Purification and Functional Reconstitution of the Voltage-sensitive Sodium Channel from Rabbit T-tubular Membranes*

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The voltage-sensitive sodium channel has been purified from rabbit T-tubular membranes and reconstituted into defined phospholipid vesicles. Membranes enriched in T-tubular elements (specific [3H]nitrendipine binding = 41 ± 9 pmol/mg of protein, n = 7) were isolated from fast skeletal muscle. After solubilization with Nonidet P-40, the sodium channel protein was purified to >95% of theoretical homogeneity based on the specific activity of [3H]saxitoxin binding. Two subunits of M, ~260,000 and 38,000 were found; these bands co-distributed with the peak of [3H]saxitoxin binding on sucrose gradients.

The purified protein was reconstituted into egg phosphatidylcholine vesicles and retained the ability to gate specific 22Na+ influx in response to activation by batrachotoxin or veratridine. All activated fluxes were blocked by saxitoxin and tetrodotoxin. On sucrose gradients, the distribution of protein capable of functional channel activity paralleled the distribution of specific [3H]saxitoxin binding and of the M, 260,000 and 38,000 components. The cation selectivity for the reconstituted, batrachotoxin-activated channel was Na+ > K+ > Rb+ > Cs+, with flux ratios of 1:0.13:0.02:0.008.

Nine of 25 monoclonal antibodies raised against the rat sarcolemmal sodium channel cross-reacted with the rabbit T-tubular sodium channel in a solid-phase radioimmunoassay. Six of these antibodies showed specific binding to immunoblot transfers of T-tubular membrane proteins. Each labeled a single band at M, ~260,000 corresponding in mobility to the large subunit of the sodium channel.

Most nerve and muscle cells are characterized by their ability to produce action potentials and to propagate these regenerative signals along their surface membranes. In mature cells, these stereotyped signals are the result of a transient increase in membrane conductance to sodium ions (1, 2); the ubiquitous sodium channel that controls these currents has been characterized in situ with both biophysical and electrophysiological techniques (3).

Although undertaken more recently, biochemical characterization of this voltage-dependent sodium channel has progressed rapidly during the past 6 years (4). Voltage-sensitive sodium channels have been purified from the electroplax organ of the electric eel (5, 6), from rat skeletal muscle (7) and brain (8), and from chick heart (9). In each case, the purified protein contained a very large glycoprotein subunit (10–12); the mammalian channels also appeared to contain one or more smaller subunits (11, 12). The channels from rat muscle and brain and from eel have now been reconstituted into defined lipid vesicles, and each has been shown to retain the ability to gate cation fluxes in response to pharmacological activation (13–16).

In skeletal muscle, action potentials arising in the sarcolemma are conducted into the muscle fiber interior along elements of the T-tubular system, where depolarization of the T-tubules ultimately leads to calcium release from the sarcoplasmic reticulum and muscle contraction (17). Although membranes of the T-tubules are direct extensions of the sarcolemma, there is evidence that these membranes differ from sarcolemma in both protein and lipid composition (18, 19). While voltage-sensitive sodium channels are present in both the sarcolemma and the T-tubules, recent studies have suggested that these channels may differ in their affinity for various neurotoxins and their derivatives (20, 21).

Our previous work with the voltage-sensitive sodium channel from rat skeletal muscle was based on a membrane preparation enriched in sarcolemma (22, 23). In that preparation, which involved prolonged extraction of the membranes with lithium bromide, we encountered persistent problems with proteolysis of the sodium channel large glycoprotein subunit (24). Because of this difficulty, an alternate source of mammalian muscle sodium channel was sought.

Recent studies with rabbit skeletal muscle have shown that membrane preparations from this species can be selectively enriched in T-tubular elements (18, 23). These T-tubular membranes can be isolated without prolonged extraction at high ionic strength and probably represent a more homogeneous membrane population than do other surface membrane preparations from muscle (18, 22, 23). Although sodium channel density in T-tubules is lower than in the sarcolemma, this is more than offset by the larger surface area of the T-system.

In an effort to better define the properties of the mammalian muscle sodium channel, we purified and characterized the rabbit T-tubular sodium channel and compared its properties to those previously reported for the sodium channel from rat muscle and rat brain. We find that, at least in regard to its physical and biochemical properties and its behavior after functional reconstitution, the T-tubular sodium channel closely resembles that found in the rat skeletal muscle and brain. Rabbit T-tubular membranes represent an excellent
source of mammalian muscle sodium channels for biochemical study.

METHODS AND RESULTS

For "Physical Properties of the Solubilized Channel" and "Channel Purification," see the miniprint on p. 6347.

Subunit Composition of the Purified Protein—SDS-PAGE of the T-tubular membrane preparation and of peak fractions from a typical purification are shown in Fig. 1. The WGA-Sepharose peak derived from the GS-550 fraction contained a predominant high molecular weight component migrating at about 260,000 (Fig. 1, lane 6) along with a number of smaller components. This component was usually present in the GS-250 WGA-Sepharose peak, but a second diffuse component migrating at about M, 150,000 was more prominent (Fig. 1, lane 5). On the sucrose gradient, derived from the GS-550 fraction, the peak of protein concentration coincided with the peak of [3H]STX binding; this peak fraction contained a major diffuse band at ~260,000 and a single low molecular weight component at 38,000 (Fig. 1, lanes 8 and 9). In the GS-250 sucrose gradient, however, the fractions containing the highest STX binding did not coincide with the protein peak. The major protein peak in the GS-250 gradient was located in a lower density region of the gradient and contained on SDS-PAGE predominantly the diffuse M, 150,000 component and two smaller peptides at M, 50,000 and 35,000 (Fig. 1, lane 7).

The relationship of these various bands on SDS-PAGE to the peak of [3H]STX binding activity was examined in more detail after subjecting the peak WGA-Sepharose fractions derived from both the GS-250 and the GS-550 fractions to more prolonged centrifugation on sucrose gradients (16 h, 175,000 × g) in order to spread the components throughout the gradient (Fig. 2). Analysis of individual fractions for the GS-250 and GS-550 gradients indicated that in both cases the M, 260,000 and 38,000 bands co-distributed with the peak of saxitoxin binding and the presumptive location of the sodium channel. In the GS-550 sucrose gradient, these two components coincided with the major peak of protein in the gradient itself (Fig. 2, C and D). In the GS-250 gradient, however, the majority of the protein sedimented in lighter fractions of the gradient and was represented on the gel by a diffuse band at M, ~150,000 and two additional bands at M, 50,000 and 35,000 (Fig. 2, A and B). The molecular weight and migratory characteristics of these three bands are comparable to those reported by Curtis and Catterall (37) for the nitrendipine binding component of the voltage-dependent calcium channel purified from this T-tubular membrane preparation. The distribution of these bands was clearly distinct from the two components co-migrating with the sodium channel STX binding activity, although their relative predominance in the GS-250 fractions resulted in contamination of the peak sodium channel fractions in that gradient.

The large subunit of the sodium channel appeared to be very sensitive to a calcium-activated protease in rabbit muscle. Sodium channel purifications carried out on membranes isolated in the presence of 2 mM EGTA rather than 10 mM EGTA in the initial homogenization buffers (in addition to the protease inhibitors listed above) showed significant loss of the M, 260,000 subunit of the channel. A new band running diffusely between 140,000 and 200,000 was prominent in these gels and probably represented the product of proteolysis of the M, 260,000 subunit as demonstrated for the rat sarclemmal sodium channel (24). Traces of this component can be seen in Fig. 2, B and D.

Reconstitution—The purified sodium channel protein was reconstituted into egg phosphatidylcholine vesicles. After reconstitution, channels could be activated by either batrachotoxin or veratridine to produce specific influx of various cations into the vesicles (Fig. 3).

The apparent Kd for activation of the sodium channel with batrachotoxin at 36 °C was 2 μM (Fig. 3B). Both tetrodotoxin and saxitoxin were able to block about 40–50% of the batrachotoxin-stimulated 22Na+ influx when applied externally. These toxins could be trapped inside the vesicles by addition to the medium prior to reconstitution (13). When present both inside and outside of the channel-containing vesicles, 100% of the batrachotoxin-stimulated influx was blocked. Thus, all batrachotoxin-activated channels could be blocked by saxitoxin or tetrodotoxin, although channel insertion into vesicles occurred with nearly random orientation. The ratio of batrachotoxin-stimulated influx to control influx was considerably higher with the rabbit channel than that previously reported with the sodium channel from rat sarclemma (14). In nine reconstituted preparations, the ratio of stimulated to control 22Na+ influx at 15 s averaged 8.8 ± 2.8.

Cation selectivity of the purified rabbit sodium channel was studied after channel activation with batrachotoxin or veratridine using either manual or quenched flow methods as previously described (13, 14). For batrachotoxin-activated channels, the selectivity sequence was Na+ > K+ > Rb+ > Cs+ (Fig. 3C). The values for the half-time of vesicle filling (Na+ = <50 ms, K+ = 390 ms, Rb+ = 2.6 s, Cs+ = 6.5 s) were comparable to those that we had previously determined in the
purified, reconstituted rat sarcolemmal sodium channel. After activation with veratridine, the half-times for the equilibration of these vesicles with the same isotopes were more than 2 orders of magnitude longer than with batrachotoxin activation. In this regard, the reconstituted rabbit channel again behaved comparably to the rat sarcolemmal channel (14).

In order to assess the relationship between the [H]STX binding peak and functional channel activity after reconstitution, the protein from each fraction of a final GS-550 sucrose gradient was reconstituted in parallel and the resultant vesicles were examined for their ability to generate specific batrachotoxin-activatable "Na⁺" fluxes (Fig. 4). A single peak of functional activity was seen which co-sedimented with the peak of [H]STX binding activity in the sucrose gradient. Over this region of the gradient, the magnitude of the activated flux, expressed either as the per cent total vesicle volume accessible to "Na⁺" through opened sodium channels or as specific "Na⁺" uptake, paralleled the total toxin binding activity in each fraction. No batrachotoxin-activated Na⁺ influx was seen in fractions that did not contain specific [H]STX binding.

Cross-reactivity with Monoclonal Antibodies against the Rat Channel—A series of monoclonal antibodies prepared against the rat sarcolemmal sodium channel was tested for cross-reactivity with the rabbit channel. Fractions from a GS-250 sucrose gradient were evaluated in a solid-phase radioimmunoassay for reactivity with these antibodies using 125I-rabbit anti-mouse F(ab')₂ to detect the bound antibody. The specificity of each of these monoclonals for the sodium channel in rat has been previously documented (24). The nonspecific immunoglobulin MOPC21 was used as a negative control.

Of the 25 anti-rat channel monoclonal antibodies tested, nine exhibited a peak of immunoreactivity that coincided with the peak of STX binding in the gradient (Fig. 5A). These antibodies showed no reactivity with other areas of the GS-250 gradient containing larger amounts of protein not associated with the sodium channel. The control immunoglobulin MOPC21 and the monoclonal antibodies not reacting with the sodium channel peak showed no reactivity with any fraction. The nine monoclonal antibodies that did cross-react with the channel were further evaluated using immunoblot
**Fig. 3.** Functional reconstitution of the T-tubular sodium channel. A, $^{86}$Rb$^+$ influx into vesicles containing the purified sodium channel. The purified channel was reconstituted into egg phosphatidylcholine vesicles and incubated for 45 min at 36 °C with $5 \times 10^{-6}$ M batrachotoxin (BTX) (O) in ethanol (0.5% final concentration in the vesicle suspension) or with ethanol alone (A). Specific batracho-
toxin-activated influx (●), defined as total minus control influx, occurred with a halftime of about 3 s in this example. Fluxes were measured after equilibration of the vesicles at 22 °C. B, dose-dependent activation of the purified, reconstituted channel by batrachotoxin. Vesicles containing the purified channel were incubated for 45 min at 36 °C with concentrations of batrachotoxin between $1 \times 10^{-5}$ and $1 \times 10^{-6}$ M prepared in ethanol or with an equivalent amount of ethanol alone. Influx of $^{22}$Na$^+$ was then measured after 15 s, a period sufficient for all vesicles containing opened channels to equilibrate with this cation. Inset, Hill plot of the same data indicating an apparent $K_d$ of 2 μM and a Hill coefficient of 1.2. C, relative rate of uptake of monovalent cations into vesicles containing the reconstituted channel activated by batrachotoxin. The total volume accessible to each cation through activated channels was comparable, and uptake is expressed as percent maximal uptake after correction for nonspecific influx. Measurements were made at 22 °C following activation of vesicles with batrachotoxin ($5 \times 10^{-6}$ M at 36 °C for 45 min). ●, $^{42}$K; O, $^{86}$Rb$^+$; □, $^{137}$Cs$^+$. $^{22}$Na$^+$ uptake was complete prior to 100 ms, the earliest time point resolvable.

**Fig. 4.** Analysis of the distribution of protein capable of functional channel activity after reconstitution in the fractions of a sucrose gradient prepared from a GS-550 fraction after centrifugation at 175,000 × g for 12 h. A, sucrose concentration (□), protein concentration (△), and specific [H]+STX binding (●) is shown for each fraction of the gradient. A single peak of STX binding is seen which corresponds to the major peak of protein present. B, fractions across the gradient were reconstituted into egg phosphatidylcholine and assayed for specific batrachotoxin (BTX)-stimulated tetrodotoxin-blocked $^{22}$Na$^+$ uptake. Only fractions 6-10 demonstrated specific uptake, and in these fractions the level of stimulatable influx expressed either as the absolute space accessible through batrachotoxin-activated channels (O) or the per cent of the total intravesicular volume accessible through batrachotoxin-activated channels (●) paralleled the level of [H]+STX binding in the gradient fractions.

Transfers of T-tubular membrane proteins (Fig. 5C). Six antibodies produced detectable binding to the transfers; in each case, a band at $M_r$ 260,000 was labeled that co-migrated with the large glycoprotein subunit of the purified channel.
positive antibodies are shown (F/H4, previously described (24). Bound antibody was detected using solid-phase radioimmunoassay; [3H]STX binding, without significant reactivity with other fractions from a GS-250 sucrose gradient. Although both are surface membranes in that they are exposed to the extracellular space and are physically in continuity, there is evidence that their protein and lipid composition is not the same (18, 19). We have shown in the rat that the relative contribution of sarcolemma and T-tubules in a membrane preparation depends on the physical methods used to disrupt the muscle (22) and that most rat membrane preparations contain a mixture of both elements. In rabbit muscle, however, methods have been developed and documented for selectively enriching membrane preparations in T-tubular elements (18, 23). The recent introduction of [3H] nitrendipine as a ligand for the dihydropyridine-binding component of the voltage-sensitive calcium channel, which is thought to be specifically located in T-tubules (38), now provides a convenient assay for this membrane element in a given preparation (34).

These T-tubular preparations from rabbit provide a rapid means of isolating large quantities of a defined membrane population that contains the voltage-sensitive sodium channel while avoiding the prolonged salt extraction steps that appear to contribute to channel proteolysis in the rat muscle preparations (11, 24). The voltage-dependent sodium channel can be solubilized and purified from these membranes with methods similar to those previously used for the rat (7, 11).

The apparent subunit composition of the purest preparations of rabbit sodium channel included only a large protein migrating at Mr ~260,000 and a single smaller component of Mr 38,000. The large subunit had identical electrophoretic mobility to the large subunit of the rat channel as now identified in muscle homogenates using monoclonal antibodies (24). Several of these antibodies against the rat sodium channel large subunit cross-react well with the rabbit large subunit on Western blot transfers, and the location of the immunoreactive bands from the two membrane sources are the same on these transfers.

We have recently shown that the subunit of the rat sarcolemmal channel that migrates between Mr 140,000 and 200,000 after purification (11) is the result of limited proteolysis of a larger Mr 260,000 subunit. This proteolysis occurs very early in the course of the preparation of sarcolemmal membranes (24). A similar component between Mr 140,000 and 200,000 was seen in the rabbit preparation when EGTA levels of 2 mM or lower were used during the initial stages of muscle homogenization and membrane isolation, suggesting that the rabbit sodium channel is also sensitive to a calcium-activated protease. In addition to the large subunit, both the rat and the rabbit channels contain a small subunit at Mr 38,000. The 45,000-dalton band variably present in the rat sarcolemmal channel was not seen in the rabbit preparation.

In the rabbit T-tubular preparation, proteolysis of the channel appears to be more easily controlled than in membrane preparations from rat skeletal muscle. This may be due in part to the elimination of the prolonged lithium bromide extraction that is an essential step in the rat membrane preparation. The tendency of T-tubular elements to vesiculate with their internal surfaces facing outward, exposed to the solutions containing the protease inhibitors, may also be a factor. Rat sarcolemmal membranes form right-side-out vesicles; these may trap activated proteases and allow them to act on the channel before they can be inactivated.

Subunit composition of the rabbit channel also closely resembles that reported for the rat brain, with the large and small subunits corresponding to the α and β subunits in that preparation (12). A counterpart in rabbit muscle for the β3

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DISCUSSION

The surface membrane of skeletal muscle can be divided into two components, the sarcolemma and the T-tubular system. Although both are surface membranes in that they are exposed to the extracellular space and are physically in continuity, there is evidence that their protein and lipid composition is not the same (18, 19). We have shown in the rat that the relative contribution of sarcolemma and T-tubules in a membrane preparation depends on the physical methods used to disrupt the muscle (22) and that most rat membrane preparations contain a mixture of both elements. In rabbit muscle, however, methods have been developed and documented for selectively enriching membrane preparations in T-tubular elements (18, 23). The recent introduction of \([3H]\) nitrendipine as a ligand for the dihydropyridine-binding component of the voltage-sensitive calcium channel, which is thought to be specifically located in T-tubules (38), now provides a convenient assay for this membrane element in a given preparation (34).

These T-tubular preparations from rabbit provide a rapid means of isolating large quantities of a defined membrane population that contains the voltage-sensitive sodium channel while avoiding the prolonged salt extraction steps that appear to contribute to channel proteolysis in the rat muscle preparations (11, 24). The voltage-dependent sodium channel can be solubilized and purified from these membranes with methods similar to those previously used for the rat (7, 11).

The apparent subunit composition of the purest preparations of rabbit sodium channel included only a large protein migrating at \(M_r \sim 260,000\) and a single smaller component of \(M_r 38,000\). The large subunit had identical electrophoretic mobility to the large subunit of the rat channel as now identified in muscle homogenates using monoclonal antibodies (24). Several of these antibodies against the rat sodium channel large subunit cross-react well with the rabbit large subunit on Western blot transfers, and the location of the immunoreactive bands from the two membrane sources are the same on these transfers.

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In the rabbit T-tubular preparation, proteolysis of the channel appears to be more easily controlled than in membrane preparations from rat skeletal muscle. This may be due in part to the elimination of the prolonged lithium bromide extraction that is an essential step in the rat membrane preparation. The tendency of T-tubular elements to vesiculate with their internal surfaces facing outward, exposed to the solutions containing the protease inhibitors, may also be a factor. Rat sarcolemmal membranes form right-side-out vesicles; these may trap activated proteases and allow them to act on the channel before they can be inactivated.

Subunit composition of the rabbit channel also closely resembles that reported for the rat brain, with the large and small subunits corresponding to the α and β subunits in that preparation (12). A counterpart in rabbit muscle for the β2

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subunit, however, was not found. The large subunits of all the mammalian sodium channels now seem similar to the single large subunit of the eel sodium channel (10). The nature of the smaller components in the mammalian preparations remains to be clarified; the possibility that they are the result of persistent proteolysis in spite of elaborate precautions remains real.

The purified rabbit channel, containing only the M, 260,000 and the 38,000 subunits, can be functionally reconstituted into defined lipid vesicles. The protein retains its capacity to gate cation fluxes in response to activation by batrachotoxin or veratridine. These fluxes are blocked specifically by tetrodotoxin or saxitoxin, and the batrachotoxin-activated channel exhibits high selectivity for sodium over other monovalent cations. In these respects, the channel behaves in a manner comparable in their size and migratory behavior to the three subunits reported by Curtis and Catterall (37) for the nitreneppeptide-binding component of the calcium channel protein.

The channel purification from T-tubular membranes may be a method to be clarified; the possibility that they are the result of persistent proteolysis in spite of elaborate precautions remains real.

The major contaminating protein seen during the sodium channel purification from T-tubular membranes may be a component of the voltage-activated calcium channel. This channel appears to be preferentially located in the T-tubules (38), is blocked by dihydropyridines (39), and can be identified by its ability to bind the radioligand [3H]nitrendipine (34). The peptide composition of the peak fractions in our preparation that contained this contaminant protein included a diffuse band at M, ~150,000 and two smaller sharp bands at M, 50,000 and 35,000 on SDS-PAGE. These bands are comparable in their size and migratory behavior to the three subunits reported by Curtis and Catterall (37) for the nitrendipine-binding component of the calcium channel protein isolated from a similar rabbit muscle preparation.

Our results indicate that the voltage-sensitive sodium channel isolated from rabbit muscle membranes enriched in T-tubular elements probably does not differ significantly in its physical characteristics, subunit composition, or functional properties after reconstitution from that isolated from rat skeletal muscle. Monoclonal antibodies against the rat sodium channel that identify the large glycoprotein subunit on immunoblots cross-react with the rabbit channel, again suggesting significant conservation of structure in this channel protein. Rabbit T-tubular membrane preparations provide an excellent source of sodium channels for further study of this channel in mammalian muscle.

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REFERENCES

1. Hodgkin, A., Huxley, A., and Katz, B. (1952) J. Physiol. (Lond.) 116, 424-448
2. Adrian, R. H., Chandler, W. K., and Hodgkin, A. L. (1970) J. Physiol. (Lond.) 208, 607-644
3. Armstrong, C. M. (1975) Annu. Rev. Biophys. 7, 179-210
4. Barchi, R. L. (1984) Trends Biochem. Sci. 9, 358-361
5. Agnew, W. S., Levinson, S. R., Brabson, J. S., and Raftery, M. A. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2060-2061
6. Norman, R. L., Schmid, A., Lomfort, A., Barhanin, J., and Lazdunski, M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4104-4108
7. Barchi, R. L., Cohen, S. A., and Murphy, L. E. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1306-1310
8. Hartshorne, R. P., and Catterall, W. A. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4620-4624
9. Lomfort, A., and Lazdunski, M. (1984) Eur. J. Biochem. 141, 651-660
10. Miller, J. A., Agnew, W. S., and Levinson, S. R. (1983) Biochemistry 22, 462-470
11. Barchi, R. L. (1983) J. Neurochem. 40, 1377-1385
12. Hartshorne, R. P., and Catterall, W. A. (1984) J. Biol. Chem. 259, 1667-1675
13. Weigle, J. B., and Barchi, R. L. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3651-3655
14. Tanaka, J. C., Eccleston, J. F., and Barchi, R. L. (1983) J. Biol. Chem. 258, 7519-7626
15. Tamkun, M. M., Talverheim, J. A., and Catterall, W. A. (1984) J. Biol. Chem. 259, 1676-1688
16. Rosenberg, R., Tomiko, S., and Agnew, W. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1239-1243
17. Franzini-Armstrong, C., and Peachey, L. D. (1981) J. Cell Biol. 91, 1665-1685
18. Rosenblatt, M., Hidalgo, C., Vergara, C., and Ikemoto, N. (1981) J. Biol. Chem. 256, 8140-8148
19. Lau, Y. H., Caswell, A. H., Brunschwig, J. P., Baerwald, R. J., and Garcia, M. (1979) J. Biol. Chem. 254, 549-546
20. Jaimovich, E., Ildefonse, M., Barhanin, J., Rougier, O., and Lazdunski, M. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3866-3900
21. Jaimovich, E., Chicheportiche, R., Lomfort, A., Lazdunski, M., Ildefonse, M., and Rougier, O. (1983) Pfluegers Arch. Eur. J. Physiol. 397, 1-5
22. Barchi, R. L., Weigle, J. B., Chalikian, D. M., and Murphy, L. E. (1979) Biochem. Biophys. Acta 550, 59-76
23. Moczydlowski, E. G., and Latore, R. (1983) Biochem. Biophys. Acta 732, 412-420
24. Casadei, J., Gordon, R., Lampson, L., Schotland, D., and Barchi, R. L. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6227-6231
25. Markwell, M., Hass, S., Tolbert, N., and Bieber, L. (1981) Methods Enzymol. 72, 296-303
26. Weigle, J. B., and Barchi, R. L. (1978) FEBS Lett. 91, 310-314
27. Laemmli, U. K. (1970) Nature 227, 680-685
28. Shing, Y., and Ruoho, A. (1983) Anal. Biochem. 110, 171-174
29. Martin, R. G., and Ames, B. N. (1961) J. Biol. Chem. 236, 1372-1379
30. Neer, E. J. (1974) J. Biol. Chem. 249, 6527-6531
31. Clarke, S. (1975) J. Biol. Chem. 250, 5459-5469
32. Barchi, R. L., and Murphy, L. E. (1981) J. Neurochem. 36, 2097-2100
33. Oku, N., Kendall, D. A., and MacDonal, R. C. (1982) Biochem. Biophys. Acta 691, 332-340
34. Fosset, M., Jaimovich, E., Delpont, E., and Lazdunski, M. (1983) J. Biol. Chem. 258, 6086-6092
35. Barchi, R. L., and Weigle, J. B. (1979) J. Physiol. (Lond.) 295, 383-396
36. Hartshorne, R. P., Coppersmith, J., and Catterall, W. A. (1980) J. Biol. Chem. 255, 10572-10575
37. Curtis, B. M., and Catterall, W. A. (1984) Biochemistry 23, 2113-2118
38. Almers, W., Fink, R., and Palade, P. T. (1981) J. Physiol. (Lond.) 312, 177-207
39. Chiaramida, D. J., and Stefani, E. (1983) J. Physiol. (Lond.) 335, 29-40
T-tubular Membrane Sodium Channels

Supplementary Material to the Purification and Functional Reconstitution of the Voltage-sensitive Sodium Channel from Rabbit T-tubular Membrane

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METHODS

Isolation of T-tubular membranes

Membranes enriched in T-tubular elements were isolated from the fast skeletal muscle of male New Zealand rabbits using the method described by Nomura and Later (28) except for the final centrifugation. In order to isolate protoplasmic membranes from other membrane types, the T-tubular membranes were isolated by differential centrifugation after disruption of the sarcoplasm. In detail, the protocol was performed as follows: the isolated T-tubular membranes were incubated with a 1 M Tris buffer; all subsequent steps were carried out in the presence of the same inhibitor; in addition to EGTA, 50 mM Tris buffer (pH 6.5-7.5) was added to each of the solutions used in the procedures. The T-tubular membranes were then collected at the interface between the washout buffer and stored at -20°C until use.

Purification of the sodium channel protein

T-tubular membranes were solubilized in 5 mg/ml buffer containing 20 mM sodium chloride, 0.5 mM phenylmethylsulfone (pH 7.4), 0.5 mM EGTA, 1 mM EDTA, 20% NP-40, 0.02 mg/ml protease inhibitor (buffer A) containing 1 mg/ml Tris buffer (pH 6.5-7.5) containing 1 mM EGTA, 0.1 mM protamine S-30, 0.1 mg/ml protease inhibitor. The homogenate was homogenized at 4°C in a Potter-Elvehjem homogenizer and centrifuged at 105,000 g for 60 min in a SS-34 rotor. Membranes were collected at the interface between the washout buffer and stored at -20°C until use.

Table 1: Physical properties of mammalian skeletal muscle sodium channels

| Stroke | S | E | Apparent N | Ry | N_s | N_t |
|-------|---|---|-----------|---|-----|-----|
| Rate 1 | 9.4 | 6.6 | 0.89 | 38000 |
| Rate 2 | 8.6 | 6.6 | 0.89 | 29100 |

Conditions for the optimal solubilization of T-tubular membranes and stabilization of the sodium channel protein were evaluated using approaches similar to those described previously. Solubilization was optimized with 150 mM sodium chloride and 0.5 mM phenylmethylsulfone in the gradient buffer. The T-tubular membranes were then incubated for 2 h at room temperature in the gradient buffer. The solubilization buffer was 50 mM sodium chloride, 0.5 mM EGTA, 1 mM EDTA, 20% NP-40, 0.02 mg/ml protease inhibitor (buffer A) containing 1 mg/ml Tris buffer (pH 6.5-7.5) containing 1 mM EGTA, 0.1 mM protamine S-30, 0.1 mg/ml protease inhibitor. The homogenate was homogenized at 4°C in a Potter-Elvehjem homogenizer and centrifuged at 105,000 g for 60 min in a SS-34 rotor. Membranes were collected at the interface between the washout buffer and stored at -20°C until use.

Electrophysiological properties of the purified channel. The amperometric response of the solubilized rabbit sodium channel was determined by chronomemography on a Hyperion C430 as described in a series of standard solutions. The channel was exposed to 50 mM sodium chloride, 0.5 mM EDTA, 20% NP-40, 0.02 mg/ml protease inhibitor (buffer A) containing 1 mg/ml Tris buffer (pH 6.5-7.5) containing 1 mM EGTA, 0.1 mM protamine S-30, 0.1 mg/ml protease inhibitor. The homogenate was homogenized at 4°C in a Potter-Elvehjem homogenizer and centrifuged at 105,000 g for 60 min. Membranes were collected at the interface between the washout buffer and stored at -20°C until use.

RESULTS

Six different ion channel preparations prepared from rabbit muscle are specified in Table 1. These ion channel preparations were optimized in several laboratories using similar approaches. In each experiment, the sodium channel was solubilized with detergent in a solution containing 150 mM sodium chloride, 0.5 mM phenylmethylsulfone, 20% NP-40, and detergent. The sodium channel was then incubated for 2 h at room temperature in the gradient buffer. The solubilization buffer was 50 mM sodium chloride, 0.5 mM EGTA, 1 mM EDTA, 20% NP-40, 0.02 mg/ml protease inhibitor (buffer A) containing 1 mg/ml Tris buffer (pH 6.5-7.5) containing 1 mM EGTA, 0.1 mM protamine S-30, 0.1 mg/ml protease inhibitor. The homogenate was homogenized at 4°C in a Potter-Elvehjem homogenizer and centrifuged at 105,000 g for 60 min. Membranes were collected at the interface between the washout buffer and stored at -20°C until use.

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Sodium channel preparations prepared from rabbit muscle are specified in Table 1. These ion channel preparations were optimized in several laboratories using similar approaches. In each experiment, the sodium channel was solubilized with detergent in a solution containing 150 mM sodium chloride, 0.5 mM phenylmethylsulfone, 20% NP-40, and detergent. The sodium channel was then incubated for 2 h at room temperature in the gradient buffer. The solubilization buffer was 50 mM sodium chloride, 0.5 mM EGTA, 1 mM EDTA, 20% NP-40, 0.02 mg/ml protease inhibitor (buffer A) containing 1 mg/ml Tris buffer (pH 6.5-7.5) containing 1 mM EGTA, 0.1 mM protamine S-30, 0.1 mg/ml protease inhibitor. The homogenate was homogenized at 4°C in a Potter-Elvehjem homogenizer and centrifuged at 105,000 g for 60 min. Membranes were collected at the interface between the washout buffer and stored at -20°C until use.