Sodium Channel, Sodium Pump, and Sodium-Calcium Exchange Activities in Synaptosomal Plasma Membrane Vesicles*  

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Two mechanisms of Na⁺ influx have been observed using synaptosomal plasma membrane vesicles purified by density gradient centrifugation from a synaptosomal hypotonic lysate. First, a 5-fold increase in uptake over basal Na⁺ entry occurs with 0.2 mM veratridine. The veratridine-dependent Na⁺ uptake is partially inhibited by 2 μM tetrodotoxin with an apparent time dependency of action (half-maximal inhibition in approximately 20 min). Second, a larger Na⁺ accumulation (approximately 15-fold over basal) was observed with 2.5 mM ATP, this effect being dependent on internal K⁺ loading of vesicles although inhibited by high external K⁺. The two uptake processes are believed to represent operation of the plasma membrane voltage-sensitive Na⁺ channel, and the Na⁺-pumping (Na⁺ + K⁺)-ATPase, respectively. Both Na⁺ flux mechanisms appear to operate in a single population of vesicles since opening of the Na⁺ channel with veratridine diminishes the ATP-dependent accumulation of Na⁺ by over 75%. An inverted orientation of the plasma membrane vesicles is likely to account for the functioning of the ATP-dependent Na⁺ pump and may also account for the low sensitivity and time dependency of the inhibitory action of tetrodotoxin on Na⁺ channel opening. Na⁺ accumulated by the Na⁺ pump was rapidly effluxed by 10 mM external Ca²⁺ via the Na⁺-Ca²⁺ exchange mechanism which (together with an ATP-dependent Ca²⁺-accumulating mechanism) was recently characterized in the vesicles (Gill, D. L., Grollman, E. F., and Kohn, L. D. (1981) J. Biol. Chem. 256, 184-192). This result, together with the observed inhibition of Ca²⁺ influx via (Na⁺-Ca²⁺)-exchange due to veratridine-mediated Na⁺ flux, strongly suggests that the Na⁺ pump, Na⁺ channel, and both Ca²⁺ transport mechanisms function in a single population of inverted plasma membrane vesicles.

The synaptic plasma membrane contains several key ionic flux mechanisms which ultimately modulate the level of intracellular Ca²⁺ essential to the coupling of depolarization with neurotransmitter release (see Refs. 1 and 2). The influx and efflux of Ca²⁺ ions across the membrane is intimately associated with changes in the flux of Na⁺ ions (2-4). Thus, both the Na⁺ gradient and the resting membrane potential arising from the function of the Na⁺ pump are driving forces for the extrusion of Ca²⁺ via the electrogenic Na⁺-Ca²⁺ antiporter (3-5). The depolarization arising from Na⁺ conductance via Na⁺ channels facilitates the influx of Ca²⁺ through voltage-sensitive Ca²⁺ channels (2, 3, 5). Also, internal Na⁺ may considerably modify the efflux of Ca²⁺ ions via an ATP-dependent Ca²⁺ pump mechanism (6). Therefore, analysis of the Na⁺ flux mechanisms of the synaptic plasma membrane has important consequences to determining the control of Ca²⁺-mediated excitation-release coupling in the nerve terminal.

Using a preparation of synaptosomal membrane vesicles, the Ca²⁺ transport mechanisms believed to originate from the plasma membrane were recently characterized in detail (6). By "flux-reversal" procedures, the two major Ca²⁺ fluxes (Na⁺-Ca²⁺-exchange and ATP-dependent Ca²⁺ transport) were demonstrated to coexist within a single population of vesicles within this preparation. Since Na⁺-Ca²⁺ exchange is known to operate across the squid axonlemma (7) and outer synaptosomal membrane (8, 9), both vesicle activities were suggested to originate from the synaptic plasma membrane (4, 6).

The present report identifies some parameters of Na⁺ transport across the same vesicle membranes which are characteristic of those fluxes associated with the plasma membrane. Thus the vesicles contain a veratridine-sensitive Na⁺ channel, and an (ATP + K⁺)-dependent Na⁺ transport mechanism. The function of both Na⁺ transport systems suggests they are operating in a single population of inverted plasma membrane vesicles. Furthermore, Ca²⁺-dependent flux-reversal of Na⁺ accumulated by the Na⁺ pump indicates that this mechanism operates in the same population of vesicles as the Na⁺-Ca²⁺ exchanger, supporting the contention that both the previously characterized Ca²⁺ transport mechanisms also operate in the plasma membrane.

EXPERIMENTAL PROCEDURES

Preparation of Synaptosomal Plasma Membrane Vesicles—Vesicles were isolated from guinea pig cerebral cortex as described previously (6). This method involved isolation of synaptosomes followed by hypotonic lysis based on the procedure of Kanner (10). Experiments on Na⁺ flux described in this report used a fraction further purified by sucrose-density gradient centrifugation and referred to previously as "light membrane vesicles" (6). This fraction corresponds closely to the synaptic plasma membrane fraction characterized in detail by Cotman (11), and is subsequently referred to in this report as synaptosomal plasma membrane vesicles or vesicles.

The vesicles were finally resuspended in 0.32 M sucrose, 1 mM K⁺, 5 mM Na⁺, 1 mM MgCl₂, 20 mM HEPES, pH 7.4, and stored as intact or 1 mg/ml under experimental conditions.

Na⁺ Flux Experiments—For Na⁺ influx studies, 100 μl aliquots of synaptosomal plasma membrane vesicles (0.6 mg of protein) were thawed at room temperature and diluted with 1.3 ml of pre-equilibration medium (150 mM KCl, 1 mM MgCl₂, 5 mM MOPS (pH 7.4) for 10 min at 37 °C. The vesicles were then centrifuged (15,000 x g, 5 min) and the pellet resuspended with 300 μl of pre-equilibration medium. Na⁺ uptake commenced upon addition of 5 μl of resuspended...
vesicles (approximately 15 µg of protein) to 100 µl of external medium containing 140 mM choline chloride, 5 mM MgCl₂, 0.2 mM EGTA, 5 mM MOPS-Tris, pH 7.4. Na⁺ uptake commenced on addition of 5 µl of pre-equilibrated vesicles to 100 µl of external medium containing 140 mM choline chloride, 5 mM MgCl₂, 0.2 mM EGTA, 5 mM MOPS-Tris, pH 7.4, 1 mM NaCl with 0.01 Ci/mmol of ²²Na either without other additions (○), with 50 µM veratridine (●), or with 50 µM veratridine and 2 µM tetrodotoxin (▲). Uptake proceeded for the time shown at 25 °C and was terminated by addition of 2.0 ml of ice-cold 150 mM choline chloride followed by immediate vacuum filtration through cellulose acetate membrane filters (0.5-µm pore size). After one repeated wash, filters were dissolved in scintillant and counted. Results are expressed as the amount of Na⁺ accumulated by vesicles with nonspecific Na⁺ adsorption to filters (approximately 0.2% of total Na⁺) subtracted.

Miscellaneous Procedures and Materials—Protein measurements on vesicles were performed by the method of Lowry et al. (12) against a standard of crystalline bovine serum albumin after 1-h treatment with 0.3 m NaOH. The internal volume of the vesicles was measured using the [methoxy-¹⁴C]inulin/H₂O method previously described (6). ATP was neutralized to pH 7.4 with Tris before addition.

²²NaCl was from New England Nuclear; ATP (Tris salt, vanadate-free), choline chloride, and veratridine were from Sigma; tetrodotoxin was generously provided by Dr. J. W. Daly, National Institutes of Health, Bethesda, MD; filters (type EHWP) used for the separation of vesicles were from Millipore Corp.

**RESULTS AND DISCUSSION**

In addition to the Ca²⁺ transport mechanisms characterized in detail in this preparation of synaptosomal membrane vesicles (6), two distinct mechanisms of Na⁺ transport also occur. Both Na⁺ flux mechanisms are characteristic of the plasma membrane and parameters of their function suggest their operation in inverted vesicles.

The first such mechanism is a veratridine-sensitive accumulation of Na⁺. Uptake of Na⁺ using tracer ²²Na in the medium was increased approximately 5-fold over basal uptake when 50 µM veratridine was present (Fig. 1). This effect indicates operation of the voltage-sensitive Na⁺ channel of excitatory plasma membranes, the opening of which is enhanced by veratridine (13). This uptake was inhibited at least partially by tetrodotoxin, the specific blocker of Na⁺ channel function in neural membranes (14). However, it was noted that the veratridine-dependent Na⁺ accumulation was considerably less sensitive to tetrodotoxin than has generally been reported for neural tissues where concentrations in the nanomolar range are normally effective (see Ref. 15). Two explanations were possible for the noncomplete action of tetrodotoxin. First, the veratridine-sensitive Na⁺-accumulating vesicles may be heterogeneous with regard to sidedness resulting in only a fractional sensitivity to tetrodotoxin. Alternatively, the likely inverted nature of the vesicles previously (6) and subsequently alluded to, may alone account for their relative insensitivity to tetrodotoxin. It is known that the low hydrophobicity of the toxin molecule precludes an effect on its external site of action when administered, for example, internally to dialyzed squid axons (16). The internally oriented active sites of inverted vesicles might be similarly inaccessible to tetrodotoxin. However, as seen in Fig. 1, although tetrodotoxin at 2 µM inhibited the veratridine effect by approximately 50% after 30 min, it was noted that after only 5 min little inhibition had occurred. This increased effectiveness of tetrodotoxin with time could represent a slow or partial entry of the toxin molecule into inverted vesicles. Such an interpretation of the effect of tetrodotoxin is strengthened by examining the effects of preincubating vesicles with tetrodotoxin prior to their addition to the uptake medium containing ²²Na and veratridine (Table I). In this experiment, 2 µM tetrodotoxin inhibited veratridine-dependent Na⁺ accumulation by no more than 25% when present only during the 10 min uptake in the presence of 50 µM veratridine. However, when added to vesicles during a 20- or 40-min preincubation, tetrodotoxin caused a proportionately greater decrease in veratridine-dependent Na⁺ uptake. After 40 min of preincubation, tetrodotoxin caused almost a 60% reduction of the subsequent veratridine-mediated accumulation of Na⁺. These results suggest an abnormally slow time dependence of the effect of tetrodotoxin which may be associated with its slow access to an internal site of action. The data do not, however, totally exclude the possible existence of noninverted vesicles. Other reports using brain membranes (17) or unpurified vesicles (18) have indicated a more rapid tetrodotoxin reversal of Na⁺ channel opening, the difference probably arising from the relative fractions of right-side out vesicles in the various preparations. The relatively rapid action of veratridine in the present experiments may imply an action on the external surface (originally internal) of inverted vesicles, although the lipid solubility of the molecule (19) would facilitate its passage into vesicles even if this were not the case. Another implication for the effect of veratridine on inverted vesicles would be the bidirectionality of Na⁺ conductance through the Na⁺ channel.

In addition to the function of this apparent Na⁺ channel, vesicles also accumulate Na⁺ in the presence of ATP, an activity which more convincingly characterizes their plasma membrane origin and inverted orientation. In this case, using 2.5 mM ATP, the uptake of Na⁺ above basal equilibration was approximately 3-fold greater than in the presence of 50 µM veratridine (Fig. 2). Unlike the effect of the latter agent, the ATP-mediated Na⁺ accumulation was dependent on pre-equilibration of the vesicles in K⁺-containing medium. Use of pre-equilibration medium (which determines the internal ionic composition of vesicles) containing 150 mM LiCl or choline chloride, or use of KCl in the external as well as pre-equilibration media, in either case precluded significant ATP-dependent Na⁺ accumulation in vesicles (data not shown). Thus the effect of ATP was dependent on internal K⁺ and either mediated by an outward K⁺ flux or simply inhibited by...
Table I

Time dependence of the effect of tetrodotoxin on sodium accumulation by synaptosomal plasma membrane vesicles

<table>
<thead>
<tr>
<th>Incubation conditions for sodium uptake</th>
<th>Sodium uptake above control</th>
<th>Sodium of veratridine control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Veratridine</td>
<td>4.06 ± 0.63</td>
<td>100.0</td>
</tr>
<tr>
<td>B. Veratridine + TTX (no preincubation)</td>
<td>3.03 ± 0.57</td>
<td>74.6</td>
</tr>
<tr>
<td>C. Veratridine + TTX (20-min preincubation)</td>
<td>2.50 ± 0.23</td>
<td>61.6</td>
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<tr>
<td>D. Veratridine + TTX (40-min preincubation)</td>
<td>1.74 ± 0.14</td>
<td>42.8</td>
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</tbody>
</table>

Table II

Effects of veratridine and ATP on sodium uptake into synaptosomal plasma membrane vesicles

<table>
<thead>
<tr>
<th>Sodium uptake conditions</th>
<th>Sodium uptake above control (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Veratridine (50 μM)</td>
<td>3.16 ± 1.03</td>
</tr>
<tr>
<td>B. ATP (2.5 mM)</td>
<td>11.72 ± 0.53</td>
</tr>
<tr>
<td>C. Veratridine (50 μM) + ATP (2.5 mM)</td>
<td>5.15 ± 0.59</td>
</tr>
</tbody>
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Synaptosomal Plasma Membrane Sodium Fluxes

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Fig. 3. Ca$^{2+}$-dependent reversal of Na$^+$ pump-mediated Na$^+$ accumulation in synaptosomal plasma membrane vesicles. Uptake of Na$^+$ commenced upon addition of 5 µl of vesicles pre-equilibrated for 10 min at 37°C in 150 mM KCl, 1 mM MgCl$_2$, 5 mM MOPS-KOH, pH 7.4, to 100 µl of external medium (140 mM choline chloride, 5 mM MgCl$_2$, 0.2 mM EGTA, 5 mM MOPS-Tris, pH 7.4, 1 mM NaCl with 0.01 Ci/mmol of Na$^+$) either with (●) or without (○) 2.5 mM ATP (Tris salt). Uptake was terminated after the appropriate time at 25°C by addition of 2.0 ml of ice-cold 150 mM choline chloride followed by rapid filtration. Tubes incubated 15 min or longer received either 10 µl of external medium (●) or 10 µl of external medium containing 100 mM CaCl$_2$ instead of choline chloride (○) giving a final Ca$^{2+}$ concentration of approximately 10 mM. These additions were made 15 min after Na$^+$ uptake began. For the 15-min time points, the additions were made rapidly by dilution and filtration. Details of the conditions for incubation and filtration are given in "Experimental Procedures."

Fig. 4. Diagrammatic representation of the Na$^+$ and Ca$^{2+}$ flux mechanisms observed in inverted synaptosomal plasma membrane vesicles. Experimental evidence for the function of (1) (ATP + Mg$^{2+}$)-dependent Ca$^{2+}$ transport, (2) Na$^+$-Ca$^{2+}$ exchange, and (3) possible voltage-sensitive Ca$^{2+}$ channel activity, is described in Ref. 6. As detailed in this report, the vesicles also accumulate Na$^+$ via (4) a veratridine-sensitive Na$^+$ channel, and (5) an (ATP + K$^+$)-dependent Na$^+$-pumping mechanism. Evidence to suggest the function of these mechanisms in a single population of inverted plasma membrane vesicles are described in the text.

Diagnosis of a Ca$^{2+}$-dependent phospholipase. Although a high concentration of Ca$^{2+}$ was used, the dose dependency of the Ca$^{2+}$-mediated Na$^+$ flux has not yet been determined. The almost complete efflux of Na$^+$ from vesicles upon addition of Ca$^{2+}$ suggests that nearly all vesicles containing the Na$^+$ pump also contain the Na$^+$-Ca$^{2+}$ exchange mechanism. Similar inferences regarding the location of Na$^+$-Ca$^{2+}$ exchange in cardiac sarcolemmal vesicles have been reported by Pitts (22).

The studies presented in this report, together with previously published data (6), strongly indicate that the vesicles accumulate and release Ca$^{2+}$ and Na$^+$ via specific mechanisms, the characteristics of which suggest the function of inverted plasma membrane sacs. The operation of these mechanisms is depicted diagrammatically in Fig. 4. Thus, previous studies (6) revealed the accumulation of Ca$^{2+}$ via three mechanisms: (a) an (ATP + Mg$^{2+}$)-dependent, vanadate-sensitive Ca$^{2+}$ transport process, thought to represent a reversed Ca$^{2+}$-extruding plasma membrane (Ca$^{2+}$ + Mg$^{2+}$)-ATPase analogous to that functioning in axolemma (23), cardiac sarcolemma (24), or erythrocyte plasma membrane (25); (b) a fully reversible Na$^+$-Ca$^{2+}$ exchange mechanism also believed to function in excitable cell plasma membranes as an important Ca$^{2+}$ extrusion mechanism (1-9, 24); (c) a membrane potential-sensitive Ca$^{2+}$ flux possibly reflecting a reversed operation of the voltage-dependent Ca$^{2+}$ channel, which functions in the intact terminal to permit Ca$^{2+}$ entry in response to depolarization (1-5, 26). In this report, vesicles are shown to transport Na$^+$ through either a veratridine-dependent, partially tetradotoxin-inhibited mechanism believed to represent the voltage-sensitive Na$^+$ channel of the neural membrane, or through the (ATP + K$^+$)-dependent Na$^+$ pump mechanism operating in the same vesicle membrane.

Since the Na$^+$-Ca$^{2+}$ exchange mechanism directly reverses each of the ATP-dependent flux mechanisms and is itself inhibited by veratridine-mediated Na$^+$ flux, it is probable that these four defined flux mechanisms operate in a single population of inverted plasma membrane vesicles. Although such structures may constitute the majority of sealed vesicles capable of accumulating ions, the experiments do not preclude the existence of other particulate structures in the preparation. Subsequent studies on the binding and influence of tetanus toxin on transport suggest that noninverted vesicles constitute a small proportion of the material. The use of such vesicles retaining so many of the functions of the native plasma membrane allows more precise identification of the mechanism and regulation of transmembrane ion fluxes and the processes by which intracellular and extracellular effectors may modulate their activity (4, 6).

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REFERENCES

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