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Spontaneous Opening at Zero Membrane Potential of Sodium Channels from Eel Electroplax Reconstituted into Lipid Vesicles

Daniel S. Duch* and S. Rock Levinson
Department of Physiology, C240, University of Colorado Medical School, Denver, Colorado 80262

Summary. The voltage-dependent sodium channel from the eel electroplax was purified and reconstituted into vesicles of varying lipid composition. Isotopic sodium uptake experiments were conducted with vesicles at zero membrane potential, using veratridine to activate channels and tetrodotoxin to block them. Under these conditions, channel-dependent uptake of isotopic sodium by the vesicles was observed, demonstrating that a certain fraction of the reconstituted protein was capable of mediating ion fluxes. In addition, vesicles untreated with veratridine showed significant background uptake of sodium; a considerable proportion of this flux was blocked by tetrodotoxin. Thus these measurements showed that a significant subpopulation of channels was present that could mediate ionic fluxes in the absence of activating toxins. The proportion of channels exhibiting this behavior was dependent on the lipid composition of the vesicles and the temperature at which the uptake was measured; furthermore, the effect of temperature was reversible. However, the phenomenon was not affected by the degree of purification of the protein used for reconstitution, and channels in resealed electroplax membrane fragments or reconstituted solely into native eel lipids did not show this behavior. The kinetics of vesicular uptake through these spontaneously-opening channels was slow, and we attribute this behavior to a modification of sodium channel inactivation.

Key Words sodium channels · reconstitution · electric eel · tetrodotoxin · sodium transport · lipid vesicles

Introduction

The voltage-dependent sodium channel mediates the rising phase of action potentials. This channel has been purified as a tetrodotoxin-binding protein from the electroplax of *Electrophorus electricus* (Miller, Agnew & Levinson, 1983) and shown to be a compositionally complex glycoprotein comprised of a single polypeptide chain. There is strong evidence that this single polypeptide can mediate the major functions of native sodium channels (Rosenberg, Tomiko & Agnew, 1984*a*; Levinson et al., 1986*b*; Duch & Levinson, 1987), including selective ion permeability, activation and inactivation. This integral, transmembrane transport system appears to have three biochemically-distinct domains (Levinson, Duch & Thornhill, 1986*a*): (1) a polypeptide core that is believed to comprise the actual physical structure mediating the passage of ions through the membrane (i.e., the ionophore); (2) a number of carbohydrate "trees" that extend away from the external surface of the channel and which may have a role in increasing the transport rate of sodium through the channel (Miller et al., 1983); and (3) the lipid that immediately surrounds the channel, both covalently and noncovalently associated (Levinson et al., 1986*b*), which may affect the steady state and kinetic behavior of voltage-dependent gating. In order to understand the roles each of these distinct domains may have in the molecular mechanism of sodium channel function, it is necessary to examine the influence of these domains under controlled conditions.

Sodium channels from electroplax and other tissues have been successfully reconstituted into lipid vesicles and bilayers, thus demonstrating that the channel ionophore can be removed from its native environment and still retain its major properties (Talvenheimo, Tamkun & Catterall, 1982; Weigele & Barchi, 1982; Krueger, Worley & French, 1983; Green, Weiss & Andersen, 1984; Moczydlowski, Garber & Miller, 1984; Rosenberg et al., 1984*a, b*; Duch & Levinson, 1985; Hartshorne et al., 1985; Levinson et al., 1986*b*). These systems are ideal for studying the interactions of the various functional domains of the channel since the highly purified protein can be enzymatically or chemically modified or placed in lipids of defined composition, and the functional consequences then determined.

* *Present address:* Department of Anesthesiology, Cornell University Medical College, New York, New York 10021

In order to demonstrate the function of sodium channels reconstituted into lipid vesicles, we have measured the uptake of isotopic sodium by such vesicles in the presence of highly selective pharmacological agents which either open or block the channel. The use of such agents is necessary for two main reasons: First, the nonspecific leak of sodium into the vesicle is usually significant and must be separated from the flux, which is specifically mediated by reconstituted channels. Second, flux measurements are most conveniently obtained and readily interpreted with identical buffers on either side of the vesicular membrane. Under these conditions the transmembrane potential is zero and sodium channels are all expected to be in the inactivated state and unable to conduct ions. Thus, in order to observe channel-dependent fluxes, the toxins veratridine and batrachotoxin are used to persistently activate the channel at zero potential, while the specific sodium channel blockers tetrodotoxin (TTX) or saxitoxin are used to separate the resultant isotopic uptakes into channel-dependent and nonspecific leak components.

However, in the course of our studies we observed that a considerable component of the leak flux (i.e., the uptake of sodium by vesicles in the *absence* of veratridine) was blocked by TTX. This unexpected result showed that at zero membrane potential a certain proportion of reconstituted sodium channels were capable of opening spontaneously. Below we report that the fraction of channels that exhibited this anomalous behavior was dramatically influenced by the lipid composition of the vesicles in which they were inserted. We hypothesize that inactivation gating is highly sensitive to the lipid environment immediately surrounding the sodium channel.

Materials and Methods

Materials

Soy bean lipids (asolectin), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from egg yolk, and bovine brain phosphatidylserine (PS) were purchased from Sigma Chemical Co. The asolectin was extracted with acetone before use in order to remove neutral lipids, as described by Kagawa and Racker (1971). All other materials were as described in the companion paper (Duch & Levinson, 1987).

Purification and Reconstitution of the TTX Binding Protein (TTXR)

Purification and reconstitution of the TTXR from eel electric organ were done as described (Duch & Levinson, 1987). Fractions from various steps of the purification process were reconstituted and studied to determine if the degree of purification affected the transport behavior observed.

Preparation of Membrane Fragments

Membrane fragments were prepared from the crude homogenate (Duch & Levinson, 1987). A 50% volume of ice-cold sodium phosphate buffer, 150 mM, pH 6.8, was added to the crude material, and the mixture was rehomogenized with a Potter-Elvehjem apparatus, placed on ice in a rosette cell, and sonicated using a micro-tipped Branson 350 sonifier with a control setting of 7 at 10% pulse for 10 min. The mixture was then centrifuged at 2°C in a J2-21 Beckman centrifuge at 12,000 × *g* for 20 min. The supernatant was again sonicated and centrifuged as above before use. Membrane fragments were also prepared as above without the sonication steps and used in the experiments. Aside from a slightly higher variability in the results obtained from unsonicated membranes, no significant differences in the final results were found between the membranes prepared with or without sonication.

^{22}Na Uptake Assays

Uptake of ^{22}Na by the reconstituted vesicles was measured by the method of Epstein and Racker (1978) (*see* Duch & Levinson, 1987). Vesicles or membrane fragments were incubated with the appropriate toxins at experimental temperatures for 30 min before uptake measurements were carried out. Unless noted otherwise, the four toxin mixtures used had a final concentration of: (i) 150 μM veratridine (vesicles plus veratridine, abbreviated *VV*), (ii) 2 μM TTX (*VT*), (iii) 150 μM veratridine + 2 μM TTX (*VVT*), or (iv) the toxin buffers only, with no toxins (*V*).

Measurement of Sodium Channel Orientation

Measurement of sodium channel orientation in reconstituted vesicles was also as described previously (Duch & Levinson, 1987).

Morphometric Measurement of Vesicle Size

As previously described by Aurora et al. (1985), a drop of vesicle-containing solution was placed on a Formvar-coated copper grid for 15 sec and then blotted well on Whatman #1 filter paper. Immediately following, a drop of 5% uranyl acetate was placed on the grid for 5 sec and then blotted as above. After airdrying for 10 min, the negatively-stained vesicles were observed and photographed using a Phillips 200 electron microscope operated at 80 kV and 22,858× magnification. Vesicle sizes were measured directly from the resulting electron micrographs and the mean diameters calculated from the volume weighted distribution of diameters.

Results

Measurement of Sodium Channel Characteristics Using Isotopic Fluxes

The isotopic uptake experiments reported in this paper were conducted with zero potential across

the vesicular membrane (i.e., with identical ionic composition of the inner-and extra-vesicular buffers). Under these conditions, the voltage-dependent sodium channel was initially expected to be in an "inactivated" state in which no flux of ions through the channels can occur. As in previous studies (Talvenheimo et al., 1982; Tanaka, Eccleston & Barchi, 1983; Rosenberg et al., 1984*a*; Tamkun, Talvenheimo & Catterall, 1984), channel-dependent ionic uptake was detected using veratridine to activate the channels persistently, thus allowing ion flux through them into the vesicles. In addition to the activating toxin, the channel-blocking agent tetrodotoxin was employed to inhibit specifically the channel-mediated fluxes. TTX acts independently of veratridine, binding noncompetitively to a separate site on the channel where it is believed to occlude the ion pore (Catterall, 1980).

Previous studies (Talvenheimo et al., 1982; Tanaka et al., 1983; Rosenberg et al., 1984*a*; Tamkun et al., 1984), have utilized these two toxins to characterize reconstituted channels by measuring the uptake of ^{22}Na by vesicles under three conditions: (i) Uptake by vesicles with no toxins present (hereafter referred to as *V*); in previous work, this condition has been taken to define the nonspecific baseline "leak" of ^{22}Na across the vesicular membrane. (ii) Uptake by vesicles in the presence of veratridine (*VV*); when compared to the baseline (i.e., *VV-V*) this condition has been used to define the uptake of ^{22}Na through functional reconstituted channels. (iii) Uptake by vesicles with both veratridine and TTX added to the external buffer (*VVT*); this condition was used to define the "sidedness" of the reconstituted channels. When applied to the external solution, TTX will only block channels in the same "right-side-out" orientation as they were in the cell membrane, whereas veratridine is known to activate channels of either orientation (Catterall, 1980). Thus the ratio *(VVT-V)/(VV-V)* has been used in previous work to define the proportion of "inside-out" channels.

Typical results from a single experiment in the present work are shown in Fig. 1. In panel *A*, uptake curves for the three conditions described above are shown, along with the uptake obtained using BTX. Both BTX and veratridine caused a rapid equilibration of tracer sodium with the internal volume of the vesicles. In addition, as found in previous studies (Tanaka et al., 1983), BTX caused a much more rapid accumulation of isotope than did veratridine; this difference is apparently a reflection of the longer channel open times in BTX (Garber & Miller, 1987; Levinson et al., 1986*b*). Lastly, TTX eliminated most of veratridine-induced flux, suggesting that a high proportion of channels were oriented "right-side" out in these vesicles.

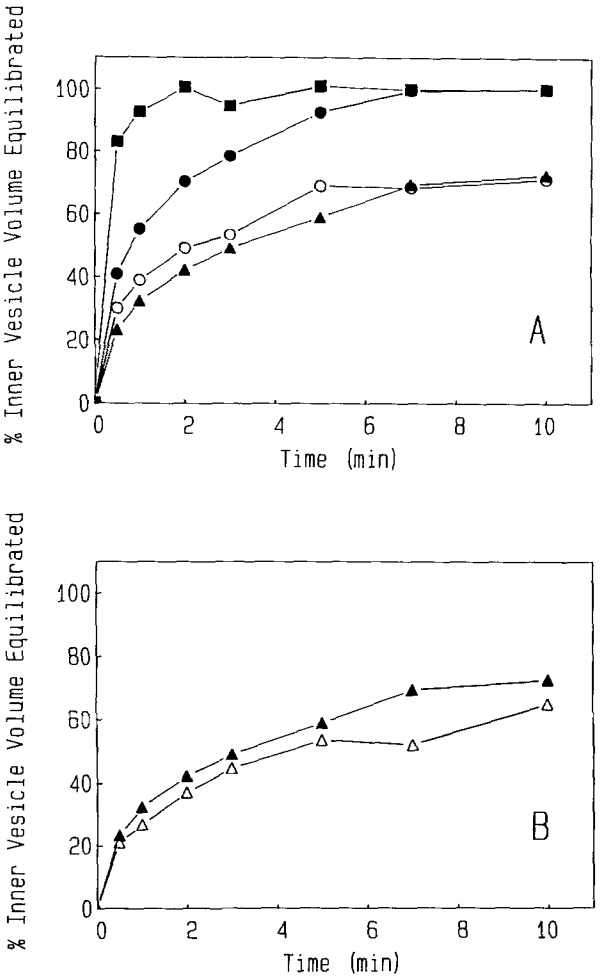

Fig. 1. ^{22}Na uptake into asolectin/PC vesicles containing purified eel sodium channels. Vesicles were incubated at 26°C with the given toxins and assayed as described in the text. (*A*) ■, 5 μM BTX; ●, 150 μM veratridine (condition *VV*); ○, 150 μM veratridine and 2 μM TTX (*VVT*); ▲, vesicles only (no additions, *V*). Uptakes are expressed as percentages of the final equilibrium attained and are the results of duplicate determinations. (*B*) The uptake into vesicles with TTX added. ▲, vesicles with no additions (same data as *A*); △, vesicles with 2 μM TTX

No significant differences in uptake curves or in the characteristics described in the following sections were seen among sodium channels which were purified to different degrees as described in the methods (*data not shown, see* Duch, 1986). The purity of these preparations was measured by the specific activity of TTX binding, which ranged about sevenfold range to a high of nearly 2700 pmol TTX bound/mg protein for highly purified material. Thus it was concluded that degree of purity did not affect functional characteristics, and most experiments utilized protein partially purified through the ion exchange step (sp act approx. 500 pmol TTX bound/mg protein) to conserve time and material.

Open Channels Observed in the Absence of Activating Toxins

In addition to the three standard conditions, the isotopic uptake was also measured for vesicles to which only tetrodotoxin had been added to the external buffer (referred to as condition *VT*). Since sodium channels should have been in an inactivated state at zero membrane potential, this condition was expected to produce results essentially the same as unmodified vesicles (condition *V*). However, the results of Fig. 1*B* show that externally applied TTX also reduced the ^{22}Na uptake in the *absence* of veratridine, demonstrating that a certain fraction of reconstituted channels could open spontaneously in the absence of activating toxins. The channel population responsible for this phenomenon will be referred to hereafter as "spontaneously opening channels" (or abbreviated SOCs), while the normally behaved veratridine-activated channels will be abbreviated VACs.

Measurement of the Fraction and Number of Spontaneously Opening Sodium Channels (SOCs)

In order to characterize the spontaneously-activated fluxes, it was necessary to develop a measure of their magnitude according to the following considerations. First, the presence of spontaneously opening channels weakens the validity of the previously used definitions of channel-dependent uptake (*VV-V*) and channel orientation ((*VVT-V*)/(*VV-V*)) because the uptake through such channels means that the condition *V* is no longer an appropriate baseline for such calculations. Second, the condition *VT* is also not a rigorous baseline, since it is reasonable to expect that SOCs will orient themselves inside-out to the same extent as veratridine-activated channels; thus the contribution of inside-out SOCs means that the "true" baseline channel-independent leak is less than condition *VT*. While in theory one could establish this baseline by incorporation of TTX inside the vesicles during their formation, in practice this was not economical due to the large quantities of ^{3}H-TTX needed in the dialysis buffers. Instead, we assumed that the proportions of right-side-out veratridine-activatable channels and right-side-out SOCs were the same. Then the fraction of SOCs relative to all functional channels was simply (*V-VT*)/(*VV-VVT*). For greatest accuracy, the uptake values used for these calculations were taken at 3 min or longer (i.e., when the vesicles were close to equilibration), except where otherwise specified. At this time the channel-specific uptake is highest relative to the nonspecific leak, and errors due to modest differences in the initial rate of equilibration between vesicles with SOCs and those with VACs were minimized.

Isotopic uptake measurements were used as an index of the number of SOCs and VACs. These numbers were obtained from the inner vesicular volume that was isotopically equilibrated through TTX-blockable uptake (i.e., (*V-VT*) for SOCs, or (*VV-VVT*)-(*V-VT*) for veratridine-activated channels). This is because in nearly all the experiments, the proportion of total inner vesicular volume loaded though either channel type was well under 100%; for SOCs the maximal proportion was 8.9% (in 0% PC), while for VACs 23.8% (at 50% PC, not corrected for a small proportion of inside-out channels—*see* Fig. 3). Thus, to a good approximation, vesicles had either just one functional channel or no functional channels at all. Therefore, changes in the absolute number of functional channels should have yielded approximately proportional changes in the absolute inner vesicular volume loaded by these channels (i.e., each additional channel was statistically more likely to be reconstituted into an unoccupied vesicle rather than one with another channel), providing that any concomitant changes in vesicle size were also corrected for (*see* Discussion).

Effects of Replacing Asolectin with Egg Phosphatidylcholine

The lipid composition of the vesicles strongly influenced the proportion of SOCs and other characteristics of both reconstituted sodium channels and the vesicles themselves. Since historically we first became aware of SOC behavior in asolectin vesicles, our first experiments were to replace varying fractions of asolectin with egg phosphatidylcholine (PC) while maintaining the total lipid concentration constant. The ^{22}Na uptakes were determined for each condition in Fig. 1, and the proportion of SOCs was calculated according to the above method, along with the total internal volume of the vesicles (expressed as the percent of the external buffer solution). The proportion of inside-out channels was determined by the TTX binding assay. The resultant uptake curves are shown for 0% PC (100% asolectin) and 50% PC in Fig. 2. Comparison of the uptakes in Fig. 2*A* (50% PC : 50% asolectin) with those of Fig. 2*B* (100% asolectin) shows that veratridine-stimulated uptake was greater for the lipid mixture (i.e., *VV-V*), while the 100% asolectin vesicles show a far greater *proportion* of spontaneous uptake. In fact, the great majority of channel-mediated uptake in 100% asolectin vesicles was apparently

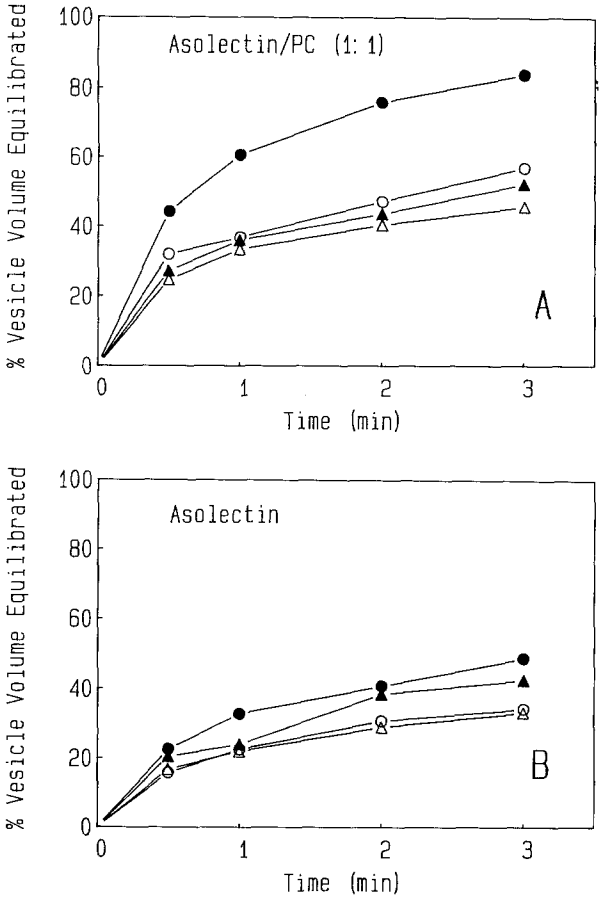

Fig. 2. ^{22}Na uptake by asolectin/PC and asolectin vesicles containing electroplax sodium channels. ●, vesicles with 150 μM veratridine (*VV*); ○, vesicles with 150 μM veratridine and 2 μM (*VVT*); ▲, vesicles with no additions (*V*); △, vesicles with 2 μM TTX (*VT*). All experiments were conducted at 26°C. (*A*) Uptake by asolectin/PC reconstituted vesicles. The results are the average of six separate preparations, each measured in duplicate. (*B*) Uptake through asolectin vesicles. The results are the average of five separate preparations, each measured in duplicate or triplicate. Error bars were excluded for clarity. The average coefficient of variation (*C.V.*) for *V* in the asolectin/PC experiment was 0.114, and for *VT* was 0.10; for the asolectin flux, the *C.V.* for *V* was 0.113, for *VT* it was 0.144

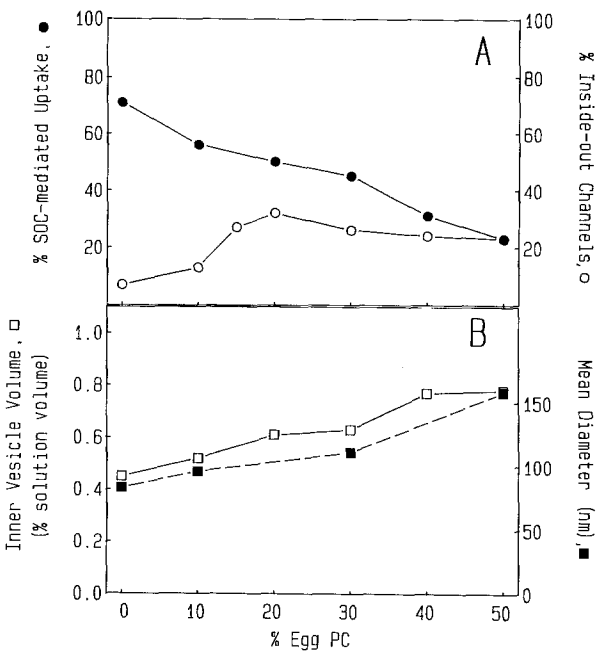

Fig. 3. Effects of replacement of asolectin with egg PC on sodium channel and vesicle characteristics. CHAPS-solubilized channels were purified through the DEAE step in asolectin buffers containing PC in the proportions shown (expressed as weight percentages). Uptake measurements were made as in Fig. 2, and the percentages of SOC-mediated uptake (●) and inside-out channels (○) are shown in *A*, while the total inner (included) volume of the vesicles (□) and mean vesicle diameter (■) are given in *B*

through SOCs, since veratridine induced very little additional isotopic uptake.

Each individual point in Fig. 2 was examined for significant differences between the two spontaneous uptake conditions (*V* and *VT*). At all points except the 1-min time point of the asolectin uptake and the 2-min time point of the asolectin-PC uptake, differences were significant ($P < 0.05$, unpaired *t* test). This difference was further examined using a Chi square test of the null hypothesis that $V = VT$ at all time points (that is, that the probability of $V \geqq VT$ is equal to the probability that $VT \geqq V$ throughout the experiment). For *both* asolectin and asolectin-PC vesicles, the differences of the observed results from those expected with the null hypothesis were highly significant ($P < 0.001$), indicating that *VT* was significantly different from *V*.

A summary of the effects of varying the vesicular fraction of phosphatidylcholine on the proportion of SOCs for all such experiments is shown in Fig. 3. First, it can be seen that the fraction of total vesicle uptake mediated by spontaneous channel openings (calculated as described above) decreased as the fraction of PC present increased. In these experiments, the amount of channel protein in the vesicles, as measured by TTX binding activity, was not significantly altered by the fraction of PC present until this fraction became greater than 50%, and all buffers had activities of between 13 and 16 pmol TTX bound/mg lipid. However, when the fraction of PC was 60% or greater, lower recoveries of the TTX-binding protein resulted, and the vesicles that formed during reconstitution began to aggregate and precipitate out of solution. This made the data obtained at high PC concentrations unreliable, and experiments utilizing PC above 50% are not reported here. These problems are apparently

due to the nature of the lipid/detergent micelles formed by CHAPS and PC, since PC with nonionic detergents (e.g. Lubrol or Triton) stabilizes the binding protein exceptionally well (Agnew et al., 1981).

Second, the internal volume of the vesicle population exhibited a significant and monotonically increasing dependence on PC content (Fig. 3, open squares). The mean vesicle diameters measured closely paralleled this trend (Fig. 3, filled squares); in fact, when both vesicle diameter and total volume were normalized to their respective values at 0% PC, the lines very nearly superimposed (calculations *not shown*). This is precisely what would be expected if all of the increase in total vesicle volume with increasing PC content were due to increased vesicle diameter. Under the experimental conditions of constant lipid concentration, the vesicular surface area should have been nearly the same in all experiments; in these circumstances, simple algebra predicts that *total* vesicle volume will be proportional to the *first* power of mean vesicle diameter. The close correspondence of the observed relations with this prediction strongly suggests that the changes in total vesicle volume are solely a consequence of changes in vesicle size, and not due to changes in vesicle "leakiness" or other factors. The consequences are discussed below.

Third, the reconstitution of sodium channels from CHAPS/asolectin solutions resulted in a highly asymmetric orientation of TTX-binding sites. Figure 3 (open circles) shows that the sidedness of channel insertion was also dependent on the PC content. In 100% asolectin, TTX-binding sites were nearly all right-side out, but in 20 to 50% PC-containing vesicles the proportion of inside-out channels increased to about a quarter of all TTX-binding sites. The causes of the observed asymmetry and its variation with lipid composition are unknown but like vesicle size may also be related to the mechanism of vesicle formation from the micellar phase (Tanford, 1980). In any case, these mechanisms were not investigated in the present study, and the minor contribution of this population has been excluded from the calculations as described above.

In order to assess whether any differences found in channel behavior between asolectin and asolectin-PC reconstituted channels could be due to an alteration of the channels during purification in the unique buffers of each system, detergent-solubilized channels were first purified in 100% asolectin (0% PC) buffers and then diluted with the appropriate detergent/lipid buffers to give the desired final lipid composition for reconstitution. No significant differences were found between these reconstituted channels and those purified and reconstituted entirely in a specific buffer. This suggests that the SOC phenomena was dependent only on the final lipid composition of the vesicles and not on the history of exposure to other lipids during purification.

Effects of Temperature on Spontaneous Uptake

The poor time resolution of isotopic uptake experiments limited the information obtainable about the functional modifications underlying spontaneous channel openings. However, some conclusions could be drawn indirectly from the temperature dependence of the phenomena (*see* Discussion). Such an experiment, using 50% PC/asolectin vesicles, is shown in Fig. 4. It can be seen that as the temperature decreased from 30 to 20°C, the component of spontaneous uptake decreased markedly (*compare* filled *vs.* open triangles), while the VAC component appeared constant (*compare* filled *vs.* open circles). To examine whether this effect of temperature was reversible, vesicles were heated at 30°C for 30 min, then divided and either examined for uptake at that temperature or cooled to 20°C for an additional 30 min before uptake measurements were made. No significant differences were found between the measured uptakes of vesicles preheated to 30°C before incubation at 20°C and those incubated only at 20°C. Also the total inner-vesicular volume measured in both this experiment and the experiments shown in Fig. 5 did not change with temperature, indicating that vesicle volume was stable throughout the experiment.

The fractions of vesicle volumes accessible through SOCs at all three temperatures were compared using an unpaired *t*-test. In these comparisons *V-VT* for all time points at a given temperature were averaged. The average *V-VT* for the 30°C experiment was 11.3 ± 1.6% of the total inner vesicular volume; for channels at 26°C, 8.2 ± 2.5%; and at 20°C it was 0.7 ± 2.4% (SEM). It was found that there was no significant difference between the 30 and 26°C experiments ($P > 0.1$), but between 30 and 20°C, and 26 and 20°C, differences were significant at the $P < 0.05$ level.

Temperature-Dependent Uptake by PC/PS/PE Vesicles

In order to examine whether some component peculiar to the asolectin preparation was responsible for SOC behavior, channels were purified and reconstituted using phospholipids from other tissues in a proportion different from that found in asolectin (*cf.* Erdahl, Stotywho & Privett, 1973). It was found that the channel could be purified in PC/PS/PE mixtures (3 : 1 : 1) with no significant loss of channel

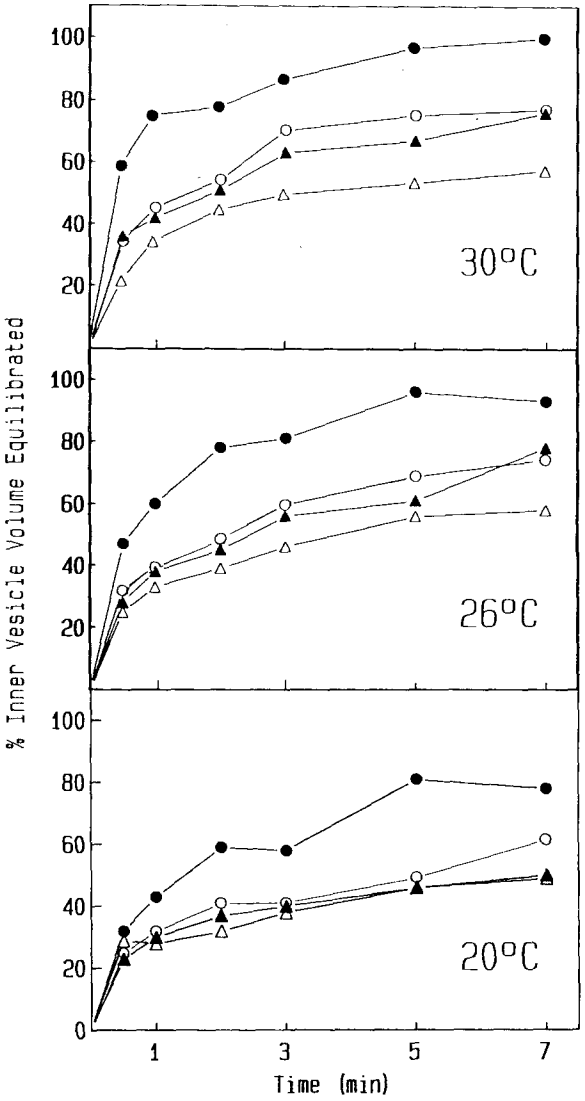

Fig. 4. Temperature dependence of the spontaneous opening behavior in asolectin/PC vesicles. Channels were purified through the DEAE step in asolectin/PC (50% PC) buffer, then reconstituted. After reconstitution, there were 33 pmol TTX binding/ml solution. The reconstituted vesicles were incubated at the given temperatures for 30 min with 150 μM veratridine (●, *VV*), 150 μM veratridine + 2 μM TTX (○, *VVT*), 2 μM TTX (△, *VT*), or no additions (▲, *V*) before the measurements were made. *Upper panel:* Uptake by vesicles at 30°C, average of duplicate determinations. *Middle panel:* Uptake by vesicles at 26°C. Results are the average of four separate determinations. *Lower panel:* Uptake by vesicles at 20°C. The results are the average of the separate measurements described above

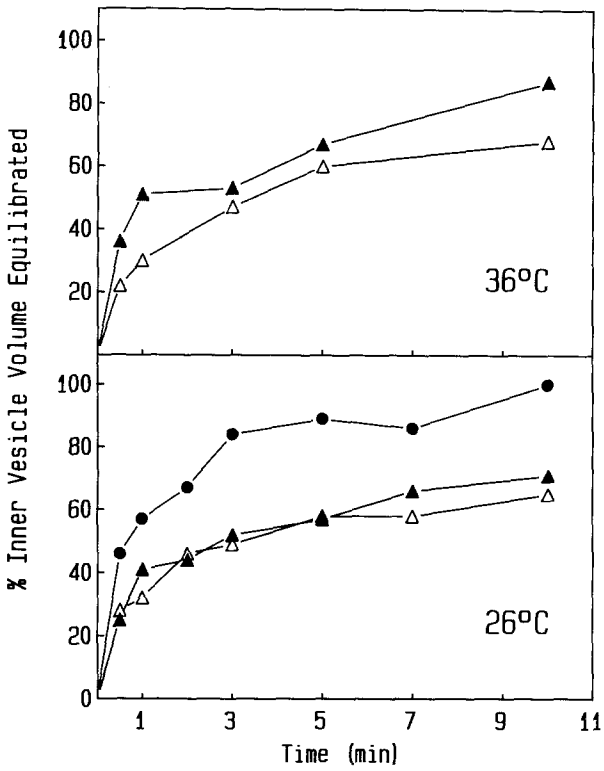

Fig. 5. Temperature dependence of the spontaneous opening behavior in vesicles comprised of PC(3):PS(1):PE(1) vesicles. Channels were purified through the DEAE step in the same buffer as the other experiments described, but with 2 mg/ml PC/PS/PE instead of the other lipids. Reconstitution was carried out by dialysis as described. Reconstituted solutions had 39 pmol TTX-binding/ml solution. The reconstituted vesicles were incubated at the given temperatures for 30 min with 150 μM veratridine (●, *VV*), 2 μM TTX (△, *VT*), or no additions (▲, *V*) before uptake measurements were made. *Upper panel:* Uptake by vesicles at 36°C, average of duplicate determinations. *Lower panel:* Uptake by vesicles at 26°C, average of duplicate determinations

stability during the purification step and no aggregation of vesicles after reconstitution. The results of such an experiment are shown in Fig. 5. It can be seen that through 5 min of uptake at 26°C, no significant SOC behavior was present in these vesicles. By contrast, in the experiment at 36°C, SOC-mediated uptake was evident throughout the experiment. The differences in *V-VT* between the uptakes at the two temperatures were examined as in Fig. 4. The results indicate that the difference in SOC flux between the two experiments was significant, $P < 0.05$. Once again, the results indicate that the SOC flux was temperature dependent. Moreover, by comparing the uptakes at 26°C in Figs. 4 and 5, it can be seen that the temperature range over which it was expressed is different for the two lipid mixtures used.

Uptake by Vesicles Formed from Electroplax Membrane Fragments

To find out if unpurified sodium channels *in situ* exhibit the same type of behavior, uptake through vesicles prepared from resealed electroplax membrane fragments was examined. Membranes were

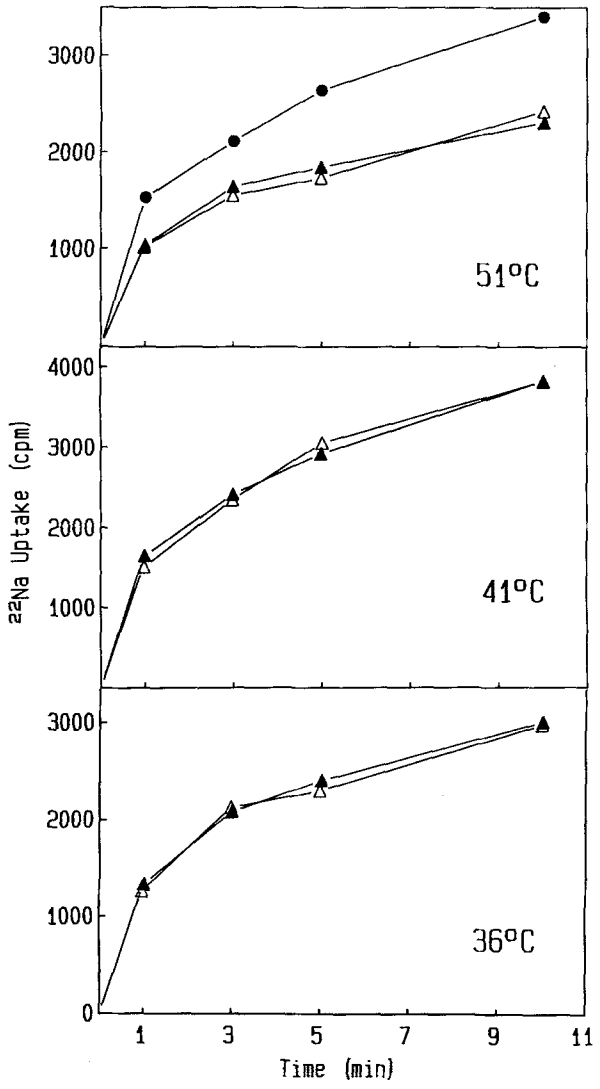

Fig. 6. ^{22}Na uptake by resealed electroplax membrane fragments. Resealed membrane fragments were prepared as described in the text. They were incubated for 30 min at the given temperatures with 150 μM veratridine (●), 2 μM TTX (△), or no additions (▲). *Upper panel:* Uptake by membrane vesicles at 51°C, average of four determinations from four separate membrane preparations. *Middle panel:* Uptake by membrane vesicles at 41°C. Results are the average of four determinations from four separate membrane preparations. *Lower panel:* Uptake by membrane vesicles at 36°C; the results are again the average of four determinations with four separate membrane preparations

prepared as described in Materials and Methods and used the same day. Because membranes became very leaky when incubated at high temperatures, the integrity of the membrane preparations at the higher temperatures was examined by equilibrating the membrane-derived vesicles with ^{22}Na overnight, then incubating them in the equilibration buffer at the respective temperatures for various times and measuring the ability of the vesicles to retain the acquired ^{22}Na at 4°C on the ion exchange column. These vesicles were able to retain all internal sodium after 30 min exposure to 51°C, whereas at 61°C the vesicles retained very little isotope after 10 min exposure (*data not shown*). Uptake experiments were therefore conducted at temperatures up to 51°C. Results are shown in Fig. 6; no indication of SOC-dependent uptake was found at any temperature tested.

Uptake by Unpurified Channels in Electroplax Lipid Vesicles

The results with resealed electroplax membrane vesicles suggested that the phenomenon of spontaneous uptake might be caused by exposure to the detergent CHAPS. To obtain information about this possibility, electroplax membranes were extracted with CHAPS, then either diluted 50/50 with CHAPS-asolectin buffer (to a final concentration of 1.5 mg/ml asolectin) or left as extracted and reconstituted by dialysis against 100 mM sodium phosphate buffer, pH 6.8. The reconstituted channels were examined for SOC-mediated uptake at both 26 and 36°C. It can be seen that there was no apparent spontaneous opening behavior in channels reconstituted in their undiluted native lipids at either temperature (Fig. 7), but that there was a significant uptake through channels diluted with asolectin at both temperatures (Fig. 8). Values of (*V-VT*) for channels reconstituted in native lipids alone were never significantly different from zero; however, in the asolectin-supplemented experiments, (*V-VT*) was significantly different from zero at all times except the 5-min time point at 26°C. A Chi-squared test of *V-VT* was applied to the results from both temperatures as described for Fig. 2. *V-VT* in native lipids alone was not significantly different from zero at either temperature ($P < 0.7$, 26°C; $P < 0.4$, 36°C). In asolectin-supplemented lipids, however, highly significant differences were found ($P < 0.02$ in both experiments). Finally, in comparing the data for asolectin-supplemented vesicles (Fig. 8), it can be seen that there was a significant increase in the fraction of channel-dependent uptake that was mediated by SOCs when the temperature was raised from 26°C to 36°C. In fact, at 36°C, almost all of the uptake was through spontaneously opening channels.

Discussion

These results lead to three main conclusions regarding the behavior of electroplax sodium channels reconstituted into lipid vesicles: (1) There are sig-

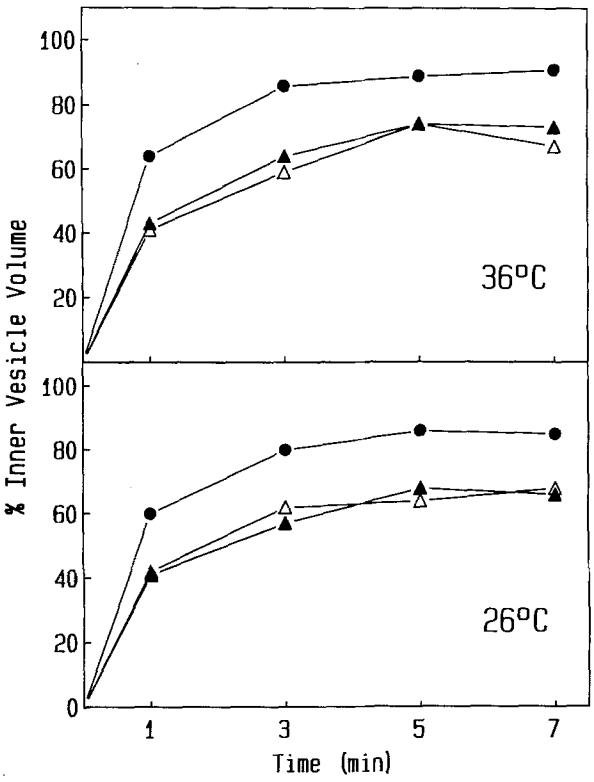

Fig. 7. Uptake by sodium channels in vesicles prepared with electroplax lipids only (unsupplemented). ●, 150 μM veratridine; △, 2 μM TTX; ▲, no additions. *Upper panel:* Uptake at 36°C. *Lower panel:* Uptake at 26°C. Results are the average of four determinations

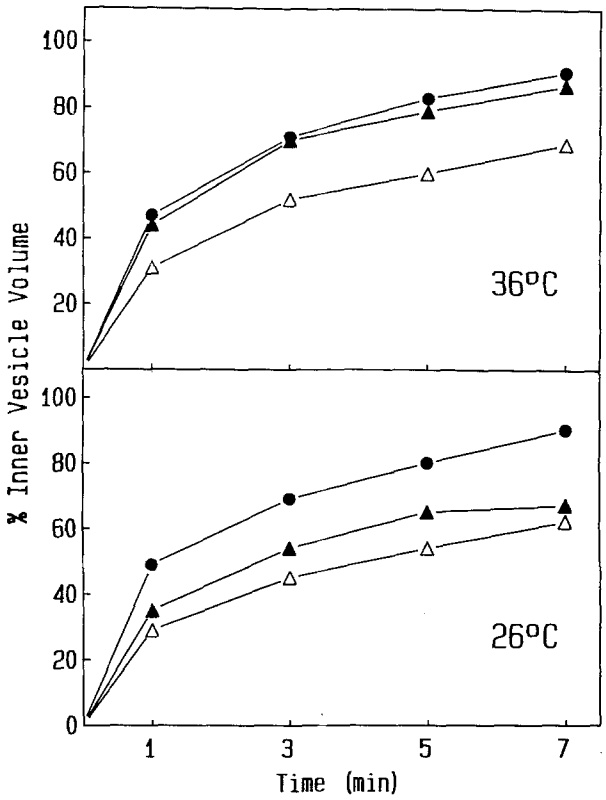

Fig. 8. ^{22}Na uptake by unpurified channels in electroplax lipids supplemented with asolectin. Channels were extracted from electroplax membrane fragments as described in the text and then were incorporated into vesicles comprised of the extracted eel lipids supplemented with 1.5 mg/ml asolectin (extract diluted 1 : 1 with buffer containing 3 mg asolectin/ml). Vesicles were incubated for 30 min with 150 μM veratridine (●), 2 μM TTX (△), or with no additions (▲) before measurements were made. All results are the average of four determinations from two separate extractions. *Upper panel:* Uptake at 36°C. *Lower panel:* Uptake at 26°C

nificant spontaneous openings of reconstituted channels at zero membrane potential; (2) This phenomenon is dependent on the lipid composition of the vesicles; (3) The phenomenon can be greatly affected by temperature, depending on lipid composition. Below we address first questions about the lipid dependence of the phenomenon of spontaneous activation, then how lipid environment might alter sodium channel function to account for our observations. Lastly, we consider the significance of our findings to both reconstitution studies and previous physiological observations of channel behavior.

Added Egg PC Decreases the Absolute Number of SOCs

The effect of added PC on the observed fraction of these channels suggested that the lipid environment of the vesicles might in some way be responsible for their expression. To investigate this further it was necessary to determine whether the changes in SOC *fraction* were due to changes in the *numbers* of either spontaneously-opening or veratridine-activated channels, or both.

As discussed in Results, in principle one can estimate the numbers of channels from the relative magnitudes of the isotopic uptakes they mediate. However, in the present experiments the relative number of conducting channels obtained in this manner must be corrected for changes in the volume of a single vesicle when PC composition is varied. Under the conditions of constant total vesicle membrane area used in our experiments, changes in the total inner volume of vesicles were reflected in proportionate changes in the mean diameter of the vesicles (Fig. 3). Since the mean *individual* vesicle volume is proportional to the cube of the diameter, an increase in diameter of 1.9-fold in 50% PC relative to 0% PC corresponds to an increase of 6.9-fold

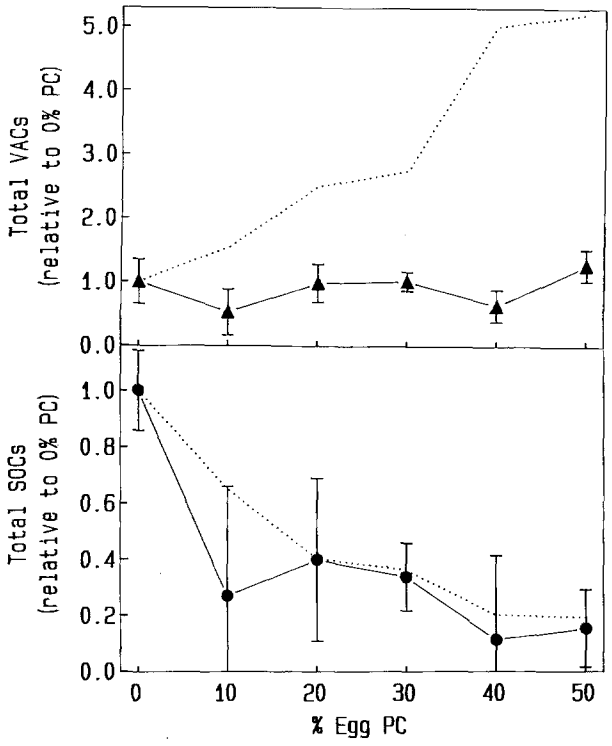

Fig. 9. Effect of replacement of asolectin with egg PC on the number of SOCs (lower panel) and VACs (upper panel). Changes are expressed relative to the number of channels in 0% PC (100% asolectin) as 1.0. The dotted lines in both panels show the changes predicted if all of the changes observed in the *fraction* of SOCs shown in Fig. 3 were due to a decrease in the *number* of SOCs (lower panel) or an increase in the number of VACs (upper panel). For further details, *see* the text

in the volume of an average vesicle formed from 50% PC, relative to those of 0% PC. The consequence of such changes in vesicle physical parameters is that each functional channel in a 50% PC vesicle will yield nearly a sevenfold greater isotopic uptake than a channel in a 0% PC (100% asolectin) vesicle. Thus, one must correct for these rather dramatic changes in *single* vesicle volume in order to estimate accurately changes in channel number as lipid composition is varied.

Figure 9 shows how the number of channels changes with PC composition relative to 0% PC, with corrections for the effect of vesicle volume changes as described above (using the data of Fig. 3). The number of SOCs shows a significant and dramatic decrease from 100% asolectin when PC is present in any amount. On the other hand, the number of veratridine-activated channels show no significant change with PC composition. The dashed lines in the figure show the changes in channel number *expected* if all of the changes in SOC fraction were due to either a decrease in the number of SOCS (lower panel) or the number of VACs (upper panel). It is clear from the data in this figure that probably all of the change in the *fraction* of SOC-mediated uptake can be attributed to a decrease in the number of SOCs rather than an increase in the number of VACs.

Alterations in Sodium Channel Function Underlying Spontaneous Opening Behavior

Sodium channels *in vivo* are nearly always found to be in the closed, inactivated state at zero membrane potential; thus, finding a significant proportion of channels spontaneously opening in our studies was clearly unexpected and suggests that the inactivation process has been altered in some way. The channels that exhibited this behavior seemed to be a stable subpopulation, since the vesicle uptake mediated by this component equilibrated within minutes to new steady state usually below that activated by veratridine.

The methods employed in this study could only resolve time-dependent changes in ^{22}Na uptake on the order of 10 sec and had no provision for determining the effects of transmembrane voltage on these uptakes. In spite of these limitations, some preliminary conclusions may be made regarding the altered function of spontaneously-opening channels. Before describing these conclusions, though, one should consider the time needed for flux through a sodium channel to equilibrate the vesicles. From morphometric studies (Fig. 3, *see also* Duch, 1986), we have found that most of the inner volume in experiments using 50% PC (which formed the largest vesicles) is contributed by vesicles having a mean diameter of about 150 nm. For each such vesicle containing a single channel with normal permeability, a channel open time of only some tens of milliseconds is necessary for equilibration to occur (Tanaka et al., 1983); thus a channel that was perpetually open would have instantaneously equilibrated its vesicle, i.e., long before the first measurement of uptake was made (at 30 sec).

However, it is obvious that both veratridine-stimulated and spontaneous uptake exhibited very slow equilibration times (e.g. Fig. 2). Thus one might hypothesize that neither veratridine-activated nor spontaneously opening channels were perpetually activated, but rather had only a small probability of being open (i.e., were on average open for a small fraction of any given time interval). In fact, recent electrical recordings of sodium channels reconstituted into lipid bilayers have shown that veratridine-activated channels have such short open times (Garber & Miller, 1987; Levinson et al., 1986*b*), suggesting that SOCs might be gated in a similar fashion.

While it is possible that SOCs also have an altered permeability to sodium, this is highly unlikely to be the explanation for the slow rate of SOC-mediated uptake, since an approximately 100-fold decrease in permeability would be required to explain the observed uptake kinetics. This possibility is further found to be improbable when the temperature dependency of the channel-dependent leak flux is examined. As has been described (*see* Hille, 1984), flux through aqueous pores is relatively temperature insensitive, having a Q_{10} of around 1.3. Therefore, in a channel that is persistently open, a change of 6-10°C should not significantly affect the rate of uptake through the channel. As shown in Figs. 4 and 5, however, the spontaneous uptake virtually disappeared with such small temperature changes. Thus the steep temperature dependence of the SOC phenomenon argues that gating is affected rather than permeability to sodium. Lastly, the argument given above that SOCs still are mainly in the closed state is an indication that such channels retain the structures responsible for normal channel inactivation, but that steady-state gating behavior was altered in these experiments. It thus seems reasonable to assume that the spontaneous-activation phenomenon represents a subpopulation of channels with altered inactivation such that channels may open for brief periods at zero membrane potential.

Properties of Lipids that may Affect Channel Behavior

Because no systematic changes in lipid parameters (head groups, fatty acid length, saturation, etc.) were made in the present experiments, it was not possible to determine which substructures or specific properties of lipids were important in moderating SOC behavior. The dramatic effect of temperature on the phenomenon would seem to suggest that microviscosity or fluidity changes (e.g. as opposed to surface potential effects) were involved.

Naturally, in light of the limited lipid substitutions employed in our studies, one cannot exclude other mechanisms for SOC behavior, such as the specific interactions of particular lipids with a gating domain of the channel. In addition, it might be thought that even after exhaustive dialysis, differences in the concentration of residual detergent in vesicles of various lipid compositions may have caused the variations of SOC behavior seen. However, this seems to be an unlikely explanation, since planar lipid bilayers exposed to these vesicles show no obvious increases in conductance noise that is a sensitive indicator of the presence of detergent (Levinson et al., 1986*b*; E. Recio-Pinto and B. Urban, *unpublished*). In addition, one other study has briefly mentioned the presence of TTX-blockable background ^{22}Na uptake in vesicles derived from Lubrol-PX extracts of electroplax sodium channels (Tomiko et al., 1986). Although such behavior was not characterized, these observations show that the SOC phenomenon can be elicited with a chemically distinct detergent from CHAPS, further suggesting that detergent is not directly involved.

Relationship to Other Studies

There are reports of native channel behavior in other tissues that are consistent with the channel behavior described here. Incomplete inactivation has been reported in squid giant axons by Chandler and Meves (1970*a–c*) and Shoukimas and French (1980). A description of channels reopening after they had inactivated has been given by Kunze et al. (1985) in experiments examining the slow inactivation of cardiac Na currents, as well as in patch-clamp studies of frog muscle (Patlak & Ortiz, 1986). Finally, the muscle fibers of patients suffering from hyperkalemic periodic paralysis have been shown to demonstrate a steady, TTX-blockable increase in sodium conductance during potassium depolarization (Lehmann-Horn et al., 1983). Further investigations will be needed to assess whether or not the spontaneous opening seen in the experiments reported here correspond to these or any other observations of native sodium channel behavior.

The results of the experiments presented here suggest that the lipid environment of reconstituted sodium channels can have profound effects on the inactivation gating of the channel. This is an important result in and of itself, because it indicates that caution must be used when interpreting the measurements of sodium channel parameters in reconstituted systems if these results are to give information about the native sodium channel. It also means that an understanding of how the sodium channel functions on a molecular level will require more than the knowledge of the primary, secondary and tertiary structures of the polypeptide comprising the channel; an understanding of how the channel molecule interacts with its lipid environment will also be necessary.

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