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Reconstituted voltage-sensitive sodium channel from *Electrophorus electricus*: Chemical modifications that alter regulation of ion permeability

(action potential/ion flux/inactivation/*N*-bromoacetamide/Pronase)

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ABSTRACT **At equilibrium, voltage-sensitive sodium channels normally are closed at all potentials. They open transiently in response to changes in membrane voltage or chronically under the influence of certain neurotoxins. Covalent modifications that result in chronic opening may help identify molecular domains involved in conductance regulation. Here, the purified sodium channel from electric eel electroplax, reconstituted in artificial liposomes, has been used to screen for such modifications. When the liposomes were treated with the alkaloid neurotoxin batrachotoxin, sodium-selective ion fluxes were produced, with permeability ratios $P_{Na} > P_{Tl} > P_K > P_{Rb} > P_{Cs}$. When the liposomes were treated with either of two oxidizing reagents (*N*-bromoacetamide or *N*-bromosuccinimide), or with Pronase or trypsin, ion-selective fluxes also were stimulated. These were blocked by tetrodotoxin and the anesthetic QX-314 in a manner suggesting that only modification of the cytoplasmic protein surface resulted in stimulation. Limited exposure to trypsin resulted in strong flux activation, with the concomitant appearance of peptide fragments with masses of ≈130, 70, and 38 kDa and fragments with masses of 45 and 24 kDa appearing later. We propose that characterization of these fragments may allow identification of channel domains important for inactivation gating.**

Voltage-sensitive sodium channels conduct the inward sodium currents of the action potential in many types of electrically excitable cells, including most neurons and muscles and some types of electroplax (1, 2). Sodium channel proteins have been isolated biochemically from electric eel electroplax (3), mammalian muscle (4), mammalian brain (5), and avian heart (6). Each purified protein appears to be formed of a large glycopeptide (260–295 kDa) either alone (electroplax and avian heart) or together with one (muscle) or two (brain) smaller peptides of 33–39 kDa. The muscle, brain, and electroplax proteins have each been functionally reconstituted in lipid vesicles (7–11). The cDNAs for the large electroplax peptide and for the corresponding large α subunits of two brain proteins have been cloned and sequenced (12). These cDNAs are closely homologous. In addition, mRNA transcribed *in vitro* from the rat brain clones directs expression of fully functional sodium channels in *Xenopus* oocytes (13). Thus, it appears that, even for the multipeptide brain proteins, the large subunits contain molecular domains that form the ion pore, ion-selectivity mechanisms, gating elements, and sites of drug and toxin interaction.

Functional reconstitution studies with purified sodium channels have advanced sufficiently to permit experiments aimed at identifying specific functional domains within the molecule. We here describe experiments with the reconstituted electroplax protein aimed at identifying molecular domains involved in conductance regulation. These were suggested by electrophysiological studies of native membranes. Internal perfusion of nerve and muscle cells with Pronase, *N*-bromoacetamide (NBA), and a variety of other oxidizing and peptide-cleaving reagents produces sodium currents that fail to inactivate (1, 14–17). These irreversible effects are presumably due to covalent modifications. They are selective in that only inactivation gating and processes closely associated with it (e.g., gating charge immobilization and interaction with "use-dependent" blockers) are modified; activation voltage dependence, kinetics, permeation selectivity, and channel pharmacology are otherwise not affected. All of the effective reagents appear to act from the cytoplasmic membrane surface, suggesting that inactivation is controlled by a portion of the protein exposed to the cell interior.

We have studied the modification of the electroplax protein in vesicles under conditions in which they should be closed due to inactivation or slow inactivation. Four agents—NBA, *N*-bromosuccinimide (NBS), Pronase, and trypsin—act to produce chronically conducting forms of the channel. Radiotracer uptake is similar in magnitude (30–100%) to that elicited by the alkaloid neurotoxin batrachotoxin (BTX), is highly ion selective, and is sensitive to tetrodotoxin (TTX) and the anesthetic QX-314. These agents appear to act at sites that face the cell interior *in situ*. Mild treatment with trypsin rapidly activates the ion-selective flux and simultaneously produces new peptide fragments with masses of ≈130, ≈70, and 38 kDa. Further reaction results in notable peptides of 45 and 24 kDa. A preliminary account of these results has been presented elsewhere (18).

MATERIALS AND METHODS

Materials. TTX was from Y. Kishi (Harvard University, Cambridge, MA) and was tritiated as previously described (3). BTX was the generous gift of J. Daly (National Institutes of Health). QX-314 from Astra Pharmaceutical was the gift of R. Aldrich (Stanford University, Stanford, CA). Radiotracers were from Amersham (^{22}NaCl, ^{86}RbCl) or New England Nuclear (^{42}KCl, ^{137}CsCl, ^{201}Tl$_2$NO$_3$). Egg phosphatidylcholine from Sigma was used in channel purification; phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine from Avanti Polar Lipids (Birmingham, AL) were added during final reconstitution. NBA, NBS, trypsin treated with 1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), and Pronase E were from Sigma. Pancreatic trypsin inhibitor was from Cooper Biomedical (Malvern, PA). *Electrophorus electricus* were from World Wide Scientific Supply (Orlando, FL).

Abbreviations: BTX, batrachotoxin; TTX, tetrodotoxin; NBA, *N*-bromoacetamide; NBS, *N*-bromosuccinimide; FTS, freeze–thaw–sonication; TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone.

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Sodium Channel Purification and Reconstitution. Purification and reconstitution have been described in detail (9, 10). Purified channel protein (7–15 ml, 0.8–2.1 nmol of TTX-binding sites at 800–1300 pmol/mg of protein) was dialyzed three times against 1 liter of 84 mM Na_2SO_4/1 mM EGTA/10 mM Hepes–NaOH, pH 7.4, for 1 hr. Sonicated liposomes of phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (5:4:1 final molar ratio) were added to the retentate; final concentrations were 10 mg of lipid per ml and 0.09–0.12 mg of protein per ml. After 30 min on ice, Bio-Beads SM2 (Bio-Rad) were added at 0.3 g/ml to adsorb detergent. The sample was agitated for 5 hr at 4°C, filtered, and stored on ice.

BTX-Modified Channel Radiotracer Flux Assays. Aliquots of the vesicle suspension (in the buffer described above) were subjected to freeze–thaw–sonication (FTS) to produce large vesicles as described (9–11). These were incubated for 30 min at 30°C with BTX (2.5 μM in 1% ethanol), in the presence or absence of blockers such as TTX or QX-314; controls with 1% ethanol alone were similarly prepared. Vesicles were then diluted 10- or 20-fold into isoosmolar Tris·H_2SO_4, pH 7.4, containing pairs of radiotracers (^{22}Na + ^{42}K, ^{22}Na + ^{86}Rb, ^{22}Na + ^{201}Tl, ^{42}K + ^{137}Cs, or ^{201}Tl + ^{137}Cs) at 2–5 μCi/ml each (1 Ci = 37 GBq). At intervals, 200 μl of the suspension was applied to a small column of Dowex 50X8-100 cation-exchange resin, and eluted with 2 ml of isoosmolar sucrose solution, as described (9, 10). The eluted internalized tracer was measured by liquid scintillation counting. Dual label and quench compensation programs of an LKB 1219 Rackbeta liquid scintillation counter were optimized for each pair of radiotracers. Several factors, including large vesicle size, low external sodium concentration, and the presence of a diffusion potential, slow the rate of equilibration and enhance maximal tracer uptake (10). Radiotracer efflux experiments (unpublished data) indicated that the sodium concentration gradient (and, therefore, the diffusion potential) was stable for more than 1 hr. In other controls, assays of long-term samples demonstrated that maximal uptake was fully stable for more than 1 hr before beginning a gradual decline over many hours.

Chemical and Proteolytic Modification of Reconstituted Channels. Initial experiments with NBA (cf. Fig. 2) were performed as follows. FTS vesicles were preincubated with or without toxins and anesthetics, as indicated, then diluted 20-fold into Tris·H_2SO_4 buffer containing ^{22}Na, ^{86}Rb, and 500 μM NBA. At intervals, aliquots were removed for Dowex chromatography and measurement of radioactivity. In subsequent experiments (Figs. 3–6), FTS vesicles were diluted 10- or 20-fold into Tris·H_2SO_4 buffer without radiotracers or modifying reagents. As the control, after 10–20 min, duplicate 225-μl samples of the diluted suspension were added to tubes containing pairs of radiotracers. After 1 min, 200 μl of this material was applied to a Dowex column, eluted, and assayed for radioactivity. Radiotracer uptake from the duplicates was averaged, and taken as the zero-time control. To the remaining suspension were added solutions of NBA, NBS, Pronase E, or trypsin. At intervals, 225-μl aliquots were removed, and 1-min tracer uptake assays were conducted as for the controls. In peptide cleavage experiments, sodium dodecyl sulfate/polyacrylamide gel electrophoresis (NaDodSO$_4$/PAGE) was conducted as described by Laemmli (19).

RESULTS

BTX Activates Cation-Selective Influx. Reconstituted vesicles treated with BTX were selectively permeable to alkali cations (Fig. 1 *A* and *B*). In the absence of neurotoxin (Fig. 1*A*) a low level of uptake was observed that was slightly selective for ^{22}Na. This small selective signal was completely blocked by external QX-314. In parallel samples treated with BTX, ^{22}Na uptake was markedly enhanced (note scales in Fig. 1 *A* and *B*).

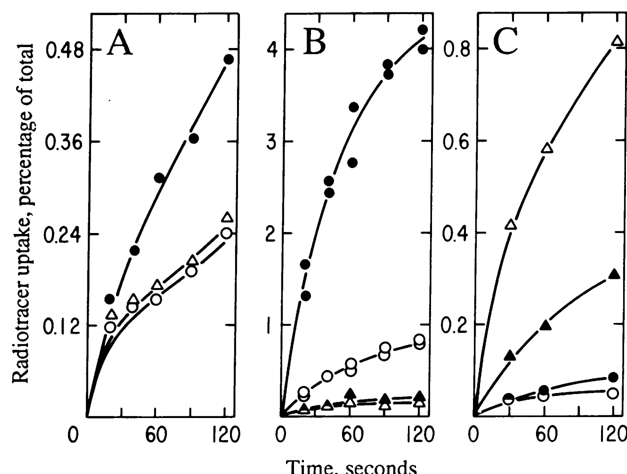

FIG. 1. (*A* and *B*) Ion selectivity of BTX-stimulated tracer uptake. FTS-treated vesicles were diluted 10-fold into Tris·H_2SO_4 buffer containing pairs of radiotracers. ●, ^{22}Na; ○, ^{42}K; ▲, ^{86}Rb; and △, ^{137}Cs. (*A*) Low weakly selective tracer uptake exhibited by control samples preincubated with 1% ethanol vehicle. (*B*) High strongly selective uptake after preincubation with 2.5 μM BTX. *Net* BTX-activated uptake is shown: control uptake has been subtracted from uptake in the presence of BTX. Uptake selectivity for this experiment, estimated as described in the text, was $P_{Na} = 1.0$, $P_K = 0.14$, $P_{Rb} = 0.04$, and $P_{Cs} = 0.03$. (*C*) Sensitivity of BTX-activated ^{22}Na uptake to blockage by 1 μM TTX and TTX plus 3 mM QX-314. Vesicles were preincubated with BTX alone (△), BTX with TTX (▲), BTX with TTX plus QX-314 (●), or TTX plus QX-314 alone (○), then diluted 20-fold into Tris·H_2SO_4 buffer containing ^{22}Na and ^{86}Rb. BTX-stimulated ^{86}Rb uptake was low (as in *B*) and was proportionately reduced by TTX, QX-314, or both.

Influx of the other tracers was stimulated to a lesser degree, in the sequence Na > K > Rb > Cs (Fig. 1*B*). When tracer accumulation at 1 min was taken as an index of relative cation permeabilities, the estimated permeability ratios were $P_{Na} = 1.00$, $P_K = 0.22$, $P_{Rb} = 0.06$, $P_{Cs} = 0.04$ ($n = 4$). Thus, the electroplax protein exhibited the selectivity sequence expected for sodium channels modified by BTX (20).

BTX-activated ^{22}Na uptake was blocked by TTX and QX-314 (Fig. 1*C*). External 1 μM TTX (acting on channels facing outside-out) blocked approximately 70% of the BTX-stimulated flux. QX-314, at 3 mM (acting on channels facing inside-out) completely blocked the remaining influx, as previously observed for QX-222 (9). As illustrated elsewhere, 1 μM TTX added before the freeze-thaw step completely suppressed the BTX signal (9, 10).

These observations provided a rationale for detecting chemical modifications that could result in chronic channel activation by damaging the inactivation elements. In the absence of toxin, the proteins may be in the inactivated or slow-inactivated state. (*i*) Reagents that damaged inactivation should stimulate radiotracer influx. (*ii*) The flux should be strongly selective for ^{22}Na over ^{86}Rb. If these modifications affected only gating, (*iii*) the proteins should retain sensitivity to TTX and local anesthetics. Such modifications should occur at the cytoplasmic protein surface and thus (*iv*) fluxes should be unaffected by external TTX and be completely blocked by external QX-314 or QX-222. We tested these predictions with oxidation reagents and proteases.

Effects of NBA and NBS. We found that incubating vesicles with 500 μM NBA produced a 2- to 3-fold increase in ^{22}Na uptake over controls (Fig. 2*A*). Uptake of ^{86}Rb was not stimulated. External TTX (1 μM) did not inhibit the NBA-stimulated flux (Fig. 2*B*). We further found that external QX-314 (3 mM) caused nearly complete block of the stimulated influx (Fig. 2*C*). Dose–response curves for QX-314

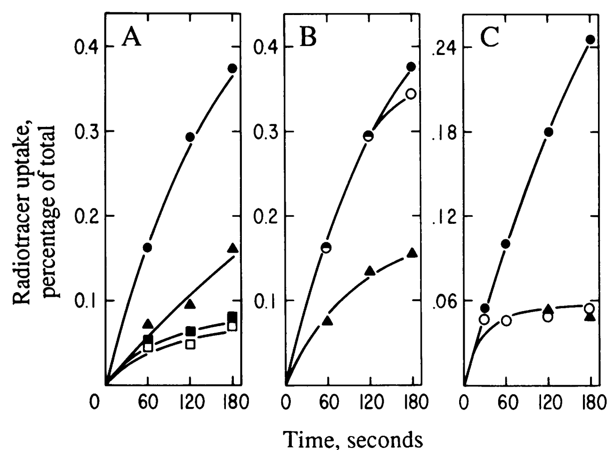

FIG. 2. Rapid activation of sodium-selective uptake by 500 μM NBA. (*A*) ^{22}Na uptake in the presence (●) or absence (▲) of 500 μM NBA and ^{86}Rb uptake in the presence (■) or absence (□) of NBA. (*B*) Insensitivity of NBA-activated ^{22}Na uptake to external 1 μM TTX. ●, NBA alone; ○, NBA in the presence of TTX; ▲, TTX alone. (*C*) Complete block of ^{22}Na uptake by 3 mM external QX-314. ●, NBA alone; ○, NBA in the presence of QX-314; and ▲, QX-314 alone.

(unpublished data) showed $K_{1/2} \approx 0.3$–0.5 mM. These findings suggested that NBA stimulated the sodium-selective flux by modifying sites on the intracellular protein surface.

The magnitudes of NBA-induced fluxes were compared with those caused by BTX (unpublished data). NBA signals were 30–50% of the fraction of the BTX signal that was insensitive to external TTX (i.e., mediated by channels facing inside-out, $n = 3$). NBA did not alter the maximum signal produced by BTX nor the ability of external 1 μM TTX to block 50–70% of the BTX-stimulated influx.

We next characterized the time dependence of the effects of NBA in more detail. In these experiments NBA was added at time zero, and at intervals aliquots were removed and tested in 1-min ^{22}Na and ^{86}Rb uptake assays. A triphasic time course was observed (Fig. 3). In the first phase (0.2 min) a progressive activation of ^{22}Na but not ^{86}Rb influx was observed. In the second phase (2–6 min), this influx declined precipitously to control levels. The third phase was a slow increase in a nonselective leak that failed to distinguish between the two ions. This complex time course suggested that several sites on the protein were being modified in sequence. The rates of the reactions were markedly shifted by changing the NBA concentration (Fig. 4). At 5 mM, the reaction proceeded most rapidly, and only the second, declining, phase was observed. At lower concentrations, NBA-stimulated uptake rose to similar maximal levels and then declined.

To identify the specific structural and functional changes resulting from chemical modification treatments, it is important to be able to titrate the reaction to different extents with limiting amounts of reagent. This proved to be impractical with NBA, which reacts slowly and requires high concentrations for a reasonable time course. We therefore tested NBS, a more rapidly reacting oxidant (20, 21). NBS stimulated ion-selective uptake at concentrations 1/100th of those required for NBA. At the lowest concentrations tested ($<\approx 2$ μM), little activation was detected. High (≈ 100 μM) NBS concentrations produced time courses like those seen with NBA: flux first rapidly increased and then subsequently declined over several minutes (unpublished results). At ≈ 5 μM a brief lag was seen, followed by rapid stimulation of flux, equal to that elicited by ≈ 1 mM NBA, but it did not decline (Fig. 4). Apparently, at intermediate NBS-to-protein ratios, the reagent is consumed before the declining phase of the reaction develops. Therefore, NBS may be used to titrate the modifications to different extents. NaDodSO$_4$/PAGE of samples treated with sufficient NBS to fully activate flux revealed no peptide cleavage. This suggested that oxidation reactions alone may cause activation, as discussed below.

Effects of Pronase and Trypsin. Treatment with Pronase also produced an initial stimulation and then a loss of sodium-selective flux (Fig. 5*A*). Here the second, falling, phase was somewhat delayed relative to the initial rise, and thus Pronase activated a higher peak level of flux than NBA or NBS, equalling 100% of the BTX-activated flux not blocked with external TTX. At low Pronase concentrations (<25 μg/ml) the activated phase persisted for 10–15 min. Trypsin also rapidly activated a large sodium-selective influx at concentrations of 1–10 μg/ml (Fig. 5*B*). At the higher concentrations, flux stimulation was followed by a gradual decline as seen with Pronase.

The fluxes stimulated by Pronase (unpublished observations) and trypsin (Fig. 6*A*) were blocked by external plus internal TTX (1 μM) and by external QX-314 (3 mM) but not

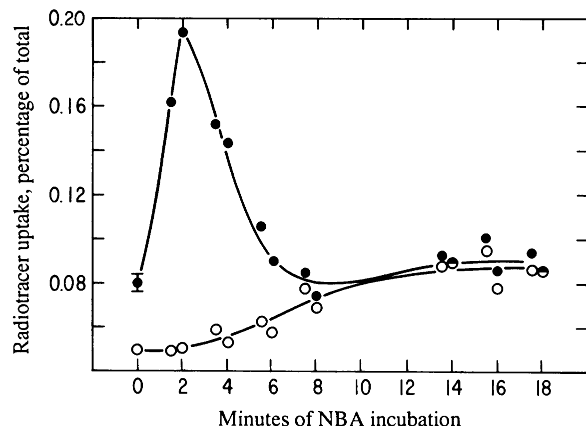

FIG. 3. Time course of flux activation by 1.1 mM NBA. FTS vesicles were diluted 20-fold into Tris·H$_2$SO$_4$ buffer and incubated at room temperature for 10 min. Controls, not shown here, indicated that tracer uptake with or without 2.5 μM BTX stabilized in 5 min and remained high for at least 1 hr. ^{22}Na (●) and ^{86}Rb (○) uptake was determined in 1-min assays, before or after the addition of NBA to 1.1 mM.

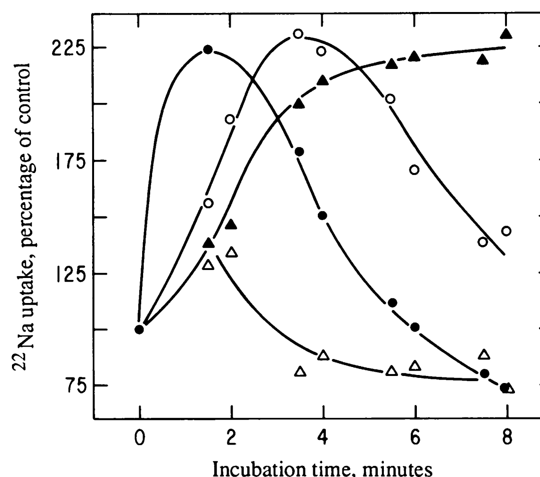

FIG. 4. Dependence of ^{22}Na uptake on NBA and NBS concentration. ^{86}Rb uptake, also determined here in dual label assays, was low and is omitted for clarity. FTS vesicles were diluted 20-fold into Tris·H$_2$SO$_4$ buffer and incubated at room temperature for 10–20 min. Tracer uptake was determined before and after the addition of NBA at 5 mM (△), 1.1 mM (●), or 0.5 mM (○) or of NBS at 5 μM (▲).

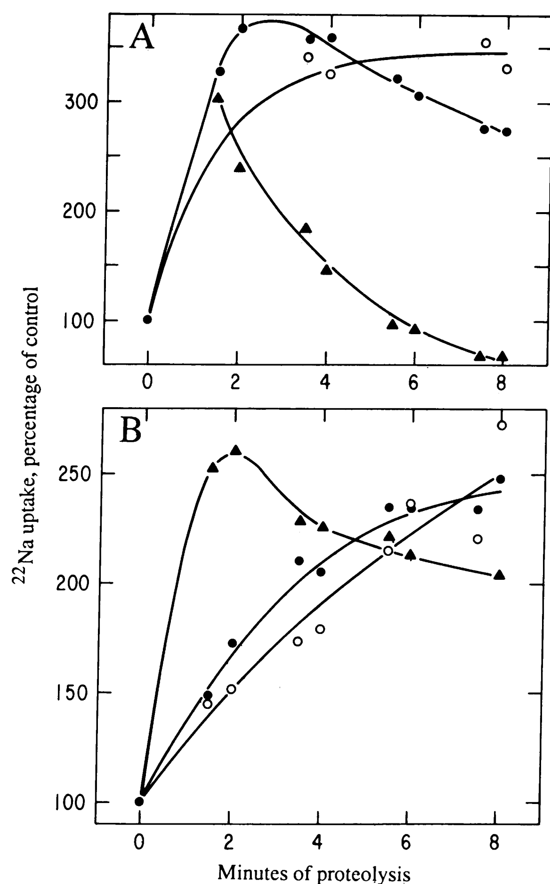

FIG. 5. Dependence of ^{22}Na uptake on Pronase E and TPCK-treated trypsin concentration. ^{86}Rb uptake was uniformly low and is not shown. (*A*) FTS vesicles were diluted 20-fold into Tris·H_2SO_4 and incubated at room temperature for 10–20 min. One-minute uptake of ^{22}Na was determined before or after the addition of Pronase E at 0.5 mg/ml (▲), 0.1 mg/ml (●), or 0.025 mg/ml (○). (*B*) ^{22}Na uptake into 10-fold diluted vesicles before and after the addition of TPCK-treated trypsin at 7.5 μg/ml (▲), 2.5 μg/ml (●), or 1.25 μg/ml (○).

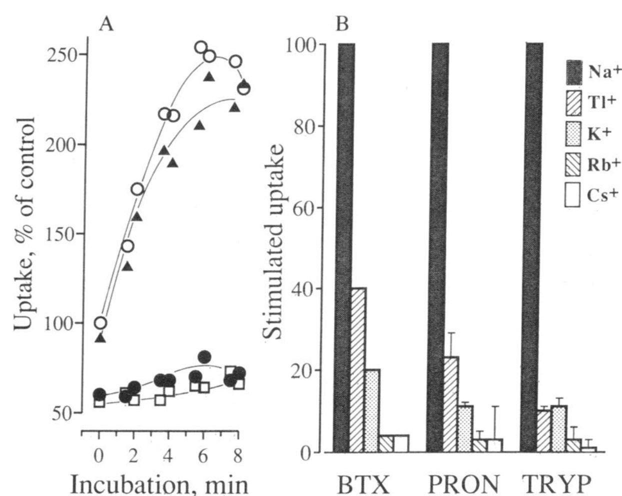

FIG. 6. (*A*) Sensitivity of trypsin-stimulated flux to external TTX, external QX-314, and external plus internal TTX. Aliquots of vesicles were subjected to FTS. In some samples, 1 μM TTX was added before FTS. The vesicles were then diluted 20-fold into Tris·H_2SO_4 buffer containing no blockers (control), 1 μM TTX, or 3 mM QX-314. After 15 min at room temperature, 1-min ^{22}Na uptake assays were conducted either before or at intervals after the addition of trypsin to 2.5 μg/ml. ○, Control; ▲, external TTX; ●, external plus internal TTX; □, external QX-314. (*B*) Cation selectivity of reconstituted sodium channels modified with BTX or by brief incubation with Pronase or trypsin. FTS vesicles were incubated for 45 min at 30°C with or without 2.5 μM BTX, diluted 10-fold into Tris·H_2SO_4 buffer, and incubated at room temperature for 10–20 min. For BTX-treated samples and controls, duplicate 1-min uptake assays were performed with pairs of radiotracers listed in *Materials and Methods*. Net BTX-activated uptake was determined and is expressed as a percentage of ^{22}Na uptake. To determine the selectivity of protease-treated channels, additional samples of the FTS vesicles were first diluted 10-fold and incubated at room temperature for 10–20 min. One-minute dual label radiotracer assays were performed before or 1.5, 2.0, 3.5, or 4.0 min after the addition of Pronase E at 50 μg/ml or TPCK-trypsin at 7.5 μg/ml. Uptake before the addition of enzymes was subtracted from protease-treated uptake to determine net stimulated flux. Data for each tracer (normalized to ^{22}Na uptake) are the average (with SD) of the four time points.

external TTX. Thus, as with NBA and NBS, the functional modifications appear to occur at the cytoplasmic protein surface. In addition, we compared in parallel experiments the cation selectivity of channels treated with the proteases and with BTX (Fig. 6*B*). We found selectivity to be somewhat greater for both Pronase- and trypsin-activated material than for that treated with BTX. Tl^+ and K^+ appeared to be significantly less permeant in the protease-treated samples than after BTX treatment, as would be expected if gating, but not permeation selectivity, had been affected (22).

Peptide Cleavages During Trypsin Activation. Trypsin is a pure enzyme of defined specificity that can be used to stimulate flux at extremely low concentrations, and it can be rapidly inhibited. For these reasons, experiments aimed at correlating the protease-stimulated ion flux with peptide cleavage were conducted with trypsin. At intervals after adding trypsin to a suspension of vesicles, aliquots were transferred to tubes containing pancreatic trypsin inhibitor. These samples were then either assayed for tracer uptake or analyzed by $NaDodSO_4$/PAGE and densitometry (Fig. 7).

In the starting material, sodium channel glycopeptide appeared as a broad band of ≈260 kDa, at >90% purity, as judged by densitometry. After 40 sec of incubation with trypsin, sodium-selective flux was activated to approximately 60% of maximum. Material in the large peptide was reduced by less than half. Three new fragments were observed, including a diffuse band of 110–150 kDa, which is probably heavily glycosylated (3). A prominent 38-kDa band was also seen, as was a smaller band at 70 kDa. Because these fragments may total 260 kDa, the apparent size of the uncleaved starting material, as few as two cleavages could account for these fragments.

At 160 sec, activation was 90% of maximum, the large peptide was reduced by more than half, the 110- to 150-kDa band was increased, the 70-kDa band was reduced, and two new fragments appeared at 45 and 24 kDa. The 38-kDa band was increased slightly. Conceivably the 70-kDa band is cleaved to the two new species observed.

At 280 sec, flux remained high, and the large peptide was further reduced, as was the 110- to 150-kDa band. The 38-kDa peptide was undiminished, and traces of the 70-kDa and 24-kDa peptides could be discerned. This basic fragmentation pattern has been observed reproducibly. These results suggest that at early stages of the reaction, where flux is strongly activated (60–90%), minimal damage to the protein has occurred.

DISCUSSION

These studies reveal that the reconstituted electroplax sodium channel, activated with BTX, mediates ionic fluxes that are blocked by TTX and QX-314 and are selective among alkali cations in the sequence $P_{Na} > P_{Tl} > P_K > P_{Rb} > P_{Cs}$. Under the conditions used, BTX is thought to stimulate flux by removing inactivation and slow-inactivation gating. Four protein modifying reagents—NBA, NBS, Pronase, and trypsin—also were able to stimulate ion-selective fluxes ($P_{Na} >> P_{Rb}$)

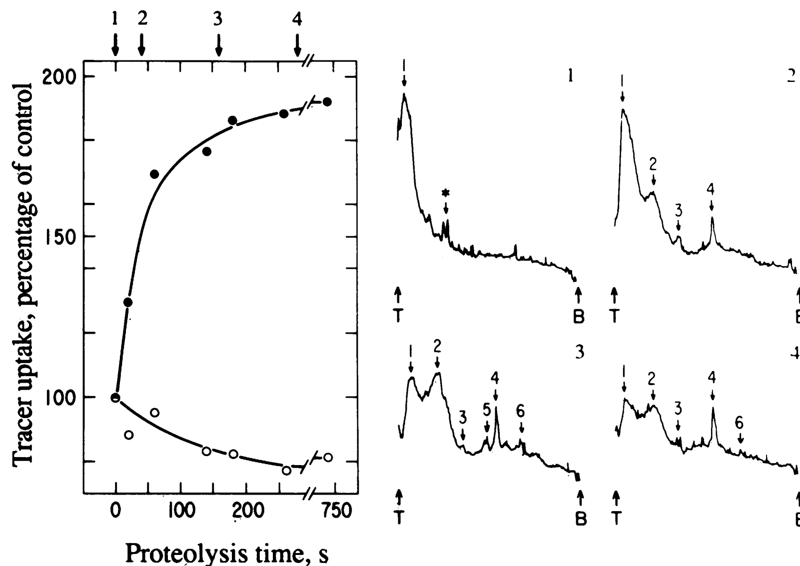

FIG. 7. Correlation of trypsin-stimulated radiotracer uptake with NaDodSO$_4$/PAGE. FTS vesicles were slowly (5–10 min) passed over a small column of Tris Dowex (ratio of column volume to vesicle suspension volume of 1:6) to exchange external Na^+ for $Tris^+$ and create a sodium concentration gradient. Before, or at the indicated intervals (numbered arrows) after the addition of trypsin (5 μg/ml), samples were diluted 10-fold into Tris·H_2SO_4 buffer containing excess pancreatic trypsin inhibitor and radiotracers [for 1-min uptake assays of $^{22}Na^+$ (●) and $^{86}Rb^+$ (○)] or trypsin inhibitor alone (for NaDodSO$_4$/PAGE). Samples were run on a 6–20% linear gradient gel, stained with silver, and subjected to scanning densitometry. Tracing numbers correspond to the intervals on the uptake plot. Peaks indicated on densitometry tracings on the right are as follows: 1, sodium channel glycopeptide, ≈260 kDa; *, ≈100-kDa contaminant, presumably the α subunit of Na,K-ATPase; 2, ≈130-kDa fragment; 3, 70-kDa fragment; 4, 38-kDa fragment; 5, 45-kDa fragment; and 6, 24-kDa fragment. T and B are top and bottom.

into the vesicles. The selectivity sequences of the BTX and protease-treated vesicles were compared and found to have the same order. However, protease-treated samples appeared somewhat more strongly sodium selective than those treated with BTX. Blockade by external QX-314 and internal plus external TTX, but not external TTX alone, together with the magnitude of the stimulation, indicates that only proteins with exposed cytoplasmic domains were activated. Further, action of all reagents followed a multistep time course of ion-selective flux activation, followed by loss of permeability and, where tested, an eventual slow rise in nonselective leak.

These results are similar in many respects to those of previous physiological studies. Each of the four reagents tested here has been shown to selectively remove inactivation (14–17). In some reports this was accompanied by up to 50% reduction in peak sodium currents (14, 17), and prolonged exposure resulted in irreversible loss of sodium currents and an eventual increase in nonspecific membrane leak.

The protein-modifying reagents differ in their mechanisms and amino acid specificities. NBA and NBS oxidize cysteine, methionine, tryptophan, tyrosine, and histidine side chains; under some conditions oxidation of aromatic amino acids may be followed with variable efficiency by rearrangement and peptide cleavage (20, 21). No peptide cleavage was observed during flux activation with NBS, suggesting oxidation alone may be responsible for the effects observed here. Pronase is a mixture of enzymes, but inactivation removal has been previously associated with an alkaline proteinase b component, specific for sites containing arginine. Trypsin is specific for sites involving arginine or lysine. Thus, the activation of fluxes may result from damage to any of several sites in a region of the peptide exposed to the cytoplasmic surface and particularly vulnerable to modification.

The pattern of peptide fragments produced by limited exposure to trypsin suggests that flux stimulation occurs after minimal damage to the protein. Perhaps as few as two or three cleavages are required. More extensive studies will be required to locate the cleavage sites and to associate specific cleavages with flux activation. Single channel recording experiments will be required to determine the precise biophysical properties of each protein form.

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