

Modification of a Voltage-Gated K^+ Channel from Sarcoplasmic Reticulum by a Pronase-Derived Specific Endopeptidase

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ABSTRACT A K^+ -selective membrane conductance channel from rabbit sarcoplasmic reticulum (SR) is studied in an artificial planar phospholipid bilayer. Membranes containing many such channels display voltage-dependent conductance, which is well described by a two-state conformational equilibrium with a free energy term linearly dependent on applied voltage. Pronase-derived alkaline proteinase b (APb), when added to the side of the membrane opposite to the SR vesicles (*trans* side), reduces the voltage dependence of the K^+ conductance. Single-channel fluctuation experiments show that after APb treatment, the channel is still able to undergo transitions between its open and closed states, but that the probability of forming the open state is only slightly voltage-dependent. In terms of the conformational model, the enzyme's primary effect is to reduce the effective gating charge of the opening process by over 80%; a second effect of APb is to reduce the internal free energy of opening from +1.2 to +0.4 kcal/mol. The kinetics of APb action are strongly voltage-dependent, so as to indicate that the enzyme can react only with the channel's open state. The results imply that the channel contains a highly charged polypeptide region which moves in the direction perpendicular to the membrane plane when transitions between the open and closed states occur. A lysine or arginine residue in this region becomes exposed to the *trans* aqueous solution when the channel is in its open conformation.

The use of proteolytic enzymes to modify proteins involved in ion conduction across electrically excitable membranes has become a valuable technique in studying these biochemically elusive systems (Sevcik and Narahashi, 1975). The most dramatic example of this approach is the effect of pronase on the Na^+ channel of the squid axon membrane. Pronase specifically destroys the channel's inactivation process while leaving intact the activation mechanism and, by implication, the conducting structure of the channel (Rojas and Armstrong, 1971; Armstrong et al., 1973). Rojas and Rudy (1976) showed that this activity of pronase (a mixture of at least seven proteolytic enzymes) resides in alkaline proteinase b (APb), a trypsin-like endopeptidase with extremely high specificity for lysine and arginine residues (Narahashi, 1970;

Olafson and Smillie, 1975). These studies have led to a model of the Na^+ channel as containing localized structures (usually called "gates") which normally cause the channel to open or close in response to changes in applied voltage. Pronase is postulated to remove one of these, the "inactivation gate," by cleavage at a critical basic amino acid.

In this report we will describe the effect of pronase on a voltage-gated K^+ channel from sarcoplasmic reticulum (SR) of mammalian skeletal muscle. This channel is studied by incorporating SR vesicles into a planar phospholipid bilayer and performing voltage-clamp conductance measurements on the resulting hybrid membrane (Miller and Racker, 1976; Miller, 1978; Miller and Rosenberg, 1979 *a*). Membranes containing many channels display voltage-dependent conductance for certain monovalent cations (Miller, 1978). In membranes containing only a small number of channels, unitary conductance fluctuations are observed (Miller, 1978), showing that the channel exists in only two electrically distinguishable states, "open" and "closed." The channel is blocked by Ca^{++} (Miller, 1978) and Cs^+ (Coronado and Miller, 1979), and it is modified by sulfhydryl ligands (Miller and Rosenberg, 1979 *a*). We refer to this system as a K^+ channel, since it is about four times more conductive to K^+ than to Na^+ ,¹ and since K^+ is the overwhelmingly abundant cation on both sides of the SR membrane *in vivo* (Somlyo et al., 1977). We will show that alkaline proteinase b purified from pronase affects the process by which the channel undergoes transitions between its open and closed states; we will throughout refer to this process as the "gating" of the channel. A preliminary report of this work has been presented (Miller and Rosenberg, 1979 *b*).

MATERIALS AND METHODS

Biochemical

Preparation of SR vesicles from rabbit back and leg muscle, and of phospholipids from beef heart has been described (Miller and Rosenberg, 1979 *a*). Mixed soy phospholipids (asolectin) were purchased from Sigma Chemical Co., St. Louis, Mo., and used without further purification. Pronase "type VI" from Sigma, a mixture of proteolytic enzymes of *Streptomyces griseus*, was dialyzed overnight against 10 mM Na-acetate, 5 mM CaCl_2 (pH 5.3), and was stored in aliquots at -70° . Clostripain and submaxillaris protease were obtained from Boehringer-Mannheim Biochemicals, Indianapolis, Ind., and trypsin from Sigma. Protein was measured according to Lowry et al. (1951), with bovine serum albumin as standard and 25 mM Na-deoxycholate included in the reaction.

Alkaline proteinase b was purified from crude pronase by chromatography on carboxymethyl-cellulose (Whatman CM-11, Whatman Inc., Clifton, N.J.), followed by chromatography on carboxymethyl-Sephadex (Pharmacia Fine Chemicals, Piscataway, N.J.) (Narahashi, 1970). Fractions were assayed for trypsin-like amidase activity using α -*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as substrate (Erlanger et al., 1961). Total recovery of activity was 70–80%, and the specific activity was 15–20-fold higher after purification. The resulting preparation displayed two bands on sodium dodecyl sulfate-polyacrylamide gels, a major band corresponding to APb, and

¹ Coronado, R. Unpublished observation.

a faint band corresponding to carboxypeptidase; this latter enzyme was inactive under our conditions, since it had been denatured during chromatography by prolonged exposure to EDTA (Narahashi, 1970). Amidase activity of the final preparation was totally inhibited by *N*- σ -*p*-tosyl-L-lysine chloromethyl ketone (TLCK) using the preincubation conditions of Rojas and Rudy (1976). The enzyme preparation was concentrated by membrane filtration and was stored at 10–20 mg/ml in 100- μ l aliquots at -70° .

Electrical

The planar bilayer system, described in detail previously (Miller and Rosenberg, 1979 *a*), was of the Mueller and Rudin (1969) type. The system consisted of two identical aqueous solutions separated by a phospholipid bilayer, about 0.8 mm in diameter. The electrical conductance of the membrane was measured under voltage-clamp conditions at room temperature, 20–22 $^{\circ}$. The aqueous phase of the system was: 10 mM morpholinopropane sulfonic acid (MOPS) 0.1 mM EDTA, containing either 0.1 M K^{+} -glucuronate, Cs^{+} -glucuronate, or Tris- Cl^{-} , for measurement of K^{+} , Cs^{+} , Cl^{-} conductances, respectively (Miller, 1978). Solutions were adjusted to pH 7.0 with Tris base.

SR vesicles were inserted into the planar bilayer by a process resembling Ca^{++} -induced membrane fusion (Miller and Racker, 1976). Two types of experiments were performed: macroscopic measurements, in which many channels (1,000–10,000) are incorporated into the membrane, with a concomitant large conductance increase (three to four orders of magnitude); and single-channel fluctuation experiments, in which the insertion of only a single SR vesicle (containing 5–50 channels) is permitted (Miller, 1978). In this latter case, the average membrane conductance increases by approximately a factor of 2, and the fluctuations in K^{+} conductance, due to the random opening and closing of individual channels, are examined at high current gain. In all experiments, the *cis* side of the membrane is defined as the side to which SR vesicles are added; voltage is defined as zero on the opposite side of the membrane, the *trans* side.

Experiments were performed using both phosphatidylethanolamine/diphosphatidylglycerol (PE/DPG) and asolectin membranes. The results were qualitatively identical with the two lipid compositions, but there were quantitative differences which made it advantageous to use asolectin membranes for displaying certain results and PE/DPG for displaying others. K^{+} channels in asolectin membranes begin to saturate at lower voltages than in PE/DPG, and so most experiments on the conductance-voltage relation were done with asolectin. On the other hand, the kinetics of enzyme action were “cleaner” in purified PE/DPG.

Theory

In order to present an analysis of the effect of proteolytic enzymes of the K^{+} channel from SR, we must develop a theoretical basis for understanding the voltage-dependent phenomena reported here. We choose the simplest model of the channel compatible with all of our data: a conformational equilibrium between two states, open and closed, in which the equilibrium constant has a voltage-dependent term. This term arises because the two conformations of the channel are assumed to differ in their spacial distribution of charge. This theory follows closely that developed to explain the behavior of the bacterial ionophore excitability-inducing material (EIM) (Ehrenstein et al., 1974; Alvarez et al., 1975; Ehrenstein and Lecar, 1977) and of a mitochondrial anion channel (Schein et al., 1976), except that in our case, the K^{+} channel is absolutely oriented in the bilayer, unable to undergo “flip-flop,” and that

the channel operates by a two-state mechanism, which is not the case for the other channels above.

If a fixed number, N , of channels is incorporated irreversibly into the bilayer, then the equilibrium fraction of channels in the open state, $\theta(V)$, at voltage V is given by (Ehrenstein and Lecar, 1977):

$$\theta(V) = [1 + \exp(\Delta G_i + zFV)/RT]^{-1}, \quad (1)$$

where F , R , T have their usual meanings, z is the effective gating charge of the opening transition, and ΔG_i is the "internal free energy of opening," i.e., that part of the free energy of channel opening not due to externally applied voltage.

In order to apply Eq. 1 to our measurements, we must consider two complications of our system. First, in addition to K^+ channels, SR vesicles also induce a small ohmic background conductance in the planar bilayer (Miller, 1978; Miller and Rosenberg, 1979 *a*). Second, since it is desirable to compare data from different membranes, a convention for normalizing the conductance is necessary. We have found it most convenient to normalize measured conductances to the background conductance, g_b . Thus, the total membrane conductance, $g(V)$ is:

$$g(V) = g_b + \gamma N \theta(V), \quad (2)$$

where γ is the value of single-channel conductance. We may define the "relative channel activity," $\Gamma(V)$, which is normalized and corrected for background, as:

$$\Gamma(V) \equiv \frac{g(V)}{g_b} - 1. \quad (3)$$

Now, combining the above equations, we arrive at an expression useful for determining the model parameters ΔG_i and z from measured conductances:

$$\ln\left(\frac{\Gamma_m}{\Gamma} - 1\right) = \Delta G_i/RT + zFV/RT, \quad (4)$$

where Γ_m is the maximum saturation value of Γ , representing the level of conductance with all channels open. Thus, a logarithmic plot of $(\Gamma_m/\Gamma - 1)$ against voltage determines both z and ΔG_i , and also tests whether the data do in fact fit such a simple theory.

In practice, g_b is measured directly at voltages more negative than -70 mV (Miller, 1978), but Γ_m cannot be directly determined since the membranes become quite unstable at highly positive voltages. For determination of Γ_m , the raw conductance data are converted to values of relative channel activity, Γ , according to Eq. 3. These values are then plotted logarithmically, as given by Eq. 4, using different values of Γ_m . Least-squares linear regression analysis is then performed on each plot, and the correlation coefficient is computed. The value of Γ_m giving the maximum correlation coefficient is then used. This method, if applied to asolectin membranes with data points up to $+85$ mV, can easily determine Γ_m to within 10% for a given membrane. Experience has shown us that while the value of Γ_m varies by as much as a factor of 3 from membrane to membrane, the calculated parameters ΔG_i and z are reproducible to within $\pm 10\%$ for a given set of conditions; furthermore, we have found that ΔG_i is sensitive to membrane lipid composition, but z is not.²

² Miller, C., C. Nakamura, and R. Coronado. Unpublished observation.

RESULTS

Macroscopic Conductance Experiments

Membranes containing many SR K^+ channels display voltage-dependent steady-state K^+ conductance (Miller, 1978). At applied voltages more negative than -50 mV, the membrane is in a low-conductance state, representing a poorly understood background conductance induced by SR vesicles. As the voltage is varied in the positive direction, the membrane conductance rises due to the increasing probability of channel opening (Miller, 1978). As applied voltage approaches highly positive values ($> +50$ mV), the beginning of saturation in the conductance is seen, as if the probability of channel opening approaches unity. This behavior is shown in Fig. 1, which also illustrates that the g - V curve is well described by a simple two-state model in which the open and closed states of the channel exist in a voltage-dependent conformational equilibrium (see Theory section). For this membrane, the effective gating charge of the conformational transition is -1.1 , which is in good agreement with the value determined from compiled data on five different membranes, -1.2 ± 0.1 SEM (The negative sign of z indicates that the opening process becomes more favored as voltage is made increasingly positive.)

Fig. 2 introduces the basic phenomenon with which this report is concerned; that pronase, when added to the *trans* compartment, modifies the K^+ conductance of a bilayer doped with SR K^+ channels. Initially, the conductance at $+50$ mV is about 4.5 times that at -50 mV; over a time of several minutes at $+50$ mV applied potential, pronase causes an increase in conductance of about 1.6-fold. In addition, after this reaction has run to completion, the conductance at -50 mV has increased four-fold. Thus, the net effect of pronase under these conditions is to increase the absolute conductance of the membrane and to reduce the relative voltage sensitivity of the conductance. If pronase is preincubated with TLCK, a specific active site inhibitor of trypsin-like proteases (Keil, 1971), no modification of membrane conductance occurs (Table I). This led us to suspect that the component of pronase responsible for the above effect is alkaline proteinase b, the only lysine/arginine-specific protease known to exist in pronase (Narahashi, 1970).

Upon fractionating the crude pronase, we recovered over 70% of the BAPNA-amidase activity and about 120% of the planar bilayer activity in a single peak corresponding to Narahashi's (1970) APb fraction. Fig. 3 confirms that purified APb added to the *trans* chamber and allowed to react with SR-doped bilayers at $+50$ mV has an effect similar to that of crude pronase. The enzyme causes an increase in $+50$ mV conductance; after the reaction has proceeded to completion, the -50 mV conductance is also seen to have increased.

We have also found (data not shown) that long periods of reaction with *trans* APb (>20 min) result in a slow decline in K^+ conductance at both $+50$ and -50 mV. This decline appears to level off at 20–40% inhibition, but the effect is variable, and we have not studied it in depth. In all experiments to be reported, reaction times with APb were < 10 min.

A striking effect of voltage on the *trans* APb reaction is shown in Fig. 4,

which presents an experiment identical to that of Fig. 3, except that the applied voltage is held at -50 mV. In this case, the enzyme causes no discernible increase in -50 mV conductance, even after 11 min of incubation with the membrane. At this time, the voltage is switched to $+50$ mV; the

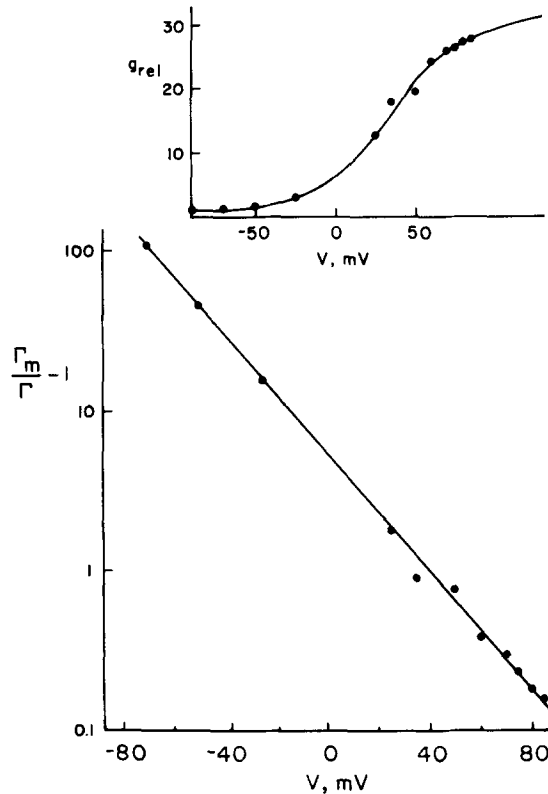


FIGURE 1. Conductance-voltage relation of a planar bilayer containing K^+ channels of SR. SR vesicles were incorporated into asolectin planar bilayers in the presence of 0.8 mM Ca^{++} until the K^+ conductance had increased by about three orders of magnitude. Incorporation was then stopped by addition of 1 mM EDTA. Steady-state conductance at a given voltage was determined by pulsing to the measuring voltage for 10 s and recording the steady-state current, as described (Miller, 1978). In the upper graph, conductance, g_{rel} , is normalized to the conductance at -80 mV, which is a good approximation to the background conductance. The solid curve is drawn according to Eqs. 1 and 2, with $G_i = +1.0$ kcal/mol, $z = -1.1$ electronic charges. These data, when plotted on a reciprocal-log plot, Eq. 4, resulted in a straight line over the entire range (lower graph). For this membrane, $g(-80) = 1.0 \mu\text{mho/cm}^2$, which is a value typical of all experiments reported here.

conductance at this new voltage immediately after the jump is identical to its value before addition of APb. However, the $+50$ mV conductance begins to increase at once, with a rate similar to that in Fig. 3. If the conductance at -50 mV is now assayed by short pulses to this voltage during the APb

reaction, it too is seen to increase over several minutes. Thus, we can conclude that the proteolytic reaction modifies both the +50 and -50 mV conductance, but that the reaction is unable to proceed at an appreciable rate at -50 mV.

In order to compare the action of APb and other proteases under various conditions, it is necessary to develop a quantitative assay of the enzyme effect on the K^+ conductance. Experience with the system has shown us that the relative rate of increase in +50 mV conductance is *not* reproducible enough for such an assay; the reason for this variation is that +50 mV is rather close

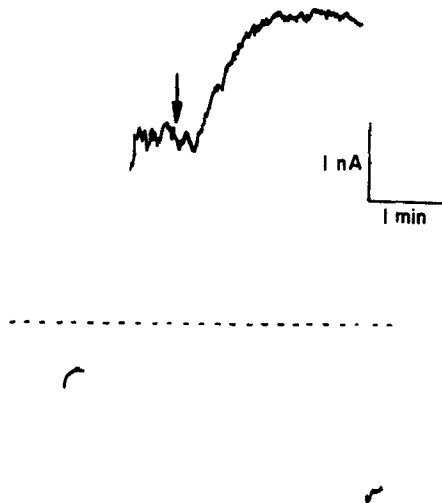


FIGURE 2. Effect of *trans* pronase on SR K^+ channels. SR vesicles were incorporated into planar bilayers composed of 70% PE/30% DPG as described in the text. After incorporation, control values of conductance were recorded, and then pronase (0.5 mg/ml) was added (at arrow) with constant stirring, to the chamber. The figure displays the current response at applied voltages of +50 mV (current trace above zero level) and -50 mV (current trace below zero level indicated by dashed line). The lag of 10–20 s after pronase addition is always seen and is due to stirring time and diffusion of enzyme through the unstirred layers.

to the “crossover voltage” of the APb effect (see below, Fig. 8), and small variations in the position of the conductance-voltage curve give rise to large changes in the rate of +50 mV conductance change. We have found, however, that the rate of change of -50 mV conductance provides an assay of enzyme activity with very low variation from membrane to membrane. In order to perform such an assay, the enzymatic reaction must be run at +50 mV, as was shown in Fig. 4.

Therefore, the protocol of the quantitative enzyme assay is as follows (Fig. 5). First, control values of conductances at +50 and -50 mV are obtained. Voltage is held at -50 mV during enzyme addition and for 40 s thereafter, to allow complete mixing of the *trans* chamber. No change in conductance occurs

during this time at -50 mV. The voltage is then jumped to $+50$ mV to begin the enzymatic reaction and hence define zero time. Every 25 s thereafter, the voltage is pulsed back to -50 mV for 5 s to assay the effect of the enzyme on

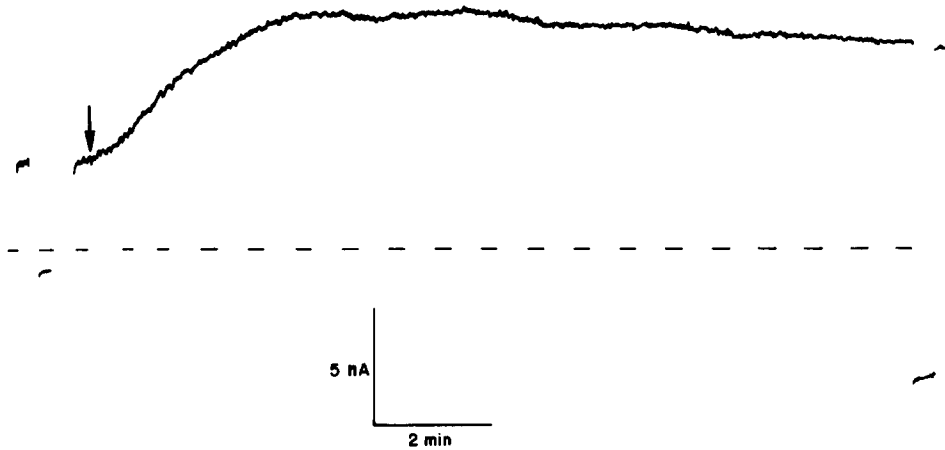


FIGURE 3. Effect of *trans* alkaline proteinase b, $+50$ mV. Conditions were as in Fig. 2, except that at the arrow, alkaline proteinase b (APb) was added to a final concentration of $40 \mu\text{g/ml}$. Throughout the enzymatic reaction, voltage was held at $+50$ mV. After 14 min, the -50 mV conductance was measured, as shown. The 20–30-s lag time after addition of enzyme is due to stirring of the chamber and diffusion through the unstirred layers to the membrane.

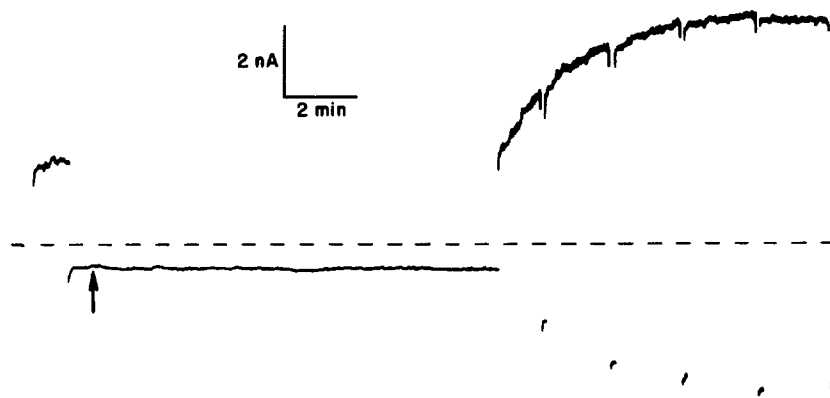


FIGURE 4. Effect of *trans* APB, -50 mV. Conditions were as in Fig. 3, except that APb ($40 \mu\text{g/ml}$) was added while the applied voltage was held at -50 mV. Voltage was held at -50 mV for the next 11 min, and then was jumped to $+50$ mV. At 1-min intervals thereafter, voltage was returned to -50 mV for 5-s pulses. Note that after the initial jump to $+50$ mV, there is no lag in the effect of enzyme.

the -50 mV conductance. The initial rate of this -50 mV conductance increase is used to define the activity of the enzyme. If $g(t)$ represents the -50 mV conductance at time t , then we define a “planar bilayer unit” as that

amount of enzyme needed to double the -50 mV conductance in one minute, i.e., such that:

$$\frac{[dg(t)/dt]_{\text{init}}}{g(0)} = 1 \text{ min}^{-1}, \quad (5)$$

where the subscript indicates that we take the initial rate of conductance

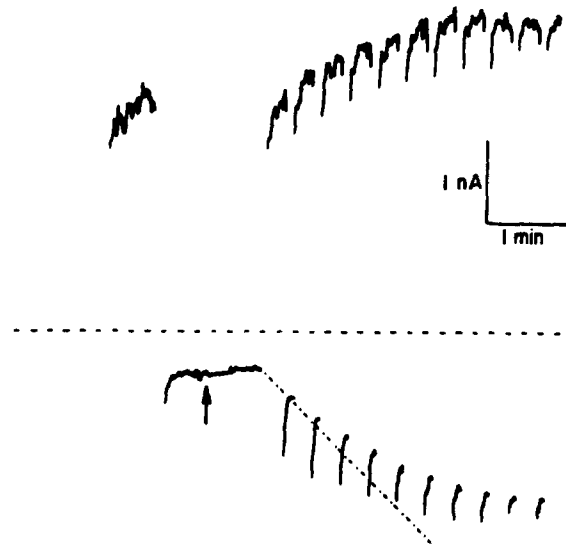


FIGURE 5. Quantitative assay of APb activity. Conditions were as in Fig. 4, except that in this experiment, APb was added (arrow) to the *trans* chamber to a final concentration of $52 \mu\text{g/ml}$. For the quantitative assay, enzyme was always added at an applied voltage of -50 mV, and this voltage was maintained for 40-s after enzyme addition, to allow diffusion through the unstirred layers to proceed to completion. Zero time is defined by the jump to a "holding voltage" of $+50$ mV. After this jump, voltage was returned to -50 mV for a 5-s "assay pulse" at intervals of 25 s. (This pulse time is sufficiently long to establish steady-state conductance.) The initial rate of conductance increase was measured (dashed line) and was used to calculate the specific activity of the enzyme, as described in the text. The initial rate measured as shown on the graph is corrected for the time spent at -50 mV, at which voltage the enzymatic reaction rate is negligible. This is a 25% correction for the pulse sequence shown here.

increase. With this definition of unit of activity, we define specific activity as activity per concentration of enzyme (milligram/milliliter) in the aqueous compartment. This definition of specific activity is justified by the fact that under a given set of conditions (temperature, lipid composition, batch of APb, etc.), the APb activity increases linearly with concentration, up to at least $80 \mu\text{g/ml}$, as shown in Fig. 6.

All of the experiments described above were done with enzyme added to the *trans* side of the membrane. It is remarkable that APb added to the *cis* chamber has no such effect on membrane conductance (Fig. 7). In the first

10 min after 40- $\mu\text{g}/\text{ml}$ APb addition, very little change in conductance at either +50 or -50 mV is seen, in contrast to the *trans* effect. For long periods of *cis* APb incubation (>20 min), we have occasionally seen a 10-30% diminution of conductance at +50 mV (data not shown), but this effect has

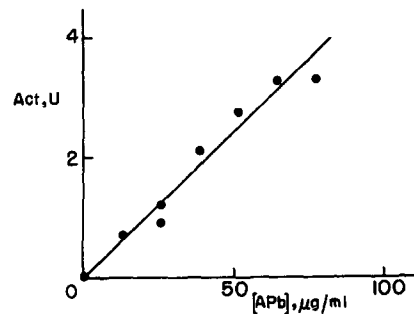


FIGURE 6. Concentration dependence of APb activity. APb activity was determined exactly as in Fig. 5, except that varying concentrations of APb, indicated on the abscissa, were added to the *trans* chamber. Each point represents a single determination on a separate membrane. Specific activity for these conditions was 48 ± 3 units per mg/ml.

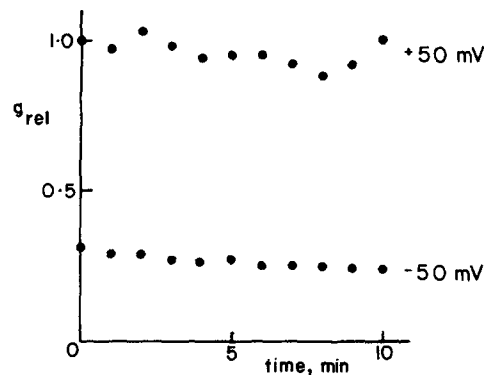


FIGURE 7. Effect of *cis* APb, +50 and -50 mV. Conditions were as in Figs. 2-4, except that at zero time, APb was added to the *cis* compartment, to a final concentration of 40 $\mu\text{g}/\text{ml}$. Conductance is plotted normalized to the value at +50 mV before enzyme addition. Data were taken from a separate membrane for each voltage.

been variable in our hands, and we have not studied it further. Pronase added on the *cis* side also fails to modify the K^+ conductance under conditions giving full effect from the *trans* side (data not shown).

Before we can assert that the action of APb is a specific modification of the K^+ channel, we must present several control experiments to rule out the

possibility that the enzyme creates a separate conductance pathway in parallel with the channel. In Table I, the planar bilayer activity is tabulated under various conditions. The following points should be noted. (a) The enzyme operates from the *trans* side of the membrane only. Besides further establishing the asymmetric orientation of the channel within the bilayer (Miller, 1978; Miller and Rosenberg, 1979 a), this experiment shows that the conductance increase is not due to the action of the enzyme on the lipid phase of the bilayer. (b) The enzyme has no effect on the Cl^- or the Cs^+ conductance induced by SR. It is known that neither of these ions can permeate the K^+

TABLE I
ENZYME ACTIVITIES UNDER VARIOUS CONDITIONS USING
PLANAR BILAYER ASSAY

Enzyme	Conductance measured	Additions	Specific activity
			<i>units per mg/ml</i>
Pronase	K^+	—	3
Pronase	K^+	TLCK	<0.3
APb	K^+	—	96
APb	K^+	TLCK	<5
APb, <i>cis</i> addition	K^+	—	<1
APb	Cs^+	—	<3
APb	Cl^-	—	<1
Trypsin	K^+	—	1.7

Specific activities of various enzyme preparations were measured in 70% PE/30% DPG membranes, via the planar bilayer assay described in the text, using a holding voltage of +50 mV. Concentrations of enzymes used were: pronase, 0.5 mg/ml; APb, 30 $\mu\text{g}/\text{ml}$; trypsin, 0.2 mg/ml. Each determination represents averages of two or three membranes. Control experiments were performed to ensure that BAPNA-amidase activity of APB was not adversely affected by the solutions used to measure Cs^+ and Cl^- conductance.

channel (Miller, 1978; Coronado and Miller, 1979), but are markers of the background conductance in the system. (c) An additional demonstration of the specificity of the APb effect is based on the fact that Ag^+ is a potent irreversible inhibitor of the K^+ channel (Miller and Rosenberg, 1979 a), but not of the background conductance. It is therefore pertinent to ask whether the additional K^+ conductance induced by APb is also inhibited by Ag^+ . Table II shows that this is the case; at -50 mV, APb stimulates the K^+ conductance 3.8-fold, and Ag^+ reduces the conductance fully to the background level. We take the results above as strong indications that APb exerts its effect by action on the K^+ channel, and not via a parallel system.

We may now investigate the mode of APb action on the K^+ channel. In Fig. 8 we study the effect on the macroscopic *g-V* curve of asolectin membranes. Before enzyme addition, a strongly voltage-dependent K^+ conductance is observed, as in Fig. 1 (which contains more extensive data in the highly

positive voltage region). For the compiled data of Fig. 8, the effective gating charge is -1.2 ; after APb treatment, the K^+ conductance has lost most of its voltage dependence, the effective gating charge being -0.19 . Notice that above -55 mV, APb actually causes a decrease in conductance. (This observation rules out entirely the possibility that the enzyme creates a parallel conductance pathway.)

Single-Channel Fluctuation Experiments

We may summarize the effect of APb as the conversion of a voltage-dependent rectifier into a nearly ohmic conductance (Fig. 8). This is merely a description of the effect, not an explanation. There are two ways in which the result of Fig. 8 could come about. One possibility is that the enzyme has the same

TABLE II
EFFECT OF Ag^+ ON APb-INDUCED K^+ CONDUCTANCE

Addition	$g(-50)$
	<i>nmho</i>
None	5.0
APb	19.0
Ag^+	3.5

An asolectin membrane containing K^+ channels was treated with $25 \mu\text{g/ml}$ *trans* APb for 10 min at $+50$ mV, after its initial conductance at -50 mV, $g(-50)$, was measured (top row). After complete reaction with enzyme, the conductance was measured again (middle row). An addition of $50 \mu\text{M}$ $AgNO_3$ was then made on the *trans* side; inhibition was complete within the stirring time of the membrane chamber (Miller and Rosenberg, 1979 *a*), and the conductance was again measured (bottom row). The conductance after Ag^+ is equal to the background conductance, measured at -80 mV.

action postulated for its effect on the Na^+ channel of squid axon: that it removes the voltage-sensitive gate. Stated in terms of the conformational model, the effect would be to cause the channels all to lock into the open state and never to be able to enter the closed state. If this explanation were true, we would expect the conductance of the APb-treated membrane to approach the saturation level of the control membrane. This is not the case; instead, the conductance after APb treatment is about half of this. We could therefore postulate that the enzyme has two effects on the channel: That it removes the gate as postulated above, and that it also reduces the single-channel conductance of the open state by about half.

Alternatively, there is a second explanation for the action of APb. It is possible that the enzyme does not remove the gate, but that it reduces its ability to sense the electric field within the membrane. In other words, the enzyme would uncouple the gate from the applied voltage, while still allowing the channel to fluctuate between its open and closed states.

We may decide between these two hypotheses by examining the action of APb via single-channel fluctuation experiments in which we directly observe transitions between open and closed states of individual channels. Previous work (Miller, 1978) has shown that the K^+ channel fluctuates between two

distinguishable states, open and closed; the closed state conductance is at most 5% of the open-state conductance.³ The applied voltage controls the probabilities of opening and closing but does not affect the conductance of the open state (Miller, 1978; Coronado and Miller, 1979). Therefore, by observing the fluctuation behavior before and after APb treatment, we can test the two

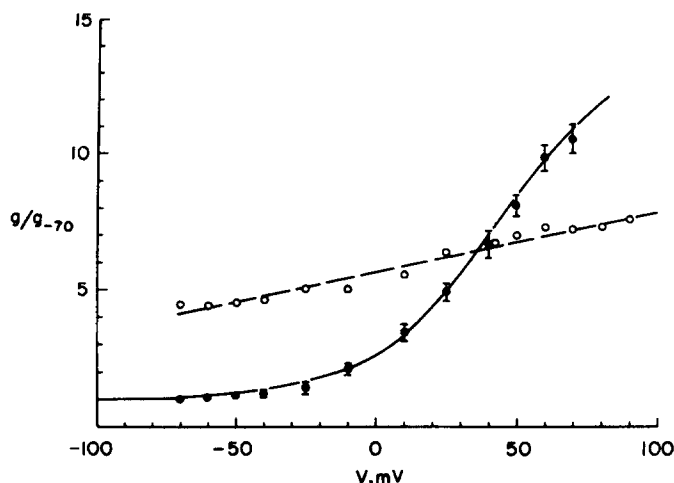


FIGURE 8. Effect of APb on conductance-voltage relation. The g - V curves for K^+ conductance in asolectin membranes before and after complete reactions with APb were measured as in Fig. 1. (●) Compiled data from eight different membranes before APb addition; (○) data from two different membranes after treatment with APb (25 μ g/ml, +50 mV, 10 min, *trans* addition). All data are normalized to the value of conductance measured at -70 mV before APb addition. Points with standard error bars represent the means of six to eight determinations; open points are individual measurements. The solid curve is drawn according to Eq. 2; the dashed curve is also drawn in this way, with the additional constraint that both asymptotes at $-\infty$ and $+\infty$ mV be the same as for the solid curve. (This is equivalent to the two assumptions that (a) the enzyme does not affect the single-channel conductance or the number of channels in the membrane, and (b) that the enzyme does not affect the background conductance.) For the g - V curve before APb, $\Delta G_i = +1.2$ kcal/mol, and $z = -1.2$; for the curve after APb, $\Delta G_i = +0.4$ kcal/mol, and $z = -0.19$.

possibilities above. The first explanation predicts that the enzyme should eliminate all fluctuations, since it simply removes the gate and opens all the channels. The second explanation predicts that at -50 mV, for instance, APb should increase the frequency of fluctuations, but that the open-state conductance should remain the same.

Fig. 9 demonstrates that the second explanation is correct. Here, single-channel fluctuations are observed at -50 mV, a voltage at which normally a given channel only rarely finds itself in the open state. In the control trace, a

³ Miller, C. Unpublished observation.

10-s segment of data is displayed. This is not a representative control trace, because of the occurrence of the very rare event of two channels opening during the same 10-s segment. This trace is displayed here to show that the channel size is well defined. After a short, 90-s APb treatment, the probability of forming an open channel is markedly increased, and occasionally two

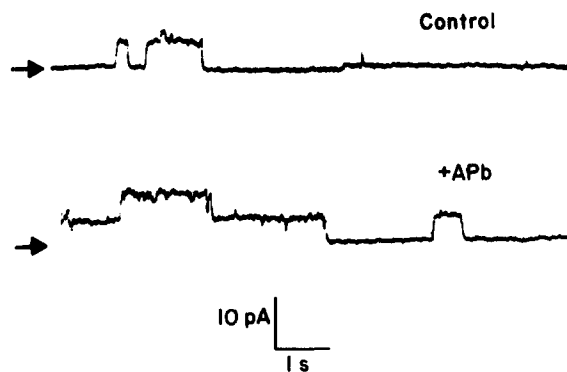


FIGURE 9. Stimulation of single-channel fluctuations by APb. Single-channel fluctuations of K^+ conductance were observed in an asolectin bilayer by adding a low concentration ($1 \mu\text{g}/\text{ml}$) of SR vesicles to the bilayer system in the presence of a low concentration (0.2 mM) of Ca^{++} . After the incorporation of a single vesicle, 0.5 mM EDTA was added to stop further incorporation, and K^+ conductance was observed at -50 mV for 70 s , a 10-s segment of which is shown in the top trace. This segment is not representative of the control traces, in that it is the only trace in which two fluctuations (rather than one or zero) were observed. After the control traces were recorded, APb ($25 \mu\text{g}/\text{ml}$) was added to the *trans* chamber, with stirring, and voltage was switched to $+30 \text{ mV}$ for 90 s , to allow the enzymatic reaction to proceed. Voltage was then returned to -50 mV , and conductance fluctuations were recorded for 40 s . The lower trace shows a representative 10-s segment of data. Arrows mark the "zero-level conductance," i.e., the conductance corresponding to all channels closed. In this experiment, the zero-level conductance was 110 pmho both before and after enzyme; it was checked by continuous recording of conductance at low time resolution on chart paper, and by addition of $20 \mu\text{M}$ CH_3HgCl to the *trans* chamber at the end of the experiment (Miller and Rosenberg, 1979 *a*).

channels are open simultaneously. All the data from this experiment are summarized in the conductance histograms of Fig. 10. These show that the APb treatment increases the probability of the appearance of a single open channel about fourfold. Enzyme treatment has no effect on single-channel conductance, which is 110 pmho before enzyme and 111 pmho after. For the data of Fig. 10, the time-averaged conductance due to channel activity increases 6.2-fold after 90 s of APb treatment. This is a larger relative increase than is seen in the macroscopic experiments, which include a background contribution that is effectively subtracted out in the fluctuation measurements.

We have also performed a few experiments at $+50 \text{ mV}$, a voltage at which

it is much more difficult to detect clean single-channel fluctuations, because of the high time-averaged conductance. Table III summarizes our measurements of single-channel conductance before and after APb treatment at both +50 and -50 mV; we conclude that, within the accuracy of our measurements

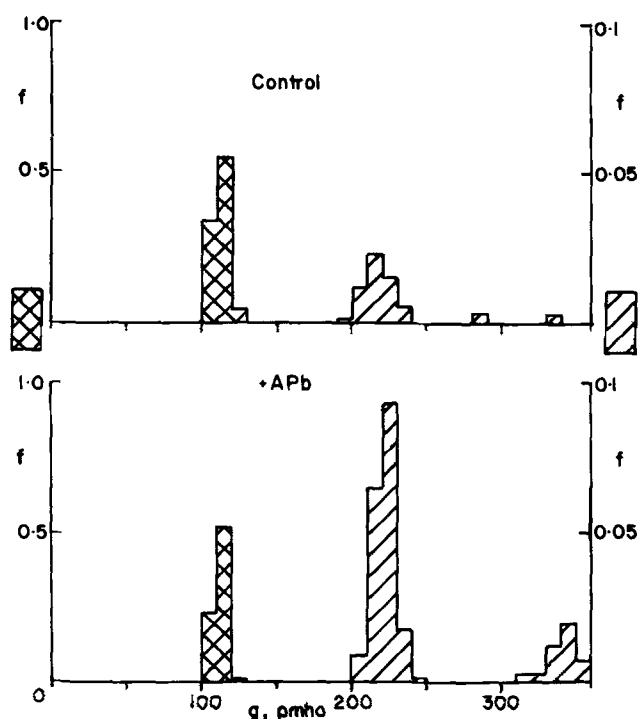


FIGURE 10. Effect of APb on conductance histograms. The data of Fig. 8 were analyzed by copying the oscilloscope photographs on xerographic transparencies, and mounting these on an enlarger with micrometer attachment. Conductance was sampled every 60 ms, and these values were tabulated. The frequency of appearance of conductance in 10-pmho intervals was then calculated, and this is plotted in the figure. Total time for control records was 70 s and for APb records was 40 s. The time-averaged conductance above the zero-level, measured by integrating the histograms, is 5 pmho before APb and 31 pmho after APb. Note that different frequency scales are used for the zero-level conductance (double-hatched bars, left-hand scale), and the higher levels (single-hatched bars, right-hand scale).

(which is poor at +50 mV), the enzymatic reaction has no effect on single-channel conductance at either voltage.

The important point to be made from the combined macroscopic and single-channel fluctuation experiments is that APb does not remove the voltage-sensitive gate. Instead, it uncouples the gate from the intramembrane electric field. After reaction with enzyme, the channel still fluctuates between open and closed states, but the probability of forming the open state is only

weakly voltage-dependent. Finally, similar single-channel experiments (not shown) using Na^+ rather than K^+ as the conducting ion show that APb treatment does not affect the K^+ -over- Na^+ selectivity of the open channel.

Voltage Dependence of APb Kinetics

An intriguing aspect of the reaction of APb with the K^+ channel is the dependence of the reaction rate on applied voltage. This behavior is quite dramatically apparent in Fig. 4, which shows a high rate of conductance increase induced by exposure to enzyme at +50 mV, but less than 5% of this rate at a holding voltage of -50 mV. The simplest explanation for this voltage

TABLE III
VALUES OF SINGLE-CHANNEL CONDUCTANCE BEFORE AND AFTER APb TREATMENT

Condition	Applied voltage	γ
	<i>mV</i>	<i>pmho</i> \pm <i>SEM</i> (<i>n</i>)
Control	-50	111 \pm 2 (75)
Control	+50	117 \pm 5 (4)
After APb	-50	112 \pm 2 (50)
After APb	+50	118 \pm 3 (14)

Values for single-channel conductance, γ , at -50 mV were calculated from the data of Fig. 9, in which the conductance in the first level was sampled randomly for 75 or 50 points. Data at +50 mV were taken in two cases in which a very small number of channels was incorporated, allowing clear unitary fluctuations to be observed at this voltage. The heights of these fluctuations were measured directly from the oscilloscope photographs, and these were averaged. APb treatment was carried out for 90 s, as in Fig. 8.

dependence is that the enzyme can react only with the open state of the channel, that the residue susceptible to cleavage is not accessible to the aqueous APb when the channel is closed. If this explanation is correct, the rate of the reaction should vary in quantitatively the same way as does the steady-state probability of forming an open channel. This latter quantity is directly proportional to the macroscopic K^+ conductance (corrected for background). Fig. 11 shows that this predicted parallel voltage dependence of enzyme activity and K^+ channel conductance is in fact observed. In the range -60 to +50 mV, both quantities vary approximately exponentially with voltage, with a 10-fold increase for each 78 (± 5) mV and 69 (± 6) mV, respectively. We consider this to be strong evidence that APb cannot react appreciably with the closed state of the channel.

DISCUSSION

We are attempting to characterize a K^+ -selective, electrically gated channel from a mammalian muscle membrane which is inaccessible to direct electrical measurement *in situ*. Our strategy for attacking this problem has been to incorporate native SR membrane vesicles into a phospholipid bilayer, and to

study the electrical properties conferred upon this artificial membrane. Using this approach, we have described some of the most basic properties of the K^+ channel. (Miller, 1978) and have found several ways of modifying these properties (Miller and Rosenberg, 1979 *a*; Coronado and Miller, 1979). In this report, we describe a profound effect of a specific endopeptidase upon the

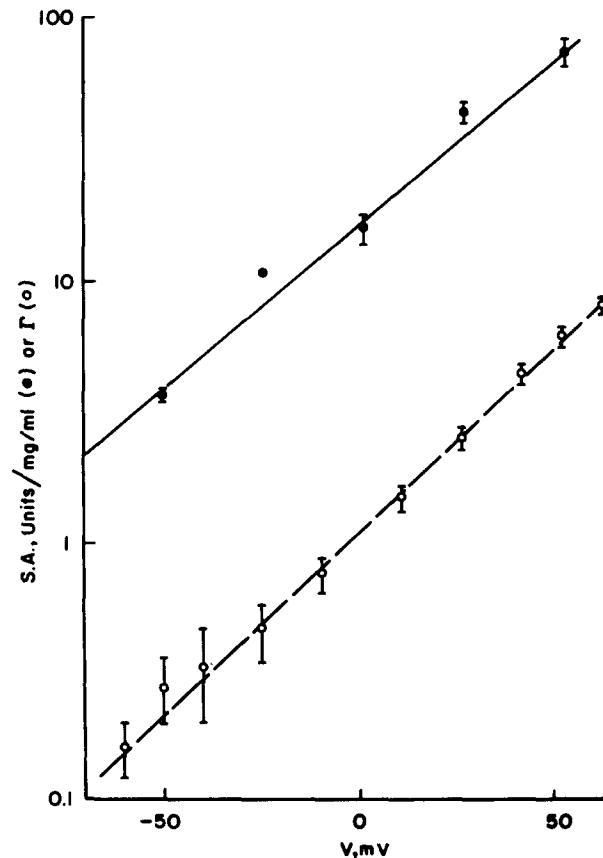


FIGURE 11. Variation of APb activity and of K^+ conductance with voltage. Measurements of K^+ conductance and APb specific activity were carried out on 70% PE/30% DPG membranes, into which SR K^+ channels had been inserted as in Fig. 1. The steady-state g - V curves were measured on eight different membranes, and relative channel activity, Γ , defined by Eq. 3, is plotted against voltage (\circ). Each point represents the mean \pm SE of three to five determinations. In a parallel series of experiments under identical conditions, the specific activity of APb was measured as described in Fig. 6 and in the text, except that the holding voltage was varied. Specific activity is plotted against holding voltage (\bullet). Points represent mean \pm SE of three to five determinations, each one on a different membrane. The point without error bars is a single determination. Data are plotted logarithmically for convenience of display; there is no theoretical significance to the lines drawn.

gating properties of the channel. In order to facilitate the interpretation of the APb effect, it was necessary to present a simple two-state conformational model for the channel; therefore, before we discuss the effect of the enzyme, we should review the postulates upon which Eq. 1 is based.

The crucial assumptions of this theory are: (a) that the channel operates by a mechanism involving only a single "open" state, (b) that channels are incorporated irreversibly into the membrane and are not lost to the torus or to the aqueous phase, (c) that the single-channel conductance is voltage-independent, (d) that the channels are asymmetrically oriented in the membrane, and (e) that a simple conformational equilibrium, rather than a subunit aggregation mechanism, can account for the formation of an open channel.

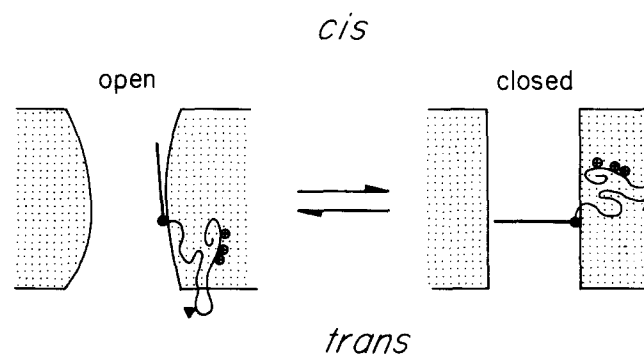


FIGURE 12. Model of SR K^+ channel gating. A schematic representation of the "open" and "closed" states of the channel is given, showing a highly charged region of the gate which is susceptible to cleavage by APb at dark triangle. The APb-susceptible region is drawn to be exposed to the aqueous phase on the *trans* side only when the channel is in the open conformation. The electrical charges on the gate are drawn such that the equilibrium will be driven towards opening as the *cis* side is made increasingly positive with respect to the *trans* side.

The first four of these assumptions have been verified directly (Miller, 1978; Miller and Rosenberg, 1979 *a*; Coronado and Miller, 1979; Coronado and Miller⁴); the last assumption is difficult to test directly, but we consider it to be valid because neither the steady-state conductance parameters (Miller, 1978) nor the kinetic constants of the gating process (Labarca and Miller⁵) are dependent on the total concentration of channels in the membrane. Until faced with evidence against this simple model, we will use it for the analysis of channel behavior.

Effect of Alkaline Proteinase b

Our results demonstrate that APb derived from pronase attacks the K^+ channel from the *trans* side of the membrane and as a result of the cleavage renders the channel's opening and closing probabilities much less sensitive to applied voltage. The enzyme leaves the open-state conductance unchanged.

⁴ Coronado, R., and C. Miller. Unpublished observation.

⁵ Labarca, P., and C. Miller. Unpublished observation.

The single-channel fluctuation experiment of Fig. 9 shows that after APb treatment the channel is still capable of undergoing transitions between the open and closed states, and so we conclude that the enzyme has reduced the coupling of the channel's gating process to the intramembrane electric field. In terms of the conformational model, the primary effect of APb is to reduce the effective gating charge from -1.2 to -0.19 . The residual voltage dependence after enzyme treatment is always present, and is not eliminated by longer reaction times, so we consider it to be a real expression of a non-zero gating charge in the modified state. The enzyme also affects the internal free energy change, ΔG_i , reducing this parameter from $+1.2$ to $+0.4$ kcal/mol. This effect may be a result of small differences in chemical interactions between the control and enzyme-treated channels.

It is not difficult to imagine how a proteolytic enzyme might reduce the effective gating charge of the channel. Fig. 12 offers a schematic model of the conformational differences between the open and closed states. If a large contribution of the protein's dipole moment comes from a highly charged structure which moves within the membrane when the conformational change occurs, then the cleavage of this part of the protein would still allow the gate to open and close, but the coupling of this movement to the electric field would be lost.

We do not seriously propose that the gate is a localized structure which plugs up the channel as shown. However, the analysis of the APb effect leads us to conclude that there does exist some kind of structure, close to the *trans* face of the membrane, coupled to the movement of a considerable electric charge. This charged structure must move in a direction perpendicular to the membrane as the channel opens and closes.

The present results do not rule out a more complicated model of APb action. It might be argued that the K^+ channel possesses two gates in series, one which is voltage-dependent and one which is voltage-independent. It could then be argued that APb does remove the voltage-dependent gate, thus rendering the channel voltage-independent but still able to open and close. This explanation would be consistent with our single-channel results. This model is untenable, however, because it predicts that at highly positive voltages (at which the voltage-sensitive gate would be open most of the time) the gating of the unmodified channel should be dominated by the voltage-independent gate. This is not the case; at $+50$ – $+80$ mV in membranes containing only one channel, we see that the channel is open nearly all the time, and only occasionally closes.¹ The frequent fluctuations predicted by the two-gate model are not observed.

Voltage Dependence of the APb Activity

The above discussion refers to the voltage dependence of the final effect of the APb reaction with the K^+ channel. The rate at which the reaction proceeds is also sensitive to the applied voltage, as is shown in Fig. 11. The simplest explanation of this result is that the enzyme can react only with the open state of the channel. This hypothesis is supported strongly by the parallel dependence upon voltage of the enzymatic reaction rate and the probability of the

appearance of the open state. In other words, the reaction is second-order, the two kinetic components being the enzyme (as evidenced by the constancy of specific activity with enzyme concentration) and the channel's open state. We take this observation as a demonstration of the occurrence of a fairly extensive conformational change as the channel fluctuates between its two states. Fig. 12 is drawn to show schematically how the APb-susceptible region of the channel might be exposed to the aqueous phase only in the open state. As a final note, we should mention that we have previously presented evidence indicating that mersalyl added on the *trans* side of the membrane irreversibly inactivates the channel by reacting with a sulfhydryl group accessible only when the channel is open (Miller and Rosenberg, 1979 *a*).

Enzyme Specificity

Because of the high specificity of APb (Narahashi, 1970; Olafson and Smillie, 1975) we can identify the region modified as containing a lysine or arginine residue. That it is this particular enzyme, and not a co-purifying contaminant, which gives the effect is shown by the complete inhibition of the reaction by TLCK, a specific active-site inactivator of trypsin-like proteases; furthermore, purified trypsin gives the same effect as APb, although its specific activity is much lower (Table I) than that of the bacterial enzyme. We have attempted and failed to modify the channel using the arginine-specific enzymes clostripain (Mitchell and Harrington, 1970) and submaxillaris protease (Schenkein et al., 1977). This negative result cannot be taken as evidence that a lysine rather than arginine residue is attacked by APb, since these enzymes may not be accessible to the vulnerable site. Finally, we should mention that our results taken along with the previous identification of a critical sulfhydryl group (Miller and Rosenberg, 1979 *a*), lead us to claim for the first time that the K^+ channel is a protein. This is not an entirely trivial assertion, since it is known that certain phospholipids (Hayashi et al., 1978), lipid vesicles (Düzgünes and Ohki, 1977), and polyethers (Schlieper and DeRobertis, 1977) can induce in planar bilayers ion-selective conductances and single-channel-like conductance fluctuations.

Finally, we should mention the similarity between APb action on the SR K^+ channel and on the inactivation process of the squid axon Na^+ channel. In both cases, cleavage at an exposed arginine or lysine residue modifies the gating properties. It has been postulated that the enzyme actually removes the Na^+ channel's inactivation gate, (Rojas and Armstrong, 1971; Armstrong et al., 1973; Rojas and Rudy, 1976). This is clearly not the effect of APb on the SR K^+ channel, since we observe single-channel fluctuations after enzyme treatment.

It is not impossible, however, that the action of APb on the Na^+ channel of squid axon is not the removal of the gate, but rather an uncoupling of the gate from the electric field. If after enzyme treatment the inactivation gate were able to close, say 20% of the time, independently of applied voltage, no shutting-off of Na^+ conductance would be observed in a voltage-clamp experiment; instead, the steady-state conductance would be 20% lower than the theoretical maximum. This idea is consistent with the failure of pronase

to increase the steady-state conductance fully to the level predicted by Hodgkin-Huxley theory (Rojas and Rudy, 1976; Bezanilla and Armstrong, 1977). Since it is not yet possible to perform single-channel experiments on the squid axon, direct tests of this possibility cannot be made; nor have any noise spectra, which might be able to detect the effect, been published for pronase-treated axons. Therefore, we submit the "uncoupling" hypothesis as a possible mechanism of pronase action on the Na⁺ channel.

We have a serious reservation about our suggestion, however. Armstrong and Bezanilla (1977) have argued convincingly that the Na⁺ channel's inactivation process is not voltage-dependent, in contrast to its formulation according to Hodgkin and Huxley (1952). If this were the case, our explanation of the pronase effect could not be correct. The resolution of the question must await further improvements in electrophysiological techniques, or the reconstitution of the active Na⁺ channel in planar bilayer membranes, to enable the performance of experiments, such as those reported here, for the K⁺ channel of SR.

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