J. Membrane Biol. 111, 253–264 (1989)

The Journal of
Membrane Biology


# Reconstituted Voltage-Sensitive Sodium Channels from Eel Electroplax: Activation of Permeability by Quaternary Lidocaine, N-bromoacetamide, and N-bromosuccinimide

Edward C. Cooper and William S. Agnew
Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06510

**Summary.** We have investigated the ion permeability properties of sodium channels purified from eel electroplax and reconstituted into liposomes. Under the influence of a depolarizing diffusion potential, these channels appear capable of occasional spontaneous openings. Fluxes which result from these openings are sodium selective and blocked (from opposite sides of the membrane) by tetrodotoxin (TTX) and moderate concentrations of the lidocaine analogue QX-314. Low concentrations of QX-314 paradoxically enhance this channel-mediated flux. N-bromoacetamide (NBA) and N-bromosuccinimide (NBS), reagents which remove inactivation gating in physiological preparations, transiently stimulate the sodium permeability of inside-out facing channels to high levels. The rise and subsequent fall of permeability appear to result from consecutive covalent modifications of the protein. Titration of the protein with the more reactive NBS can be used to produce stable, chronically active forms of the protein. Low concentrations of QX-314 produce a net facilitation of channel activation by NBA, while higher concentrations produce block of conductance. This suggests that rates of modifications by NBA which lead to the activation of permeability are influenced by conformational changes induced by QX-314 binding.

**Key Words** action potential · ion channel · inactivation gating · local anesthetic · chemical modification

## Introduction

Most studies of the functional properties of purified sodium channels reconstituted into liposomes or planar bilayers have made use of alkaloid type neurotoxins (e.g., batrachotoxin (BTX), veratridine) to increase channel open probability (*see* Miller, 1986). These are powerful toxins that strongly modify the channel's biophysical and pharmacological properties (Khodorov, 1978).

Chemical modification can also be used to activate channel conductance. In electrophysiological studies, chemical modifiers and proteases eliminate sodium channel fast inactivation gating, but leave other biophysical and pharmacological properties essentially unchanged (Armstrong, Bezanilla & Rojas, 1973; Cahalan, 1978; Oxford, Yu & Narahashi, 1978; Patlak & Horn, 1982; Horn, Vandenberg & Lange, 1984). We have recently used chemical reagents and proteases to activate conductance by sodium channels purified from *Electrophorus electicus* and reconstituted into liposomes (Cooper, Tomiko & Agnew, 1987; Agnew et al., 1988). N-bromoacetamide (NBA), N-bromosuccinimide (NBS), pronase and trypsin each stimulated ion-selective fluxes that were blocked by tetrodotoxin (TTX) and the lidocaine derivative QX-314. Our studies indicated that only those channels exposed to modification at their cytoplasmic surface were activated. More recently (Shenkel, et al., 1989), it was observed that NBS and trypsin-modified proteins incorporated into planar bilayers exhibit conductance, gating, and pharmacological properties resembling those of channels in native membranes modified by these reagents.

We here focus on some unexpected observations made with the reconstituted preparation. We observe that one component of background tracer uptake is markedly sodium selective and blocked by TTX and QX-314, suggesting that channel occasionally open spontaneously. NBA and NBS produce multiple effects, enhancing sodium-selective uptake in brief treatments, then abolishing it as time increases. NBS, which is active at 10 to 100-fold lower concentrations than NBA, can be used to titrate the reaction, leaving the channels in a chronic state of activation. This suggests that the rising and falling phases of the flux activation result from multiple modification reactions; further, stable, conducting NBS modified preparations are suitable for characterization in planar bilayer or patch-clamp recording studies. We further observe that, under

some conditions, QX-314 paradoxically increases rather than decreases tracer flux. This lidocaine analogue, at low doses, stimulates TTX-sensitive tracer flux, in the absence of other channel activators. Further, at low concentrations, QX-314 enhances the rate at which NBA activates flux. This suggests that QX-314 binding produces a conformational change in the channel protein, which results in altered sensitivity to NBA modification.

## Materials and Methods

### Materials

Live *Electrophorus electricus* were from World Wide Scientific Supply (Orlando, FL). TTX was from Y. Kishi (Harvard University) and was tritiated, purified, and calibrated as previously described. BTX was the kind gift of J. Daly (NIH). QX-314, from Astra Pharmaceuticals, was the gift of R. Aldrich (Stanford University). Radiotracer $^{22}$NaCl and $^{86}$RbCl was purchased from Amersham. Egg phosphatidylcholine from Sigma Chemical was added to buffers used in purification procedures. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine from Avanti Polar Lipids (Birmingham, AL) was added to the purified channel suspension prior to detergent removal. Biobeads SM2 were from Biorad. Other reagents were from Sigma.

### Purfication and Reconstitution Methods

Two purification strategies were used. The first, previously described in detail (Tomiko et al., 1986), used DEAE-sephadex ion exchange chromatography followed by size fractionation on Sepharose 6B. This yielded specific activities of 800–1300 pmol [3H]TTX binding sites per mg protein, and purity by SDS-polyacrylamide gel electrophoresis was 80–90%. In the second method, lectin-affinity chromatography (James, Emerick & Agnew, 1989) was used after ion exchange, and gel filtration was omitted. This method resulted in a degree of purity similar to the first, but yield and specific activity were markedly increased. Reconstituted vesicles were formed by adding sonicated liposomes of PE : PS : PC (in a 5 : 4 : 1 weight ratio) to the purified channel solution, followed by detergent adsorbtion with Biobeads SM2 (as previously described, Tomiko et al., 1986). Reconstitution was performed in 10 mM HEPES-NaOH, 84 mM $Na_2SO_4$, 1 mM EGTA, pH 7.4. The osmolarity of the reconstituted vesicle solution was ~200 mOsmol, measured by vapor pressure osmometry.

### Radiotracer Uptake Assays

Aliquots of the reconstituted vesicle suspension were subjected to freeze-thaw-sonication (FTS) to produce large vesicles (*see* Agnew, Tomiko & Rosenberg, 1986). For experiments with BTX, TTX, or QX-314, vesicles were pre-incubated for 30 min at 30°C with the toxin(s) or drug. Iso-osmolar Tris $SO_4$ was prepared by titrating Tris base to pH 7.4 with $H_2SO_4$, and dilution to ~200 mOsmol (~0.170 M Tris).

Two tracer assay protocols were used. In the "uptake time course" protocol, vesicles were diluted 10- or 20-fold into iso-osmolar Tris $SO_4$ containing either tracer $^{22}$Na alone, or both $^{22}$Na and $^{86}$Rb. At intervals the uptake time course was sampled by passing aliquots of the diluted suspension over small columns of Dowex cation exchange resin (Rosenberg, Tomiko & Agnew, 1984*a*). Preliminary experiments indicated that >99.99% of free $^{22}$Na and $^{86}$Rb was bound by the resin; internalized tracer was eluted with the vesicles and measured by liquid scintillation counting. In the second "flux" protocol, 1-min uptake experiments were conducted. Preincubated vesicles were diluted into iso-osmolar tris $SO_4$ *without* radiotracers. Control experiments showed that after a small rundown in the first 5 min after dilution, tracer flux into the vesicles was stable for >1 hr. In experiments reported here, 10–20 min were allowed for rundown, before beginning flux assays. For controls, two aliquots of diluted vesicles were transferred to tubes containing tracers. After 1 min, the vesicles were passed over Dowex columns, and the internalized tracer was counted. The average of these two 1-min assays was taken as the control flux before chemical modification. These duplicates typically differed by 5–20%. Following the control assays, chemical reagents were added to the diluted vesicle stock. at intervals, aliquots of this mixture were assayed in 1-min flux assays. The time course of the permeability modification was followed by plotting the flux *vs.* the duration of modification.

### Basis of Radiotracer Uptake

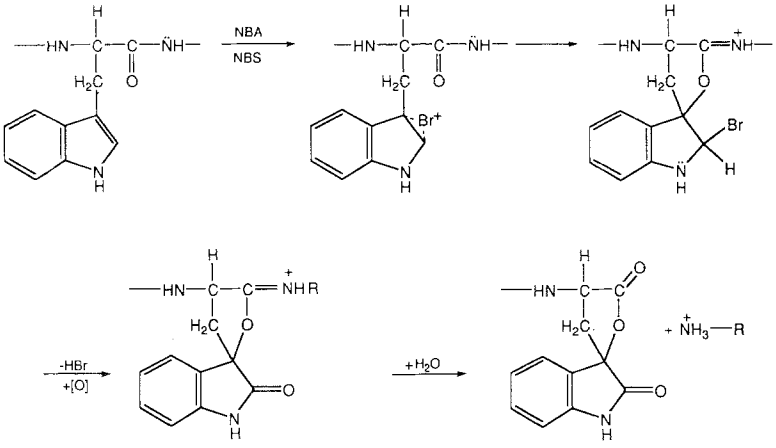
The radiotracer fluxes measured here occur as a result of electoneutral cation exchange, as described in detail previously (Tomiko et al., 1986), and are only briefly commented on here.

In the experiments reported here, liposomes were prepared in 84 mM $Na_2SO_4$, 10 mM HEPES, NaOH, pH 7.4, and 1 mM EGTA, and diluted 10- or 20-fold into an iso-osmolar Tris $SO_4$ buffer. Under these conditions, diffusion potentials are expected to develop across the liposomal membranes that will reflect the concentration gradients and relative permeabilities of the permeant ions.

In "leaky" vesicles that are permeant to all ions, all concentration gradients should dissipate quickly, diffusion potentials should be minimal, and tracer uptake should be rapid and nonselective. Sealed vesicles lacking channels should be relatively impermeable to all ions. Uptake of $^{22}$Na and $^{86}$Rb will be slow and reflect the cation selectivity of the bare lipid membrane. Sealed vesicles containing open sodium channels will exhibit markedly selective permeability to sodium and will develop an inward-negative diffusion potential; this is simply given (in V) as:

$$E_m = 0.058 \log ([Na]_o/[Na]_i). \quad (1)$$

Channels oriented inside-out in the liposomal membranes would experience this potential as a strong depolarization ($E_{m,\text{channel}} > 0$), and outside-out oriented channels would experience an equal, but opposite potential ($E_{m,\text{channel}} > 0$).These vesicles, containing open channels, should exhibit high permeability to tracer $^{22}$Na. Uptake of other cation tracers relative to $^{22}$Na will reflect channel selectivity. Such diffusion potentials have previously been measured with voltage-sensitive dyes in liposomes treated with batrachotoxin, and the permeation selectivity of the potentials demonstrated (Agnew et al., 1988). Net tracer uptake should continue until the ratio of internal to external tracer con-




**Scheme 1.** Proposed mechanism of NBA or NBS side chain modification and peptide cleavage (after Witcop, 1961)

centration equals $[Na]_i/[Na]_o$. Because $[Na]_i/[Na]_o$ is initially 10 to 20, uptake of all ions should plateau when the ratio of internal to external tracer concentration is 10 to 20 times a factor reflecting dissipation of the concentration gradient.

### Chemical Modifications

N-bromoacetamide and N-bromosuccinimide are oxidizing reagents, whose mechanism and reaction products differ from those of the familiar haloacetamide alkylating reagents. NBA and NBS are sources of highly electrophilic bromonium ions ($Br^+$), that are capable of interacting with side chains of tryptophan (Scheme 1), tyrosine, histidine, and sulfur containing amino acids (Witkop, 1961; Ramachandran & Witkop, 1967).

Under appropriate conditions (pH 3–4, 70% acetic acid), selective modification of tryptophan residues is observed, which may be followed by rearrangement and peptide cleavage (Ramachandian & Witkop, 1967). At neutral pH, however, the reagents are less selective for tryptophan, and peptide cleavage is not seen (Spande et al., 1966; Shechter, Patchornik & Burnstein, 1976).

## Results

### Channel-Mediated Fluxes in the Presence and Absence of Activating Neurotoxins

It is readily possible to measure sodium channel-mediated flux stimulated by activating neurotoxins. Figure 1 illustrates the time course of $^{22}Na$ and $^{86}Rb$ uptake by a reconstituted vesicle preparation. Freeze-thawed-sonicated (FTS) vesicles were incubated for 30 min at 30°C with 2.5 $\mu$M BTX (Fig. 1*B* and *D*) or 1% EtOH vehicle (Fig. 1*A* and *C*), and then diluted 10-fold into iso-osmolar Tris $SO_4$ buffer containing the two radiotracers. The first minutes of tracer accumulation are illustrated in Fig. 1*A* and *B*. BTX stimulated a large increase in the rate of $^{22}Na$

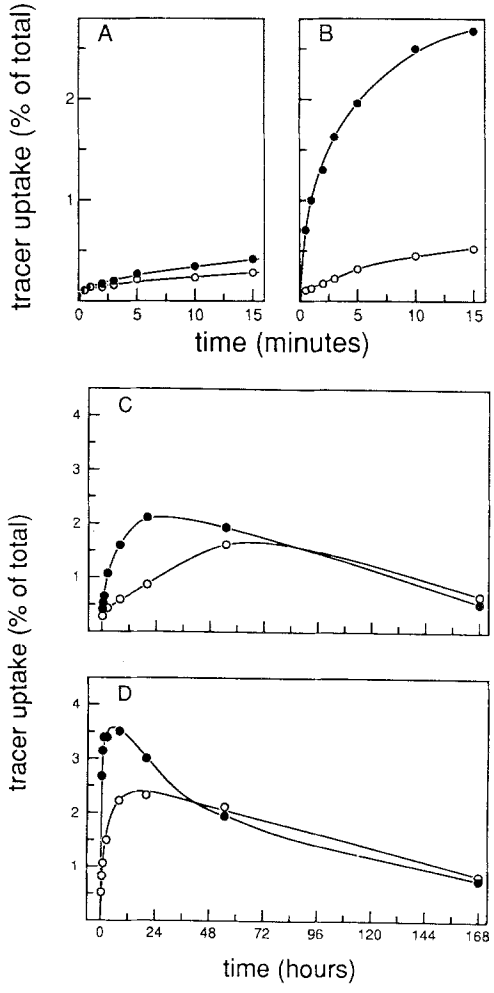



**Fig. 1.** Effect of BTX on $^{22}Na$ and $^{86}Rb$ uptake. Freeze-thaw-sonicated (FTS) vesicles were pre-incubated with 2.5 $\mu$M BTX (*B*, *D*) or ethanol vehicle (*A*, *C*) for 30 min at 30°C, then diluted 10-fold into iso-osmolar Tris $SO_4$ buffer containing $^{22}Na$ (●) and $^{86}Rb$ (○). At the indicated times, aliquots were removed, and internalized tracer was determined as described in Materials and Methods

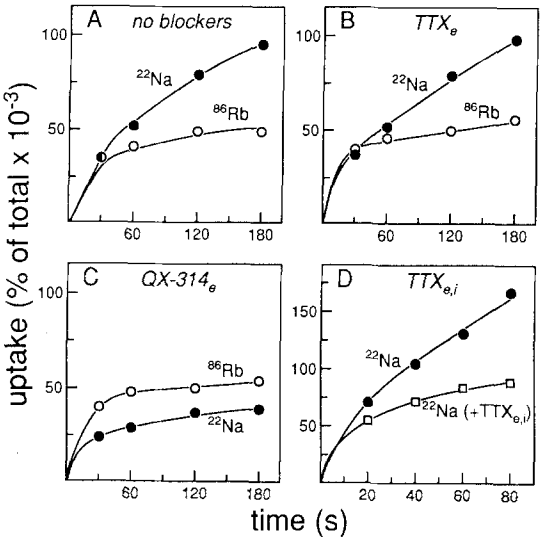



**Fig. 2.** Sensitivity of tracer uptake to TTX and QX-314. (*A–C*) Reconstituted vesicles were subjected to FTS; aliquots were then pre-incubated for 30 min at 30°C either alone or with TTX or QX-314. Afterwards, each aliquot was diluted 20-fold into iso-osmolar Tris $SO_4$ containing radiotracers, and, where indicated, TTX or QX-314. Uptake of $^{22}$Na (●) and $^{86}$Rb (○) by (*A*) control vesicle, (*B*) by vesicles treated with external 1 μM TTX and (*C*) by vesicles treated with 3 mM QX-314. (*D*) In a separate experiment, two aliquots of vesicles were subjected to FTS either with (□) or without (●) 1 μM TTX added. Each aliquot was then diluted 20-fold into iso-osmolar Tris $SO_4$ containing $^{22}$Na

uptake over untreated controls. BTX also stimulated $^{86}$RB uptake, though to a much lesser degree. When BTX-stimulated tracer uptake in 1 min was taken as an initial rate, $P_{Na}/P_{Rb}$ was estimated to be ~40 for this experiment.

At longer times, the tracer uptake exhibited the biphasic course discussed in Methods (Fig. 1*C* and *D*). In the BTX-activated sample, $^{22}$Na uptake reached a level of >3.5% within minutes, plateaued at this high level, and then declined to <1.0% with a time constant of several hours. The rapid $^{22}$Na uptake resulted from the selective permeability of BTX-activated channels. The slow decline is the expected consequence of gradually dissipating gradients of the less permeant Tris and $SO_4$ ions, and net efflux of Na.

It was also evident that $^{22}$Na uptake exceeded $^{86}$Rb uptake, even in the control samples not treated with BTX (Fig. 1*A*). This suggested, as previously concluded by Duch and Levinson (1987), that reconstituted channels are capable of occasional spontaneous openings. At longer times, it became clear that the sodium-selective "background" uptake proceeded with very slow kinetics (Fig. 1*C*). Furthermore, after several hours this background uptake approached levels reached in the first minutes by the BTX-treated sample, suggesting that a large fraction of the channel-containing vesicles experienced this slow equilibration.

Additional experiments provided evidence that the slow, sodium-selective background uptake was mediated by inside-out oriented sodium channels. For the experiment illustrated in Fig. 2*A–C*, aliquots of a FTS vesicle preparation were incubated either alone, or with external TTX or QX-314, then were given uptake assays. The control sample (Fig. 2*A*) exhibited typical sodium-selective uptake. The sample pre-incubated with 1 μM TTX (Fig. 2*B*) exhibited permeability identical to the control: Neither $^{22}$Na or $^{86}$Rb uptake appeared sensitive to this high external TTX concentration. In the sample incubated with external 3 mM QX-314 (Fig. 2*C*), uptake of $^{22}$Na was reduced, so that uptake of $^{86}$Rb exceeded uptake of $^{22}$Na slightly. (Pure liposomes of PE/PC/PS, lacking the sodium channel protein, were also observed to be slightly more permeable to $^{86}$Rb than $^{22}$Na; *data not shown.*) Although uptake by the reconstituted vesicles was insensitive to external TTX, 1 μM TTX added before FTS reduced $^{22}$Na uptake about the same extent as external QX-314 (Fig. 2*D*).

Thus, it appeared that baseline radiotracer uptake by the reconstituted vesicles included two components. The first, a true background leak, was nonselective or slightly Rb selective, and was not sensitive to TTX or QX-314. The second was sodium selective, and sensitive to *external* QX-314 and *internal* TTX, and therefore seemed likely to result from the openings of inside-out-facing sodium channels. These channels appeared to open infrequently, because the sodium-selective component of the background uptake was very slow compared to that stimulated by BTX (Fig. 1). Because inside-out oriented channels would experience a strongly depolarized membrane potential throughout the assay period, they might be expected to be inactivated and nonconducting. The high peak level of the background sodium-selective uptake seen in Fig. 1*C* suggested, however, that a significant fraction of the inside-out channels were occasionally opening spontaneously.

## Multiple Effects of NBA and NBS

As described previously (Cooper et al., 1987), treatment of the reconstituted preparation with mM concentrations of NBA or NBS rapidly increased sodium-selective flux, but prolonged treatment had additional effects. For example, Fig. 3 illustrates the time dependence of treatment with 1.1 mM NBA. Before NBA addition, $^{22}$Na flux exceeded

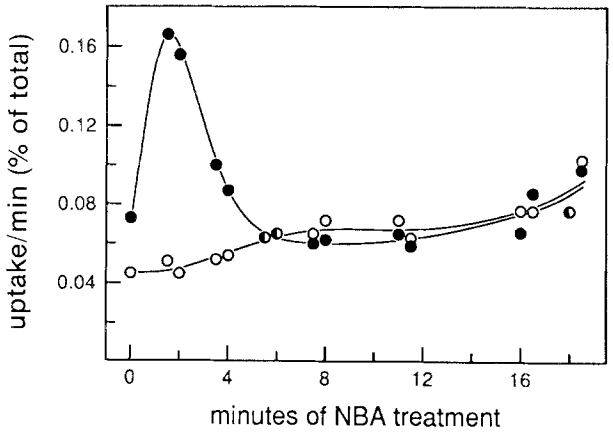



**Fig. 3.** Effect of different periods of NBA treatment on $^{22}Na$ (●) and $^{86}Rb$ (○) flux. FTS vesicles were diluted 20-fold into isoosmolar Tris $SO_4$. One-minute tracer uptake assays were performed before or at intervals after the addition of 1.1 mM NBA, as described in Materials and Methods

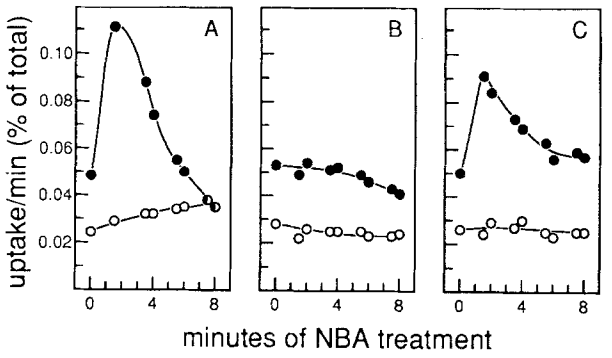



**Fig. 4.** Effect of tryptophanyl alanine (TA) on NBA flux activation. Vesicles were diluted 20-fold, then subjected to 1 min $^{22}Na$ (●) and $^{86}Rb$ (○) flux assays, before and after the addition of 1.1 mM NBA. (*A*) No TA was added. (*B*) 5 mM TA was added before the control uptake time points were taken. (*C*) TA was added 15 sec after the addition of NBA

$^{86}Rb$ flux by 70%. During the first 2 min of NBA treatment, $^{22}Na$ flux more than doubled, while $^{86}Rb$ flux remained near control levels. During minutes 3 to 6, $^{22}Na$ flux declined, and $^{86}Rb$ flux rose slowly, so that at 6 min, flux was nonselective and at a level intermediate between control levels for the two tracers. Further treatment resulted in slow increases in nonselective flux.

## Titration of Flux Activation

Additional experiments suggested that the late phases of the NBA treatment time course, which abolished sodium-selective flux, were due to further covalent modification of the channel protein. First, we attempted to stop the NBA reaction abruptly by quenching with the model substrate, tryptophanyl alanine (Fig. 4). Each panel shows the $^{22}Na$ and $^{86}Rb$ uptake before and after addition of 1.1 mM NBA. In Fig. 4*A*, tryptophanyl alanine (TA) was not added. In Fig. 4*B* and *C*, 5 mM TA was added either before NBA (*B*) or 15 sec after NBA (*C*). In the absence of TA (Fig. 4*A*), NBA elicited the expected three phases of flux activation. Prequenching with TA (Fig. 4*B*) abolished all effects of NBA. When TA was added very early after initiating the reaction (i.e., 15 sec after NBA, Fig. 4*C*), all three phases were significantly inhibited. TA quenched later phases of the reaction more effectively than the early phase: peak $^{22}Na$ uptake at 2 min was reduced only slightly but the slower decline in $^{22}Na$ and increase in $^{86}Rb$ uptake were more completely inhibited. These results suggested that each of the three effects of NBA were due to successive reactions with the channel protein. It was also apparent that quenching with Ta was too slow for precise control of the extent of the reaction.

With micromolar concentrations of N-bromosuccinimide (NBS), it was possible to limit the extent of modification much more successfully (Fig. 5). NBA and NBS are thought to have identical reaction mechanisms and specificity, but NBS reacts much more rapidly (Ramachandran & Witkop, 1967; Spande et al., 1966). Both reagents selectively remove inactivation gating from sodium channels in squid axon; NBA has been more often used because it is more soluble in saline solutions (Oxford et al., 1978; D. Eaton, *personal communication*). Figure 5*A* shows that the activation of $^{22}Na$ flux could be titrated with 1–5 μM NBS. At 4.8 μM NBS, $^{22}Na$ flux was nearly fully activated by 6 min, and remained at peak levels for the following 10 min observed. At higher concentrations, rapid activation of $^{22}Na$ flux was followed by decline as with NBA (Fig. 5*B*).

The flux stimulated by NBS, like that stimulated by brief NBA treatment was strongly sodium selective (Fig. 5*C*). The $^{86}Rb$ fluxes stimulated by NBA and NBS were too small to compare quantitatively with $^{22}Na$. This suggested that the chemically modified channels were more sodium selective than those treated with BTX.

## Interactions with QX-314

Many local anesthetics and related compounds block sodium currents and alter channel gating (Strichartz, 1973; Hille, 1977; Yeh & Narahashi, 1977; Cahalan, 1978; Cahalan & Almers, 1979; Cahalan et al., 1980; Yeh & Tangey, 1985; Moc-

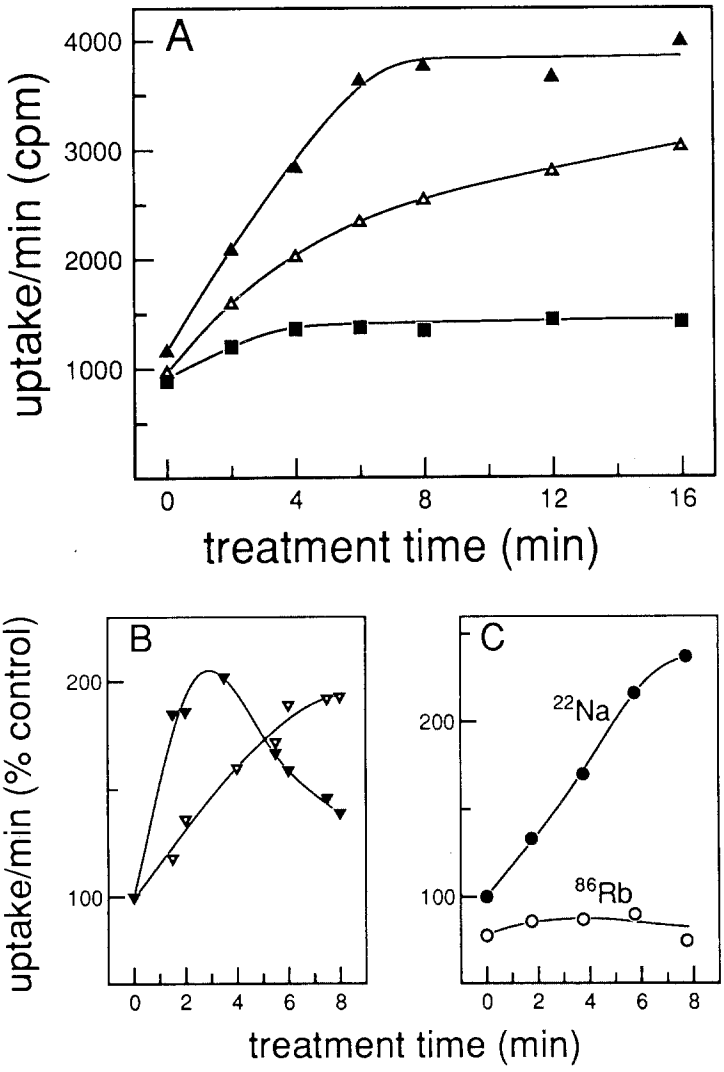



**Fig. 5.** NBS activates sodium-selective tracer flux. (*A*) FTS vesicles were diluted 20-fold into Tris $SO_4$. One-minute flux assays were conducted either before or after the addition of 1.0 μM NBS (■), 2.5 μM NBS (△), or 4.8 μM NBS (▲). (*B*) FTS vesicles from a different reconstitution preparation than shown in *A* were diluted 10-fold into Tris $SO_4$ buffer. After 20 min tracer uptake was assayed before or after the addition of 150 μM NBS (▼) or 15 μM NBS (▽). (*C*) Effect of 5 μM NBS on $^{22}$Na (●) and $^{86}$Rb (○) fluxes. Vesicles were diluted 20-fold prior to assay

zydlowski, Uehara & Hall, 1986; Green, Weiss & Anderson, 1987). Previously, we and others have used the membrane impermeant lidocaine derivatives QX-314 and QX-222, at saturating concentrations, to block purified reconstituted channels that were activated by BTX or chemical modifiers (Rosenberg et al., 1984*a*, Cooper et al., 1987). Results were consistent with the simple model of block resulting from binding to a single type of cytoplasmic site: when QX-314 or QX-222 was added to the vesicle exterior, a component of tracer uptake that was insensitive to external TTX was inhibited. As we will describe, further studies with QX-314 yielded results that could not be explained as simple, open channel block.

*QX-314 Enhances Flux Activation by NBA*

At concentrations of 10 to 100 μM, QX-314 markedly increased the rate at which NBA stimulated $^{22}$Na flux. For the experiments illustrated in Fig. 6, aliquots of reconstituted vesicles were pre-incubated either without QX-314 (Fig. 6*A*), or with 10 μM to 3 mM QX-314 (Fig. 6*B*). After pre-incubation, each sample was diluted into Tris $SO_4$ buffer containing $^{22}$Na, $^{86}$Rb, QX-314 (at the concentrations present during pre-incubation), and except for controls, 500 μM NBA.

In samples pre-incubated without QX-314 (Fig. 6*A*), NBA stimulated a twofold increase in $^{22}$Na uptake by 90 sec, but had little effect on $^{86}$Rb uptake. In the presence of 10–100 μM QX-314, NBA-stimulated $^{22}$Na uptake *exceeded* that produced by NBA alone; at higher concentrations of QX-314, $^{22}$Na uptake was reduced in a dose-dependent manner (Fig. 6*B*, *C*). $^{86}$Rb uptake was not significantly altered by NBA, regardless of the QX-314 concentration (Fig. 6*C*). These effects are seen most clearly in Fig. 6*C*, where the uptake of $^{22}$Na and $^{86}$Rb, at 90 sec, are shown for all QX-314 concentrations; complete

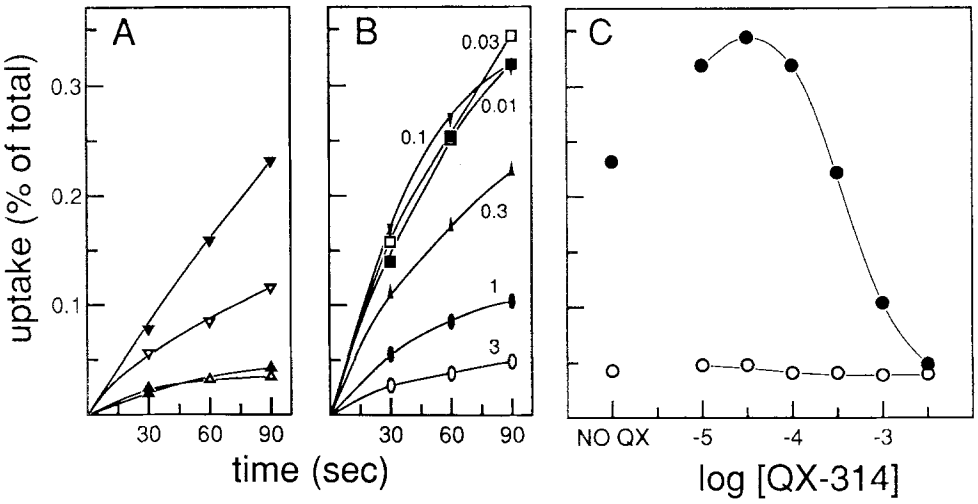



**Fig. 6.** Effect of different concentrations of QX-314 on stimulation of tracer uptake by NBA. (*A*) Control vesicles (pre-incubated without QX-314) were diluted 20-fold into Tris $SO_4$ buffer containing $^{22}Na$ and $^{86}Rb$ with or without 500 $\mu$M NBA. Uptake time courses were performed for each condition as described in methods: (▼) $^{22}Na$, NBA treated; (▽) $^{22}Na$, no NBA; (▲) $^{86}Rb$, NBA treated; (△) $^{86}Rb$, no NBA. (*B*) Uptake by vesicles treated with NBA in the presence of different QX-314 concentrations. FTS vesicles were pre-incubated with 0.01–3.0 mM QX-314 for 30 min at 30°C. The vesicles were then diluted 20-fold into Tris $SO_4$ buffer containing $^{22}Na$, $^{86}Rb$, QX-314 at the concentrations present during pre-incubation, and 500 $\mu$M NBA. At intervals afterward, internalized tracer was determined. Time courses of $^{22}Na$ uptake at QX-314 concentrations of 0.01 mM (■), 0.03 mM (□), 0.10 mM (▼), 0.30 mM (▲), 1 mM (●), and 3 mM (○) are indicated. (*C*) Concentration dependence of effects of QX-314. $^{22}Na$ (●) and $^{86}Rb$ (○) uptake stimulated by NBA at each QX-314 concentration (90 sec) can be compared with uptake in the absence of drug (NO QX)

time courses of $^{22}Na$ uptake are shown in Fig. 6*B*. Again, 3 mM QX-314 reduced $^{22}Na$ uptake to below control levels, so that uptake was nonselective.

Two hypotheses might explain the enhanced uptake produced by co-incubation with NBA and low concentrations of QX-314. First, QX-314 might alter, by a conformational mechanism or by protection, the rate at which sites on the channel react with NBA. Alternatively, reversible QX-314 binding might itself promote the open state. Further experiments were conducted to test these hypotheses.

In the experiment illustrated in Fig. 7, the time dependence of flux activation by 500 $\mu$M NBA was studied, using vesicles pre-incubated with or without 25 $\mu$M QX-314. Before the addition of NBA, QX-314 caused a slight increase in uptake (*discussed below*). The initial rising phase of the NBA time course was strongly enhanced by QX-314, but the slower declining phase was not much affected. As a result, peak stimulation was increased. The fact that the rising, but not the falling, phase of NBA modification was affected by QX-314 suggested that the drug selectively increases the rate of a reaction responsible for flux activation.

Figure 8*A–B* illustrates an experiment in which the NBA concentration was lowered to highlight the effects of QX-314. Aliquots of vesicles were pre-incubated with or without 25 $\mu$M QX-314. Uptake time courses were then conducted with and without

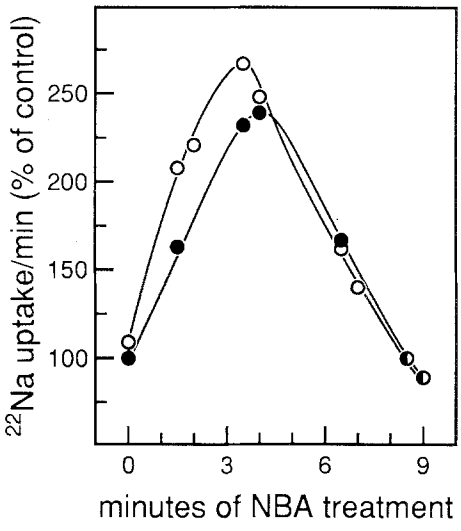



**Fig. 7.** Effect of 25 $\mu$M QX-314 on NBA treatment time course. FTS vesicles were diluted 20-fold into Tris $SO_4$ buffer, with (○) or without (●) 25 $\mu$M QX-314. One-minute $^{22}Na$ flux assays performed, either before or at intervals after the addition of 500 $\mu$M QX-314

100 $\mu$M NBA. As indicated (Fig. 8*A*), 100 $\mu$M NBA alone produced only a slight, delayed increase in TTX-sensitive $^{22}Na$ uptake. In the sample treated with both 25 $\mu$M QX-314 and NBA, $^{22}Na$ uptake was markedly enhanced. These effects of NBA and QX-314 were not seen when vesicles were incubated with 1 $\mu$M TTX before freeze-thaw-sonication (Fig.

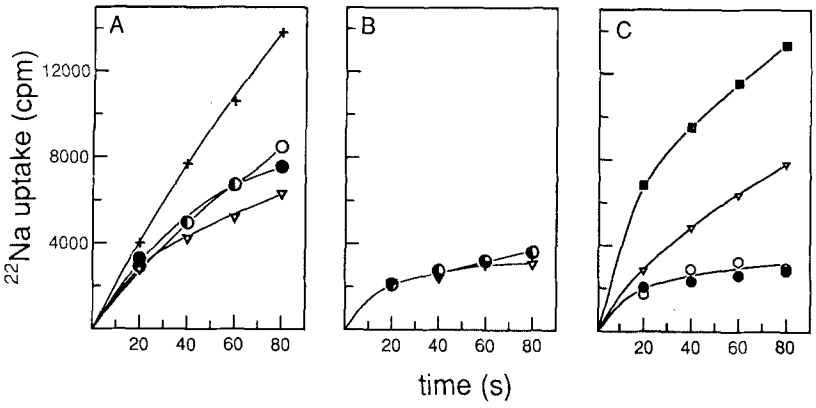



**Fig. 8.** (*A, B*) QX-314 enhancement of NBA-stimulated $^{22}$Na uptake is TTX sensitive. Vesicles were subjected to FTS, either without (*A*) or with (*B*) 1 $\mu$M TTX added before the freeze step. After sonication, aliquots of the TTX-treated and nontreated vesicles were pre-incubated for 30 min at 30°C with and without 25 $\mu$M QX-314. Aliquots were then diluted 20-fold into Tris $SO_4$ buffer containing $^{22}$Na and, where indicated, 100 $\mu$M NBA, 1 $\mu$M TTX, and/or QX-314. Uptake time courses were conducted for each incubation condition: (▽) no QX-314, no NBA; (●) QX-314 only; (○) NBA only; (+) both NBA and QX-314. (*C*) Transient stimulation of tracer uptake by QX-314 alone. Vesicles were subjected to FTS with and without 1 $\mu$M TTX. Aliquots of the $TTX^+$ and $TTX^-$ treated vesicles were then pre-incubated with or without 400 $\mu$M QX-314 for 30 min at 30°C. Each aliquot was then diluted 20-fold into $Tris_2SO_4$ buffer containing $^{22}$Na, 1 $\mu$M TTX (for samples treated with TTX prior to FTS) but no additional QX-314. At 20-sec intervals, uptake of $^{22}$Na was determined as described in Materials and Methods. $^{22}$Na uptake *vs.* time for each condition: (●) TTX alone; (○) TTX and QX-314; (▽) no TTX, no QX-314; (■) QX-314 only

8*B*). The level of uptake exhibited by all TTX-treated samples was about half the control uptake (no TTX, QX-314, or NBA, inverted triangles in Fig. 8*A*), indicating that the untreated channels were spontaneously opening.

### *QX-314 Activates Sodium Channel Flux*

Vesicles exposed to 25 $\mu$M QX-314 alone consistently exhibited higher $^{22}$Na uptake than controls (e.g., Fig. 8*A*). This small effect was abolished when 1 $\mu$M TTX was equilibrated with the vesicle interior by freeze-thaw-sonication (Fig. 8*B*). In order to test for more pronounced channel-opening activity at higher concentrations, that might be masked by QX-314's rapid blocking activity, a non-equilibrium assay was used (Fig. 8*C*). Aliquots of vesicles were subjected to FTS either with or without 1 $\mu$M TTX, preincubated with 400 $\mu$M QX-314, then diluted 20-fold into Tris $SO_4$ buffer containing $^{22}$Na, but no QX-314. As in Fig. 8*A–B*, the total QX-314 concentration during the uptake assay was ~20 $\mu$M, but in Fig. 8*C*, drug binding was not at equilibrium at the beginning of the time course. TTX-sensitive uptake was much more strongly increased under nonequilibrium binding conditions (Fig. 8*C*) than under equilibrium binding conditions (Fig. 8*A,B*). Nearly all of the increase elicited by QX-314 in Fig. 8*C* occurred during the first 20 sec after dilution into buffer-containing tracers. In subsequent 20-sec periods, flux in the presence of QX-314 returned to a level only slightly above control, as revealed by comparing the slopes of QX-314 and control time courses. The effects illustrated in Fig. 8*C* may be explained by invoking two or more distinct binding sites for QX-314, with similar affinities but different dissociation kinetics, and opposing effects on permeability. Alternatively, dissociation of the drug from channels in a blocked state (under strongly depolarized membrane voltage) might leave them in an open state that inactivates slowly (*cf.* Armstrong & Bezanilla, 1977; Oxford & Yeh, 1985; Yeh et al., 1986; *see* Discussion).

### *QX-314 Causes Simple Dose-Dependent Block of NBA-Activated Channels*

From the previous experiments it appeared that QX-314 may itself promote the open state and enhance the rate at which the channels react with NBA. Additional experiments showed that QX-314 did not enhance flux through channels *after* chemical activation. In the experiment illustrated in Fig. 9, vesicles were first treated with 5 $\mu$M NBS for 15 min, in the absence of QX-314. As previously shown (Fig. 6*A*), this concentration of NBS produced stable, peak activation of flux. Aliquots of the NBS-treated vesicles were next incubated for 2 min with various concentrations of local anesthetics, then subjected to 1-min tracter uptake assays. As shown, QX-314 block of the activated channels followed simple, monophasic dose dependence, with an $IC_{50}$ of ~0.3 mM. At no concentration did uptake exceed control levels.

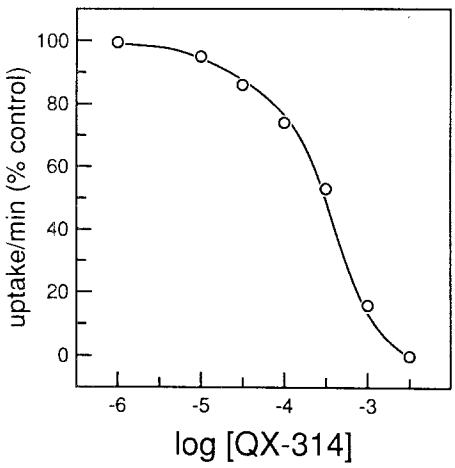



**Fig. 9.** Concentration dependence of QX-314 inhibition of NBS-stimulated $^{22}Na$ uptake. FTS vesicles were diluted 20-fold into Tris $SO_4$ buffer. Tracer uptake assays were performed in duplicate before or 15 min after beginning treatment with 5 $\mu$M NBS. QX-314 was then added to aliquots of the NBS-treated vesicles at concentrations between 1 $\mu$M and 3 mM. After 2 min, $^{22}Na$ uptake assays were performed for each QX-314 concentration. Data shown is the average of two determinations. Uptake in the presence of 3 mM QX-314, which was taken as an estimate of complete inhibition of "channel-mediated" flux, equalled 56% of control (no QX-314, no NBS) uptake in both experiments. Uptake in the presence of each QX-314 concentration is expressed as a percentage of "channel-meditated" uptake observed in the absence of the drug

## Discussion

In native membranes, sodium current inactivation can be eliminated selectively by controlled modification of the intracellular surface with various proteases and chemical reagents (Armstrong et al., 1973; Rojas & Rudy, 1976; Bezanilla & Armstrong, 1977; Oxford et al., 1978). We have previously screened four such agents, NBA, NBS, pronase and trypsin, with reconstituted sodium channels purified from eel electroplax, to attempt to reproduce these observations in vitro. The ultimate aim is to identify sites of covalent modification associated with specific changes in channel function. Here we describe in detail several properties of the reconstituted preparation influenced by binding of the lidocaine analogue QX-314 and by reaction with NBS and NBA.

We conclude that electroplax sodium channels, reincorporated into liposomes and under the influence of strong depolarizing diffusion potentials, appear capable of occasional spontaneous openings. Progressive modification of the cytoplasmic surface of the protein with NBA and NBS produces, in sequence, a chronically opened form of the protein, a closed form, and perhaps a form which is nonselectively permeable to cations. These modified states appear to result from multiple reactions with the reagents, and the more reactive NBS can be used to prepare stable, chronically opened preparations of the channel. We also observe that QX-314, at low concentrations, stimulates a small increase in premeability of the sodium channel. At these concentrations, QX-314 markedly stimulates the rates at which NBA activates sodium channel-mediated flux. This suggests that the reactivity of sites involved in flux activation is increased by conformational changes attending local anesthetic binding.

### Evidence for Rare, Spontaneous Openings

In dual label radiotracer flux experiments, we found evidence that reconstituted *E. electricus* sodium channels open spontaneously during maintained depolarizations (e.g., Fig. 2). Uptake of tracer $^{22}Na$ exceeded that of $^{42}K$, $^{86}Rb$, or $^{137}Cs$ (Cooper et al., 1987). Such $^{22}Na$-selective uptake was blocked by 3 mM external QX-314, but not by 1 $\mu$M external TTX (Fig. 3). Thus, inside-out oriented channels appear to have a small, nonzero probability of opening under our experimental conditions. The sodium-selective uptake resulting from spontaneous openings is extremely slow, but peak levels are about the same as those resulting from maximal activation by BTX (Fig. 2*C*,*D*). This suggests that the flux results from rare openings by nearly all of the channels rather than by frequent openings by a small population of damaged or partially denatured channels. This was somewhat unexpected because, usually, inactivation mechanisms are thought to reduce sodium channel opening proability to zero during maintained depolarizations (Hodgkin & Huxley, 1952). Nevertheless, under some conditions steady-state inactivation may be incomplete (Shoukimas & French, 1980; Oxford & Yeh, 1985; Patlak & Ortiz, 1985, 1986).

These observations are in good agreement with the findings of Duch and Levinson (1987), despite many differences in purification, reconstitution, and tracer assay methods. Several factors may contribute to the background, channel-mediated fluxes. Duch and Levinson found that these fluxes were affected by temperature and the lipid composition of the reconstituted vesicles. Ionic conditions may play a role: both studies used internal cations (high [Tris] and high [Na]) that have been shown to partially prevent inactivation in squid giant axon (Oxford & Yeh 1979, 1985). In the present study, only inside-out channels contributed to the background flux, suggesting that strong membrane depolarization is also required.

### NBA and NBS Have Multiple Effects and Appear to Act at Multiple Sites

The effects of NBA and NBS on tracer uptake by the reconstituted vesicles depended strongly on time and reagent concentration (Figs. 3 and 5; *see also* Cooper et al., 1987). With both reagents, distinct phases were observed during the modification time course experiments. We wanted to determine whether the observed phases resulted from covalent modification, or whether noncovalent interactions (for example, block by reactions products; *see* Huang, Tanguy & Yeh, 1987) or processes intrinsic to the protein (e.g., slow inactivation) played a role. It appeared that block by reaction products was not involved, since incubation of vesicles with NBA and excess tryptophanyl alanine, an alternative substrate, abolished all effects on tracer flux. Experiments with the more reactive NBS provided the most direct evidence that the effects were due to covalent modifications. When limiting concentrations of NBS were used, only the rapid increase in $^{22}Na$ uptake was observed. At higher concentrations of NBS, $^{22}Na$ uptake rose and declined as with NBA. Thus, additional chemical modifications seemed necessary to elicit the declining phase of the time course. These results suggested that NBA and NBS were capable of reacting with several different sites on the sodium channel protein, and that reaction at different sites had different effects on channel behavior. The NBS treatment appears to be selective in that permeation selectivity (Na > Rb) and sensitivity to TTX and QX-314 blockade were maintained during the initial phases of the reactions. This is consistent with the expectation that sites involved in conductance regulation (e.g., inactivation) were specifically modified.

The ability to produce chemically-stable, functionally-altered channels with limiting, defined concentrations of NBS permits more detailed biochemical and biophysical characterization of the modified protein. As previously reported (Cooper et al., 1987), gel electrophoresis indicates that NBS does not cleave the Na channel peptide. More recently, the gating, conductance, and pharmacological properties of NBS-modified, purfied, reconstituted channels have been studied in planar bilayer (Shenkel et al., 1989).

### Interactions with QX-314

We observed three distinct effects of QX-314: block of activated channels, flux enhancement, and enhancement of the effects of NBA.

When channels were activated to peak levels with NBS and then exposed to QX-314, monophasic dose-dependent block of tracer flux, with an $IC_{50}$ of ~0.3 mM was observed (Fig. 9). These results were as expected for rapid blockade of opened sodium channels.

When unmodified reconstituted channels were exposed to low QX-314 concentrations (~25 $\mu$M), a slight, TTX-sensitive enhancement of $^{22}Na$ flux was observed (Figs. 7 and 8*A*,*B*). This effect was quite unexpected. QX-314 might be expected to bind to spontaneously opening, inside-out channels, but such binding was expected to result only in reduced uptake. Even if QX-314 binding increased the fraction of channels in the open state by mass action, the additional "open" channels should be blocked and nonconducting. The slight enhancement in flux we observed at low QX-314 concentrations might be expected if the channel possessed a second, longer-lived open state, and if QX-314 enhanced the probability of entering this second open state. Yeh and coworkers (1986) obtained evidence for such an effect in studies of the action of QX-314 on single sodium channels in membrane patches from neuroblastoma cells. In controls, open times were distributed with a mean of 2–3 msec at a test potential of −50 mV. In the presence of 100 $\mu$M QX-314, overall open probability was reduced, but openings were distributed bimodally, with one mean about the same as control, and a second about fivefold longer (Yeh et al., 1986). The voltage and concentration dependence of this effect on single channel kinetics has not as yet been described.

The enhancement of flux by QX-314 was not seen when uptake was measured in the presence of high QX-314 concentrations (e.g., Fig. 2*C*). It appeared that free drug rapidly blocked the open channels; at saturating concentrations, the increased open probability caused by QX-314 was masked. To separate the two effects, we pre-equilibrated vesicles with a high QX-314 concentration, then measured uptake after rapidly diluting the vesicles (and free QX-314) in the flux assay medium (Fig. 8*C*). Uptake in this nonequilibrium assay was strongly enhanced: the level of stimulation in the first 20 sec was five times higher than in a control (Fig. 8*A*) equilibrated at steady state with essentially the same final concentration of QX-314. This suggests that local anesthetic binding may induce a significant and long-lived change in the conformation of elements of the protein involved in gating.

QX-314 also appeared to increase the rate at which NBA covalently modified certain sites on the cytoplasmic surface of the channel. When a low concentration of QX-314 was present during treatment with NBA, the rate of flux activation was increased. The stimulation by QX-314 and NBA together was markedly greater than the sum of the effects of the two agents acting alone. QX-314 did

not affect the rate at which NBA caused reductions in uptake during prolonged incubations. Thus, QX-314 may shift the conformation of the sodium channel so that covalent reactions that produce flux activation are more rapid, without affecting secondary modifications that close the conductance pathway. It is tempting to speculate that this conformational shift is from an inactivated to an open (or open but blocked) state. In voltage-clamp experiments, weak membrane depolarization sufficient to inactivate most channels slowed the rate at which NBA eliminated sodium current inactivation (Salgado, Yeh & Narahashi, 1985). Thus, the site or sites of modification associated with inactivation removal may be partially protected in the inactivated state. The inside-out oriented reconstituted channels sensitive to NBA modification are likely to be inactivated. By promoting opening (or opening and block), QX-314 may increase susceptibility to modification by NBA.

We thank A.M. Correa and S. Shenkel for helpful discussions, W.M. James for the use of affinity chromatography methods and reagents, and Catherine Fobes for assistance in preparation of the manuscript. E.C.C. was supported by the NIH GM 07205 (Medical Scientist Training Program, National Institute of General Sciences). This research was supported by N.I.H. grants NS 17928, and HL 38156, and a grant from the National Multiple Sclerosis Society.

## References

Agnew, W.S., Cooper, E.C., James, W.M., Tomiko, S.A., Rosenberg, R.L., Emerick, M.C., Correa, A.M., Zhou, J.Y. 1988. Voltage sensitive sodium channels: Molecular structure and function. *In:* The Molecular Biology of Ion Channels. W.S. Agnew, T. Claudio, and F.J. Sigworth, editors. pp. 329–365. Academic, New York

Agnew, W.S., Tomiko, S.A., Rosenberg, R.L. 1986. Reconstitution of the sodium channel from *Electrophorus electicus*. *In:* Ion Channel Reconstitution. C. Miller, editor. pp. 307–322. Plenum, New York

Armstrong, C.M., Bezanilla, F. 1977. Inactivation of the sodium channel: II. Gating current experiments. *J. Gen. Physiol.* **70:**567–590

Armstrong, C.M., Bezanilla, F., Rojas, E. 1973. Destruction of sodium conductance inactivation in squid axons perfused with pronase. *J. Gen. Physiol.* **62:**375–391

Bezanilla, F., Armstrong, C.M. 1977. Inactivation of the sodium channel: I. Sodium current experiments. *J. Gen. Physiol.* **70:**549–566

Cahalan, M.D. 1978. Local anesthetic block of sodium channels in normal and pronase treated squid giant axons. *Biophys. J.* **23:**285–311

Cahalan, M.D., Almers, W. 1979. Interactions between quaternary lidocaine, the sodium channel gates, and tetrodotoxin. *Biophys. J.* **27:**39–56

Cahalan, M., Shapiro, B.I., Almers, W. 1980. Relationship between inactivation of sodium channels and block by quaternary derivatives of local anesthetics and other compounds. *In:* Progress in Anesthesiology, Vol. 2, Molecular Mechanisms of Anesthesia. B.R. Fink, editor. pp. 17–33. Raven, New York

Cooper, E.C., Tomiko, S.A., Agnew, W.S. 1987. The reconstituted voltage sensitive sodium channel from *Electrophorus electricus:* Chemical modifications that alter regulation of ion permeability. *Proc. Natl. Acad. Sci. USA* **84:**6282–6286

Duch, D.S., Levinson, S.R. 1987. Spontaneous opening at zero membrane potential of sodium channels from eel electroplax reconstituted into lipid vesicles. *J. Membrane Biol.* **98:**57–68

Green, W.N., Weiss, L.B., Anderson, O.S. 1987. Batrachotoxin-modified sodium channels in planar lipid bilayers. Ion permeation and block. *J. Gen. Physiol.* **89:**841–872

Hille, B. 1977. Local anesthetics: Hydrophilic and hydrophobic pathways for the drug-receptor reaction. *J. Gen. Physiol.* **69:**497–515

Hodgkin, A.L., Huxley, A.F. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol. (London)* **117:**500–544

Horn, R., Vandenberg, C.A., Lange, K. 1984. Statistical analysis of single sodium channels: Effects of N-bromoacetamide. *Biophys. J.* **45:**323–335

Huang, J.M.C., Tanguy, J., Yeh, J.Z. 1987. Removal of sodium inactivation and block of sodium channels by chloramine-T in crayfish and squid giant axons. *Biophys. J.* **52:**155–163

James, W., Emerick, M., Agnew, W.S. 1989. Affinity purification of the voltage-sensitive sodium channel from *E. electricus*. *Biochemistry (in press)*

Khodorov, B.I. 1978. Chemicals as tools to study nerve fiber sodium channels: Effects of batrachotoxin and some local anesthetics. *In:* Membrane Transport Processes. Vol. 2. pp. 153–174. D.C. Tosteson, Y.A. Ovchinnikov, and R. Latorre, editors. Raven, New York

Miller, C. (Editor) 1986. Ion Channel Reconstitution. Plenum, New York

Moczydlowski, E., Uehara, A., Hall, S. 1986. Blocking pharmacology of batrachotoxin activated sodium channels. *In:* Ion Channel Reconstitution. C. Miller, editor. pp. 405–428. Plenum, New York

Oxford, G.S., Yeh, J.Z. 1979. Interference with sodium inactivation gating in quid axons by internal monovalent cations. *Biophys. J.* **25:**195*a*

Oxford, G.S., Yeh, J.Z. 1985. Interactions of monovalent cations with sodium channels in squid axon: I. Modification of physiological inactivation gating. *J. Gen. Physiol.* **85:**583–602

Oxford, G.S., Yu, C.H., Narahashi, T. 1978. Removal of sodium channel inactivation in squid axons by N-bromoacetamide. *J. Gen. Physiol.* **71:**227–247

Patlak, J., Horn, R. 1982. Effect of N-bromoacetamide on single sodium channel currents in excised membrane patches. *J. Gen. Physiol.* **79:**333–351

Patkak, J., Ortiz, M. 1985. Slow currents through single sodium channels in adult rat heart. *J. Gen. Physiol.* **86:**89–104

Patlak, J., Ortiz, M. 1986. Two modes of gating during late $Na^+$ channel currents in frog sartorius muscle. *J. Gen., Physiol.* **87:**305–326

Ramachandran, L.K., Witkop, B. 1967. N-bromosuccinimide cleavage of peptides. *Methods Enzymol.* **11:**283–299

Rojas, E., Rudy, B. 1976. Destruction of the sodium current inactivation by a specific protease in perfused nerve fibers from *Loligo*. *J. Physiol. (London)* **262:**501–531

Rosenberg, R.L., Tomiko, S.A., Agnew, W.S. 1984*a*. Reconstitution of neurotoxin-modulated ion transport by the voltage-regulated sodium channel isolated from the electroplax of *Electrophorus electricus*. *Proc. Natl. Acad. Sci. USA* **81:**1239–1243

Rosenberg, R.L., Tomiko, S.A., Agnew, W.S. 1984*b*. Single-channel properties of the reconstituted voltage-regulated Na channel isolated form the electroplax of *Electrophorus electricus Proc. Natl. Acad. Sci. USA* **81**:5594–5598

Salgado, V.L., Yeh, J.Z., Narahashi, T. 1985. Voltage-dependent removal of sodium inactivation by N-bromoacetamide and pronase. *Biophys. J.* **47**:567–571

Shechter, Y., Patchornik, A., Burnstein, Y. 1976. Selective chemical cleavage of tryptophanyl peptide bonds by oxidative chlorination with N-chlorosuccinimide. *Biochemistry* **15**:5071–5075

Shenkel, S., Cooper, E.C., Agnew, W.S., Sigworth, F.J. 1989. Purified, modified sodium channels are active in planar bilayers in the absence of hatrachotoxin. *Biophys. J.* **55**:230 (*Abstr.*)

Shenkel, S., Cooper, E.C., James, W.M., Agnew, W.S., Sigworth, F.J. 1989. Purified, modified voltage-sensitive sodium channels are active in planar bilayers in the absence of neurotoxin. *Proc. Natl. Acad. Sci. USA* (*in press*).

Shoukimas, J.J., French, R.J. 1980. Incomplete inactivation of sodium currents in nonperfused squid axon. *Biophys. J.* **32**:857–862

Spande, T.F., Greene, N.M., Witkop, B. 1966. The reactivity towards N-bromosuccimimide of tryptophan in enzymes, zymogens, and inhibited enzymes. *Biochemistry* **5**:1926–1932

Strichartz, G.R. 1973. The inhibition of sodium currents in myelinated nerve by quaternary derivatives of lidocaine. *J. Gen. Physiol.* **62**:37–57

Tomiko, S.A., Rosenberg, R.L., Emerick, M.C., Agnew, W.S. 1986. Fluorescence assay for neurotoxin modulated ion transport by the reconstituted voltage-activated sodium channel isolated from eel electric organ. *Biochemistry* **25**:2162–2174

Wang, G.K., Brodwick, M.S., Eaton, D.C., Strichartz, G.R. 1987. Inhibition of sodium currents by local anesthetics in chloramine-T-treated squid axons. The role of channel activation. *J. Gen. Physiol.* **89**:645–667

Witkop, B. 1961. Non-enzymatic methods for the preferential and selective cleavage and modification of proteins. *Adv. Prot. Chem.* **16**:221–322

Yeh, J.Z. 1978. Sodium inactivation mechanism modulates QX-314 block of sodium channels in squid axons. *Biophys. J.* **24**:569–574

Yeh, J.Z., McCarthy, W.A., Quant, F.N., Yamamoto, D. 1986. Single channel analysis of the action of Na channel blockers 9-aminoacridine and QX-314 in neuroblastoma cells. *In:* Molecular Mechanisms of Anesthesia. S.H. Roth and K.W. Miller, editors. pp. 227–241. Plenum, New York

Yeh, J.Z., Narahashi, T. 1977. Kinetic analysis of pancuronium interaction with sodium channels in squid axon membranes. *J. Gen. Physiol.* **69**:293–323

Yeh, J.Z., Tanguy, J. 1985. Na channel activation gate modulates slow recovery from use-dependent block by local anesthetics in squid giant axons. *Biophys. J.* **47**:685–694

Received 7 February 1989