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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).

Contribution No. 2656, Lab. of Genetics, Univ. of Wisconsin, Madison, WI.

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 Unpublished results.

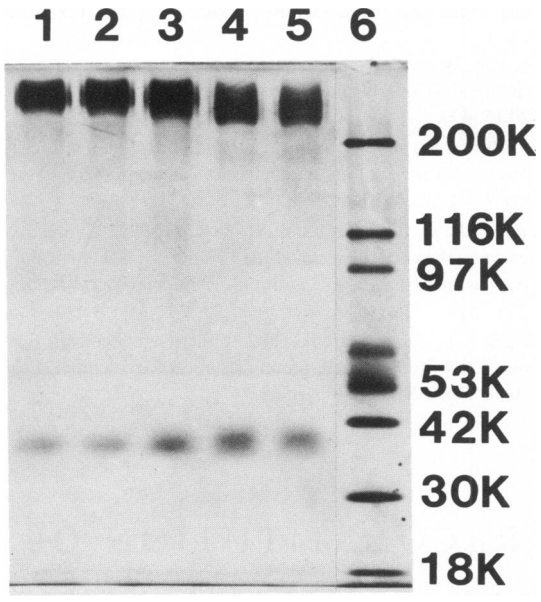

FIGURE 1 Analysis of the subunits of the sodium channel by SDS gel electrophoresis. Purified sodium channels were denatured in SDS without reduction with β-mercaptoethanol (*lanes* 1 and 2) or with 0.1 mM (*lane* 3), 1.1 mM (*lane* 4) or 6.6 mM (*lane* 5), and analyzed by polyacrylamide gel electrophoresis. Protein standards and their molecular weights are indicated in *lane* 6. From reference 6.

Purification and Subunit Composition of the Sodium Channel

The solubilized sodium channel has been purified 1,500-fold to essential homogeneity by chromatography on DEAE-Sephadex, hydroxylapatite, and wheat germ agglutinin-Sepharose followed by velocity sedimentation through sucrose gradients (5, 6). Analysis of the most highly purified preparations by SDS gel electrophoresis followed by sensitive silver staining reveals only two protein bands with molecular weights of 260,000 and 38,000 (Fig. 1, lane 5). These molecular weights correspond closely to those of the sodium channel subunits covalently labeled by scorpion toxin in synaptosomal membranes (1, 5). These subunits migrate as a stoichiometric complex coincident with saxitoxin binding activity on velocity sedimentation through sucrose gradients (5, 6). Thus, sodium channel subunits of similar size are identified by both solubilization and purification of the saxitoxin binding activity and by covalent labeling with scorpion toxin.

Studies of the effect of reduction of disulfide bonds on the migration of the sodium channel subunits show that there are two nonidentical β subunits with similar molecular weights (Fig. 1). As the concentration of β-mercaptoethanol is increased from lane 1 to 5, the protein band migrates more rapidly, consistent with a reduction in molecular weight of ~28,000; the stain intensity in the β protein band doubles. These results suggest that, in the absence of reduction, the α protein band contains a disulfide-linked complex of two polypeptides, α, M_r = 260,000; β2, M_r = 37,000. After reduction, the β2 subunit dissociates from α, the α subunit band migrates more rapidly, and the β1 and β2 subunits migrate as a poorly resolved doublet with twice the stain intensity (6). This is confirmed by preparative separation of the complex of α and β2 from β1 by gel filtration in SDS under nonreducing conditions and analysis of the resulting high and low molecular weight fractions by SDS gel electrophoresis (6). The two β subunits separated in this way have apparent molecular weights of 39,000 for β1 and 37,000 for β2 (6). They are distinguished by two additional properties: only β1 is covalently labeled by scorpion toxin, and only β2 is covalently linked to the α subunit by disulfide bonds (6).

In the analysis above, it was assumed that the α and β subunits identified in intact synaptosomes by photoaffinity labeling are identical to those in the purified sodium channel preparation because they are the same size. Recently, we have prepared antisera against the purified sodium channel. This antiserum specifically precipitates the α and β subunits covalently labeled by scorpion toxin from detergent extracts of synaptosomes (7). These experiments confirm the identity of the sodium channel subunits in the purified preparation and those labeled in situ. Evidently, the sodium channel from rat brain consists, both in situ and after purification, of a stoichiometric complex of three subunits: α, β1 and β2.

Reconstitution of Functional Sodium Channels from Pure Components

To determine whether the purified saxitoxin receptor contains all the functional components of the sodium channel, we have developed methods to incorporate the purified protein into phospholipid vesicles and to assess its ion-transport and neurotoxin-binding activities. As a first step we showed that incorporation of cholate-solubilized,

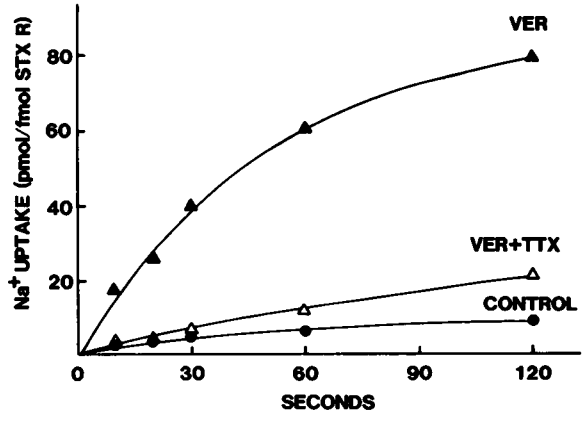

FIGURE 2 Sodium influx mediated by the purified and reconstituted sodium channel. Sodium influx into reconstituted phosphatidylcholine vesicles containing purified sodium channels was measured for the indicated times in the presence of 100 μM veratridine (▲), 100 μM veratridine plus 1 μM tetrodotoxin both inside and outside the vesicles (△), or in control medium with no additions (●). From reference 11.

but unpurified, sodium channel preparations into phosphatidylcholine vesicles results in full recovery of the functional properties of the sodium channels (8). These results indicate that no irreversible loss of sodium channel function occurs following detergent solubilization.

The sodium channel is purified after solubilization in Triton X100. To incorporate partially purified channel preparations into phospholipid vesicles, the purified protein was supplemented with excess phosphatidylcholine in Triton X-100 and the detergent was removed by adsorption to polystyrene beads (Bio Beads). The resulting reconstituted vesicles contain sodium channels as assessed by saxitoxin binding (9). More importantly, the channel recovers its native thermal stability at 36°, transports sodium after activation by veratridine, and is blocked by appropriate concentrations of tetrodotoxin (9). These experiments established a reconstitution procedure for purified sodium channels.

We have now applied this reconstitution procedure to essentially homogeneous preparations (Fig. 1, references 6 and 10) in which >90% of the protein is the α, $\beta 1$, and $\beta 2$ subunits of the sodium channel. The resulting vesicles contain approximately one sodium channel protein per vesicle. They have thermostable saxitoxin binding activity with a K_D of 3 nM, as in native membranes. Veratridine increases the initial rate of $^{22}Na^+$ influx into vesicles 10- to 20-fold (Fig. 2). The increased rate of sodium influx is blocked by tetrodotoxin with K_I of 15 nM as in native membranes. The ion transport by the reconstituted sodium channel is selective. The relative permeability of the vesicles containing veratridine-activated sodium channels to Rb^+ and Cs^+ is 0.25 and 0.12 compared to Na^+. These selectivity ratios compare favorably with those of neurotoxin-activated sodium channels in intact membranes. The results show that the purified sodium channel complex consisting only of the α, $\beta 1$, and $\beta 2$ subunits is sufficient to mediate neurotoxin-activated, selective ion conductance (11).

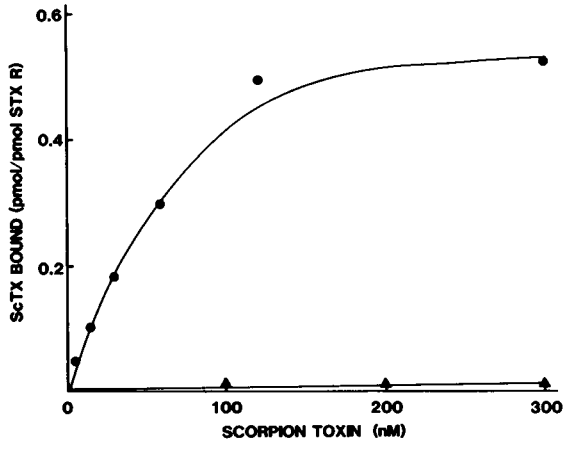

FIGURE 3 Scorpion toxin binding by the purified and reconstituted sodium channel. Specific scorpion toxin binding to purified sodium channels incorporated into phosphatidylcholine vesicles (▲) or phosphatidylcholine vesicles containing brain lipids (•) was measured in a rapid filtration binding assay. From reference 11.

While incorporation of the purified sodium channel into phosphatidylcholine vesicles restores veratridine-activated sodium transport, binding of scorpion toxin is not recovered (Fig. 3, ▲, and reference 11). In contrast, if a protein-free preparation of rat brain lipids is added to the reconstitution mixture, specific scorpion toxin-binding is recovered (Fig. 3, •). From 0.5 to 0.85 mol scorpion toxin are bound per mole of saxitoxin, indicating that most of the sodium channels that are successfully incorporated into these mixed lipid vesicles recover scorpion toxin binding. Incubation of the purified sodium channel at 36° for 2 min prior to reconstitution destroys both saxitoxin and scorpion toxin-binding activity, confirming that the purified channel and not the brain lipid is the receptor site for scorpion toxin. Evidently, scorpion toxin binding by the sodium channel is particularly sensitive to its lipid environment and requires the presence of one or more brain lipids in addition to egg phosphatidylcholine (11).

CONCLUSIONS

The voltage-sensitive sodium channel has been purified to essential homogeneity from rat brain and shown to consist of a stoichiometric complex of three subunits: α, $\beta 1$, and $\beta 2$. This complex is sufficient to mediate the functional activities of the sodium channel that can be tested using biochemical methods. Further experiments are required to determine whether the channel will undergo voltage-dependent activation and inactivation.

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VOLTAGE-ACTIVATED K CHANNELS IN EMBRYONIC CHICK HEART

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Voltage clamp experiments indicate the presence of a time-dependent potassium current activated at potentials positive to −50 mV and involved in repolarization of the action potential of chick embryonic ventricle (1). This potassium current, called I_X, is comparable to the delayed rectifier in Purkinje fibers (I_{X1}, reference 2), adult ventricle (I_K, reference 3), and SA node (I_K, reference 4). In each of these preparations the current we will call I_K is activated in the range between −50 and +10 mV, is time dependent with a time constant in the range of hundreds of milliseconds, and is an inward rectifier. The reversal potential reported for I_X is variable. In Purkinje fibers it was found to reverse at −65 mV in 4 mM K_O and was thus considered not completely specific for K ions (2). In ventricle, however, the delayed rectifier reversed at −90 mV in 3 mM K_O (3). The delayed rectifier in nerve has been shown to have a single channel conductance in the range of 12–17 pS (5, 6). There are few data on single channels in heart cell membrane to compare with macroscopic data on the delayed rectifier. We report single channel measurements on a current in 10-d chick embryonic ventricle that is activated in the same range as I_X, reverses at −88 mV in 4 mM K_O, and appears to rectify inwardly above 0 mV.

RESULTS

Although we have not measured the relative ion selectivity of the channel, Figs. 1 and 2 indicate that it is highly selective for K. First, the reversal potential for the current is near the K equilibrium potential. For 140/4 mM K gradient in the cell-attached experiments (Fig. 1 *a–c*), the Nernst potential at room temperature is −90 mV. The measured reversal potential is −87 mV (Fig. 2). Second, in outside-out patches where the K gradient is controlled (Fig. 1 *d*), the channel has the expected conductance and kinetics if the data are shifted along the voltage axis by the K Nernst potential.

The open state of this channel shows the phenomenon known as flickering, that is, the open state appears to occur in groups separated by closed times that are long compared to those within a group. It is evident from Fig. 1 that the mean length of these groups varies from tens of milliseconds at −30 mV to hundreds of milliseconds at 20 mV. Although the fraction of time spent in the open state increases dramatically as the membrane is depolarized, the length of time the channel actually stays open is rather brief. Open time histograms fit one exponential but closed time histograms are the sum of at least two exponentials (see legend, Fig. 3).

The open channel $i(V)$ relationship shown in Fig. 2 *a* appears to rectify inwardly at large positive voltages. It is unclear at the present time whether this is a real effect due to open channel conductance or whether this apparent depression of the currents at higher voltages results from increased flickering. The $i(V)$ data fit extremely well between −70 and 0 mV to a simple ohmic relationship given by $i = \gamma (V-E)$ where $\gamma = 62$ pS and $E = -87$ mV. A quadratic curve that fits all the points better than a straight line is given in Fig. 2 for comparison. The apparent curvature of the $i(V)$ data is not due to a varying patch-seal resistance. This is shown in Fig. 2 *b*, where the background current through the patch-seal resistance is measured. The background current is the current that flows through the parallel combination of the seal resistance and the patch membrane when the channel is closed. These points are fit to a quadratic function of voltage for comparison with Fig. 2 *a*, but they essentially lie on a straight line with a slope of 41 pS or 24 GΩ.

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