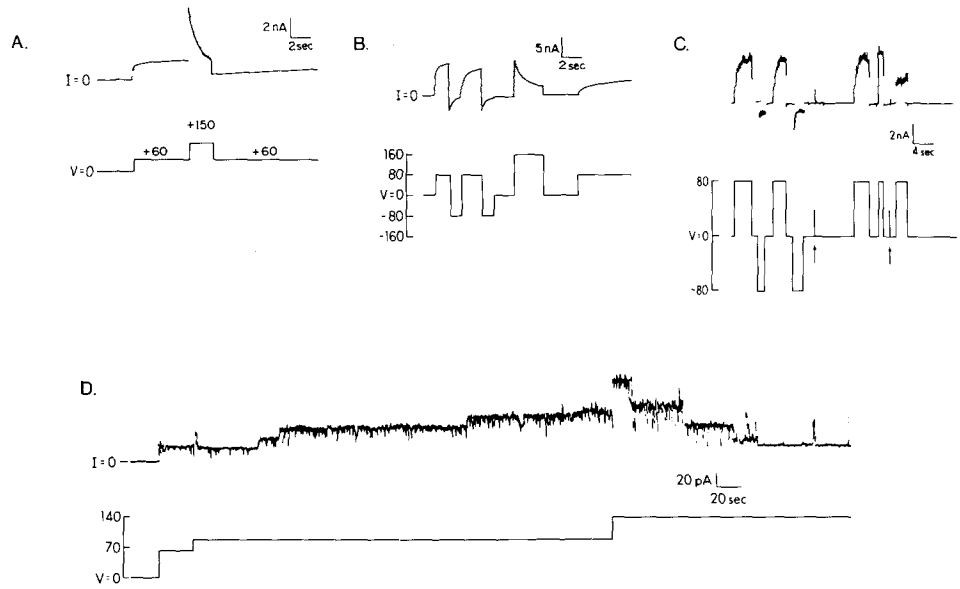

Fig. 3. Inactivation of colicin channels at large positive voltages. (*A*) Inactivation and its effect on subsequent turn-on rate. An asolectin membrane separated a solution of 1 M KCl, 5 mM $CaCl_2$, 12 mM DMG, pH 3.5, containing 70 ng ThCF, from a solution of 1 M KCl, 5 mM $CaCl_2$, 5 mM DMG, pH 4.5. The conductance had leveled off at +60 mV when the voltage was pulsed to +150 mV, which caused most of the channels to turn off (inactivate). When the potential was restored to +60 mV, they failed to turn on again with the usual kinetics (as for the original +60 mV pulse). (*B*) Closed channels first turn on before inactivating. An asolectin membrane separated a solution of 1 M KCl, 5 mM $CaCl_2$, 12 mM DMG, pH 3.5, containing 100 ng ThCF, from a solution of 1 M KCl, 5 mM $CaCl_2$, 5 mM DMG, pH 4.5. Positive and negative 80-mV pulses (which turn the channels on and off) were followed by a +160 mV pulse at a time when the channels were all off. Channels first turned on (at a rate too fast to resolve here) and began to inactivate. (*C*) A short inactivating pulse has no effect on closed channels. A diphytanoyl phosphatidylcholine/phosphatidylinositol (4:1) membrane separated symmetric solutions of 1 M NaCl, 5 mM $CaCl_2$, 10 mM DMG, pH 3.5. The *cis* compartment contained 5 ng of ThCF. At the beginning of the record channels were turned off and on with positive and negative 80-mV pulses. When the channels were off, the membrane was subjected to a pulse of +320 mV for 3 msec (first arrow). The channels turned on normally in response to a subsequent +80 mV pulse. The 3-msec pulse was then repeated with the channels on (second arrow), which inactivated about half of them. (*D*) Inactivation at the single channel level. An asolectin membrane separated symmetric solutions of 1 M KCl, 5 mM $CaCl_2$, 5 mM DMG, pH 3.5. The *cis* compartment contained 2 ng of TrCF. Individual channels turned off (inactivated) when the voltage was stepped to +140 mV

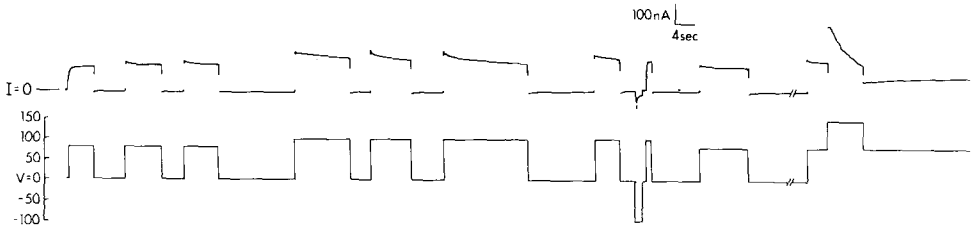

Fig. 4. Recovery from inactivation. An asolectin membrane separated symmetric solutions of 1 M KCl, 5 mM $CaCl_2$, 10 mM DMG, pH 3.4. 20 ng ThCF was added to the *cis* compartment before the section of record shown. The record shows a series of pulses of +80 and +100 mV. These potentials caused some inactivation, but the conductance recovered between the pulses. In contrast, a pulse to +150 mV prevented the conductance from reaching its former level when the potential was returned to +80 mV. *See* text for a more detailed description

3*D*), and therefore is not dependent on cooperative interaction among channels. Both whole colicin E1 and its active C-terminal fragments made by cleavage with enzymes or cyanogen bromide (Cleveland et al., 1983) are capable of inactivation.

Figure 4 demonstrates the existence of at least two different inactivated states. At the first +80 mV pulse, the conductance reaches a steady state; associated with that steady state is inactivation that is not manifested by a bi-phasic response, because of the overlap of the turn-on and inactivation kinetics. That inactivation has occurred is evidenced by the subsequent responses when the voltage is pulsed to 0 mV for a few seconds and then returned to +80

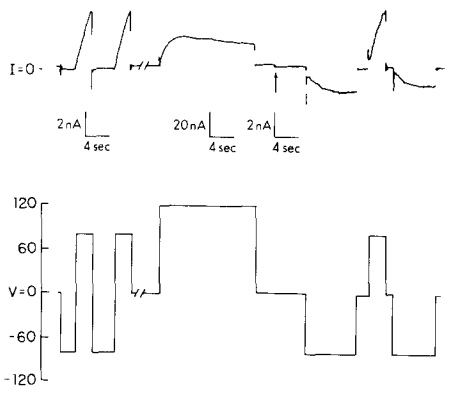

Fig. 5. Induction of reversed turn-on of conductance. An asolectin membrane separated symmetric solutions of 100 mM NaCl, 5 mM $CaCl_2$, 5 mM DMG, pH 3.5, with 10 ng ThCF added to the *cis* compartment. Note that the current scale changes twice; first at the break in the record, and then at the arrow. *See* text for an exegesis of the record

mV. The conductance is initially slightly higher than before, indicating a recovery at 0 mV from inactivation, but then it slowly declines (inactivates). Pulses of +100 mV lead to more inactivation, but recovery is still nearly total. Pulses to 0 mV for various times show that recovery from this "shallow" inactivated state is rapid; this contrasts to the "normal" off state, from which channels turn on very slowly at 0 mV. At larger voltages, more of the conductance inactivates, but into what appears to be a "deeper" inactivated state, from which channels do not recover at 0 mV under these conditions. Channels in the deeper inactivated state induced by higher voltages not only do not turn on at 0 mV, they turn on again only slowly, if at all, at normal positive stimulating voltages. This is seen in Fig. 4 by the failure of the conductance to rapidly turn on at +80 mV following the +150 mV inactivating pulse (*see also* Fig. 3*A-C*). The shallow inactivated state is further distinguished from the normal off state by the fact that the channels are protected from *cis* pepsin destruction in this state. (*Trans* pepsin does not destroy channels in either state.] In one experiment, for example, channels held 45 sec in the shallow inactivated state (at +80 mV) in the presence of *cis* pepsin all reopened within a second at 0 mV; that is, they had not been destroyed by the enzyme. In contrast, when these channels were subsequently turned off (at negative voltage) for the same length of time, only 20% of them could be reopened by positive voltages—the other 80% having been destroyed.

Reversal of Turn-On

Under the same conditions that allow inactivation, some fraction of the E1-induced conductance can reverse its voltage dependence, so that it appears that some of the colicin has been translocated to the *trans* side of the bilayer. This is shown in Fig. 5. At the beginning of the record, the colicin-induced conductance turns on and off (to essentially zero conductance) in response to positive and negative 80 mV pulses. Later, when the same membrane is clamped at +120 mV, some of the conductance inactivates. However, a subsequent pulse to −80 mV does not turn the conductance totally off, as it did at the beginning of the record. Instead, after a fast turn-off of most of the channels, the conductance increases again, leveling off at a value of about 15% of the prior, "normal" conductance observed at +80 mV. The subsequent +80 mV pulse leads to a fast turn-off of the reversed conductance, followed by (actually, concurrent with) the turn-on of the normally behaving channels. Note that the reversed conductance does not result simply from a failure of all the channels to turn off at −80 mV (as sometimes does occur in membranes as they age), but rather reflects a new, voltage-dependent subset of channels that appear to have been translocated across the membrane. Except for the signs of the voltages that turn them on and off, these channels behave in all other respects like normal channels and can also be inactivated by large "on" (in this case, negative) voltages (*data not shown*). The amount of reversed conductance depends upon the particular conditions of the experiment and on the voltage history of the membrane. NaCl media favor reversal compared to KCl. The phenomenon appears only subsequent to positive pulses that inactivate at least some of the original conductance, but in no case does all of the inactivated conductance reappear as reversed conductance, and often none of it does. In fact, the relationship between inactivation and reversed conductance is not entirely clear. Whole colicin E1 and its various C-terminal channel-forming fragments all exhibit this phenomenon, although the shorter fragments appear to do so more readily.

The reversed conductance could be due to some of the channels actually physically crossing the membrane and reappearing on the *trans* side, or it could reflect a more subtle rearrangement of the protein in the membrane. The experiment shown in Fig. 6 attempts to address this question. Normal E1 channels are destroyed by *cis* pepsin only when they are off and are not destroyed at all by *trans* pepsin (*see* above). Using this observation as a test for "*cis*ness," we measured the susceptibility of reversed conductance to pepsin to see if the responsible channels behave as if they are on the *trans* side of the membrane. In the experiment, a population of colicin channels was first run through a pulsing regime designed to produce some reversed conductance. The normal conductance was then turned off

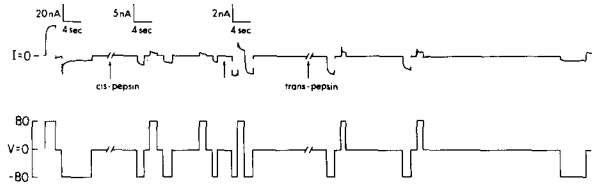

Fig. 6. The effect of pepsin on reversed conductance. An asolectin membrane separated symmetric solutions of 1 M NaCl, 5 mM $CaCl_2$, 20 mM DMG, pH 3.5, with 100 ng ThCF in the *cis* compartment. At the beginning of the first break in the record (which lasted 3 min), 5 mg pepsin was added to the *cis* compartment. At the beginning of the second break in the record (which lasted 1 min), 5 mg pepsin was added to the *trans* compartment. (During the 1-min break at 0 mV, most of the conductance remained on and was not affected by the pepsin, as evidenced by the fact that the current at −80 mV after the break is almost as large as that just before.) The unlabeled arrow marks the second change in current scale. (Because the current record is highly filtered, the transients are damped, so that the fast part of the turn-off in going from −80 to +80 mV is missed). *See* text for a fuller description

with a pulse of −80 mV (the turn-on of the reversed conductance is barely visible at the low gain), and pepsin was then added to the *cis* compartment. Within 3 min (the length of the break in the record) this resulted in the destruction of all the normal conductance, leaving only the small reversed conductance, that appears to be protected from pepsin action from the *cis* compartment. Pepsin was then added to the *trans* compartment, and gradually destroyed these channels when they were in the off state. In similar experiments, we found that *trans* pepsin had no apparent effect on the reversed conductance as long as it was turned on. This is exactly the behavior expected if the reversed conductance is due to channels that have crossed the bilayer.

Discussion

Colicin E1 and several of its C-terminal fragments form voltage-gated channels in lipid bilayer membranes. Although it has not been possible to determine the number of gating charges, n, from equilibrium or steady-state conductance measurements, kinetic data suggest an approximate value of $n = 4$ (*data not shown*). Schein et al. (1978) estimated a value of $n = 5$ to 7 for colicin A channels, which are thought to be gated by a similar mechanism. These results mean that several formal charges must cross the transmembrane field to gate the channel. Conceptually, the simplest way to accomplish this is for several charged amino acid residues in the protein to be driven across the membrane by the field. This, however, requires a major translocation of the protein, exposing different domains to the *cis* and *trans* solutions in the different channel states, and appears *a priori* unlikely on energetic grounds. Nevertheless, in this paper we present evidence that this apparently is what occurs in colicin channel gating.

The principal evidence for this conclusion comes from experiments utilizing enzymatic digestion by proteases as a probe for exposed protein on either side of the bilayer when the channel is in one of its particular states. These experiments were, for the most part, carried out at low pH (pH 3.5), because it is only under these conditions that the colicin E1-induced conductance reaches a steady state. The conductance can then be reproducibly turned on and off with voltage, and any changes induced by proteases are readily observed. Pepsin was the protease of choice because of the low pH.

Our basic finding is that channels in the closed state are destroyed by pepsin addition to the *cis* side but not to the *trans* side[2]; open channels are refractory to pepsin action from either side. This result is consistent with the gating mechanism proposed by Cleveland et al. (1983); namely, E1 binds to the bilayer by inserting its 35 amino acid-long hydrophobic domain into the bilayer, but does not form an open pore until more "upstream" protein is inserted in a voltage-dependent step(s). An alternative interpretation is that gating represents a more subtle change in protein conformation that just happens to expose a pepsin-cleavable site only to the *cis* side in the closed state. While we cannot rule this out, it seems unlikely in view of the other results presented here (and in the next paper). Similar experiments which exposed colicins A, Ia, and Ib to the nonspecific protease pronase gave results similar to the E1/pepsin experiments. These colicins show significant similarity to E1 (Varley & Boulois, 1984) and probably gate in the same way. Pronase (and pepsin too, for that matter) is sufficiently nonspecific that it is unlikely that a particular site on each protein is responsible for these results, especially since the actual protein sequences are quite dissimilar. More likely, any part of the protein not actually in or near the membrane is vulnerable to the enzymes.

[2] Soluble E1 can be treated with various proteolytic enzymes, including pepsin, without destruction of its channel-forming ability; in fact, such digestion leaves a C-terminal peptide of approximately 20 kD which contains the channel-forming region (Cleveland et al., 1983). Our report here that the channel-forming activity of membrane-bound protein can be destroyed by pepsin is no contradiction, since the protein is expected to have a vastly different conformation when associated with the bilayer compared to that in solution. [In fact, the shorter C-terminal channel-forming fragment generated by cleavage with cyanogen bromide is easily destroyed by proteases, suggesting that part of the colicin protein not involved in channel formation protects the "latent" channel in the soluble state (Cleveland et al., 1983).]

The Cleveland et al. (1983) interpretation of E1 gating is also consistent with channel activity and behavior in decane-containing (BLM) bilayers. Thus, colicin presumably binds to BLM's, but is faced with an even larger energy barrier to the insertion of upstream segments of the protein because of the thicker hydrophobic core of the BLM. Consequently, colicin activity is orders of magnitude lower in BLM's than in hydrocarbon-free bilayers. Once fully inserted (either by the trick of converting a hydrocarbon-free membrane to a BLM by addition of decane to the medium, or by direct insertion into a BLM at drastically reduced efficiency), the channel behaves more or less normally until it is turned off into the closed state, from which it cannot easily reinsert.

The only effect of pepsin detected in our experiments was the destruction of channel activity when the channels are closed. We cannot conclude from these results, however, that pepsin does not act on colicin when the channels are in the open state. In this state colicin is protected from pepsin action only in the sense that any change induced by pepsin was not detectable under the conditions of the experiment. Since several peptides derived from whole colicin E1, and whole E1 itself, all make very similar channels, it is quite possible that pepsin does digest away part of the protein when the channel is in the open state, but does not alter the channel-forming region of the protein. The next paper discusses this point in detail.

We have also found that (at low pH) E1 channels that are open will enter an inactivated state at large positive voltages. That these nonconducting channels are not in the usual closed state(s) achieved with negative voltages is clear from the fact that they do not turn on again with the voltage-dependent rates characteristic of closed channels. Channels inactivated by large positive voltages (>+100 mV) have very long recovery times, and may never return to the open state. Under some inactivating circumstances the voltage dependence of colicin E1 channels is reversed. That is, channels turn on in response to negative potentials and turn off in response to positive potentials. Their kinetic behavior is identical to "normal" channels, except that the voltage signs are reversed. It appears as though the channel-forming domain of the molecule has been completely translocated across the membrane, and the pepsin experiments support this. That is, these reversed channels are now attacked by *trans* pepsin, when the channels are closed, but not by *cis* pepsin. It is interesting that channels formed by a large protein such as colicin E1 appear to gate in a manner reminiscent of the mechanism proposed for channels formed by much smaller molecules, such as monazomycin (Heyer, Muller & Finkelstein, 1976), in that they respond to the field by penetrating into and through the bilayer. (An important difference, however, is that, unlike E1, many identical molecules of monazomycin, each presumably spanning the membrane, are required to form a pore. While the molecularity of the E1 channel is unknown, it is likely to be one or two molecules/channel, with each molecule contributing several transmembrane segments.) Whether there are eucaryotic channels that share this mechanism is not known. There certainly are numerous examples of a requirement for a transmembrane potential to effect insertion of proteins into bacterial and mitochondrial membranes (*see* Wickner & Lodish, 1985). In the following paper we pursue further the issue of protein translocation associated with the gating of colicin E1 channels and its relevance to the general issue of protein insertion into and through cell membranes.

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