Destruction of Sodium Conductance Inactivation in Squid Axons Perfused with Pronase

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ABSTRACT We have studied the effects of the proteolytic enzyme Pronase on the membrane currents of voltage-clamped squid axons. Internal perfusion of the axons with Pronase rather selectively destroys inactivation of the Na conductance (g_{Na}). At the level of a single channel, Pronase probably acts in an all-or-none manner: each channel inactivates normally until its inactivation gate is destroyed, and then it no longer inactivates. Pronase reduces $\bar{g}_{Na}$, possibly by destroying some of the channels, but after removal of its inactivation gate a Na channel seems no longer vulnerable to Pronase. The turn-off kinetics and the voltage dependence of the Na channel activation gates are not affected by Pronase, and it is probable that the enzyme does not affect these gates in any way. Neither the K channels nor their activation gates are affected in a specific way by Pronase. Tetrodotoxin does not protect the inactivation gates from Pronase, nor does maintained inactivation of the Na channels during exposure to Pronase. Our results suggest that the inactivation gate is a readily accessible protein attached to the inner end of each Na channel. It is shown clearly that activation and inactivation of Na channels are separable processes, and that Na channels are distinct from K channels.

INTRODUCTION

The ionic channels of nerve membrane and the gates that control ion movement through them are widely supposed to be composed of protein, but there is surprisingly little evidence on the question. (*a*) Some proteases cause marked prolongation of the action potential (Tasaki and Takenaka, 1964; Rojas and Atwater, 1967; Takenaka and Yamagishi, 1969) which suggests that they alter the channels or their gates. (*b*) Shrager et al. (1969), working with crayfish axons, found that protein crosslinking reagents cause very long duration plateau action potentials. Membrane conductance is high during the plateau, and they concluded that Na inactivation had been partially or

wholly destroyed. Formaldehyde, also a protein crosslinking reagent, has a very similar action on frog skeletal muscle (Dominguez and Hutter, 1969). (*c*) Experiments with artificial bimolecular membranes have shown that the peptide gramicidin-A can form transmembrane ionic channels (Hladky and Haydon, 1970; Urry et al., 1971; Krasne et al., 1971), and the not yet characterized protein EIM (excitability-inducing material) can confer on bilayers a form of excitability (Mueller and Rudin, 1963; Bean et al., 1969; Ehrenstein et al., 1970).

We report here strong evidence that at least one of the ionic gates, Na inactivation, is composed of protein. Internal perfusion of squid axons with the proteolytic enzyme(s) Pronase completely and rather selectively destroys inactivation of g_{Na} (Rojas and Armstrong, 1971) and has no marked effect on the channels themselves or on their activation gates. Our evidence also makes it clear that (*a*) activation and inactivation of g_{Na} are separable processes, and (*b*) Na channels and K channels are distinct from each other.

METHODS

Experiments were performed in Chile on cleaned axons from *Dosidicus gigas* and in Woods Hole using axons from *Loligo pealei*. Most of the experiments for which records are presented were performed using a chamber with three significant new design features. (*a*) At either end of the chamber the axon passed through close-fitting holes in a plastic wall, whose function was to isolate the ends and minimize the effects of irregularities outside the guard region (see Fig. 1). (*b*) The trough of the chamber could be made quite narrow (the sides of the chamber were movable) to make resistance to longitudinal current flow as high as possible. Ideally, all current flow from a space-clamped axon is radial, and high longitudinal resistance helps to eliminate longitudinal components of current. (*c*) The guard electrodes on one side were made from a silver block, with an inset central electrode for current measurement. The exposed surfaces of the block and of the central electrode were platinized. The block was in direct contact with the cold plate of a Peltier cooler (Cambion, model 7240-01) providing an efficient cooling path. Temperature was measured by a thermistor in the chamber floor, and the signal from the thermistor was used to control current through the Peltier device by means of a feedback system. This system maintained temperature easily within 0.1°C of the set level. External fluid was continuously exchanged, at several chamber volumes per minute.

Axons were perfused internally by a modification of the Tasaki method (Tasaki et al., 1962). For details see Rojas and Ehrenstein, 1965 and Bezanilla and Armstrong, 1972.

Pronase (*Streptomyces griseus* protease) was obtained from Calbiochem, San Diego, Calif. (Pronase, grade B; Pronase CB; Pronase, nuclease free) or from Sigma Chemical Co., St. Louis, Mo. (protease type VI). In most cases, a fresh solution of the enzyme was made up a few minutes before the commencement of each experiment. Names and compositions of solutions are given in Table I. In the text, the external solution will be given, then two slashes followed by the internal solution.

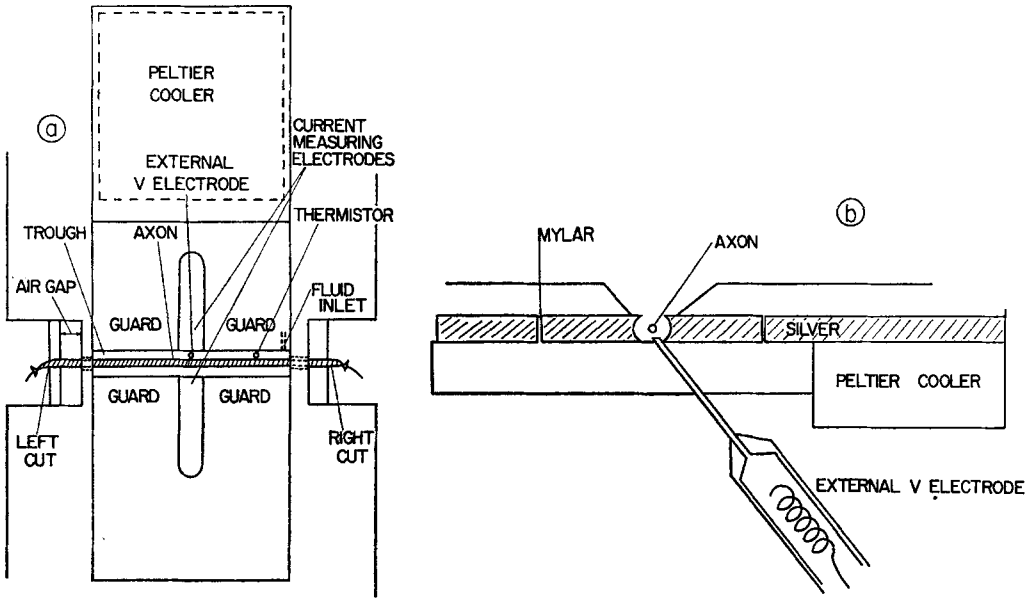

FIGURE 1. (*a*) Top view of voltage-clamp chamber. The axon is mounted horizontally and the perfusion canula enters at the cut in the right end, the dual electrode at the left cut. (*b*) Cross section through the center of the chamber.

TABLE I
SOLUTIONS

Name	Na^+	K^+	Ca^{2+}	Mg^{2+}	TEA^+	Cl^-	F^-	Br^-	Sucrose	TTX
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*M*
ASW	440	0	10	50		560				
ASW-TTX	"	"	"	"		"				$1\text{-}2 \times 10^{-7}$
60 K SW	380	60	"	"		"				
275 K		275					275		400	
275 K-50 Na-15 TEA	50	"			15		"	15	325	

Terminology

From the Hodgkin-Huxley equations, $g_{Na} = g_{Na}m^3h$ (Hodgkin and Huxley, 1952). We shall refer to m^3 as the activation factor, and to h as the inactivation factor. m^3, in this terminology, gives the fraction of the Na channels that have open activation "gates," and h gives the fraction that have open inactivation gates. Both gates must be open in order for a channel to conduct. A K channel has only a single gate, the K activation gate. ("Gate" is used for convenience. No physical picture is implied.) The ionic currents I_{Na} and I_K (potassium current) are related to the conductances

by the equations (Hodgkin and Huxley, 1952)

$$I_{Na} = g_{Na}(V - V_{Na})$$
$$I_K = g_K(V - V_K),$$

where V is membrane voltage and V_{Na} and V_K are the equilibrium potentials for Na and K ions.

RESULTS

Pronase destroys inactivation of the Na channels but leaves the activation process essentially intact, as illustrated in Fig. 2. Part *a* of the figure shows the voltage-clamp currents of a tetraethylammonium ion (TEA^+) poisoned

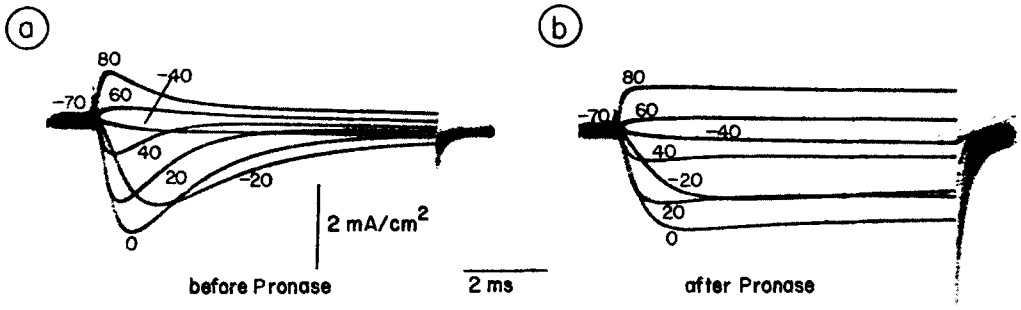

FIGURE 2. Destruction of Na inactivation by Pronase. The traces show membrane current (predominantly I_{Na}) for depolarization from −70 mV to the voltages indicated, before, and after 360 s of internal perfusion with 2 mg/ml protease VI (Sigma Chemical Co.) at 10°C. Photographs at 3°C. ASW//275 K-50 Na-15 TEA.

axon before addition of Pronase to the internal perfusion medium. For relatively small depolarizations I_{Na} (Na ion current) is inward, and it increases in magnitude with a sigmoid time-course that is determined (in the Hodgkin and Huxley description) mainly by the Na activation gates (see Methods for terminology). After a millisecond or less, most of the activation gates are open and the current is at its maximum. As the inactivation gates close, current then diminishes almost to zero. Upon repolarization, there are very small tails of inward current through those channels that are still open, but these are quickly extinguished by closing of the activation gates. For large depolarizations beyond V_{Na} current is outward in direction but shows the same activation-inactivation sequence, with the differences that (*a*) current peaks earlier, and (*b*) inactivation is less complete than for smaller depolarizations (cf. Chandler and Meves, 1970). After perfusing the axon internally with Pronase for a few minutes, Na inactivation is almost completely absent (Fig. 2 *b*). I_{Na} still activates with a sigmoid time-course, though apparently more slowly, and current magnitude is somewhat reduced, as further described below. On repolarization there are large inward tails of current, for

all of the Na channels are still open and repolarization suddenly increases the driving force, $(V - V_{Na})$. This tail current quickly decays as the activation gates close.

The destruction of Na inactivation by Pronase is progressive, and after a brief period of treatment a portion of I_{Na} inactivates normally and the remainder inactivates slowly or not at all. This is illustrated in Fig. 3 which shows I_{Na} $(V = 0)$ after 128, 448, and 724 s of perfusion with Pronase. After 128 s, inactivation is still virtually complete, while after 724 s there is no inactiva-

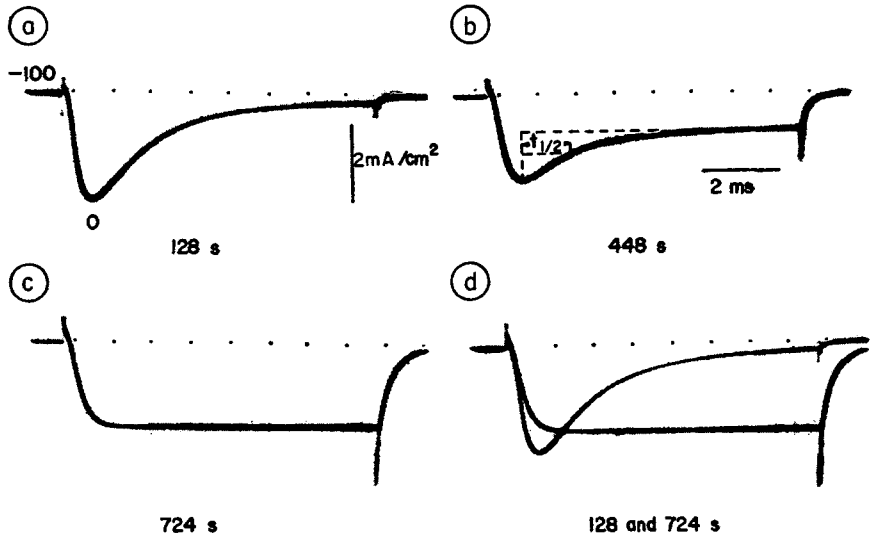

FIGURE 3. Inactivation kinetics are not affected by Pronase. All traces are for depolarizations to $V = 0$ mV from a prepulse potential of -100 mV after perfusion with 1 mg/ml Pronase CB for the times indicated, at 8°C. The half time of inactivation is the same whether inactivation is complete or partial. (*d*) Traces from *a* and *c* superimposed. ASW//275 K-50 Na-15 TEA.

tion, and one sees a large tail of inward current on repolarization which is absent in the 128 s trace. At 448 s I_{Na} inactivates partially, with a half time (see Fig. 3 for method of measurement) that is almost exactly the same as in the record at 128 s. Half times from a number of similar experiments in which inactivation was partially destroyed are listed in Table II, and in no case did Pronase significantly alter the kinetics of the remaining fraction of inactivation. The rate of action of Pronase depended on perfusion rate and was somewhat variable, but in most cases inactivation was almost completely destroyed after 10 min of perfusion with 1 mg/ml Pronase at 8°–10°C.

Pronase and the Activation Gates of the Na Channels

In all experiments except one, the absolute rate of rise of g_{Na} $(=I_{Na}/[V - V_{Na}])$ and its maximum amplitude were decreased by Pronase, as seen

TABLE II
HALF TIMES OF INACTIVATION

$t_{1/2}$	V_m	$\frac{I_{peak}}{I_{pulse\ end}}$	Temperature
			°C
0.88	0	0.01	8
0.83	0	0.53	8
0.93	0	0.05*	8
0.93	0	0.48	8
0.83	0	0.04	8
0.75	0	0.56	8
0.88	0	0.05	8
0.75	0	0.52	8
1.26	0	0.12*	8
1.20	0	0.48	8

Brackets enclose measurements from a single axon.
* Measurement taken after a short exposure to Pronase.

in Fig. 2. For reasons given in the Discussion, we think the kinetics of the activation gates were probably unaffected by Pronase, and the smaller value of dG_{Na}/dt simply reflects a decrease in maximum g_{Na}.

Closing of the activation gates can be followed by returning V to -50 mV or more negative while g_{Na} is high, and measuring the current tail that occurs after the stepback. The method and results of an experiment are shown in Fig. 4. The axon was held at $V = -70$, then depolarized to 0 mV for 1 ms, and then returned to -60 mV. The dashed traces show the result of prolonging the depolarization. In the lower traces, inactivation has been almost completely eliminated, with no discernible effect on the turn-off currents. The time constants of the tails (fitted by eye on semilog plots) in this and two similar experiments are given before and after Pronase in Table III. These time constants give an unequivocal measure of activation gate kinetics, and in none of the three experiments was there a significant change.

Another measure of activation gate behavior is the dependence of g_{Na} on membrane voltage, and this dependence can be readily determined from a current-voltage plot. The maximum value of I_{Na} before and after Pronase treatment from the experiment of Fig. 2 is plotted in Fig. 5 as a function of membrane voltage, and the points have been joined by a hand-drawn curve. At every voltage, the maximum current after Pronase is smaller than before by about the same factor, and from this and two similar experiments we conclude that the voltage dependence of g_{Na} is not affected by Pronase.

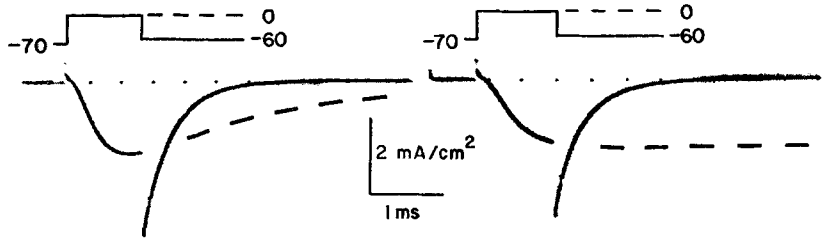

FIGURE 4. g_{Na} turn-off kinetics are not affected by Pronase. The traces show membrane current (predominantly I_{Na}) for the indicated procedures, before, and after 300 s of perfusion with 2 mg/ml protease VI (Sigma Chemical Co.) at 8°C. Photographs taken a 3°C. ASW//275 K-50 Na-15 TEA.

TABLE III
TIME CONSTANTS OF I_{Na} TAILS

τ	V_m	Temperature
μs	mV	°C
147	−70	3
121	"	"
365	−60	3
371	"	"
173	−50	3.6
173	"	"

Brackets enclose measurements from a single axon before and after Pronase.

From Fig. 5 it can also be inferred that selectivity of the Na channels is not changed by Pronase, for the voltage at which current through the channels changes from inward to outward is essentially unchanged (−56 mV before, −55 mV after), and this reversal voltage gives a good measure of the selectivity of the channels.

Pronase and Maximum g_{Na}

Simply removing inactivation would be expected to increase maximum g_{Na}, but in fact Pronase reduces it, probably by destroying or otherwise altering some of the channels. In the experiments where we followed it, the reduction of g_{Na} seems to occur in two phases, as shown in Fig. 6, in which peak I_{Na} for a depolarization to $V = 0$ mV is plotted as a function of time of exposure to Pronase. During the first phase peak I_{Na} (open circles) falls relatively rapidly to 40–90% of the control value, and the portion of I_{Na} that does not inactivate (filled circles) increases rapidly. The second phase begins when inactivation is largely destroyed (when the open and filled circles are close together), and during this phase I_{Na} amplitude falls quite slowly. In fact,

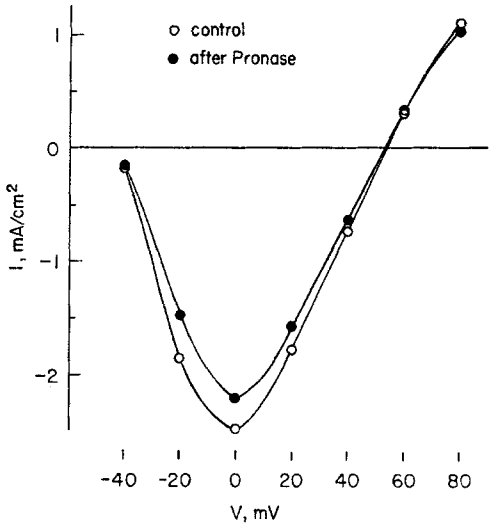

FIGURE 5. The voltage dependence of g_{Na} is not affected by Pronase. Maximum current (predominantly I_{Na}) is plotted as a function of V for the traces of Fig. 2. After Pronase the I-V curve is scaled down uniformly.

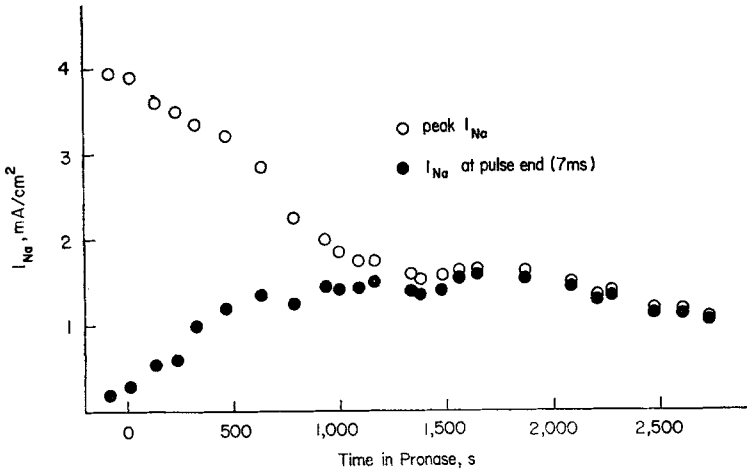

FIGURE 6. Two phases of decline of g_{Na} during Pronase action. Peak I_{Na} (open circles) declines rapidly while inactivation is being destroyed, and slowly thereafter. ASW//275 K-50 Na-15 TEA + 0.75 mg/ml Pronase CB, at 8°C.

current amplitude probably falls no more rapidly in this phase than it would in the absence of Pronase, but we have not performed the large number of control experiments necessary to give statistical weight to this supposition. A possible meaning for the two phases seen here is given in the discussion.

Pronase also Acts when the Na Channels Are Inactivated

From the preceding results, inactivation almost certainly involves a protein and it seemed conceivable that when Na channels were inactivated, the protein might change conformation sufficiently to lose its vulnerability to Pronase. We tested this possibility by immersing several axons in 60 K SW which lowered their resting potentials to about −30 mV, and then perfusing them with Pronase. At this resting potential, g_{Na} in a normal axon is almost completely inactivated. At intervals of 1–2 min we polarized the axon to −100 mV for 2 s to open the remaining inactivation gates, and then depolarized to $V = 0$ and measured I_{Na}. Despite the fact that the channels were inactivated most of the time, Pronase destroyed inactivation at about the same rate as it does in fibers held at −60 or −70 mV. We conclude that inactivation does not lessen the vulnerability of the presumed inactivation protein in Pronase.

TTX Does Not Protect the Channels from Pronase

It seemed possible, though unlikely, that blocking the Na channels with tetrodotoxin (TTX) might protect the inactivation gates, and we tested this in two axons, with the expected result. After recording currents in ASW//275 K the fibers were treated briefly with ASW-TTX//275 K, and then with ASW-TTX//275 + 1 mg ml^{-1} Pronase CB. After 15–22 min, Pronase was washed from the interior of the fiber (with 275 K-50 Na-15 TEA) and 1 or 2 min later, ASW-TTX was replaced by ASW. In one experiment at 8°C, the axon was Pronase treated for 930 s (15.5 min), and at the end of this time, inactivation was largely removed (I_{Na} at the end of an 8 ms step was 61% of peak I_{Na}). Thus Pronase acted more slowly than in the experiment of Fig. 2, but at about the same rate as in Fig. 6. In another experiment, at 10°C, inactivation was completely destroyed after 1326 s (22.1 min) of Pronase treatment in ASW-TTX. We conclude that TTX does not afford appreciable protection against Pronase action. It is also clear from these experiments that internally applied Pronase does not affect the TTX receptor.

Narahasi et al. (1967) have reported that TTX added to the internal solution does not block g_{Na}. We have confirmed this, and we also find that even at 10^{-6} M internally, TTX has no detectable effect on g_{Na} after Pronase has destroyed inactivation.

Pronase and g_K

Pronase does not have a well-defined effect on I_K. I_K amplitude as a function of time of exposure to Pronase is shown for four TTX poisoned axons in Fig. 7. In all cases there was an increase in I_K after adding Pronase to the perfu-

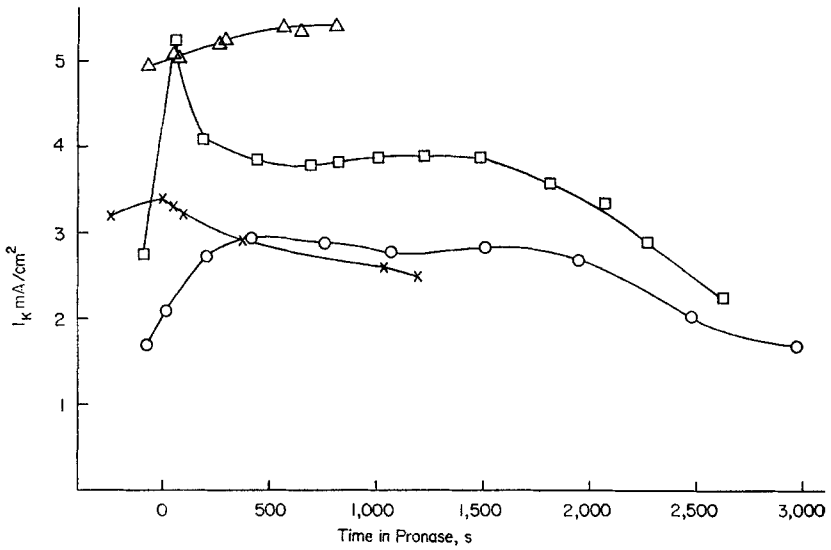

FIGURE 7. I_K from four experiments with TTX-poisoned axons is plotted as a function of time of perfusion with Pronase. I_K was measured at the end of 8-ms pulses to membrane voltages of +80 (○, △, □) or +20 mV (×). After Pronase, I_K usually increased, but this is not a Pronase-specific effect. ASW-TTX//275 K + 1 mg/ml Pronase CB. ○, △, □, 8°C. ×, 10°C.

sion medium, but the duration and amplitude of the rise, and the subsequent behavior were variable. The initial increase in I_K is not an effect specific to Pronase, for we have observed a similar increase with other proteolytic enzymes (which do not affect Na inactivation) and sometimes with no enzyme present at all (see Bezanilla and Armstrong, 1972). The increase may occur because these enzymes remove remaining axoplasm and facilitate washing of the axon interior, which removes Na^+ ions or, perhaps, some other substance that has an inhibitory effect on I_K. This phenomenon requires further investigation. After 300–400 s, I_K acquires a relatively stable value which in two experiments was maintained for 1,500–1,800 s (25–30 min), after which I_K slowly declined. For comparison, Na inactivation in most cases was almost completely destroyed after 700–800 s of Pronase at the same concentration and temperature. In two other experiments, Pronase was removed from the internal medium and TTX from the external after 930 (△) and 1,326 (×) s of exposure, and Na inactivation had been, respectively, partially and completely removed (see above).

g_{Na} and g_K Can Be High Simultaneously after Pronase

Total ionic current is given by the equation

$$I_{ionic} = I_{Na} + I_K + I_{leak}.$$

In a normal axon, I_{ionic} in the steady state after a depolarization is about equal to I_K, for I_{Na} is inactivated and I_{leak} is small. This is no longer true after Na inactivation is destroyed, for I_{Na} remains large in the steady state, and sums with I_K, as illustrated in Fig. 8. Curves A and B give respectively the peak early current (predominantly I_{Na}) and the steady-state current (end of a 12 ms pulse) as a function of membrane potential, 44 s after adding Pronase to the perfusion solution. The curves have the usual configuration except for the

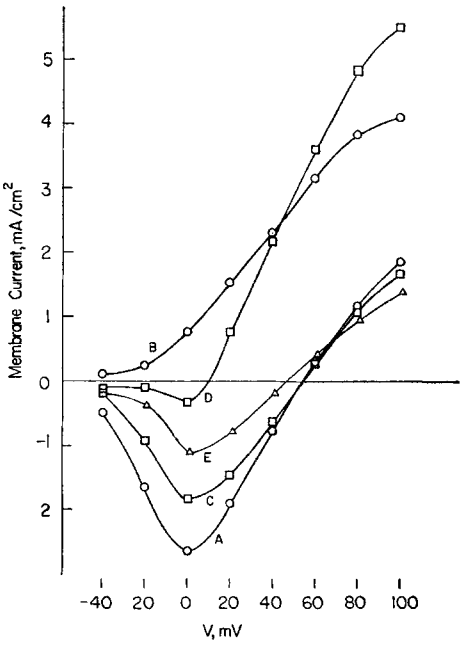

FIGURE 8. Na and K channels active simultaneously. Curves A and B show, respectively, early current (predominantly I_{Na}) and steady-state current (predominantly I_K) while inactivation is intact. B and D show the same currents after partial destruction of inactivation. Curve D can be reproduced by adding curve E to curve B. Curve E is (approximately) the steady-state Na current. ASW//275 K-50 Na + 0.5 mg/ml Pronase, 9°C.

decreasing slope of curve B above $V = +60$ mV. This is caused by the presence of Na ions in the internal medium, which have been shown to enter and obstruct the K channels (Bezanilla and Armstrong, 1972). After 492 s of Pronase (curves C and D), I_{Na} amplitude is reduced but curve C otherwise resembles curve A. The steady-state curve (D), however, is remarkably changed. Current is still inward at $V = 0$ mV, and the curve then climbs steeply as V increases and crosses curve B near the Na equilibrium potential, V_{Na}. (V_{Na} is 54 mV. The crossover is at 45 mV.) If curve B is subtracted from D, one obtains curve E, which closely resembles curve C, but scaled down by a factor of about 0.6. Our interpretation is that 492 s after Pronase, steady-

state I_{Na} is about 60% of peak I_{Na}, and steady-state I_{Na} (curve E) sums with I_K which is unaltered (curve B) to give curve D. This reconstruction has two minor, related flaws. First, curves B and D do not cross exactly at V_{Na} as would be expected; and curve E is not zero at V_{Na}. Both discrepancies could be explained quantitatively if g_K increased by 9% between determination of curve B and curve D. Measurements of I_K at V_{Na} show that g_K did increase by 7%, leaving only a very small discrepancy.

In principle it should be possible to destroy Na inactivation completely and increase total steady-state current and conductance still more, but in practice this is very difficult because of problems with control of membrane voltage along the axon, as described in the next section.

Pronase and g_{leak}

In a number of experiments we measured the current required to hold V at -65 to -75 mV, and we take this current as an indication of the leakage current. Our measurements are not highly accurate, but they do show that in most cases Pronase does not cause a large increase in g_{leak} ($=I_{leak}/[V - V_{leak}]$). Frequently we did observe large transient increases in the holding current after Pronase had acted for some time but only in axons not poisoned with TTX. We believe these increases occurred because the ends of the fiber escaped potential control and underwent some form of action potential, which, because of the absence of Na inactivation, lasted for many seconds. Cole and Moore (1960) have shown that parts of the fiber distant from the internal potential electrode can escape control (*a*) if the $I - V$ curve of the membrane has a negative resistance region, and (*b*) if the surface impedance of the axial wire is high. The surface impedance of even a well-platinized wire rises quickly when it passes sustained current in one direction, and thus both conditions for failure of control are fulfilled in a Pronase-treated axon. That is, the membrane shows negative resistance, for many seconds at a time because I_{Na} does not inactivate and, because I_{Na} is large, the axial wire must pass a sustained inward current which raises its surface impedance. Consistent with this interpretation is the absence of transient holding-current increases in TTX-poisoned axons, for by eliminating I_{Na}, TTX abolishes negative resistance.

Experiments with Injected Pronase

We were surprised initially to find that injected Pronase has little or no effect on membrane properties, even when the axoplasmic concentration is near 1 mg/ml. Similarly, Pronase can be left in the axon for several minutes during the early stage of perfusion with no effect on the membrane currents, provided there is no flow of perfusion fluid. We made use of this fact routinely by allowing Pronase to digest the axoplasm, to forestall difficulties with slow perfusion. The explanation of the ineffectiveness of injected Pronase is almost

certainly that axoplasm provides large quantities of substrate for the enzyme molecules, which are thus too occupied to attack the proteins involved in inactivation.

Efficacy of Different Pronase Preparations

Pronase is a mixture of proteolytic enzymes from *Streptomyces griseus* (Narahashi, 1970), and we thought that some preparations might lack the component or components that destroy inactivation. We have tried four preparations (Pronase, nuclease-free Pronase, and Pronase CB from Calbiochem; and Protease type VI from Sigma Chemical Co.), and all were found efficient in destroying inactivation when perfused internally. Pronase applied to the exterior of the fiber does not affect inactivation; nor does internal perfusion with Pronase that has been boiled (c.f. Rojas and Atwater, 1967).

DISCUSSION

Three conclusions and a strong presumption can be drawn from our data. The presumption is that inactivation depends on the intactness of a protein attached to the inner surface of the membrane. The conclusions are: (*a*) Activation and inactivation of the Na channels are separable processes. (*b*) The Na channels are distinct from the K channels. (*c*) The Na and K channels and their activation gates are not very vulnerable to Pronase. If these structures are composed of protein, as seems likely, they must be buried in the membrane and inaccessible to Pronase, or their exposed groups are not attacked by Pronase.

Inactivation and Membrane Protein

EVIDENCE THAT INACTIVATION INVOLVES A PROTEIN Pronase is a mixture of many enzymes (Narahashi, 1970), and one must consider the possibility that some nonproteolytic component of it is destroying inactivation. This seems extremely unlikely, for a number of other proteolytic enzymes, e.g. papain and ficin, which are crystalline enzymes, (Tasaki and Takenaka, 1964) and BPN′ and bromelin (Takenaka and Yamagishi, 1969) appear from their action in prolonging the action potential to be capable of destroying inactivation. Protein crosslinking agents (Shrager et al., 1969) also seem to destroy Na inactivation in the crayfish, and formaldehyde has the same action on frog skeletal muscle (Dominguez and Hutter, 1969). In addition to these agents which have selective action on protein, inactivation can be removed by three agents of unknown action: venom from two scorpions (Koppenhöfer and Schmidt, 1968; Narahashi et al., 1972) and *Condylactis* toxin (Narahashi et al., 1969).

IS IT A MEMBRANE OR AXOPLASMIC PROTEIN? For three reasons we think that the inactivation protein must be fixed to the membrane. (*a*) It cannot

be washed out, even when the interior of the axon is perfused with several hundred axon volumes of artificial solution. (*b*) We have perfused axons with a number of other proteolytic enzymes that seem to be effective in removing the axoplasm, but have little or no effect on inactivation. We are currently working to obtain electron microscopic confirmation of this point. (*c*) Each channel has an inactivation protein that is distinctly its own, and it no longer inactivates when this protein is altered or destroyed by Pronase. Our evidence shows that at the level of a single channel, the effect of Pronase is all-or-none, for when only a portion of inactivation has been destroyed there are clearly two populations of channels: those that have retained their inactivation gates and inactivate normally, and those that do not inactivate (except, perhaps, by a separate and very slow mechanism). This finding clearly indicates a one-for-one relation between inactivation proteins and channels, and it is hard to reconcile with a picture which places the inactivation protein in the axoplasm, with no permanent bond to the channel.

MECHANISM OF INACTIVATION Though many details remain to be worked out, we believe that at the inner mouth of each Na channel there is an inactivation gate composed of protein, which changes with a time lag from one conformation to a second after the activation gate opens, and in the second conformation it blocks the channel. This protein can be altered or removed without disturbing the activation gate (see below), and the postulated conformational change does not lessen the susceptibility of the protein to lysis by Pronase. This proposal is not entirely consistent with the Hodgkin and Huxley equations for g_{Na}, but until it is completely formulated it is convenient to continue to use their terminology.

The Activation Gates

We made three measurements that reflect the properties of the activation gates. Two of them, the turn-off rate of g_{Na} on repolarization and the voltage dependence of g_{Na}, were unaffected by Pronase. The fact that the kinetics of the I_{Na} tails on repolarization are unaffected by removing inactivation shows clearly that Hodgkin and Huxley were correct in saying that the activation gates determine the time-course of these tails. The third measurement, the rate of rise of g_{Na} after depolarization, was decreased, but this probably results from a decrease in $\bar{g}_{Na}$ (the value of the Na conductance if it were possible to open all of the Na channels simultaneously) and not from an effect on activation kinetics.

These results show clearly that activation and inactivation are separable processes. By using Pronase it is now possible for the first time to study Na activation directly, without interference from inactivation.

THE DECREASE OF MAXIMUM g_{Na} Fig. 6 shows that once the inactivation gates are destroyed, Pronase has little or no effect on the Na channels, and g_{Na} decreases slowly, perhaps entirely as a result of general deterioration of the axon. This shows that the Na channels themselves are not very vulnerable to Pronase, at least after the removal of their inactivation gates. In view of this fact, the early phase of g_{Na} decline, while the inactivation gates are being destroyed, has two possible explanations. (*a*) While attacking the inactivation gate of a channel, there is a good chance that Pronase will destroy the channel as well. Once its inactivation gate is removed, the channel is in no danger of lysis. Or (*b*) destruction of the inactivation gate of a channel decreases the conductance of the channel. At present we know of no way to decide whether Pronase is reducing the number of channels or reducing the conductance per channel. The early decline is even sharper if one considers that before Pronase g_{Na} is never more than about half of $\bar{g}_{Na}$, while g_{Na} can equal $\bar{g}_{Na}$ when there is no inactivation. Thus, if $\bar{g}_{Na}$ were plotted in Fig. 6 instead of maximum g_{Na}, the points near time zero would be considerably higher, while the points at later times, after destruction of inactivation, would be unaffected.

Lengthening of the Action Potential by Pronase.

This paper contains the first extensive presentation of voltage-clamp records for enzyme-treated axons, and it is necessary to see if these results can account for the effects of proteolytic enzymes on the action potential. During the early stage of Pronase treatment the action potential develops a long plateau, which is readily explained by the failure of g_{Na} to inactivate completely. Our voltage-clamp records show that g_K during the plateau must also be high. Repolarization is probably caused by a slow inactivation of g_{Na}, which causes V to drift slowly toward a repolarization threshold, which, roughly speaking, is the voltage at which Na activation gates begin to close. Repolarization has a regenerative character, for closing of Na activation gates causes V to go negative, which in turn causes closing of more activation gates. Takenaka and Yamagishi (1969) found that during this stage the plateau could be shortened or abolished by increasing external $[Ca^{2+}]$ or internal $[K^+]$, and we explain these effects as follows. Increasing $[Ca^{++}]$ at constant V causes closing of Na activation gates (Frankenhaeuser and Hodgkin, 1957) and thus promotes repolarization and shortens the plateau. For given permeability values, increasing internal $[K^+]$ makes V more negative, and can result in abolition of the plateau because after the action potential peak V returns to a level sufficiently negative that the Na activation gates close.

The stage during which plateau action potentials are recorded lasts a few minutes with Pronase, but may last for an hour with BPN′ (Takenaka and Yamagishi, 1969), probably because this enzyme is not very effective at

destroying Na inactivation. There ensues a phase that has not been well characterized in voltage-clamp experiments. In this phase the resting potential is low in amplitude and there are very long spontaneous action potentials of small amplitude ("flip-flop" behavior; Tasaki and Takenaka, 1964). Presumably the membrane is alternating between states of low and high Na permeability, and repolarization is caused by a slow inactivation process (c.f. Armstrong and Binstock, 1965).

Distinctness of Na and K Channels

There is substantial pharmacological evidence that Na and K channels are separate entities (for a review, see Hille, 1970), but none of this evidence is absolutely conclusive (Mullins, 1968). Mullins (1959) has suggested as an alternative to the two-channel hypothesis that there is a single set of channels that conduct first Na and then K ions, a suggestion that gains credibility from the similarity in time-course between Na inactivation and K activation. An essential step in this model is that the channels inactivate and lose their ability to conduct Na ions before they begin to conduct K ions. Our experiments show clearly that Na inactivation can be completely destroyed without diminishing g_K. Further, in the experiment of Fig. 8, a good fraction of the Na channels continued to conduct Na^+ ions well after g_K was fully activated, to a value even slightly in excess of that before Na inactivation was destroyed. Taken either singly or together, we believe that these observations make the single-channel hypothesis untenable, and prove Na and K channels are distinct from each other.

We are grateful to Mrs. Lillian Peracchia for preparing the illustrations.
This work was supported by U.S. Public Health Service Grant No. NS08951.

Received for publication 27 December 1972.

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