CATION GATING AND SELECTIVITY IN A PURIFIED, RECONSTITUTED, VOLTAGE-DEPENDENT SODIUM CHANNEL

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In excitable membranes, the voltage-dependent sodium channel controls the primary membrane conductance change necessary for the generation of an action potential. Over the past four decades, the time- and voltage-dependent sodium currents gated by this channel have been thoroughly documented with increasingly sophisticated voltage-clamp techniques. Recent advances in the biochemistry of membrane proteins have led to the solubilization and purification of this channel protein from nerve (6) and from muscle (4) or muscle-derived (1) membranes, and have provided an approach to the correlation of the channel's molecular structure with its functional properties. Each of these sodium channel preparations appears to contain a large glycoprotein either as its sole component (2) or in association with several smaller subunits (6, 3). Proof that these purified proteins represent the excitable membrane sodium channel, however, must rest with demonstration of functional cation gating.

RESULTS AND DISCUSSION

The sodium channel, identified by its capacity for high-affinity saxitoxin (STX) binding, was purified from rat sarcolemmal membranes as detailed previously (3, 4). When examined under denaturing conditions, preparations that had the highest specific activity (2,500–2,900 pm STX binding/mg protein) contained a large glycoprotein that ran anomalously as a diffuse band in the high-molecular weight region of 7–20% SDS-PAGE gradient gels, a 45,000 mol wt component, and a third component often resolved as a doublet at 37,000–38,000 mol wt (4). We have reconstituted this purified channel protein into egg phosphatidylcholine vesicles using Biobeads SM-2 (Bio-Rad Laboratories, Richmond, CA) to remove the NP-40 detergent (8). Recovery of specific STX binding in the vesicles ranged between 40% and 60%; of these binding sites, half were accessible on the external surface of intact vesicles, the remainder only after disruption of the vesicle membrane, indicating random orientation of the channels in the bilayer. In contrast to the lability of the solubilized channel protein, the reconstituted channel recovered temperature stability properties similar to those seen for this channel in intact sarcolemma. Activation of vesicles containing the reconstituted sodium channel with batrachotoxin (5×10^{-6} M) produced a rapid specific influx of $^{22}Na^+$. 50–70% of this specific influx was blocked by externally applied STX, while the remainder was blocked by the presence of STX inside the vesicles as well as in the external solution. Functional reconstitutions were also obtained using sarcolemmal phospholipids or soybean phospholipids in place of phosphatidylcholine.

The dose-response characteristics for the interaction of various toxins and pharmacologic agents with the sodium channel in situ have been extensively studied, and help to characterize this unique protein (5). Some of these functional interactions have been quantitated for the purified reconstituted channel. The dose-response curve for activation of the reconstituted channel with batrachotoxin indicated binding at a single class of sites with an apparent K_d of 1.5×10^{-6} M. Veratridine activated the reconstituted channel with an apparent K_d of 3.5×10^{-5} M. Activated flux in either case was blocked by STX; when inhibition of initial rates was measured, the K_i for STX was 5×10^{-9} M at 18°C. All channels containing a binding site for batrachotoxin that produced activation also contained a binding site for STX. The local anesthetics lidocaine and benzocaine both inhibited batrachotoxin-stimulated cation influx. The apparent K_i for lidocaine was 4×10^{-4} M, while that for benzocaine was 6×10^{-4} M, both determined in the presence of 5×10^{-6} M batrachotoxin. In each case, the demonstrated function and apparent dissociation constant for a given neuroactive agent observed with the purified, reconstituted sodium channel corresponded well to those seen with sodium channels in their native membrane environment.

Quenched-flow kinetic techniques have been adapted to the measurement of cation influx in this system (7) (Fig. 1). Using an electronically controlled drive, a small volume (20–40 μl) of batrachotoxin-activated vesicles containing the purified sodium channel was rapidly mixed (~4 ms) with an equal volume of buffer containing labeled cation to initiate the influx measurement. After a variable delay the reaction was rapidly quenched by injection into a slurry of Dowex 50W-X-8 cation exchange resin (Dow Corning Corp., Midland, MI). The dead time of the system was determined experimentally to be ~90 ms.

Using this approach, the kinetics of vesicle equilibration for $^{22}Na^+$, $^{42}K^+$, $^{86}Rb^+$ and $^{137}Cs^+$ through batrachotoxin-

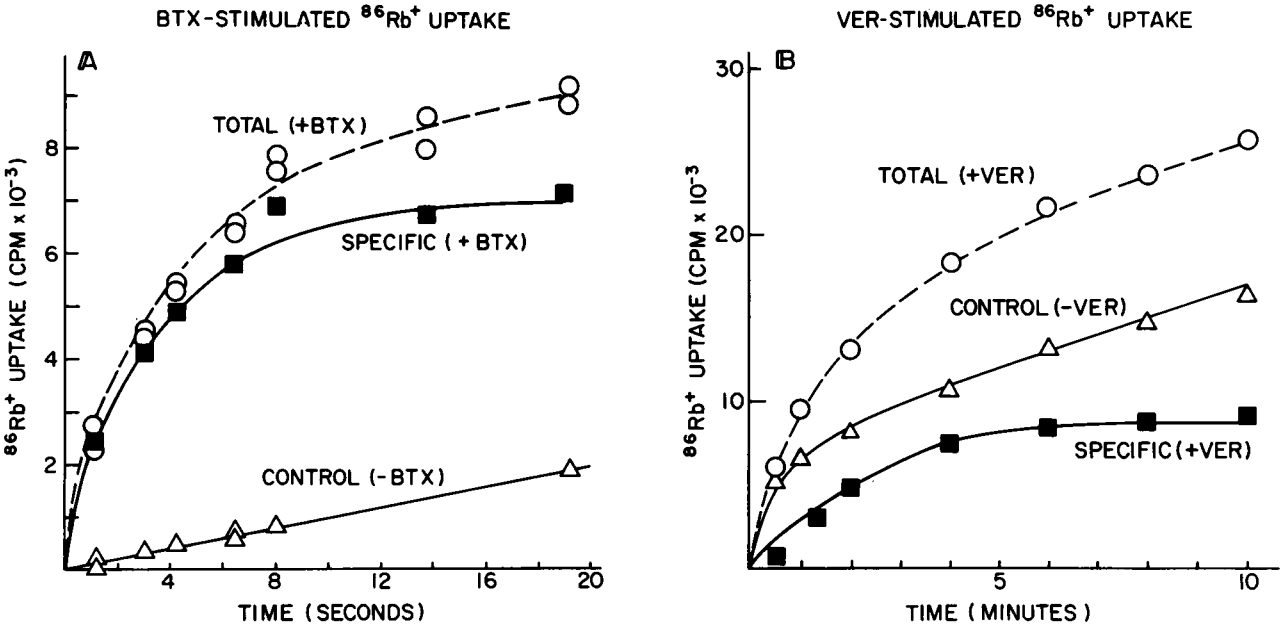

FIGURE 1 Alkaloid-stimulated $^{86}Rb^+$ influx into vesicles containing the purified sarcolemmal sodium channel. *A*, Vesicles activated with batrachotoxin (BTX, 5×10^{-6} M). Measurements were carried out with quenched-flow techniques at 18°C. Over these short intervals, nonspecific uptake appears linear. The $T_{1/2}$ for specific $^{86}Rb^+$ uptake is ~2.5 s. *B*, Vesicles activated with veratridine (VER, 5×10^{-4} M). Measurements made with manual technique at 36°C. Over longer time intervals the nonlinearity of nonspecific uptake is apparent. The $T_{1/2}$ for specific VER-stimulated $^{86}Rb^+$ uptake is 2 min at this temperature.

activated channels were determined. Influx of $^{137}Cs^+$, $^{86}Rb^+$ and $^{42}K^+$ at 18°C could be quantitated easily, yielding halftimes of 10 s, 2.5 s and 350 ms, respectively. $^{22}Na^+$ influx was nearly complete at the shortest possible measurement interval (94 ms) and an upper limit of 50 ms was assigned to its halftime. Using this upper limit, a cation selectivity series of Na^+, 1.0 : K^+, 0.14 : Rb^+, 0.02 : Cs^+, 0.005 was calculated (Table I). Veratridine stimulation (5×10^{-4} M) produced specific cation influx with halftimes several orders of magnitude slower than batrachotoxin at 36°C, (1.0, 1.2, 2.5, and 10 min, respectively for $^{22}Na^+$, $^{42}K^+$, $^{86}Rb^+$ and $^{137}Cs^+$) and with much lower apparent selectivity between cations.

Specific batrachotoxin-activated cation influx showed little temperature dependence; Arrhenius plots of initial rates for Rb^+ and Cs^+ uptake between 5° and 40°C yielded activation energies of 7.6 and 6.1 Kcal/mol. Similar measurements with veratridine-stimulated influx demonstrated a marked temperature dependence, with a calculated activation energy of 31 Kcal/mol for both cations. Flux measurements with batrachotoxin may reflect ion movement through an opened channel, while other factors, such as the probability of channel opening, may be rate-limiting with veratridine activation.

We conclude that the purified reconstituted saxitoxin binding protein from rat sarcolemma is a functional sodium channel. It can be pharmacologically activated to gate cation influxes and these cation movements can be blocked in a manner comparable to that observed for this sodium channel in situ. The purified channel also retains the ability to select between closely related cations, another characteristic of this unique membrane protein in its native environment.

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TABLE I
CATION SELECTIVITY IN THE RECONSTITUTED SODIUM CHANNEL

	Na^+		K^+		Rb^+		Cs^+	
	$T_{1/2}$*	R‡	$T_{1/2}$	R	$T_{1/2}$	R	$T_{1/2}$	R
Batrachotoxin	50 ms	1	350 ms	0.14	2.5 s	0.02	10 s	0.005
Veratridine	1.0 min	1	1.2 min	0.83	2.0 min	0.50	2.6 min	0.38

*Half-time for vesicle equilibration at 18°C for batrachotoxin (5×10^{-6} M) or at 36°C for veratridine (5×10^{-4} M).
‡Ratio of halftime for test cation to that for $^{22}Na^+$. An upper limit of 50 ms is assumed for Na^+ in batrachotoxin-stimulated vesicles.

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STRUCTURE AND FUNCTIONAL RECONSTITUTION OF THE SODIUM CHANNEL FROM RAT BRAIN

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We have employed neurotoxins as molecular probes to identify, purify, and reconstitute the ion transport activity of voltage-sensitive sodium channels from rat brain. This brief paper reviews the work in our laboratory on this problem.

RESULTS AND DISCUSSION

Identification of Sodium Channel Subunits in Intact Excitable Membranes

The protein components of the sodium channel from rat brain were initially identified by covalent labeling with a photoreactive derivative of the polypeptide scorpion toxin from *Leiurus quinquestriatus*, which binds at neurotoxin receptor site 3 on the sodium channel (1). Two polypeptides with molecular weights of 260,000 and 39,000 (by our current calibration procedures) were specifically labeled in rat brain synaptosomes. Labeling was blocked by carrying out the reaction in the presence of excess native scorpion toxin or sea anemone toxin. Depolarization of the synaptosomes with K^+ blocks specific binding of scorpion toxin to sodium channels (2) and prevents covalent labeling (1). These experiments confirm that the polypeptides of M_r = 260,000 and M_r = 37,000 are protein components of the sodium channel located at or near the scorpion toxin receptor site. They have subequently been designated the α and $\beta 1$ subunits of the sodium channel (see below).

Solubilization of the Saxitoxin Receptor of the Sodium Channel

The sodium channel can be solubilized in good yield from rat brain membranes by a variety of nonionic detergents including Triton X-100 (3). The solubilized channel binds saxitoxin and tetrodotoxin with affinity similar to that in the membrane-bound state, but the solubilized binding activity becomes unstable to incubation at 36° (3). The solubilized channel loses binding activity for neurotoxins at neurotoxin receptor site 2, the alkaloid toxin receptor site, and at neurotoxin receptor site 3, the polypeptide toxin receptor site (3).[1] The saxitoxin binding activity of the sodium channel solubilized from rat brain is stabilized by including 10 mM calcium and 1 mol of phosphatidylcholine/5 mol Triton X-100 in the solubilization solutions (3).

Molecular Size of the Sodium Channel

Analysis of the hydrodynamic properties of the solubilized sodium channel by gel filtration and sucrose gradient sedimentation in H_2O and D_2O indicated that the solubilized Triton X-100-phosphatidylcholine-sodium channel complex has a molecular weight of 601,000 (4). Assuming that the sodium channel protein has a partial specific volume of 0.73, this complex consists of the sodium channel protein, M_r = 316,000, and 0.9 g of bound Triton X-100 and phosphatidylcholine per gram of channel protein (4).

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[1] Catterall, W. A., J. A. Talvenheimo, M. M. Tamkun, D. J. Messner, R. P. Hartshorne, and R. M. Sharkey. Unpublished results.