Removal of Sodium Channel Inactivation in Squid Giant Axons by *N*-Bromoacetamide

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ABSTRACT The group-specific protein reagents, *N*-bromoacetamide (NBA) and *N*-bromosuccinimide (NBS), modify sodium channel gating when perfused inside squid axons. The normal fast inactivation of sodium channels is irreversibly destroyed by 1 mM NBA or NBS near neutral pH. NBA apparently exhibits an all-or-none destruction of the inactivation process at the single channel level in a manner similar to internal perfusion of Pronase. Despite the complete removal of inactivation by NBA, the voltage-dependent activation of sodium channels remains unaltered as determined by (*a*) sodium current turn-on kinetics, (*b*) sodium tail current kinetics, (*c*) voltage dependence of steady-state activation, and (*d*) sensitivity of sodium channels to external calcium concentration. NBA and NBS, which can cleave peptide bonds only at tryptophan, tyrosine, or histidine residues and can oxidize sulfur-containing amino acids, were directly compared with regard to effects on sodium inactivation to several other reagents exhibiting overlapping protein reactivity spectra. *N*-acetylimidazole, a tyrosine-specific reagent, was the only other compound examined capable of partially mimicking NBA. Our results are consistent with recent models of sodium inactivation and support the involvement of a tyrosine residue in the inactivation gating structure of the sodium channel.

INTRODUCTION

The widely held view that ionic channels in excitable membranes are composed of protein molecules has been brought into clear focus by the demonstration that certain proteases can modify the inactivation gating behavior of sodium channels (Rojas and Armstrong, 1971; Armstrong et al., 1973; Sevcik and Narahashi, 1975; Rojas and Rudy, 1976). In addition, a correlation between the spectral dependence of selective sodium channel destruction by ultraviolet radiation and absorption spectra of aromatic amino acids has been demonstrated (Fox, 1974; Oxford and Pooler, 1975*a*).

Such data have prompted more systematic investigations of the possible role of specific protein chemical groups in the function of both sodium and potassium channels in nerve membrane (Shrager, 1974, 1975, 1977; Keana and Stämpfli, 1974; Marquis and Mautner, 1974; Gainer et al., 1974; Inoue et al., 1976). The application of group-specific protein reagents to probe the molecular interactions within ionic channels under voltage clamp conditions is a potentially

useful approach (Shrager, 1975; Keana and Stämpfli, 1974). Such studies are not without difficulties arising from ambiguous interpretation of data obtained with reagents of overlapping chemical 'specificities' (Means and Feeny, 1971), physiological incompatibility of certain reaction conditions required for biochemical specificity (e.g., low pH, nonaqueous solvents), and membrane asymmetry of effect (Narahashi, 1974).

Recently we began a systematic investigation of the effects of certain group-specific reagents upon excitability in the internally perfused squid giant axon. By choosing reagents requiring mild aqueous reaction conditions and having access to and control of the composition of the solutions on both membrane surfaces, we attempted to minimize some of the difficulties mentioned. In this paper we report on our most dramatic finding to date, that the *N*-bromoacetamide and *N*-bromosuccinimide destroy sodium channel inactivation gating at the internal membrane surface. A preliminary report of this work has been presented at the 20th Annual Meeting of the Biophysical Society (Oxford et al., 1976).

METHODS

Experiments were performed on single giant axons (330–800 μm in diameter) isolated from *Loligo pealei* at the Marine Biological Laboratory, Woods Hole, Mass. Axons were cleaned of most adhering tissue, the axoplasm squeezed out with a tiny rubber roller after the technique of Baker et al. (1961) and replaced by an artificial internal solution infused with the aid of a micrometer syringe. Axons were then mounted in a Plexiglas chamber designed for voltage clamping by conventional techniques described previously (Wang et al., 1972; Wu and Narahashi, 1973).

Briefly, a "piggyback" double axial electrode assembly was inserted longitudinally into the axon for measurement and control of membrane potential. The potential monitoring glass capillary was 75 μm in diameter, was filled with 0.6 M KCl, and contained an electrically floating 25-μm Pt-Ir wire. The current-passing electrode was a 100-μm Pt-Ir wire plated with Pt black. Membrane current was measured by the virtual ground of an operational amplifier (model 48J, Analog Devices, Inc., Norwood, Mass.) from a platinized platinum or silver electrode situated within a region of the chamber electrically guarded (ground) to minimize longitudinal current flow. The response time of the voltage control circuit was ~2 μs (10–90% of step pulse command). Feedback compensation was used in all experiments to reduce voltage errors arising from 3–5 $\Omega \cdot cm^2$ of resistance in series with the axolemma. In some experiments the ohmic leakage current and brief surges of linear capacitative current associated with the beginning and end of command voltage steps were substantially reduced by summing the measured membrane current with a synthetic signal of equal but opposite amplitude to the leakage and capacitative components generated by an analog circuit similar to those described previously (Armstrong and Bezanilla, 1975; Hille and Campbell, 1976). Holding potentials were maintained at −80 mV between step commands.

Solutions and Reagents

The axons were perfused externally with an artificial seawater (ASW) containing ions in the following concentrations (mM): Na^+ 450, K^+ 10, Ca^{++} 50, Cl^- 576, HEPES buffer 5. External pH was adjusted to 8.0 in most experiments. The standard internal solution (SIS) contained (mM): Na^+, 50; K^+, 350; glutamate$^-$, 320; F^-, 50; sucrose, 310; phosphate buffer, 15; and was adjusted to pH 7.3 except where otherwise indicated.

In experiments designed to examine the behavior of sodium channels exclusively, the current through potassium channels was reduced or eliminated by internal perfusion of either 20 mM tetraethylammonium chloride (TEA) added to the SIS or 275 mM CsF substituted for K glutamate (isosmolarity maintained by sucrose).

N-bromoacetamide, *N*-bromosuccinimide, *N*-acetylimidazole, *o*-nitrophenyl sulfenyl chloride, 2-methoxy-5-nitrobenzyl bromide, cyanogen bromide, and diethyl pyrocarbonate were purchased from Sigma Chemical Co. (St. Louis, Mo.) and used without further purification. 2,4,6-tribromo-4-methylcyclohexadienone was obtained from Pierce Chemical Co. (Rockford, Illinois).

Temperature in all experiments was maintained between 6 and 10°C by prechilling the continuously flowing external solutions in an ice bath. In later experiments a chamber of similar design to that employed by Armstrong et al. (1973) was used, and temperature was maintained by controlling a thermoelectric cooling device (Cambion model 806-1006-01, Cambridge Thermionic Corp., Cambridge, Mass.) by an electronic feedback circuit.

CHEMISTRY OF N-BROMOACETAMIDE AND N-BROMOSUCCINIMIDE *N*-bromoacetamide (NBA) and *N*-bromosuccinimide (NBS) have been used for the selective nonenzymatic cleavage of peptide bonds in proteins at certain amino acid residues (Means and Feeny, 1971; Ramchandran and Witkop, 1967; Spande and Witkop, 1967). Only peptide bonds adjacent to (on the C-terminal side of) tryptophan (Fig. 1), tyrosine, histidine, and phenylalanine (previously reduced) are cleaved by these reagents. The common element associated with these residues responsible for this degree of specificity is the presence of a double bond in a position γ, δ-to the amide bond. In contrast to the conventional nucleophilic hydrolytic reaction, cleavage with NBA or NBS involves intramolecular nucleophilic participation from the adjacent amide group which can only occur in the case of the four residues mentioned (Mathur and Narang, 1975).

In addition to these cleavage reactions, NBA and NBS are known readily to oxidize sulfur containing amino acids such as cysteine and methionine by hydrogen abstraction. These reactions are of potential importance in view of recent evidence for a role of sulfhydryl groups in membrane excitability (Marquis and Mautner, 1974; Shrager, 1975, 1977). Allylic bromination of nonprotein groups represents another characteristic reaction of these compounds (Mathur and Narang, 1975), but this process is favored in nonaqueous solvents and is considered of little importance to the present investigation.

RESULTS

Addition of 1 mM NBA or NBS to the internal perfusion medium of voltage-clamped squid giant axons results in a rapid disappearance of the fast inactivation process of sodium channels. Fig. 2 illustrates the loss of sodium inactivation gating in two axons perfused with 20 mM TEA to reduce ionic currents through potassium channels. In these examples the effect was essentially complete after a 1-min exposure to NBA (B) or a 3-min exposure to NBS (D). In general, the action of internal NBS was somewhat slower than NBA perhaps owing to its approximately 12-fold lower solubility in aqueous solution (Mathur and Narang, 1975). For this reason NBA was used almost exclusively in the remainder of the experiments.

The action of internal NBA closely resembles the effect of internally applied Pronase (Armstrong et al., 1973). Sodium channels can apparently activate normally upon depolarization after NBA treatment and exhibit rapid tail currents (deactivation) upon return of the membrane potential to hyperpolarized levels. Removal of inactivation was consistently achieved by brief exposure

to NBA, and was maintained after removal of NBA from the perfusion medium. Prolonged exposure to NBA (>5 min) led to a progressive decline in sodium current and an increase in leakage current reflected as an irreversible depolarization under current clamp conditions.

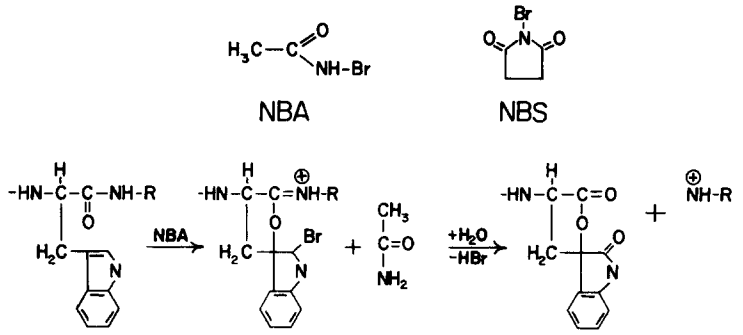

FIGURE 1. Structures of *N*-bromoacetamide (NBA) and *N*-bromosuccinimide (NBS). The reaction scheme for NBA with a tryptophanyl peptide bond is illustrated in the lower portion of the figure.

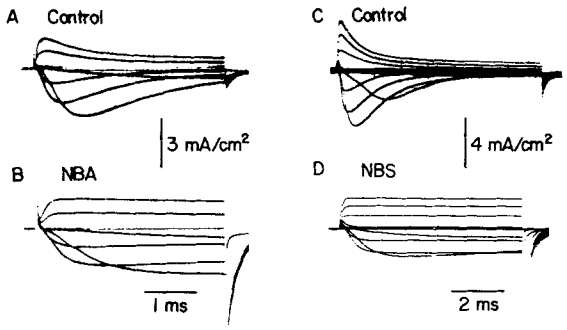

FIGURE 2. Sodium current-time families obtained before (A and C) and after (B and D) removal of inactivation by internal perfusion with NBA and NBS, respectively. Membrane potential steps were to −40, −20, 0, +20, +40, +60, and +80 mV in A and B, and to −30, −10, +10, +30, +50, +70, +90, and +110 in C and D. Holding potential was −80 mV in all cases.

NBA Destroys Sodium Inactivation in an All-or-None Manner

In a normal perfused squid axon with potassium channels pharmacologically blocked, the sodium current in response to a step depolarization rises to a peak and decays to a small residual steady-state level (Fig. 2A and C). During exposure to NBA the steady-state sodium current progressively increases (Fig. 3, top). After partial treatment with NBA the time-course of remaining sodium inactivation (standard exponential fitting analysis after asymptotic correction) is identical to control values (Fig. 3, bottom). This suggests that after brief exposures to NBA the total channel population is represented by only two groups: those channels with normal inactivation kinetics and those channels

completely devoid of normal inactivation properties. The presence of a significant fraction of channels with only retarded inactivation kinetics would be expected to contribute a detectable distortion in the remaining inactivation time course during NBA treatment.

The voltage dependence for remaining sodium inactivation behavior following partial NBA treatment is unchanged as determined by a standard double pulse procedure (Fig. 4). In this experiment the relative amplitude of peak sodium current during a step to 0 mV was determined as a function of conditioning voltage steps (70 ms) immediately preceding the test pulse. The

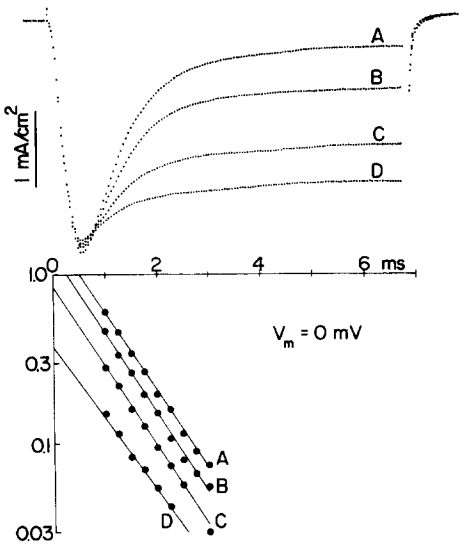

FIGURE 3. All-or-none destruction of inactivation by NBA. Upper records are sodium currents at 0 mV obtained at various times during NBA treatment and manually traced from 35-mm oscilloscope photographs. Lower graph illustrates the time-course of remaining inactivation (after correction for steady-state I_{Na}) corresponding to each trace. Logarithmic ordinate is in arbitrary units. Filled circles are data points while straight lines represent the least-squares exponential fit to each set of point.

axon was sequentially exposed to 50 μM NBA for brief periods to achieve different levels of effect. A 'foot' developed in the steady-state inactivation curve as the NBA effect progressed, indicating the appearance of a population of channels which failed to inactivate completely (at least after a 70-ms depolarization). Consideration of the steady-state inactivation curve for the total channel population reveals an apparent shift of the voltage dependence in the direction of depolarization. However, if the curves are normalized in amplitude (maximum to minimum values) to represent only the inactivating population, the apparent shift is greatly reduced. Midpoint potential values are given in the figure legend.

NBA and Maximum Sodium Current

According to the Hodgkin-Huxley model for sodium conductance, removal of the inactivation or h process alone would result in a significant increase in the peak amplitude of sodium current (I_{Na}). Some studies have demonstrated consistent increases in peak I_{Na} after internal perfusion of giant axons with a crude fraction of the enzyme Pronase (Rojas and Rudy, 1976), whereas other investigators report slight decreases in I_{Na} with crude Pronase (Armstrong et al., 1973). The results with NBA treatment reflect similar variability. In general, decreases of about 20–30% were found to accompany NBA treatment (see for example Fig. 2). On occasion the magnitude of I_{Na} would either remain constant

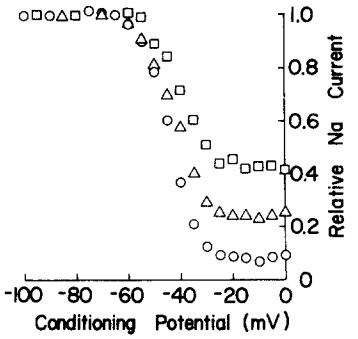

FIGURE 4. Steady-state sodium inactivation after partial NBA treatment. Data points represent peak sodium current at 0 mV as a function of conditioning prepulse (70 ms) amplitudes (normalized at −80 mV) before NBA (○), after a 2-min perfusion with 50 μM NBA (△), and an additional 2-min perfusion with 50 μM NBA (□). After further normalizing the curves (see text) the membrane potentials corresponding to 0.5 maximum inactivation were −43 mV (○), −42 mV (△), and −40.5 mV (□).

or increase by up to 15%. The small increases are still less than the prediction of the Hodgkin-Huxley model perhaps reflecting a total destruction of some channels by NBA (see Discussion) as previously suggested for Pronase (Armstrong et al., 1973).

NBA and Sodium Channel Activation

As reported previously for internal perfusion with Pronase (Armstrong et al., 1973), activation properties of the sodium channels are not significantly altered by NBA. Fig. 5 illustrates the time-course of sodium currents corresponding to depolarizations to −40, −30, −20, and −10 mV from a holding potential of −80 mV before (A) and after (B) internal perfusion with 1 mM NBA. The kinetics of opening the sodium channels are not dramatically altered by the NBA treatment. The apparent slowing in this figure is eliminated if the current amplitudes are normalized by extrapolating the inactivation phase time-course back to the beginning of the voltage step and scaling the current after NBA treatment by the ratio of the extrapolated current value at $t = 0$ before NBA to the value

after NBA. This normalization procedure allows for the kinetics of normal sodium inactivation assuming a Hodgkin-Huxley (1952) type of conductance mechanism.

Activation kinetics can also be measured by observing the time-course of sodium tail currents upon repolarization (deactivation). Fig. 5 illustrates the time-course of sodium tail currents at −80, −70, and −60 mV before (C) and after (D) NBA treatment. The decay rates of sodium tail currents are virtually identical before and after NBA treatment. Time constants for the traces in Fig. 5C and D are given in the figure legend. The tail currents at −70 and −60 mV are, however, somewhat larger after removal of inactivation despite only a 6%

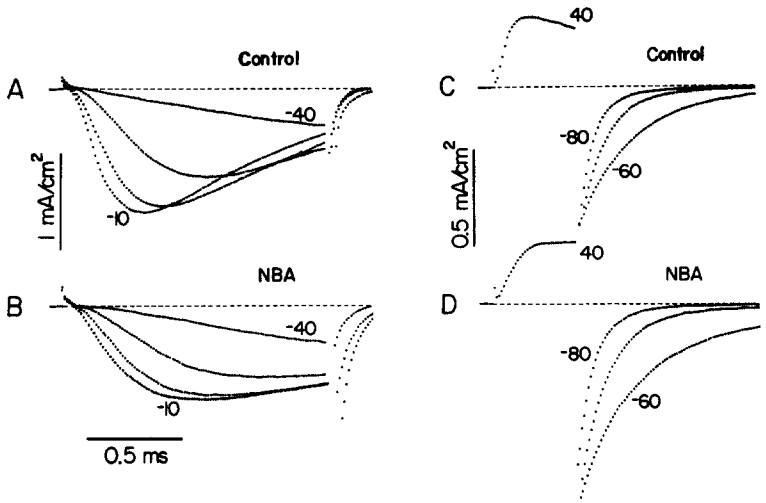

FIGURE 5. Activation and deactivation of sodium channels in NBA. Time-course of sodium current turn-on before (A) and after (B) NBA treatment. Sodium tail currents before (C) and after (D) NBA treatment. Axons were bathed in 1/3 normal sodium seawater (2/3 sodium substituted by tetramethylammonium) and internally perfused with 275 mM CsF replacing the K glutamate. Time constants (μs) for the decay of sodium tails in C and D are (Control/NBA) 221/217 at −80 mV, 242/241 at −70 mV, and 318/337 at −60 mV.

increase in sodium current at the end of the 40 mV voltage step. In addition, the time-course appears to be slowed by NBA on casual observation. There are two factors which we feel contribute to this appearance. First, in the absence of inactivation, a steady-state inward sodium current is present at low depolarizations; thus, the tail currents for −70 and −60 mV decay to non-zero asymptotic values make the tails seem larger and slower. Secondly, preliminary examination of the instantaneous sodium current-voltage relation in NBA-treated axons suggests a change in shape of the curve consistent with a relative increase in sodium conductance for membrane potentials more negative than ~ −10 mV (Oxford, unpublished observation; Yeh and Narahashi, 1977). Tail currents observed upon repolarization to potentials more positive than these are appreciably contaminated by normal inactivation gating before NBA treatment and

exhibit larger steady-state inward sodium currents after NBA treatment, thus making detailed comparison more difficult.

Results such as those of Fig. 5 were, by far, the rule in our experiments. On occasion, however, NBA perfusion apparently prolonged the time-course of sodium tail currents by a factor of 2–3. So far similar effects have not been observed after removal of inactivation with Pronase. These rare effects are perhaps related to difficulties in maintaining spatial homogeneity of membrane potential in the face of prolonged inward sodium currents in normal sodium ASW, inasmuch as NBA appears consistently to destroy more of the inactivation mechanism than does Pronase. Further experiments are needed to clarify this point. Recently, more quantitative experiments on sodium activation have been performed in low external sodium concentrations. In these experiments sodium

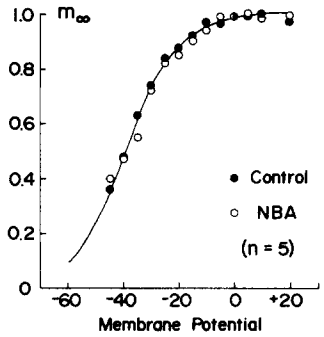

FIGURE 6. Steady-state sodium activation, m_∞ of the Hodgkin-Huxley model, before and after NBA treatment. Data points represent mean values (n = 5) of data obtained as described in text. Solid curve is drawn according to $\alpha_m \cdot (\alpha_m + \beta_m)^{-1}$ after Hodgkin and Huxley (1952) but displaced by 5 mV in the hyperpolarizing direction.

activation time constants (τ_m of the Hodgkin-Huxley formulation) and sodium deactivation time constants (tail current measurements) were found to be unchanged by either NBA or Pronase treatment over a wide range of membrane potentials. These data have appeared in preliminary form (Oxford and Yeh, 1977) and will be reported in detail at a later time.

The steady-state voltage dependence of sodium activation was not significantly altered by perfusion with NBA (Fig. 6). Control values of steady-state activation (m_∞) were derived by extrapolating the time-course of sodium inactivation (I_{Na}) back to the beginning of each depolarizing step, computing a conductance (G_{NA}') from the resulting values of current, and taking the cube root of G_{Na}' (maximum values normalized to one). Direct measurements of m_∞ from steady-state sodium currents were performed after removal of inactivation by NBA. The data are adequately described (solid line) by the empirical formulae of Hodgkin and Huxley (1952) for α_m and β_m, the rate constants governing transitions between closed and open channel states, shifted by 5 mV in the hyperpolarizing direction.

As another test of the retention of normal activation properties, the response

of sodium channels to changes in external calcium concentration was determined in a few cases after internal NBA treatment. Sodium conductance-voltage curves in external solutions containing either 10, 50, or 100 mM Ca^{++} were normalized, and shifts of the curves along the voltage axis (relative to the values in 50 mM Ca^{++}) were measured at 10% of the maximum sodium conductance. The shifts (Fig. 7) were essentially identical before (●) and after (○) removal of sodium inactivation by NBA. A least-squares fit to the data yields a line with a slope of 9.1 mV/*e*-fold change in Ca^{++} concentration, consistent with previous values of 8.7 mV for *Rana* nodes (Hille, 1968), 9.4 mV for *Loligo forbesi* axons (Frankenhaeuser and Hodgkin, 1957), and 9.8 mV for *Myxicola* axons (Schauf, 1975).

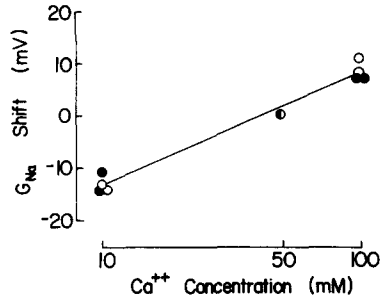

FIGURE 7. Effect of external calcium on sodium activation. Shifts of peak G_{Na} curves (measured at 0.1 maximum G_{Na}) along the voltage axis as a function of external calcium concentration. Measurements were made before (●) and after (○) NBA perfusion. Straight line is a least-squares fit with a slope of 9.1 mV/*e*-fold calcium change.

Specific Groups Involved in the NBA Effect

As mentioned in the Introduction, interpretation of the effects of protein reagents on excitability in terms of key specific amino acid residues is clouded to some degree by the range and overlap of specificity of the reagents. NBA, which is highly reactive with a number of amino acid side chains, is no exception. However, by making use of the overlap in reactivities of a number of other reagents, we attempted to narrow the possible amino acid candidates involved in the destruction of sodium inactivation by a process of elimination. By applying a series of reagents to the internal axon surface reported to exhibit greater specificity for groups involved in "side reactions" with NBA, we sought to determine whether such reagents (*a*) could mimic the effects of NBA or (*b*) could antagonize or prevent the effects of a subsequent exposure to NBA. Negative results with respect to both points with a given reagent was taken as suggestive that the particular residue for which that reagent is most specific is not intimately involved in the action of NBA in removing sodium channel inactivation. The key residues to be tested were tryptophan, tyrosine, and histidine (NBA cleaves adjacent peptide bonds), and cysteine and methionine (NBA oxidizes sulfur-containing amino acids).

Caution must necessarily be exercised when evaluating the results of this

approach, inasmuch as comparisons of relative effectiveness among the various reagents may be significantly influenced by different dose dependencies and unknown microenvironmental constraints imposed by the association of the target protein with the membrane. Nevertheless, such experiments may provide limited insight into the molecular architecture of the inactivation mechanism and so were performed using near-equimolar concentrations of reagents as a first approximation to ideal conditions.

Reactions with sulfhydryl groups of cysteine residues were examined by perfusion with *N*-ethylmaleimide (NEM), 5,5'-dithiobis(2-nitrobenzoic acid)

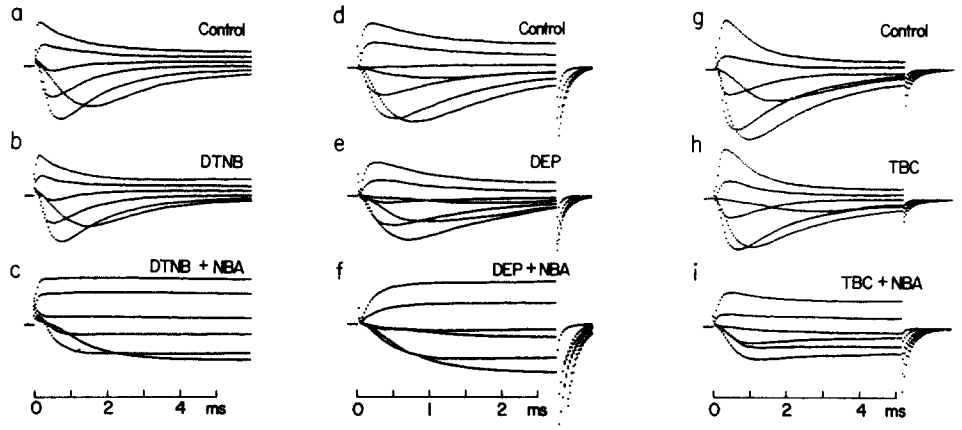

FIGURE 8. Absence of interaction between sulfhydryl, histidyl, or tryptophanyl group reagents and the sodium inactivation gate. Three sets of sodium current-time families represent (from top to bottom) control currents, the effects of internal perfusion with a particular protein reagent, and the effects of NBA perfused in combination with the other protein reagents. The reagents shown in this figure are 2 mM DTNB (a, b, c), 1 mM DEP (d, e, f), and 5 mM TBC (g, h, i). Membrane potential steps were in 20-mV increments between −20 and +80 (a, b, c), −40 and +80 (d, e, f), and −30 and +70 mV (g, h, i). Normal ASW (a–f) or 1/3 normal sodium seawater (g–i) bathed the axons. Potassium channels were blocked by perfusion with either 20 mM TEA (a–c) or 275 mM Cs (d–i). The vertical bar in each case represents 1 mA/cm^2. See text for further details.

(DTNB), or *p*-chloromercuriphenyl sulfonic acid (PCMBS). These reagents are known to react specifically with protein sulfhydryl groups under mild aqueous conditions (Means and Feeny, 1971). The effect of internal perfusion with 2 mM DTNB is illustrated in Figure 8b. After 30 min, the peak sodium currents were reduced by only 14% while inactivation remained normal. A subsequent 1-min exposure to 1 mM NBA plus 2 mM DTNB yielded the usual loss of inactivation (Fig. 8c). Similar results were observed with NEM and PCMBS although a somewhat larger reduction in I_{Na} was observed with these reagents alone (~30%).

Diethyl pyrocarbonate (DEP) has been shown to react specifically with histidine residues at pH 6.0 (Muhlrad et al., 1967; Ovádi et al., 1967) and has been used by Shrager (1974) to modify potassium channels in the crayfish axon. DEP was added to the internal perfusate at 1 mM (pH 6.3) by dilution of a stock

solution in dimethyl sulfoxide (DMSO). The final DMSO concentration of 0.5% was without effect on sodium currents in control experiments. A 30-min exposure to DEP reduced peak sodium currents by 27% on average (n = 2) as illustrated in Fig. 8e. Again, a subsequent treatment with 1 mM NBA plus 1 mM DEP failed to prevent the characteristic removal of inactivation (Fig. 8f).

Cyanogen bromide (CNBr) has been used for the selective cleavage of peptide bonds adjacent to methionine residues in proteins (Gross and Witkop, 1962). The reaction usually requires harsh acidic conditions. However, selective cleavage of methionyl peptide bonds has been achieved at pH 6.0 (Cahnmann et al., 1966). At pH 6.3, a 30-min exposure to 5 mM CNBr internally perfused was without effect upon sodium currents and did not prevent the destruction of inactivation by NBA.

Attempts at modification of tryptophan residues with 2-methoxy-5-nitrobenzyl bromide (MNBB) or 2-hydroxy-5-nitrobenzyl bromide (HNBB) (Koshland et al., 1964) were difficult owing to the rapid aqueous hydrolysis of the compounds ($t_{\frac{1}{2}}$ = ~1 min in water; Means and Feeny, 1971). Despite this difficulty MNBB (0.1 mM) was found to suppress sodium current slightly when applied internally, but greatly reduced potassium current in a reversible manner resembling the action of TEA. No obvious alteration in sodium inactivation behavior occurred with MNBB nor did it antagonize the action of NBA.

o-Nitrophenylsulfenyl chloride (*o*-NPS) has been shown to modify tryptophan residues selectively to yield a 2-thioether derivative under acidic conditions (Scoffone et al., 1968). *o*-NPS was added to the internal perfusate by dilution of a stock solution in DMSO to final concentrations of 1 mM NPS and 0.5% DMSO. *o*-NPS had no effect upon sodium currents during a 30-min exposure nor did it antagonize the effect of NBA.

The reactivity of 2,4,6-tribromo-4-methyl-cyclohexadienone (TBC) with peptide bonds has been directly compared to that of NBS in synthetic tripeptides containing either histidyl, tyrosyl, or tryptophanyl peptide bonds (Burstein et al., 1967). TBC was found to cleave tryptophanyl peptide bonds only under the same conditions in which NBS reacted with all three types of peptide bonds. When 5 mM TBC in DMSO (0.5% final DMSO concentration) was internally perfused in the squid axon, sodium currents were reduced by 22% (n = 2), but inactivation properties were unaltered (Fig. 8h). However, inactivation was still sensitive to NBA after TBC treatment (Fig. 8i).

Under mild aqueous conditions *N*-acetylimidazole (NAI) has been found to react selectively with tyrosine residues by acetylating the phenol group (Riordan and Vallee, 1963; Riordan et al., 1965). When perfused inside the squid giant axon, NAI (1 mM, pH 7.3) substantially reduced sodium current and destroys much of the inactivation mechanism (Fig. 9). The reduction in current is partially reversed by perfusion with NAI-free solution (Fig. 9b), but the dramatic loss of sodium inactivation is irreversible resembling an incomplete treatment with NBA.

NBA and Slow Sodium Inactivation

In addition to the fast sodium inactivation process described by the h parameter of the Hodgkin-Huxley model, voltage-dependent attenuation of sodium conductance has also been reported which exhibits much slower kinetics (Adelman

and Palti, 1969*a*, *b*; Chandler and Meves, 1970; Schauf et al., 1976; Fox, 1976; Rudy, 1975, 1977; and Shrager, 1977). As a preliminary examination of the effect of NBA upon slow sodium inactivation, we measured sodium currents after removal of fast inactivation with NBA during depolarizing step pulses applied from holding potentials (1 min.) between −80 and 0 mV. The sodium current during the test steps declined for progressively depolarizing holding potentials (relative to −80 mV) along a sigmoid curve (Fig. 10) with a half-saturation value corresponding to a holding potential of −37 mV. This value is essentially the same as the value of −35 mV found for slow inactivation in *Loligo forbesi* (Rudy, 1977). Although more detailed studies of the slow inactivation process are needed before its interaction with NBA becomes clear, it is apparent that some form of slow inactivation is retained despite removal of the fast inactivation by NBA.

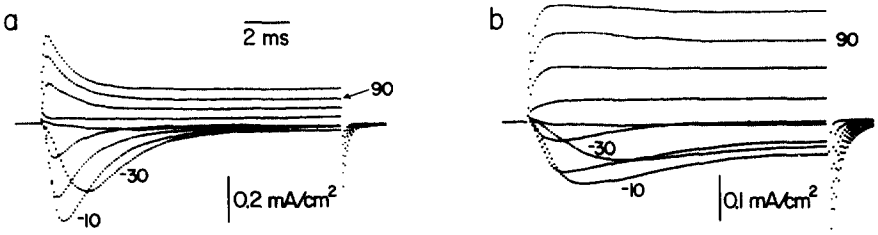

FIGURE 9. *N*-acetylimidazole inhibits sodium channel inactivation. Sodium current-time families before NAI (a) and after a 15-min exposure to 20 mM NAI followed by an 18-min internal rinse with NAI-free perfusate (b). Membrane potential steps were in 20 mV increments from −50 to +110 mV. 275 mM CsF replaced K glutamate in the internal perfusion medium.

NBA and Potassium Channels

In a few experiments the effects of internal NBA upon potassium conductance (G_K) were examined when sodium channels were blocked by 10^{-6} M tetrodotoxin (TTX) and the internal perfusate was normal SIS. In contrast to the apparently small effect of Pronase upon G_K (Armstrong et al., 1973), we found that, at exposure times just sufficient to remove sodium inactivation completely, G_K was reduced by 20–40%. The kinetics of potassium channel activation were not affected as time constant (τ_n) values were unaltered by NBA at all potentials examined.

External Application of NBA

When applied externally 1 mM NBA or NBS progressively reduced the action potential amplitude and depolarized the axon by 20–30 mV (45-min exposures). Under voltage clamp conditions the peak sodium current was reduced by ~80% after 20–30 min of exposure while potassium current (I_K) was much less affected. Prolonged exposure resulted in a rapid increase in leakage current and decline in I_K. No changes in sodium inactivation time course were observed during external NBA treatment.

DISCUSSION

N-bromoacetamide and *N*-bromosuccinimide have been shown to effectively remove the normal fast inactivation of sodium channels when perfused inside squid axons at moderate concentrations and near neutral pH. The effect is irreversible for up to 7 h after removal of the compounds from the perfusion medium and is quite rapid, being essentially complete after a few minutes of exposure.

The voltage-dependent activation of sodium channels is essentially unchanged after destruction of inactivation by NBA as demonstrated by (*a*) the kinetics of sodium current turn-on during step depolarizations, (*b*) the kinetics of sodium tail currents after repolarization to voltage levels favoring the closed state of the

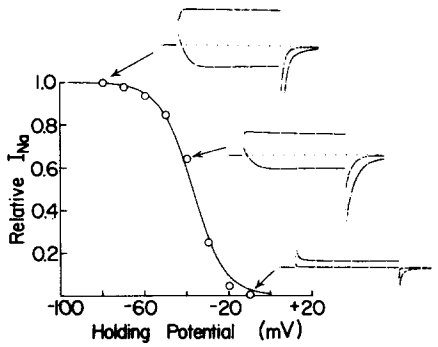

FIGURE 10. Slow sodium inactivation remains after NBA treatment. Data points represent sodium currents at a test potential of 0 mV from different holding potentials (normalized to I_{Na} at a holding potential of −80 mV). Smooth curve drawn according to relative $I_{Na} = [e^{(-35 - E_H)/-11.5} + 1]^{-1}$. Insets are sodium currents for depolarizations to 0 and +100 mV after NBA treatment corresponding to the indicated data points (holding potentials of −80, −40, and −10 mV).

channels, (*c*) the steady-state voltage dependence of sodium conductance, and (*d*) the sensitivity of the activation gating mechanism to changes in external calcium. In addition, the potassium conductance is somewhat reduced during NBA perfusion without significant change in time course. Also, some form of slower sodium inactivation remains after NBA perfusion as 1-min depolarizing prepulses significantly suppress G_{Na}.

The magnitude of I_{Na} after experimental treatments which selectively slow or remove sodium inactivation in axon membranes is apparently quite variable. Such is the case with NBA inasmuch as increases in current were occasionally observed, but slight decreases were seen as a rule. Attempts to observe increases in I_{Na} consistent with the prediction of the Hodgkin-Huxley model of independent activation and inactivation mechanisms have been successful qualitatively, but have consistently failed quantitatively. Increases in I_{Na} have been reported to occur after slowing of inactivation by *Condylactis* toxin (Narahashi et al., 1969) and by 2,4,6-trinitrophenol (Oxford and Pooler, 1975*b*) applied to crustacean giant axons and after removal of inactivation in squid axons by a crude fraction

of Pronase (Rojas and Rudy, 1976). Increases in I_{Na} were also observed upon internal application of beryllium ions in squid axons although corresponding changes in kinetics were not reported (Eaton and Brodwick, 1975). A number of explanations for the failure to observe the quantitatively predicted increase have been suggested including forward coupling between activation and inactivation processes (Goldman, 1975; Oxford and Pooler, 1975*b*; Bezanilla and Armstrong, 1977), total destruction of a portion of sodium channels simultaneous with loss of inactivation in the remaining population (Armstrong et al., 1973; Bezanilla and Armstrong, 1977), and enhanced pharmacological interaction between sodium channels and ion species used to inhibit current through delayed potassium channels (TEA or Cs) (Rojas and Rudy, 1976). In the latter case sodium currents in *Loligo forbesi* were found to be insensitive to internally applied TEA with intact sodium inactivation, but rendered sensitive to block by TEA after Pronase treatment. In contrast, sodium currents in the present studies were partially blocked (20%) by internal TEA (20 mM) before removal of inactivation by NBA (or Pronase). In all cases, however, no direct evidence is yet available supporting any single explanation for the discrepancies between predicted and observed amplitudes of sodium current after modification of the inactivation mechanism.

Role of Specific Amino Acid Residues in Sodium Inactivation? Clues and Reservations.

NBA and NBS react with a variety of amino acid side chains, but the cleavage of peptide bonds adjacent to tryptophan or tyrosine residues in proteins are the most characteristic reactions, often used in determination of amino acid sequence (Mathur and Narang, 1975). By examining the effects of other reagents which exhibit overlapping spectra of reactivities for other amino acid residues which also react with NBA, we attempted to clarify the specific target residue of NBA by an indirect approach. Basically we examined the possibility that several other reagents might either mimic or antagonize the effects observed with NBA when applied to the internal axon surface.

The amino acid 'scorecard' from these studies is given in Table I. The table lists the amino acids which can in some way react with NBA along with the alternative protein reagents tested with regard to their ability to modify sodium inactivation, antagonize or compete with NBA, and suppress peak sodium current. In addition, the reagents are classified as either side-chain reactive (S) or peptide bond reactive (P) towards the appropriate amino acid residue. It is apparent from the table that the effects of the other reagents on sodium inactivation were largely negligible. Specifically, treatment of the internal surface with *N*-acetylimidazole was the only case in which removal of the inactivation behavior occurred in a manner similar to NBA. Because tyrosine residues represent the only amino acid target common to both NBA and NAI, these limited results perhaps suggest that fast sodium inactivation can be destroyed by modification of a tyrosine residue (either by acetylation with NAI or peptide bond cleavage by NBA) which forms an integral component of the inactivation gating mechanism.

Unfortunately, the complicated nature of this experimental approach prohibits unambigous conclusions concerning the site of action of NBA. For example, the reaction conditions in each case were designed as a compromise between those which reportedly promote biochemical specificity and those which of themselves are compatible with normal sodium channel function. In most cases a satisfactory set of conditions was reached; however, in other cases (e.g., peptide bond reactions with CNBr which are favored in very low pH), the extent of the reactivity with appropriate membrane components cannot be assured. The reagents employed can be placed into two broad categories: those

TABLE I

PROTEIN REAGENT MODIFICATIONS OF Na INACTIVATION IN SQUID GIANT AXONS

Amino acid	Reagent	Type of reaction*	pH	Concn	Modifies inactivation‡	Antagonizes NBA action	Suppresses sodium current‡
				mM			
Cysteine	NBA	S	7.3	1	+	....	+
	NEM	S	7.3	2	−	−	++
	PCMBS	S	7.3	2	−	−	++
	DTNB	S	7.3	2	−	−	+
Methionine	NBA	S	6.5	1	+	....	+
	CNBr	P	6.3		−	−	−
Histidine	NBA	P	7.3	1	−	....	+
	DEP	S	7.3	1	−	−	+
	DEP	S	6.3	1	−	−	+
Tryptophan	NBA	P	7.3	1	+	....	+
	MNBB	S	7.3	1	−	−	+
	o-NPS	S	7.3	Saturated§	−	−	+
	TBC	P	6.5	5	−	−	+
Tyrosine	NBA	P	7.3	1	+	....	+
	NAI	S	7.3	5	+	−	+

* Peptide bond cleavage (P) or side chain reaction (S).

‡ Positive result (+) or negative result (−).

§ Concentration unknown because of low aqueous solubility.

which disrupt peptide bonds and those which somehow modify the amino acid side chain. Only in the case of sulfhydryl groups (DTNB, NEM, PCMBS) and tryptophan (TBC) were the reactions between amino acid and reagent of a similar nature to the reaction with NBA. Thus, direct comparisons of the effects of the other reagents and NBA in terms of specific residues may not be strictly appropriate.

The degree of exposure and topography of target residues associated with sodium inactivation gating is unknown. The possibility exists therefore, that certain of the reagents examined were somehow limited by steric or coulombic forces, in their access to the site(s) where NBA reacts. Such phenomena would be manifest as a negative result in Table I. One cannot argue this possibility without further information; however, in view of the relative lability of sodium inactivation in the presence of such small molecules as NBA or NAI on the one hand, and large enzymes such as pronase and trypsin on the other hand, it

seems reasonable to postulate sufficient exposure of a peptide chain such that most reactions with key amino acids could readily occur.

Thus, any speculation concerning a specific role for tyrosine residues in the sodium inactivation process necessarily rests on the assumptions that (*a*) all reagents examined had access in sufficient concentrations to the key molecules altered by NBA treatment, (*b*) the reactivities of all reagents under our experimental conditions are sufficiently representative of their known specific effects, and (*c*) the target molecule in the NBA effect is sufficiently sensitive to modification that several different types of reactions can lead to altered sodium inactivation gating. In any event, until further data are available, we can only suggest a possible role for tyrosine in sodium channel function.

Relation of Present Work to Previous Data

Much of the previous work utilizing group specific reagents to dissect the process of membrane excitability has concentrated upon the possible involvement of sulfhydryl groups. A number of sulfhydryl reagents have been shown to block action potentials in axons when applied to either external (Smith, 1958) or internal (Huneeus-Cox et al., 1966) membrane surfaces. Externally applied NEM was found to reduce sodium conductance in voltage-clamped frog nodes of Ranvier (Keana and Stämpfli, 1974). Marquis and Mautner (1974) reported an interesting potentiation of action potential block by repetitive stimulation of squid axons treated externally with PCMB, mercurochrome, NEM, DTNB, and fluorescein mercuric acetate. This effect resembles the "use dependent" inhibition of sodium currents by certain local anesthetics (Strichartz, 1973; Courtney, 1975; Yeh and Narahashi, 1976). A detailed investigation of the action of NEM on sodium channels of crayfish axons (Shrager, 1977) has recently revealed that inhibition of sodium currents by NEM is almost completely reversed by prolonged hyperpolarization of the membrane. This effect suggests that induction of a slow inactivation mechanism by NEM which may in fact be a change in voltage dependence of a process normally present in the membrane (Shrager, 1977). No changes in sodium current kinetics resembling the action of NBA were observed. Similarly, we observed only decreases in sodium current unaccompanied by changes in inactivation kinetics upon treatment with sulfhydryl reagents. Thus, the accumulated data on sulfhydryl group modification in excitable membranes fails to support the involvement of cysteine residues in the destruction of fast sodium inactivation by NBA.

Keana and Stämpfli (1974) investigated the actions of a number of other group specific reagents on the external surface of frog nodes of Ranvier. Several of them, including NBS, caused progressive declines in both G_{Na} and G_K. Unfortunately, no kinetic data is available from this study. Shrager (1977) briefly mentioned that NBS produced a rapid loss of resting potential when applied externally to crayfish axons. These data are consistent with our observations of decreased G_{Na} and depolarization in squid axons bathed in solutions containing NBS or NBA. The lack of effect of NBS or NBA in destroying the inactivation process when applied externally is consistent with the data from enzyme studies and suggests that an important component of the

inactivation gating machinery is readily accessible only from the internal membrane surface.

Few nonenzymatic treatments have been found to remove sodium inactivation in a manner similar to NBA. Internal application of iodate or periodate effectively removes the inactivation mechanism in frog node of Ranvier (Stämpfli, 1974; Conti et al., 1976). Tannic acid produces long action potentials in crayfish axons when internally perfused (Shrager et al., 1969) and indeed prolongs sodium currents under voltage clamp conditions (Shrager and Starkus, personal communication; Horn and Brodwick, personal communication). The exact mechanisms by which iodate or tannic acid act are unknown at present, by may well involve interaction with much of the same peptide substrate attacked by NBA.

Comparison with Proteolytic Enzyme Treatments

Complete destruction of fast sodium channel inactivation was first demonstrated by internal perfusion with the enzyme mixture Pronase (Rojas and Armstrong, 1971). This treatment apparently does not alter sodium channel activation properties as the kinetics of sodium tail currents and the voltage dependence of peak I_{Na} were unaffected (Armstrong et al., 1973). A partially purified fraction of Pronase, alkaline proteinase B, has produced essentially identical results when internally perfused (Rojas and Rudy, 1976) although peak sodium currents were consistently observed to increase by ~25% during treatment. The possible implications of this result have already been discussed.

Several other studies (discussed in detail by Rojas and Rudy, 1976) of proteolytic enzymes applied to the internal surface of squid axons under both current- and voltage-clamp conditions suggest that the following enzymes are all capable of removing fast sodium inactivation: subtilisin, prozyme, bromelin, papain, ficin, α-chymotrypsin, and trypsin (Tasaki and Takenaka, 1964; Takenaka and Yamagishi, 1969; Sevcik and Narahashi, 1975; Rudy, 1976). The high substrate specificity of trypsin and alkaline proteinase B for cleaving peptide bonds adjacent to lysine or arginine residues led Rojas and Rudy (1976) to suggest several possible primary structures for the inactivation "gate" which include an arginine or lysine residue (positively charged at neutral pH) which can move in response to changes in the local membrane electric field. Such a structure could be located at the inner membrane surface adjacent to the sodium ion pathway of the channel and would presumably upon depolarization move into a position so as to occlude the ion pathway in a manner analogous to tetraethylammonium ion derivatives (Armstrong, 1971; Rojas and Rudy, 1976).

Although our data are consistent with the possible involvement of a tyrosine residue in the inactivation gating structure, it is not necessarily inconsistent with previous proposals. In fact, the particular primary structure favored by Rojas and Rudy (1976, Fig. 16D) incorporates a peptide linkage between arginine (or lysine) and phenylalanine. A tyrosine residue could be substituted for the hypothetical phenylalanine resulting in a structure compatible with both sets of data. Evidence supporting the involvement of an arginine residue in the inactivation process has recently been obtained with internal application of

several arginine-specific reagents (Eaton, Oxford, Brodwick, and Rudy, unpublished observations). It is important to point out here that all of these suggested structures are highly speculative and much more data are required before an accurate molecular picture of the inactivation gate can be proposed.

In summary, it appears as though Pronase (and perhaps other enzymes) and *N*-bromoacetamide react with sodium channels in a similar manner; both destroy the inactivation process and leave activation essentially unaltered. Both agents can be used to examine the behavior of sodium activation in detail uncontaminated by inactivation in voltage ranges where previous studies have been either impossible or have relied heavily upon assumptions concerning the relation between the two processes. Such investigations are now in progress (Oxford and Yeh, 1977) and will be reported in detail later. In addition, NBA may prove useful in attempts at biochemical isolation of channel "gating molecules." Both Pronase and NBA have already proven useful in direct measurements of the interaction of drug molecules with sodium channels (Cahalan and Shapiro, 1976; Almers and Cahalan, 1977; Yeh and Narahashi, 1976, 1977).

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