

## VOLTAGE-DEPENDENT REMOVAL OF SODIUM INACTIVATION BY *N*-BROMOACETAMIDE AND PRONASE

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**ABSTRACT** When perfused internally through crayfish giant axons, pronase removed sodium inactivation more than three times as fast at  $-100$  mV as compared with  $-30$  mV. *N*-bromoacetamide, applied internally, removed sodium inactivation twice as fast at  $-100$  mV as at  $-30$  mV, and the relative rate of removal declined with membrane depolarization in proportion to steady-state sodium inactivation. We conclude that in the closed conformation the sodium inactivation gate is partially protected from destruction by *N*-bromoacetamide and pronase.

### INTRODUCTION

The proteolytic enzyme pronase and the peptide bond-cleaving reagent *N*-bromoacetamide (NBA) destroy sodium channel inactivation (Armstrong et al., 1973; Rojas and Rudy, 1976; Oxford et al., 1978) with little or no effect on the activation kinetics (Oxford, 1981). Because of their specific destruction of sodium inactivation, NBA and pronase have become useful tools for the study of sodium channels in the absence of inactivation.

While using NBA and pronase in internally perfused and voltage-clamped crayfish giant axons in a sucrose gap chamber, we noticed that inactivation was always less completely removed in the depolarized region of the axon than in the artificial node, which was clamped at  $-100$  mV. Further investigation has shown that the rate of removal of sodium inactivation by NBA is voltage dependent and that the voltage dependence is similar to that of steady-state sodium inactivation. These results suggest that the inactivated sodium channels are less sensitive than the resting channels to removal of sodium inactivation by NBA and pronase. A preliminary report of this work has been published (Salgado et al., 1983).

### METHODS

Giant axons, 175–200  $\mu$ m in diameter, were isolated from the circumoesophageal connectives of the crayfish, *Procambarus clarkii*, and were cannulated and internally perfused as described by Lund and Narahashi (1981). The double sucrose gap voltage-clamp technique originally developed by Julian et al. (1962a, b) was used. The chamber consisted of three compartments separated by two sucrose streams. The right-hand pool contained isotonic KCl solution to depolarize the membrane, and the membrane potential of the central artificial node was measured between the right and central pools. The left-hand pool was perfused with van Harrevelde's solution, and current was applied to this pool to voltage clamp the nodal membrane. The aspect of this preparation most crucial to the present work is that the length of axon in the left-hand pool can be moved into the central pool after the experiment with the first node is finished,

providing a fresh node that will have been exposed to exactly the same internal conditions as the first node, but will not have been voltage clamped, and will have been perfused externally with van Harrevelde's solution rather than a modified external solution. The solutions had the following compositions (in millimoles per liter): van Harrevelde's solution, 205 NaCl, 5.4 KCl, 10 CaCl<sub>2</sub>, 2.6 MgCl<sub>2</sub>, 3 HEPES, pH 7.5; 50 Na, K-free external solution, 160 tetramethylammonium chloride, 50 NaCl, 10 CaCl<sub>2</sub>, 2.6 MgCl<sub>2</sub>, 3 HEPES, pH 7.5; standard internal solution, 170 K-glutamate, 50 KF, 15 NaCl, 5 HEPES, pH 7.35; 50 Na, K-free internal solution, 135 Cs-glutamate, 50 CsF, 50 NaCl, 5 HEPES, pH 7.35; isotonic KCl, 265 KCl, 15 NaCl. The sucrose solution measured 500 mOsmol/kg after deionizing, and contained 10  $\mu$ M LaCl<sub>3</sub> to decrease leakage current (Pooler and Valenzano, 1983). The temperature was maintained at 9–10°C for all experiments.

### RESULTS

Fig. 1 *A* shows outward sodium currents for a step depolarization to  $+60$  mV from a holding potential of  $-100$  mV before and 5 min after beginning internal perfusion with 2 mM NBA. No inactivation was seen after NBA treatment. Fig. 1 *B* shows a sodium current from a new node formed from a part of the same axon that had been exposed externally to van Harrevelde's solution in the left-hand pool during the NBA treatment. In this case, about half of the inactivation remained. The important difference between the two nodes was the membrane potential during NBA treatment. Whereas the first node was held at  $-100$  mV during treatment, the second node had been depolarized during treatment because of the reversed potassium gradient between the 50 mM Na, K-free internal solution, and the van Harrevelde's solution in the left-hand pool. The resting potential for several axons with these solutions was found to be  $-30$  to  $-35$  mV when measured with a single sucrose gap.

For a quantitative study of the removal of inactivation by chemicals, we need to measure the fraction of channels from which inactivation has been removed. Unfortunately,

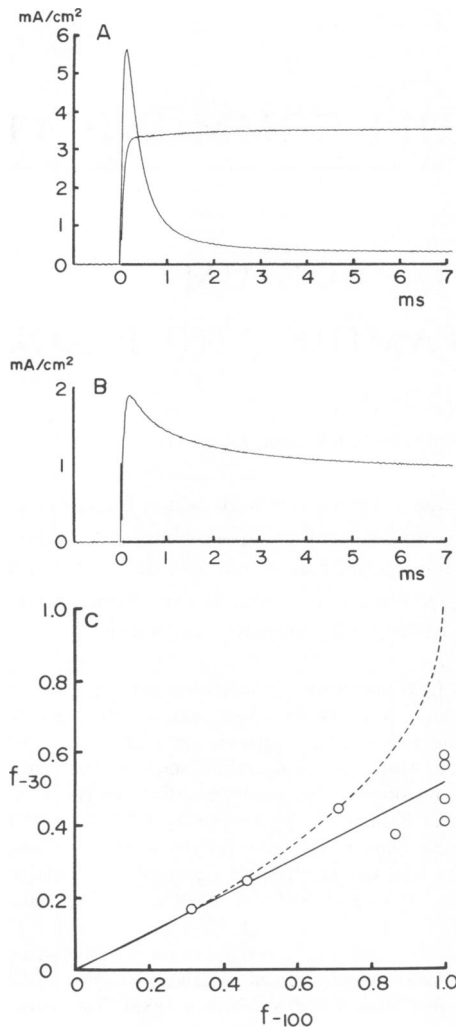


FIGURE 1 (A) Outward sodium currents elicited by step depolarizations to +60 mV from a holding potential of -100 mV before and 5 min after internal perfusion with 2 mM *N*-bromoacetamide. (B) Same as A, but in a region of membrane that had been depolarized in the left-hand pool containing van Harreveld solution during NBA perfusion. (C) Plot of fraction of sodium inactivation removed in a region of membrane in the left-hand pool at the resting potential of -30 mV ( $f_{-30}$ ) as a function of fraction of inactivation removed in a node clamped at -100 mV ( $f_{-100}$ ). Data from several axons with varying exposure to NBA.  $f$  is defined by Eq. 1. The solid line is a linear least-squares fit with a slope of 0.51. The broken curve is a plot of Eq. 3 with  $k_{-30}/k_{-100} = 0.462$  and  $f_{\max} = 1.0$ .

this fraction cannot be measured directly without the assumption of a specific model for inactivation. Therefore, we took the ratio of steady state to peak sodium current ( $I_{ss}/I_p$ ) as an approximate measure of the extent of removal of inactivation. The noninactivating fraction ( $f_0$ ) of the control sodium current thus estimated was  $0.048 \pm 0.006$  (mean  $\pm$  SD,  $n = 8$ ). The extent of removal of inactivation by a chemical, corrected for  $f_0$ , is then given by

$$f = \frac{I_{ss}/I_p - f_0}{1 - f_0} \quad (1)$$

In the following experiments,  $f$  was always measured for test pulses to +60 or +80 mV where activation was rapid and complete. In Fig. 1 A,  $f$  for the control current is 0, by definition, and  $f$  after treatment is 1.0. In Fig. 1 B,  $f$  is 0.50.

To compare the rate of NBA action at two potentials, we treated axons with low concentrations (0.5–1.0 mM) of NBA and stopped the reaction by washing with NBA-free internal solution before complete removal of sodium inactivation. The value of  $f$  was measured in the original node, which had been voltage clamped at -100 mV ( $f_{-100}$ ), and then in the new node, which had been depolarized to -30 mV ( $f_{-30}$ ) during NBA treatment as described above. The  $f_{-30}$  value was plotted against the  $f_{-100}$  value for each axon in Fig. 1 C. The  $f_{-30}$  value was always about half of the  $f_{-100}$  value, with the regression line having a slope of  $0.51 \pm 0.08$  (mean  $\pm$  SD,  $n = 8$ ). However, a linear relation seems counterintuitive. We expect a linear relation when  $f$  is small at both potentials, but when  $f_{-100}$  approached 1.0,  $f_{-30}$  should also approach 1.0 if the reaction goes to completion at both potentials.

If it is assumed that NBA reacts with channels at one rate at -100 mV ( $k_{-100}$ ) and at another rate at -30 mV ( $k_{-30}$ ), then  $f$  is a measure of the extent to which the reaction has proceeded at each potential for the same duration of exposure to NBA. If the reaction is first order with respect to the fraction of unreacted channels,  $f$  should increase approximately exponentially at each potential according to the equation

$$f/f_{\max} = 1 - e^{-k_v t} \quad (2)$$

$f_{\max}$  is the maximum value of  $f$  obtainable (1.0 for NBA),  $k_v$  is the voltage-dependent rate constant, and  $t$  is the time. This was indeed the case for both NBA and pronase (Fig. 2). For 2 mM NBA the rate constant was calculated to be  $9.1 \times 10^{-3} \text{ s}^{-1}$  at 10°C and -120 mV. For 0.5 mg/ml pronase the rate constant was  $7.8 \times 10^{-3} \text{ s}^{-1}$  at 10°C and -100 mV. NBA could always remove inactivation completely, whereas pronase removed only 69% in the case of Fig. 2 B, and in no case did it remove more than 85% (Fig. 3 C).

To find the relationship between two  $f$  values for regions of membrane that have been exposed to NBA for the same period of time at two different membrane potentials, 1 and 2, we write Eq. 2 for each potential and eliminate  $t$  between the two equations. This gives

$$f_1/f_{\max} = 1 - (1 - f_2/f_{\max})^{k_1/k_2} \quad (3)$$

This function is plotted as a broken line in Fig. 1 C with  $k_1/k_2 = 0.462$  and  $f_{\max} = 1.0$ . Eq. 3 seems to fit the data up to  $f_{-100} = 0.7$ , but for longer NBA exposure the measurements fall below the curve. The reason for this deviation is not clear. For small values of  $f$ , the theoretical curve approaches a straight line, so that for short exposure to NBA ( $f_{-100} < 0.5$ ) a fairly good estimate of the relative rate of action at the two potentials can be obtained.

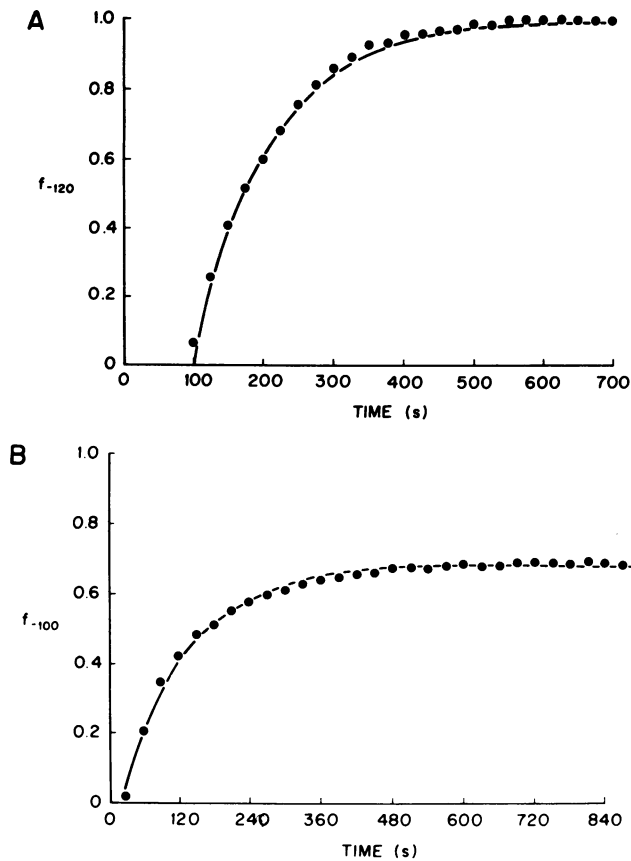


FIGURE 2 The time course of removal of sodium inactivation. (A) The dots show the value of  $f$  measured at intervals after introducing 2 mM NBA in the internal perfusate at a holding potential of  $-120$  mV. The solid curve was drawn according to the equation  $f = 1 - \exp[-(t - 95)/110]$ . (B) Similar to A, but with 0.5 mg/ml pronase at a holding potential of  $-100$  mV. The curve was calculated from the equation  $f = 0.69 \{1 - \exp[-(t - 45)/128]\}$ . The delays of 95 and 45 s in A and B, respectively, represent the time from beginning pulsing to application of the reagents to the axon.

Pronase action was also inhibited by depolarization. Fig. 3 A shows sodium currents at  $+80$  mV before and after pronase treatment, and Fig. 3 B shows a sodium current in a node that had been depolarized during pronase treatment. Fig. 3 C gives the plot of  $f_{-30}$  vs.  $f_{-100}$  for pronase. Prolonged pronase treatment removed 85% of sodium inactivation. Further treatment resulted in an increase in leakage current. The curve in Fig. 3 C was drawn according to Eq. 3 with  $f_{\max} = 0.85$  and  $k_1/k_2 = 0.30$ . The measurements appear to be fairly well fitted by the curve.

The results up to this point have shown that depolarization from  $-100$  to  $-30$  mV inhibits NBA action twofold and pronase action more than threefold. As NBA has no electrical charge, the voltage dependence must arise from the voltage dependence of gating and possibly from that of inactivation itself. It is important, therefore, to determine the voltage dependence of NBA and pronase action and compare it to the steady-state inactivation ( $h_{\infty}$ ) curve.

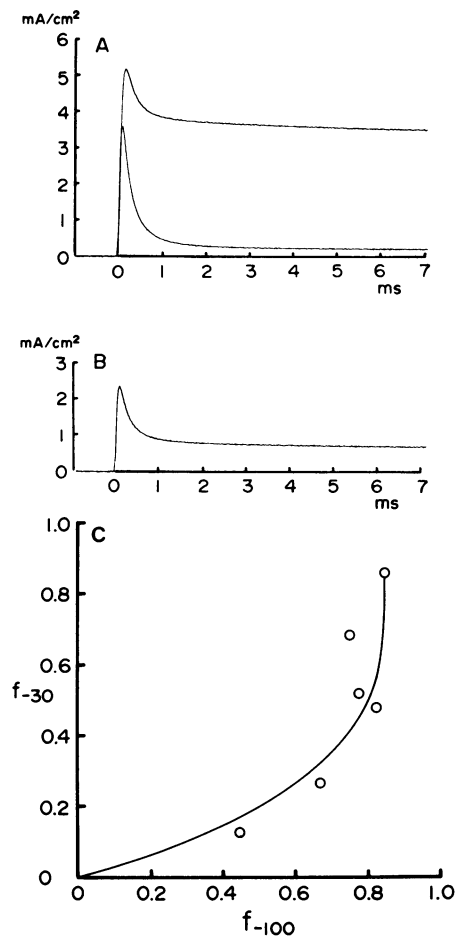


FIGURE 3 (A) Outward sodium currents elicited by step depolarizations to  $+80$  mV from a holding potential of  $-100$  mV before and after internal perfusion with 0.1 mg/ml pronase. (B) Same as A, but in a region of membrane that had been depolarized in the left-hand pool during pronase perfusion. (C) Plot of  $f_{-30}$  vs.  $f_{-100}$  as in Fig. 1 for several axons with varying exposure to pronase. The curve was drawn according to Eq. 3 with  $k_{-30}/k_{-100} = 0.298$  and  $f_{\max} = 0.85$ .

The  $h_{\infty}$  curve was determined with the standard two-pulse protocol, with a 50-ms conditioning prepulse to various potentials, followed by a test pulse to  $+60$  mV. The holding potential was  $-120$  mV. The filled circles in Fig. 4 are the average data from eight axons. The  $h_{\infty}$  value decreased to 0.5 at  $-67$  mV, and the slope was 7.3 mV per  $e$ -fold change in  $h_{\infty}$ , similar to Hodgkin and Huxley's (1952) value of 7 mV for squid axons. The open circles in Fig. 4 show  $k_v/k_{-30}$  determined as described above, by comparing a node clamped this time at a potential ( $v$ ) to that depolarized to  $-30$  mV in the left-hand pool during NBA treatment. NBA was washed out after sufficient exposure to remove less than half of the inactivation ( $f < 0.5$ ) in the clamped node. Each circle represents either a single measurement or the mean and the standard error with the number of experiments. The voltage dependence of NBA action closely follows the  $h_{\infty}$  curve.

In addition, the possible role of slow Na inactivation ( $s_{\infty}$ ) in determining the rate of NBA action was investi-

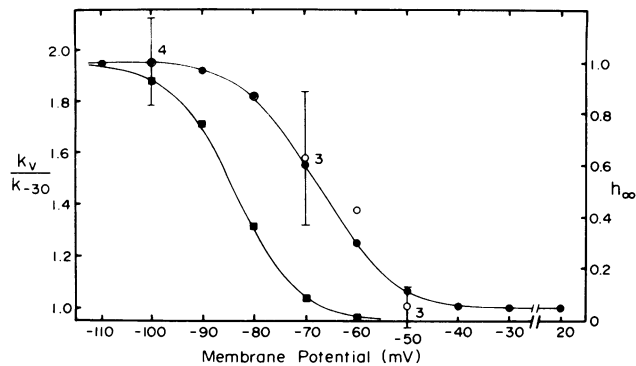


FIGURE 4 The voltage dependence of NBA action (open circles), mean  $\pm$  SEM, with the number of experiments shown), steady-state sodium inactivation ( $h_{\infty}$ ; filled circles, average of eight experiments), and slow sodium inactivation ( $s_{\infty}$ ; filled squares, average of two experiments). The open circles show the rates of removal of inactivation for nodes held at various potentials during NBA perfusion relative to the rate at  $-30$  mV determined in a single axon. NBA concentrations of 0.5, to 2 mM were used, and the treatment was terminated when  $f_v$  was between 0.2 and 0.5 where the linear approximation and Eq. 3 coincide. The curve for  $h_{\infty}$  was drawn according to  $h_{\infty} = 1/[1 + \exp(v - 67)/7.3]$  and that for  $s_{\infty}$  according to  $s_{\infty} = 1/[1 + \exp(v - 87.3)/6.0]$ , where  $v$  is membrane potential in millivolts.

gated. To measure  $s_{\infty}$ , the membrane was conditioned at various potentials for 60 s, then returned to  $-120$  mV for 30 ms to allow fast inactivation to recover before a test pulse to  $+60$  mV. The peak current obtained during the test pulse, normalized to the maximum peak current with a hyperpolarized conditioning potential was plotted against conditioning pulse potential in Fig. 4 (filled squares). The membrane potential at which  $s_{\infty}$  becomes 50% was  $-83.7$  mV, and the slope was 6.0 mV per  $e$ -fold change in  $s_{\infty}$ . In as much as the  $s_{\infty}$  curve is located 16.7 mV more negative than the  $h_{\infty}$  curve, the rate of NBA action is correlated much better with  $h_{\infty}$  than with  $s_{\infty}$ .

## DISCUSSION

We have shown that the rate of removal of sodium inactivation by NBA and pronase is voltage dependent, decreasing two and threefold, respectively, between  $-100$  and  $-30$  mV. The relationship between the rate of NBA action and the membrane potential follows the  $h_{\infty}$  curve closely. The  $s_{\infty}$  curve is located  $\sim 17$  mV more negative than the  $h_{\infty}$  curve, as has been also found by Starkus and Shrager (1978). Because the  $s_{\infty}$  curve differs from the  $h_{\infty}$  curve, and the curve for the voltage dependence of NBA action is similar to the  $h_{\infty}$  curve, we conclude that fast inactivation protects the  $h$  gate from destruction by NBA.

State dependence of NBA and pronase action is not unexpected. As these treatments hydrolyze peptide bonds in a region of the sodium channel, which is intimately involved in the inactivation process, we might anticipate that the accessibility of these particular peptide bonds will be affected by the conformational change associated with inactivation. Armstrong et al. (1973) looked for voltage

sensitivity of pronase action in squid axon, but did not find any difference between the action at  $-30$  and  $-70$  mV. Although this may be due to a species difference, it is more likely that the threefold difference in the rate of action was too small to be noticed, given the variability between axons. Such variability between axons was eliminated under our experimental conditions, since the degree of removal of inactivation was always compared at two different potentials in a single axon.

Our findings suggest that inactivation only partially protects sodium channels from the action of NBA and pronase. In the control experiments  $\sim 5\%$  of the sodium current did not inactivate. This may represent an upper limit to the probability that an inactivation gate remains open at the depolarized potential. If NBA and pronase only react with open  $h$  gates, the rate of action in the depolarized region of the axon should be only one-twentieth that in the polarized node. The observed rate was much higher than this, suggesting that NBA and pronase do react with closed inactivation gates, but only at about one-half (NBA) or one-third (pronase) the rate at which they react with open gates.

A difference in the fast Na inactivation between crayfish axons and squid axons has been reported before. Swenson (1980) showed that the kinetics of fast Na inactivation in the crayfish axon are fivefold faster than in the squid axon. With regard to chemical sensitivity, an additional difference in the fast Na inactivation is revealed in this paper. Pronase treatment is known to completely remove the fast Na inactivation in squid axons (Armstrong et al., 1973; Rojas and Rudy, 1976), whereas in crayfish axons such treatment could only remove 85% of the fast Na inactivation. A similar result was reported by Starkus and Shrager (1978). The nature of this partial resistance to pronase remains to be studied.

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