Communication

Reconstitution of Neurotoxin-stimulated Sodium Transport by the Voltage-sensitive Sodium Channel Purified from Rat Brain*

(Received for publication, June 2, 1982)

Jane A. Talvenheimo, Michael M. Tamkun, and William A. Catterall†

From the Department of Pharmacology, University of Washington, Seattle, Washington, 98195

Incorporation of the saxitoxin receptor of the sodium channel solubilized with Triton X-100 and purified 250-fold from rat brain into phosphatidylcholine vesicles is described. Fifty to 80% of the saxitoxin receptor sites are recovered in the reconstituted vesicles ($K_d = 3 \text{ nM}$). Unlike the detergent-solubilized saxitoxin receptor, the reconstituted saxitoxin binding activity is stable to incubation at 36 °C. Approximately 78% of the reconstituted saxitoxin receptor sites are externally oriented and 25% are inside-out. The initial rate of $^{22}\text{Na}^+$ uptake into reconstituted vesicles is increased up to 3- to 4-fold by veratridine with a $K_d$ of 11 μM. Seventy percent of this increase is blocked by external tetrodotoxin (TTX) with a $K_d$ of 10 nM. All of the veratridine-stimulated $^{22}\text{Na}^+$ uptake is blocked when TTX is present on both sides of the vesicle membrane, or when tetracaine is added to the external medium. The apparent binding constants for veratridine, saxitoxin, and TTX are essentially identical to those in intact rat brain synaptosomes. The results demonstrate reconstitution of sodium transport, as well as neurotoxin binding and action, from substantially purified sodium channel preparations.

The voltage-sensitive sodium channel has been studied extensively using neurotoxins. Sodium channels in peripheral nerve, neuroblastoma cells, and synaptosomes possess three distinct receptor sites for neurotoxin action (for review see Ref. 1). Neurotoxin receptor site 1 binds TTX and saxitoxin, which block ion flux through the channel. The alkaloid toxins, veratridine, aconitine, and batrachotoxin, bind to neurotoxin receptor site 2 and cause persistent activation of the sodium channel. Neurotoxin receptor site 3 binds the polypeptides scorpion toxin and sea anemone toxin, which inhibit channel inactivation and enhance activation by alkaloid toxins.

Considerable progress has been made in identifying and isolating the sodium channel components from the eel electric organ (2), and from mammalian brain and muscle (3-5) using neurotoxins as specific probes. The major component of the sodium channel in eel electrophysiology (6) and rat brain (3, 4) is a large polypeptide of $M_r = 250,000$ to 270,000, designated the α subunit. In rat brain synaptosomes, two additional polypeptides, $\beta_1$ with $M_r = 39,000$ and $\beta_2$ with $M_r = 37,000$, are associated with the sodium channel (3). In order to determine whether the polypeptide components of the purified saxitoxin receptor represent the entire sodium channel, sodium channel function must be reconstituted from purified components. In previous studies, veratridine-stimulated $^{22}\text{Na}^+$ transport has been recovered by incorporating lobster nerve membrane fragments (7) or sodium cholate extracts of brain membrane (8) into liposomes. Goldin et al. (9) used a density-shift technique to demonstrate veratridine-dependent Cs$^+$ transport by cholate-solubilized brain sodium channels reconstituted into phosphatidylcholine vesicles. Neurotoxin binding and action at all three neurotoxin receptor sites, and sodium transport activity, were quantitatively recovered when cholate-solubilized but unpurified sodium channels from rat brain were reconstituted into phosphatidylcholine vesicles (10). In this communication, we report evidence for the successful reconstitution of neurotoxin-sensitive $^{22}\text{Na}^+$ flux from sodium channel components purified 250-fold from rat brain. While this work was in progress, an abstract describing similar experiments on sodium channels purified from rat skeletal muscle appeared (11).

Experimental Procedures

Materials—The phosphatidylcholine used for reconstitution was purified from egg yolks by the method of Goldin (12). Saxitoxin, obtained from the National Institutes of Health, was titrated to a specific activity of 7.96 Ci/mmol by the procedure of Ritchie et al. (13), and was purified as described previously (14). Tetracaine-HCl was donated by Dr. Bertil Takman of Astra Pharmaceuticals. Bio-Beads SM2 (Bio-Rad) were washed according to Holloway (15) and Triton X-100 (Sigma) was de-ionized prior to use. All other reagents were obtained from commercial sources.

Purification of the Saxitoxin Receptor—The saxitoxin receptor was solubilized and purified 250-fold from rat brain by a modification of the procedure described by Hartshorne and Catterall (3). The purification was carried through two steps, ion exchange chromatography and WGA-Sepharose chromatography. Adsorbed saxitoxin receptor was eluted from the WGA-Sepharose column with 150 mM N-acetylglucosamine in 67.5 mM Na$_2$SO$_4$, 0.5 mM MgSO$_4$, 25 mM Hepes, pH 7.4. The ten saxitoxin receptor preparations used in this study had an average specific activity of 400 pmol/mg prior to reconstitution and were approximately 15% pure by sodium dodecyl sulfate gel electrophoresis (3).

Reconstitution of Purified Saxitoxin Receptor into Phosphatidylcholine Vesicles—Ten percent (w/v) Triton X-100 in Na$_2$SO$_4$ medium (67.5 mM Na$_2$SO$_4$, 0.5 mM MgSO$_4$, 150 mM sucrose, 25 mM Hepes-Tris, pH 7.4), containing 4% (w/v) phosphatidylcholine and a trace amount of $[^{14}C]$phosphatidylcholine, was added to the purified saxitoxin receptor, bringing the final concentrations of Triton X-100 and phosphatidylcholine to 1.75% (w/v) and 0.68% (w/v), respectively. A volume of Bio-Beads SM2 equal to one-fifth of the sample volume was added, and the sample was rotated overnight at 4 °C. Then the Bio-Beads were replaced with an identical volume of fresh Bio-Beads, the sample was rotated an additional 2 h at 4 °C, and the Bio-Beads were removed by filtration. For some experiments, vesicles were preloaded with $^{22}\text{Na}$Cl (10 μCi/ml) or TTX (1 μM) by adding the

2 R. P. Hartshorne, and W. A. Catterall, unpublished results.

11868
2NaCl or TTX to the soluble receptor sample before adding Bio-Beads.

Measurement of $[^{3}H]$Saxitoxin Binding—$[^{3}H]$Saxitoxin binding to the soluble saxitoxin receptor was measured using a rapid gel filtration assay, as described previously (14). $[^{3}H]$Saxitoxin binding to the reconstituted channel was measured by the filtration assay described by Tamkun and Catterall (19).

Measurement of Veratridine-stimulated $^{22}Na^+$ Uptake—$^{22}Na^+$ uptake by vesicles containing the reconstituted saxitoxin receptor was measured by the method of Epstein and Racker (16). To initiate uptake, a 20-μl aliquot of the vesicle suspension in N$_2$SO$_4$ medium was diluted 10-fold with 180 μl of Tris-SO$_4$ medium (105 mM Tris-SO$_4$, pH 7.4, 0.5 mM MgSO$_4$, 150 mM sucrose, 25 mM Heps-Tris, pH 7.4) containing $^{22}NaCl$ (10 μCi/ml). The diluted sample was incubated at 36°C for the desired length of time, then applied to a Dowex AG50W-X8 (Tris form) column (10 × 0.5 cm) in a Pasteur pipette, and immediately eluted with 1 ml of 0.38 M sucrose containing 1 mg/ml of BSA. Recovery of the vesicles in the column eluate, determined by measuring the recovery of vesicles containing $[^{3}H]$phosphatidylcholine, averaged 100%. $^{22}Na^+$ uptake is terminated within 3 s as the cations are adsorbed to the resin.

Protein Measurement—Protein was measured using the method described by Peterson (17) with BSA as a standard.

RESULTS AND DISCUSSION

Binding of Saxitoxin to the Purified Saxitoxin Receptor—The purified and reconstituted saxitoxin receptor binds $[^{3}H]$ saxitoxin with high affinity (Fig. 1A). When plotted according to Scatchard (18), the data describe a single high affinity site (Fig. 1B) with a $K_D$ for $[^{3}H]$saxitoxin binding of 3 nM, similar to the $K_D$ for $[^{3}H]$saxitoxin binding to intact rat brain synaptosomes (19). TTX displaces $[^{3}H]$saxitoxin from the reconstituted receptor with an inhibition constant ($K_I$) of 10 nM (not shown), which agrees well with the $K_T$ for TTX inhibition of $[^{3}H]$saxitoxin binding to synaptosomes (14). Generally, 40-65% of the purified soluble saxitoxin receptor was reconstituted following reconstitution into vesicles, yielding an average of 230 pmol of saxitoxin receptor/mg of vesicle protein. Vesicles prepared with purified saxitoxin receptor denatured by incubation at 36°C did not bind $[^{3}H]$saxitoxin.

When the saxitoxin receptor is solubilized, saxitoxin binding activity becomes labile to incubation at 36°C (Refs. 10, 14, and 20 and Fig. 2A, □). Following incorporation into phosphatidylycholine vesicles, however, the purified saxitoxin receptor quantitatively regains the heat-stability characteristic of sodium channels in intact synaptosomes (Fig. 2A, △, Ref. 8). The complete recovery of thermal stability following reconstitution suggests that all of the saxitoxin receptors are incorporated into the lipid bilayer in a configuration similar to native membranes.

In native membranes, the saxitoxin binding site on the sodium channel is located on the outer membrane surface (21). Since saxitoxin does not penetrate lipid bilayers, the $[^{3}H]$ saxitoxin binding to vesicles containing purified saxitoxin receptor represents binding only to the externally oriented saxitoxin binding sites. In order to determine whether all of the reconstituted channels are oriented with the saxitoxin binding site at the outer vesicle surface, vesicles were resolubilized by titration with Triton X-100. The results of this experiment are shown in Fig. 2B. As the Triton X-100 concentration increases from 0 to 0.16% (w/v), the number of $[^{3}H]$saxitoxin binding sites increases; at higher detergent concentrations, solubilized saxitoxin binding activity is denatured. Data from three separate experiments showed that 25% of the total reconstituted saxitoxin binding sites are exposed only after detergent treatment, suggesting that 75% of the reconstituted channels are oriented normally and 25% are inside-out in these vesicles. Thus, the total saxitoxin receptor (internal plus external) recovered in reconstituted vesicles is 53 to 80% of that in the original purified soluble preparation.

Sodium Transport Mediated by the Purified and Reconstituted Sodium Channel—The time course for veratridine-stimulated $^{22}Na^+$ uptake by vesicles containing the purified saxitoxin receptor is shown in Fig. 3A. Vesicles incubated with Na$_2$SO$_4$ medium, veratridine, or veratridine and TTX were diluted 10-fold in Tris-SO$_4$ medium containing 22NaCl at 36°C to give a final external Na$^+$ concentration of 13.6 mM. Under these conditions, 100 μM veratridine stimulates $^{22}Na^+$ accumulation 3- to 4-fold over the control. $^{22}Na^+$ uptake increases linearly up to 20 s, then slows with increasing incubation times. TTX, at a concentration of 1 μM, specifically blocked 60 to 70% of the veratridine-stimulated $^{22}Na^+$ influx, indicating that the veratridine-stimulated $^{22}Na^+$ uptake is a result of activation of the reconstituted channels by the alkaloid toxin. Samples incubated with 1 μM TTX alone gave the same rate of uptake as the control samples (data not shown). Vesicles

![Fig. 1. Binding of $[^{3}H]$saxitoxin (STX) to the reconstituted sodium channel. A, vesicles containing the reconstituted channel were incubated with the indicated concentrations of $[^{3}H]$saxitoxin for 20 min at 36°C. The amount of $[^{3}H]$saxitoxin bound was measured by filtration, as described previously (19). Binding was measured in the absence (□) or presence (○) of 1 μM TTX. B, specific binding, defined as the difference between total $[^{3}H]$saxitoxin binding and binding measured in the presence of 1 μM TTX, is presented as a Scatchard plot.](image-url)
on both sides of the vesicle membrane, and 0.1 mM tetracaine, added externally, on veratridine-stimulated $^{22}$Na$^+$ influx. For this experiment, purified saxitoxin receptor was incorporated into two identical samples of phosphatidylethanolamine vesicles, one in Na$_2$SO$_4$ medium and one in Na$_2$SO$_4$ medium containing 1 $\mu$M TTX. In the control vesicles, 70% of the veratridine-stimulated $^{22}$Na$^+$ uptake, measured at 20 s, was blocked by external TTX (Table I). When the vesicles containing 1 $\mu$M internal TTX were incubated with 1 $\mu$M external TTX prior to the addition of veratridine, veratridine stimulation of $^{22}$Na$^+$ influx was completely blocked. Similarly, the local anesthetic tetracaine, which is membrane-permeant and blocks ion transport through sodium channels (22), blocked veratridine-stimulated $^{22}$Na$^+$ uptake by the control vesicles completely when added only to the external medium. Together, these results support the conclusion that all of the veratridine-stimulated $^{22}$Na$^+$ uptake is mediated by active sodium channels.

The fraction of the internal volume of the vesicles that is accessible to active sodium channels was determined by measuring veratridine-enhanced $^{22}$Na$^+$ efflux (Fig. 3B). For this experiment, vesicles preloaded with NaCl were diluted 10-fold into Na$_2$SO$_4$ medium. Without veratridine, the rate of exchange of internal $^{22}$Na$^+$ with external Na$^+$ is slow; only 22% of the internal $^{22}$Na$^+$ is lost after 1 min. This result suggests that these vesicles are relatively impermeable to Na$^+$ and that the reconstituted sodium channels assume an inactive conformation. However, when the vesicles are incubated with 100 $\mu$M veratridine, an additional 90% of the trapped $^{22}$Na$^+$ is lost. Veratridine-enhanced $^{22}$Na$^+$ efflux is complete within 10 s, the earliest time point that could be reliably measured. Addition of 0.15% (w/v) Triton X-100 to the vesicles released all of the $^{22}$Na$^+$ (Fig. 3B), confirming that the $^{22}$Na$^+$ measured initially represents internal, readily releasable $^{22}$Na$^+$. This data indicates that about 30% of the apparent internal vesicle volume is accessible to veratridine-activated sodium channels.

**Table 1**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Veratridine-stimulated $^{22}$Na$^+$ uptake</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>340 ± 78 (n = 8)</td>
<td>0</td>
</tr>
<tr>
<td>External TTX (1 $\mu$M)</td>
<td>100 ± 51 (n = 8)</td>
<td>71</td>
</tr>
<tr>
<td>External TTX (1 $\mu$M) + internal TTX (1 $\mu$M)</td>
<td>0 (n = 8)</td>
<td>100</td>
</tr>
<tr>
<td>Tetracaine (0.1 mM)</td>
<td>0 (n = 8)</td>
<td>100</td>
</tr>
</tbody>
</table>

prepared with purified saxitoxin receptor denatured by incubation at 36 °C did not exhibit veratridine-enhanced $^{22}$Na$^+$ transport. Since veratridine is lipid-soluble, the toxin would be expected to activate inside-out channels as well as channels oriented in the normal direction. However, external TTX will only block Na$^+$ flux through channels oriented with the saxitoxin binding site facing the vesicle exterior. Thus, the fact that TTX blocks approximately 70% of the veratridine-stimulated $^{22}$Na$^+$ uptake is entirely consistent with our data from the same vesicle preparations showing that 75% of the reconstituted saxitoxin binding sites are available on the outer vesicle surface.

The data in Table I show the effects of 1 $\mu$M TTX, present...
The effect of increasing concentrations of veratridine on $^{22}\text{Na}^+$ influx through the reconstituted channel is shown in Fig. 4A. Veratridine enhances $^{22}\text{Na}^+$ uptake with a $K_D$ of 11 $\mu$M, which agrees well with the $K_D$ of 13 $\mu$M measured for veratridine stimulation of $^{22}\text{Na}^+$ uptake in rat brain synaptosomes (19). Moreover, TTX inhibits veratridine-enhanced $^{22}\text{Na}^+$ flux through the reconstituted channel with a $K_i$ of 10 nM (Fig. 4B), which agrees closely with the $K_i$ for TTX inhibition of $[^3\text{H}]$saxitoxin binding to synaptosomes (14) and to vesicles containing the reconstituted channel. These results show that neurotoxin binding and action at neurotoxin receptor sites 1 and 2 are quantitatively restored in these reconstituted sodium channels.

While the reconstituted sodium channel regains the sensitivity to veratridine and TTX that is characteristic of the native channel, we were not able to detect specific $^{125}\text{I}$-labeled scorpion toxin binding to vesicles containing the reconstituted saxitoxin receptor, nor could we detect any allosteric enhancement of veratridine action by scorpion toxin using the $^{22}\text{Na}^+$ flux assay. Thus, the procedures described do not lead to recovery of toxin binding and action at neurotoxin receptor site 3. Since $^{125}\text{I}$-scorpion toxin binding can be recovered in good yield from unpurified, Triton X-100 solubilized sodium channel preparations by these reconstitution procedures, it seems most likely that sodium channel components that are required for this activity are denatured or separated from the saxitoxin receptor during purification.

When sodium channels are solubilized with detergents, four functional changes in biochemical properties occur: TTX and saxitoxin binding at neurotoxin receptor site 1 becomes labile to incubation at 36 $^\circ$C (10, 14), neurotoxin binding and action at neurotoxin receptor site 2 is lost,4 scorpion toxin binding at neurotoxin receptor site 3 is lost (10, 14), and dissolution of the excitable membrane prevents measurement of ion flux. Our results show that the heat stability of saxitoxin binding, the binding and action of veratridine at neurotoxin receptor site 2 and the TTX- and tetracaine-sensitive sodium transport characteristic of the native sodium channel can all be recovered by incorporation of sodium channels purified 250-fold from rat brain into phosphatidylcholine vesicles. These results are consistent with the hypothesis that the purified saxitoxin receptor from rat brain consisting of the $\alpha$, $\beta_1$, and $\beta_2$ subunits (3) retains neurotoxin receptor site 2 and the ion conducting pore of the sodium channel in a form which is active after incorporation into a lipid bilayer. Additional experiments with homogeneous preparations of the saxitoxin receptor will be required to confirm this hypothesis.

Acknowledgments—We thank Cynthia Morrow for providing $[^3\text{H}]$saxitoxin. We are particularly grateful to Dr. M. Schramm, Hebrew University, for encouraging us to use Bio-Beads to remove Triton X-100.

REFERENCES