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Permeability of the Squid Giant Axon to Organic Cations and Small Nonelectrolytes

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Summary. The permeability of the Na channel of squid giant axon to organic cations and small nonelectrolytes was studied. The compounds tested were guanidinium, formamidinium, and ^{14}C-labeled urea, formamide, thiourea, and acetone. Permeability was calculated from measurements of reversal potential and influx on internally perfused, voltage clamped squid axons. The project had two objectives: (1) to determine whether different methods of measuring the permeability of organic cations yield similar values and (2) to see whether neutral analogs of the organic cations can permeate the Na channel. Our results show that the permeability ratio of sodium to a test ion depends upon the ionic composition of the solution used. This finding is consistent with the view put forward previously that the Na channel can contain more than one ion at a time. In addition, we found that the uncharged analogs of permeant cations are not measurably permeant through the Na channel, but instead probably pass through the lipid bilayer.

Key Words squid axon · voltage clamp · Na channel · influx · permeability · nonelectrolytes

Introduction

It has become increasingly clear that ion permeation through the sodium channel of nerve is not a simple process. Hille (1975*a*) showed that in myelinated nerve current carried by sodium and organic cations deviated significantly from that predicted by the "independence relation" of Hodgkin and Huxley (1952). He was able to qualitatively account for these results with a model of the sodium channel that represented the ion pathway as a series of free energy barriers and wells; the permeating ions jump from well to well with a rate that decreases with the height of the intervening energy barrier. Hille's model allowed only one ion in the pore at a time and featured a single rate-limiting barrier and one relatively deep well. Cahalan and Begenisich (1976) found that the selectivity of the sodium pore in squid axons, as determined by permeability ratios obtained from zero current potentials, depended upon the concentration of the internal permeant ion. This is one of several types of behaviors expected from pores that can be simultaneously occupied by more than one ion (Hille & Schwarz, 1978). Indeed, Begenisich and Cahalan (1980*a*,*b*) were able to quantitatively reproduce several types of experiments concerning ion permeation through squid axon sodium channels with a three barrier-two site multi-ion pore. The results of this work suggested that the sodium pore is not dominated by a single large energy barrier.

We report here experiments that further examine the permeation process in sodium pores in squid axons. We compare several methods of obtaining permeabilities and permeability ratios for sodium and the organic cations guanidinium and formamidinium. We have also measured the fluxes of some small nonelectrolytes. We find that different permeability ratios are obtained by different methods. The nonelectrolytes can cross the axon membrane, but we were unable to demonstrate any permeation through the pore by these molecules.

Our results are consistent with the view that the sodium pore is a multi-ion pore: it can simultaneously be occupied by at least two ions. The positive electronic charge on permeant cations significantly enhances their permeability compared with uncharged molecules of similar size and structure. The main pathway for nonelectrolyte permeability is probably through the lipid bilayer.

Materials and Methods

Membrane currents were measured with a 12-bit analog/digital converter controlled by a microcomputer (WOPR) of our own

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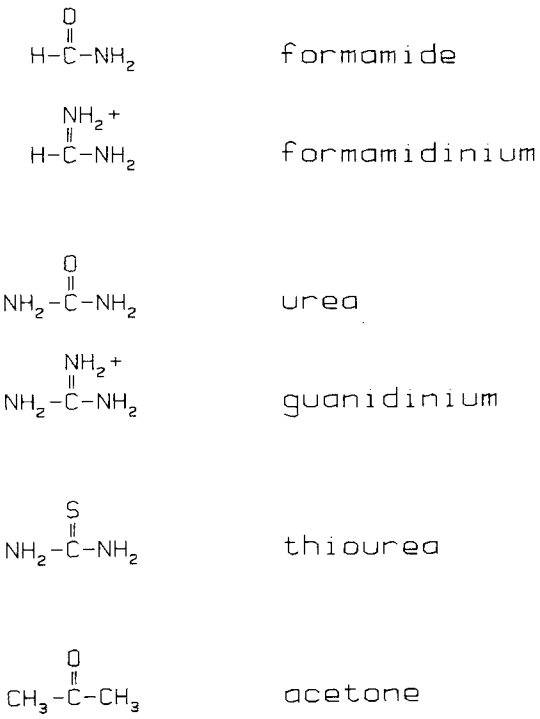

Fig. 1. Structures of the compounds used. Formamide-formamidinium and urea-guanidinium are paired to emphasize the structural similarity of the cationic and neutral molecules

design. Voltage-clamp pulse sequences in the electrical experiments were also generated by the computer. Data were stored on diskettes for subsequent analysis. Nonsodium channel currents were subtracted using an identical pulse pattern in the presence of 300 nM tetrodotoxin. All experiments were done at 10°C.

The structures of the organic compounds investigated in this study are shown in Fig. 1. These molecules are generally similar in size and structure. The guanidinium ion can, to an extent, be considered a charged analog of the nonelectrolyte urea. The carbonyl oxygen of urea is only slightly larger than the amino group of guanidinium. These same considerations apply to the formamide-formamidinium pair. Thiourea is very similar to urea but the sulfur atom (which makes this molecule less hydrophilic) forms weaker hydrogen bonds than the oxygen of urea. Acetone is even more hydrophobic than thiourea and has less hydrogen bonding capability.

Electrical Experiments

The electrical measurements reported here were made using axons from squid obtained at the Marine Biological Laboratory, Woods Hole, MA. The details of the internal perfusion and voltage-clamp techniques have previously been described (Begenisich & Lynch, 1974; Busath & Begenisich, 1982). Series resistance compensation was used in all experiments.

The standard artificial seawater (Na ASW) used in these experiments consisted of (in mM) 440 NaCl, 10 $CaCl_2$, 50 $MgCl_2$, 10 HEPES (pH = 7.5). A formamidinium solution (Form ASW) and a guanidinium seawater (Guan ASW) were made by replacing the NaCl of Na ASW with 440 mM of the acetate and chloride salts, respectively, of these compounds. The internal perfusion flow rate of 2–3 axon volumes/min should have minimized any acidification produced by the acetate seawater.

Table 1. Composition of internal solutions[a]

Name	Na	Cs	K	Form
KSIS	—	—	350	—
Na SIS I	50	150	—	—
Na SIS II	50	350	—	—
Form SIS	—	200	—	200

[a] Concentrations in mM.

The cation composition of the "standard" internal solutions (SIS) used are described in Table 1. The anions used were 50 mM fluoride and the remainder glutamate or acetate (in Form SIS only). Potassium channel currents in KSIS were blocked by 1 mM 3,4-diaminopyridine (3,4-DAP). The pH was brought to about 7.5 with 10 mM HEPES or 15 mM phosphate buffers. Glycine was added as necessary to maintain osmolarity.

In general, several methods employing electrical measurements can yield values for the relative permeabilities of two ions. The simplest method is to compare the current magnitude (at a particular voltage) when one cation is substituted for another. The method is subject to error if one of the ions has a pharmacological effect (i.e., reduces the number of active channels) or binds to a saturable site in the pore.

Hille (1971) described a method in which the change in sodium channel zero current or reversal potential (ΔV_{rev}) upon complete replacement of external sodium by another cation is used. The relevant equation is:

$$\Delta V_{rev} = RT/F \ln (P_X/P_{Na}). \quad (1)$$

P_X is the permeability of the replacement cation. A measurement of ΔV_{rev}, then, provides the means to calculate the P_X/P_{Na} ratio. The utility of this technique is that it is independent of the internal ionic composition.

In an analogous fashion internal sodium can be replaced by another cation with the composition of the external solution held constant. Under these conditions, the change in reversal potential also yields an estimate of the relative permeabilities using an equation similar to Eq. (1).

Another method is appropriate for biionic conditions in which, for example, Na is the only permeant species in the external solution and another cation (X) is in the internal solution. In this case:

$$V_{rev} = RT/F \ln (P_{Na}Na_o/P_X X_i). \quad (2)$$

The ionic conditions can be reversed to yield the following equation with one type of ion outside and sodium ions inside the cell:

$$V_{rev} = RT/F \ln (P_X X_o/P_{Na}Na_i). \quad (3)$$

In all these equations ion activities should, strictly speaking, be used rather than concentrations. However, activity coefficients for the organic cations are not available. Single ion activity coefficients can be calculated from the Debye-Hückel theory using the effective diameter of the hydrated ion. At a total salt concentration of 0.4 M the calculated activity coefficients for diameters

of 3.5 and 5 Å are 0.71 to 0.73, respectively. These coefficients are also functions of ionic strength, but are not expected to change much over the relatively small range of ionic strengths used in this study (Bockris & Reddy, 1970). Furthermore, in experiments designed to measure the change in reversal potential when sodium is replaced by a test cation, equal ionic strength solutions were used (e.g., Na SIS II and Form SIS). Consequently, we will consider all the activity coefficients equal and the above equations will be used as written.

All these methods should yield the same results if the ions cross the membrane in an independent fashion. Different values are, in general, expected for pores that violate this principle (Hille & Schwarz, 1978).

Flux Experiments

Permeabilities can also be obtained by measuring the movement of radiolabeled compounds across the membrane. Such a determination is similar to the comparison of ionic current described above. However, since only trace amounts of the permeating species are required, these measurements are less susceptible to possible pharmacological effects.

The axons used in the flux measurements were obtained from squid delivered to Rochester by the Arrive Alive Biological Supply Co., Greenport, NY. The techniques for measuring fluxes of the nonelectrolytes are similar to those described in Begenisich and Busath (1981). The chamber in which the axon was mounted had three sections, which were isolated from one another by Vaseline seals. This allows the measurement of electrical current and determination of tracer flux across the same membrane area.

Internal perfusate was collected by flowing isotonic sucrose at 1 ml/min across the cut end of the axon and into a fraction collector. Fractions were collected at 1-min intervals and counted on a Beckman scintillation counter. In some cases counts from two to four samples were averaged.

The nonelectrolytes were all ^{14}C labeled and used at concentrations of 1–4 mM with specific activities of about 30 to 150 μCi/mM. ^{14}C-guanidine-HCl (used at 1 mM) was obtained from Rose Chem Products, Los Angeles, CA; ^{14}C-urea (1 mM) from California Bionuclear, Sun Valley, CA; and ^{14}C-thiourea (1 mM), ^{14}C-formamide (3 and 4 mM), and ^{14}C-acetone (2 mM) from American Radiochemical Corp., Sanford, FL.

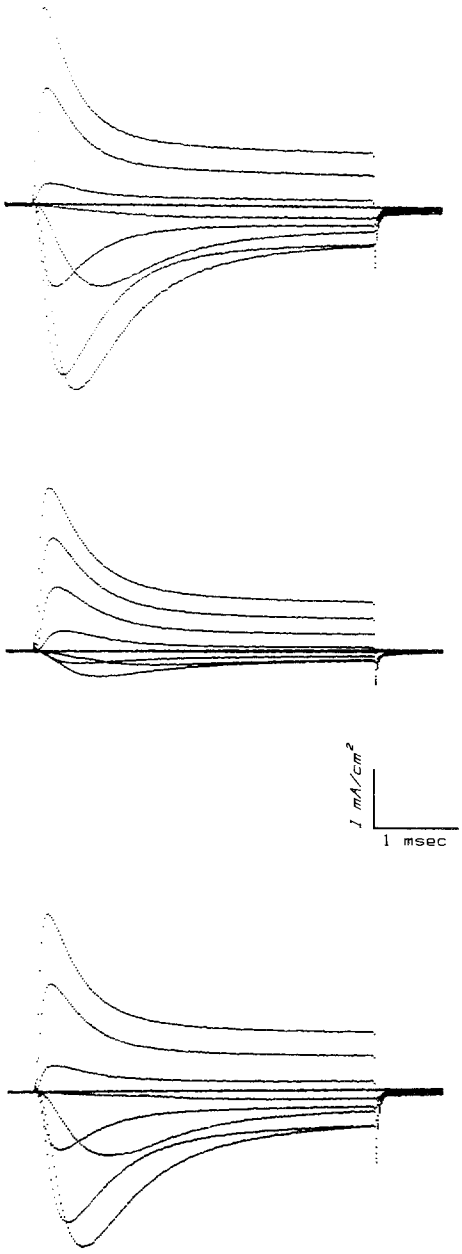

Fig. 2. Sodium channel currents in Na and Guan ASW. TTX-corrected currents are shown in response to step depolarizations of the membrane voltage from a holding potential of −69 mV. In each panel the currents from nine depolarizations separated by 20 mV are illustrated. The top panel shows control currents in Na ASW, the middle panel currents in Guan ASW, and the bottom panel currents again in Na ASW. Axon SQDRQ is perfused with Na SIS I

Results

Guanidinium

Figure 2 shows sodium channel currents recorded from an axon perfused with Na SIS I and bathed in Na ASW and Guan ASW. The large reversible reduction in inward currents and the more negative reversal potential in Guan ASW reflect the relatively low guanidinium permeability. These results are seen more clearly in Fig. 3*A*, which is a plot of peak sodium channel current as a function of membrane potential. The maximum inward current in Guan ASW is reduced to about 0.15 of the value in Na ASW.

A higher resolution plot of the same type of data is shown in Fig. 3*B* for potentials near the reversal potentials for each solution. The data are fitted (*not shown*) by quadratic functions of voltage in order to obtain reversal potential values. These values are 26.1 and 55.6 mV for Guan ASW and Na ASW,

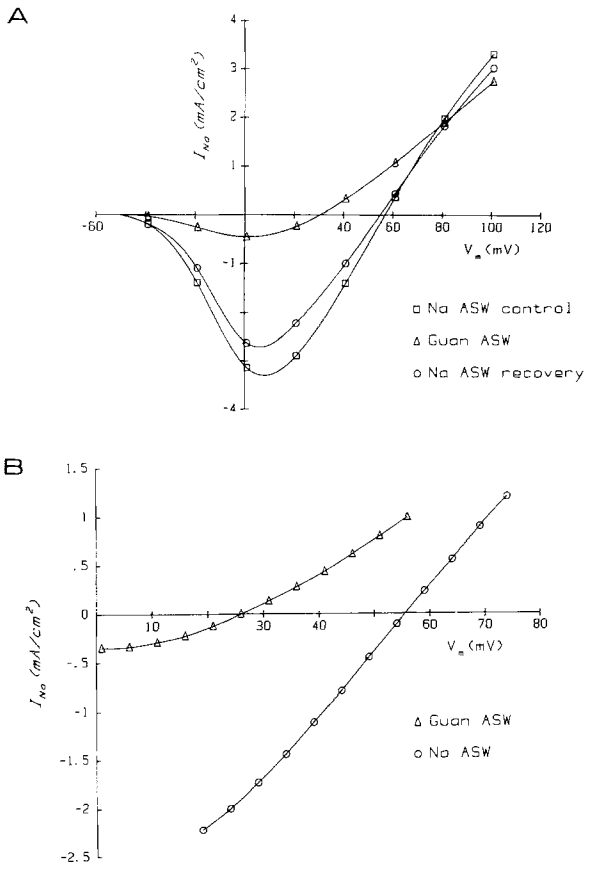

Fig. 3. Sodium channel current-voltage relations in Na and Guan ASW. (*A*): Peak currents from the records of Fig. 2 are plotted against membrane potential for Na ASW (□), Guan ASW (△), and again in Na ASW (○). Smooth curves are cubic spline functions to aid the eye and have no theoretical significance. (*B*): Sodium channel currents from the same axon are shown, but recorded at membrane potential increments of 5 mV near the reversal potential. Currents in Guan (△) and Na (○) ASW are shown. Axon SQDRQ is perfused with Na SIS I

Table 2. Determination of the guanidinium/sodium permeability ratio by various methods

External solution change method			
Exp	ΔV_{rev} (mV)	P_{Guan}/P_{Na}	Internal solution
SQDRN	32.2	0.27	K SIS
SQDRQ	29.7	0.30	Na SIS I
SQDRS	28.1	0.32	Na SIS I
SQDRS	30.7	0.29	K SIS
SQDSM	26.3	0.34	Na SIS I
Mean ± SEM		0.30 ± 0.012	
Biionic potential method			
Exp	V_{rev} (mV)	P_{Guan}/P_{Na}	Internal solution
SQDRQ	26.1	0.33	Na SIS I
SQDRS	23.3	0.29	Na SIS I
SQDRM	26.0	0.33	Na SIS I
Mean ± SEM		0.32 ± 0.013	
Flux method			
EXP	Vm (mV)	Na flux (pmol/cm² · sec)	Guan flux (pmol/cm² · sec)	P_{Guan}/P_{Na}
SQDPP	−34	220	0.1	0.20
SQDPQ	−34	218	0.14	0.28
SQDPQ	−34	343	0.19	0.24
Mean ± SEM				0.24 ± 0.023

respectively—a difference of 29.5 mV. The average reversal potential difference from several determinations in this axon was found to be 29.7 mV.

The relative permeability can be computed by solving Eq. (1) for, in this case, P_{Guan}/P_{Na}, which has a value of 0.30. This and several other determinations are listed in Table 2. An average value of 0.30 is obtained and does not significantly depend upon whether the internal solution is K SIS or Na SIS I.

The reversal potential in Guam ASW of Fig. 3*B* can be used in Eq. (2) to provide another measure of P_{Guan}/P_{Na}, since with Na SIS I internally the necessary biionic conditions are fulfilled. Such a computation yields a value of 0.33, very similar to the value obtained via the change of external solution method. This and two other determinations are listed in Table 2. An average P_{Guan}/P_{Na} value of 0.32 is obtained for these biionic conditions.

An illustration of an experiment in which the influx of radiolabeled guanidinium was measured is shown in Fig. 4. After a few minutes at a constant potential of −64 mV, 5-msec pulses to −34 mV were applied at 10 Hz for 6 min. A second stimulation period at 15 Hz produces more influx as expected. Tetrodotoxin was added at the time marked by the arrow. After a transient increase, the flux at the holding potential attains a constant (larger) value. Stimulation in the presence of TTX does not produce an increase in guanidinium influx, which demonstrates that the extra flux during stimulation occurs through sodium channels.

Integration of the sodium current during the pulses provides a measure of the Na influx. (At −34 mV essentially all the current is influx; *see* Begenisich & Busath, 1981). A measure of P_{Guan}/P_{Na} can be obtained from the ratio of the extra guanidinium flux and the sodium flux divided by their respective concentrations. The results of such calculations are shown in Table 2. An average value of 0.24 is obtained in this manner: only slightly smaller than the values from the other methods.

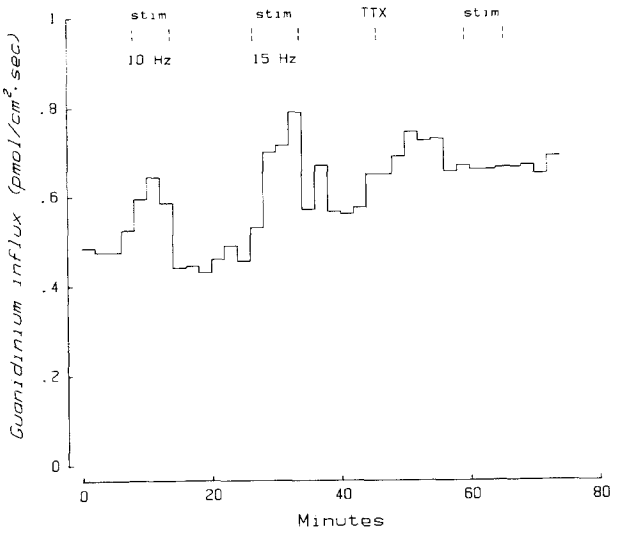

Fig. 4. Guanidinium flux through sodium channels. Influx of radiolabeled guanidinium is plotted versus time. During the periods marked *stim*, 5-msec pulses to −34 mV were applied at the frequencies shown (10 Hz for the third period). 300 nM tetrodotoxin (TTX) was applied at the time shown. Two 1-min samples were averaged for flux determination. Axon SQDPQ was bathed in Na ASW and perfused with K SIS + 1 mM 3,4 DAP

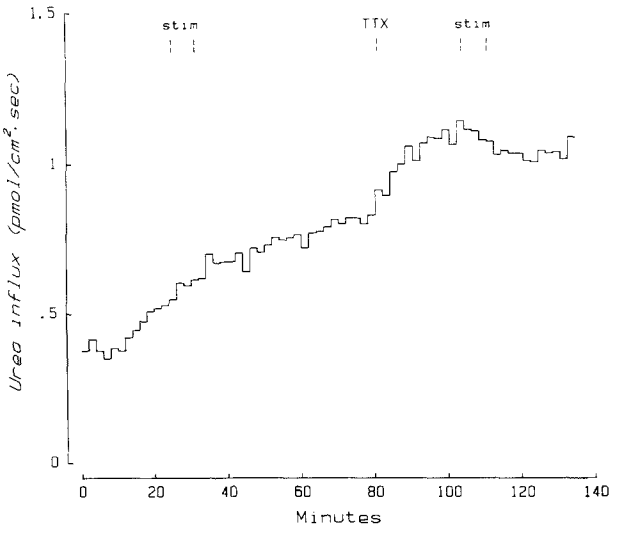

Fig. 5. Urea flux. Influx of radiolabeled urea is plotted versus time. During the periods marked *stim*, 5-msec pulses to 11 mV were applied at 10 Hz. TTX (330 nM) was applied at the time shown. Two 1-min samples were averaged for flux determination. AXON SQDOX was bathed in Na ASW and perfused with Na SIS I

Urea

Figure 5 illustrates an experiment in which the influx of urea was measured. The membrane potential was maintained at −69 mV except during the periods marked *Stim*, during which 5-msec pulses to 11 mV were applied at 10 Hz. No increase in flux is seen during these periods either in the presence or absence of TTX. The flux during the early part of this experiment (0.7 pmol/cm²-sec) is listed in Table 3 along with values from several other experiments. Also listed in the table are estimates of sodium influx obtained by integrating the current during the voltage-clamp pulses. No evidence of urea flux through sodium channels was found in any of these experiments.

An upper limit to the P_{Urea}/P_{Na} value can be estimated in the following way. The variation in the baseline flux in Fig. 5 is such that an increase in flux of 10% of the baseline might just barely have been detectable. The urea flux must, therefore, be less than 0.07 pmol/cm²-sec. The Na flux in this experiment was 220 pmol/cm²-sec. The concentration of Na was 440 mM and that of urea was 1 mM. Thus the P_{Urea}/P_{Na} ratio must be less than 0.14 ((0.07/220)(440/1)). Upper limits of 0.019, 0.055, and 0.13 were estimated in experiments SQDOU, SQDOL, and SQDOM, respectively. (Any accurate comparison of the urea and Na permeabilities must allow for the influence of membrane potential of Na ions. Most of

Table 3. Nonelectrolyte fluxes and permeabilities

Exp	Resting flux (pmol/cm² · sec)	Resting perm ×10⁻⁶ (cm/sec)	Stimulated Na flux (pmol/cm² · sec)
Urea			
SQDOL	0.4	0.4	320
SQDOM	0.8	0.8	268
SQDOU	0.3	0.3	691
SQDOX	0.7	0.7	220
Mean ± SEM		0.55 ± 0.12	
Formamide			
SQDEG	60	6.0	200
SQDPD	22	4.4	328
SQDPE	25	8.3	151
SQDPF	20	6.7	266
SQDPH	20	6.7	451
Mean ± SEM		6.5 ± 0.63	
Thiourea			
SQDOY	5.0	5.0	200
SQDOZ	2.0	2.0	338
SQDPA	5.0	5.0	161
SQDPB	2.0	2.0	230
Mean ± SEM		3.5 ± 0.87	
Acetone			
SQDPI	15	7.5	169
SQDPJ	25	12.5	294
SQDPK	15	7.5	120
SQDPL	20	10	545
Mean ± SEM		9.4 ± 1.2	

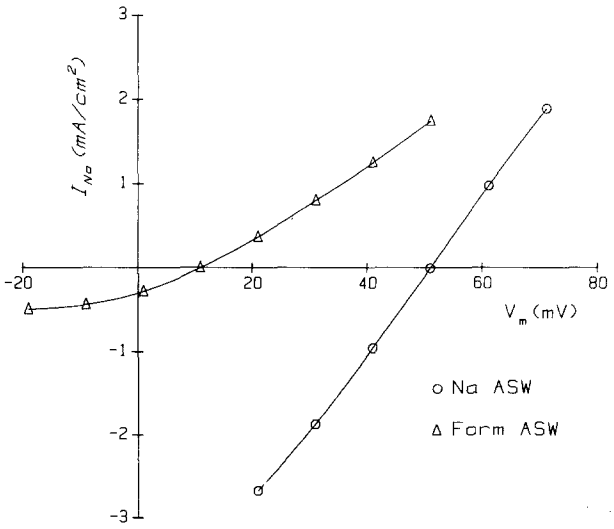

Fig. 6. Sodium channel current-voltage relations in Na and Form ASW. Peak currents in Na (○) and Form (△) ASW are shown. The solid lines are cubic spline curves. Axon SQDSL was perfused with Na SIS I

the experiments with urea were done at potentials near zero mV where the effects of voltage will be minimal.) In any case, it is clear that urea is far less permeant (if at all) through sodium channels than is guanidinium.

Formamidinium

An experiment demonstrating the change in reversal potential when Na ASW is replaced by Form ASW is shown in Fig. 6. In Na ASW the reversal potential is 50.6 mV and is 10.7 mV in Form ASW. There is then a change of 39.9 mV which, from Eq. (1), corresponds to a P_{Form}/P_{Na} of 0.20. Table 4 provides a summary of similar experiments. The mean value of P_{Form}/P_{Na} from experiments using external solution changes is 0.20, independent of the type of internal solution.

The reversal potential measured in Form ASW (Fig. 6) provides another estimate of the P_{Form}/P_{Na} ratio. The internal solution in this case is Na SIS I, so the biionic conditions appropriate for Eq. (2) are satisfied. This calculation yields a value of 0.18 very similar to the value of 0.20 obtained in this axon with the external solution change method. Results from this and three other experiments with Na SIS I are listed in Table 4. The average value of P_{Form}/P_{Na} of 0.19 is essentially the same as that obtained with the external solution change method.

The biionic potential method was applied with two other sets of solutions as shown in Table 4. P_{Form}/P_{Na} with Form ASW externally and Na SIS II inside was found to be about 0.29, the same as that with Na ASW and Form SIS. In spite of the relatively few experiments, the results are quite consistent and these values are considerably larger than the value of 0.19 to 0.20 found using the external change method and with the biionic potential method with Na SIS I.

The results above suggest that the P_{Form}/P_{Na} ratio depends on the type of solutions used. The point is made more convincingly by experiments of the type illustrated in Fig. 7. In this experiment the external solution was Na ASW and the axon was perfused with both Form SIS and Na SIS II. The change in V_{rev} for these two conditions was 15.7 mV from which a P_{Form}/P_{Na} value of 0.13 is computed using an equation similar to Eq. (1) but appropriate for internal solution changes. A value of 0.14 was found in another experiment (*see* Table 4). These two values are much smaller than those obtained using the other methods. In these same two axons

Table 4. Determination of the formamidinium/sodium permeability ratio by various methods

Exp	ΔV_{rev} (mV)	P_{Form}/P_{Na}	Internal solution
External solution change method			
SQDSF	36.3	0.23	K SIS
SQDSK	35.3	0.24	K SIS
SQDSK	43.3	0.17	Na SIS I
SQDSL	39.9	0.20	Na SIS I
SQDSM	37.6	0.22	Na SIS I
SQDTI	40.5	0.19	Na SIS II
SQDTJ	42.9	0.17	Na SIS II
Mean ± SEM		0.20 ± .011	
Biionic potential method			
Exp	V_{rev} (mV)	P_{Form}/P_{Na}	Solutions: External//internal
SQDSF	14.7	0.21	Form ASW//Na SIS I
SQDSK	8.1	0.16	Form ASW//Na SIS I
SQDSL	10.7	0.18	Form ASW//Na SIS I
SQDSM	14.7	0.21	Form ASW//Na SIS I
Mean ± SEM		0.19 ± 0.12	
SQDTI	24.1	0.30	Form ASW//Na SIS II
SQDTJ	22.3	0.28	Form ASW//Na SIS II
SQDTI	49.8	0.29	Na ASW//Form SIS
SQDTJ	49.5	0.29	Na ASW//Form SIS
Change in internal solution method			
Exp	ΔV_{rev} (mV)	P_{Form}/P_{Na}	External solution
SQDTI	4.2	0.21	Form ASW
SQDTJ	2.7	0.22	Form ASW
SQDTI	14.8	0.14	Na ASW
SQDTJ	15.7	0.13	Na ASW

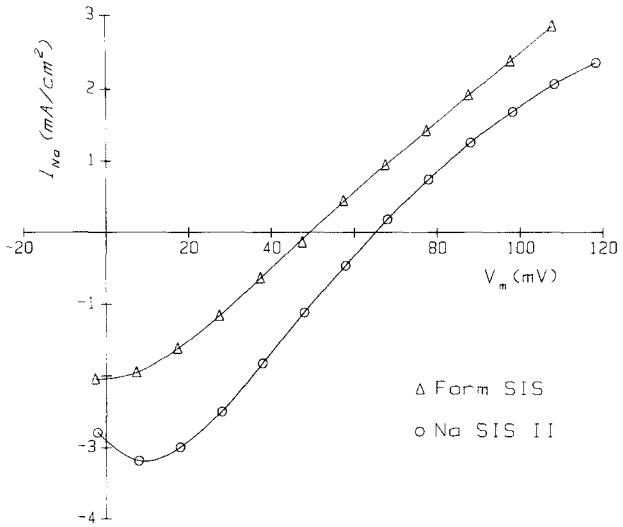

Fig. 7. Sodium channel current-voltage relations with internal Na and formamidinium. Peak currents in Form SIS (△) and Na SIS II (○) are shown. The solid lines are cubic spline curves. Axon SQDTJ was bathed in Na ASW

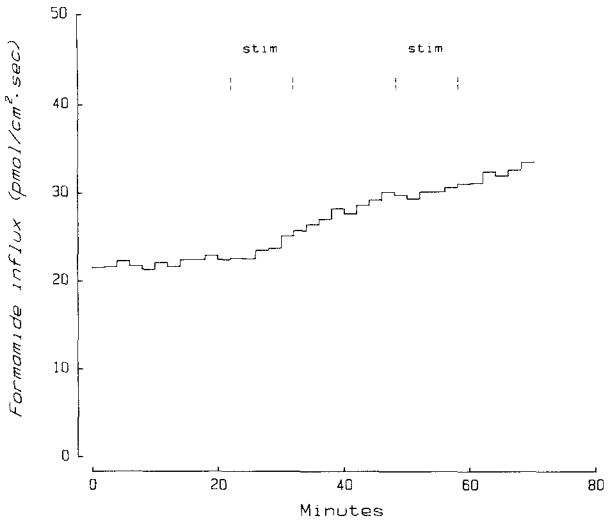

Fig. 8. Formamide flux. Influx of radiolabeled formamide is plotted versus time. During the first period marked *stim*, 5-msec pulses to −14 mV were applied at 10 Hz. Pulses to −19 mV were used during the second stimulation period. Two 1-min samples were averaged for flux determination. Axon SQDPH was bathed in Na ASW and perfused with Na SIS I

this same internal solution change technique yields much larger values of 0.21 and 0.22 (Table 2). These values are quite similar to those obtained from external changes and biionic potentials with Na SIS I. The consistency of the results from two different axons and, in particular, the large difference between P_{Form}/P_{Na} values obtained on the same axon but with different solutions, suggests that these differences are not fortuitous. Rather, it appears that the relative formamidinium/sodium permeability depends on the ionic composition of the solutions used.

Formamide

Unfortunately, since radiolabeled formamidine was not available at the time these experiments were being done, the flux method of determining formamidinium permeability could not be used. However, the structurally similar neutral molecule formamide, was available with a ^{14}C label. An example of an experiment in which the influx of formamide was measured is shown in Fig. 8. The baseline influx is rather constant initially but increases after 25 min. There was no evidence of an increase in flux during periods of repetitive application of voltage-clamp pulses in this nor in any of four similar experiments.

The large resting flux of formamide does not allow a useful estimate of an upper limit of its permeability relative to that of sodium. For example, a calculation similar to that given for urea indicates that the permeability of formamide relative to Na would have to have been greater than 0.65 to have been detected.

Other Nonelectrolytes

Table 3 lists the resting fluxes of urea and formamide and two other nonelectrolytes: thiourea and acetone. The resting flux of thiourea is near that of formamide and consequently is too large to allow determination of a meaningful upper limit for its permeability through sodium channels. This is also true for acetone, which has the largest resting permeability of all the nonelectrolytes tested.

The permeability of lipophilic substances may be limited by an aqueous unstirred layer. This is not the case for even the most permeable nonelectrolytes used in this study. An unreasonably large unstirred layer of about 1 cm would have an effective permeability equal to that of acetone (*see* Hidalgo & Latorre, 1970*a*). An unstirred layer of a few hundred μm is possible, but would constitute an effective permeability barrier several orders of magnitude too small to be significant.

The permeabilities of the nonelectrolytes are not very dependent on the electrical "leak" conductance of the axons. A plot of relative nonelectrolyte permeability as a function of relative leak conductance is shown in Fig. 9. Each type of symbol in this figure represents a different compound. The permeabilities have been scaled so that the mean

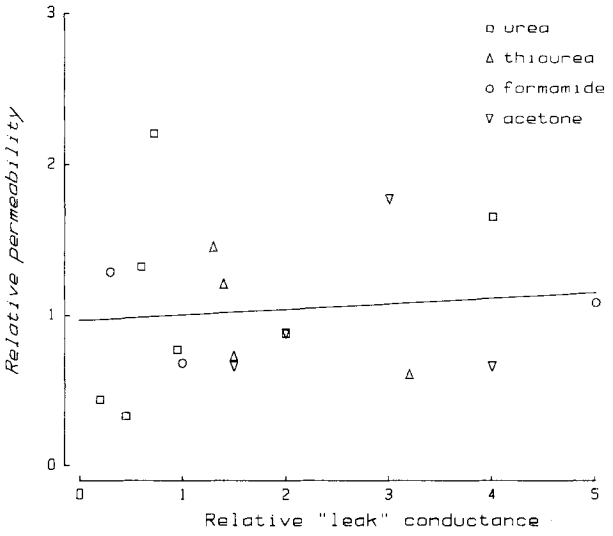

Fig. 9. Nonelectrolyte permeability and electrical conductance. The relative permeability of the nonelectrolytes (scaled so that the mean permeability of each compound is unity) is plotted against relative leak conductance determined either from currents in the presence of TTX or from a small hyperpolarizing pulse of membrane potential. Each type of symbol corresponds to a different compound, and each symbol represents a single axon. The line is a least squares fit of a linear relation between permeability and conductance

permeability for each compound is the same. While there is a large degree of scatter, there is little correlation between permeability and leak conductance. The line is a least-squares fit of a linear relation between permeability and leak conductance to all the data. There is also little or no correlation for any individual nonelectrolyte.

The values of permeability used in Fig. 9 were determined at the beginning of the experiments. As can be seen in Figs. 5 and 8, there is a tendency for the baseline flux to gradually increase during the course of an experiment. This suggests that there may be some nonelectrolyte flux through the ionic leak conductance pathway which becomes appreciable at increasingly higher leak conductance levels.

Discussion

Comparison to Previous Results

The external change, biionic potential (with Na ASW, Na SIS I or K SIS), and tracer flux methods all yield P_{Guan}/P_{Na} values near 0.25 to 0.30. No experiments were done in this study with a guanidinium internal solution, but Cahalan and Begenisich (1976) found a value of about 0.5 with 50 mM and about 1 with 200 mM guanidinium inside squid axons bathed in Na ASW.

Hironaka and Narahashi (1977) used the external change method and obtained a value of 0.2 for both P_{Guan}/P_{Na} and P_{Form}/P_{Na}, essentially the same as our values. These authors used an internal solution very similar to our K SIS. There is also a report (*unpublished observations*) of a determination of P_{Guan}/P_{Na} in voltage clamped squid axons (*see* Meves, 1970). The method used is not clear but most likely the biionic potential was measured on axons perfused with a sodium solution and bathed in a guanidinium seawater. A value near 0.25 was found, also very similar to our results under similar ionic conditions.

Hille (1971) found a value of 0.13 using an external solution change method on myelinated nerve. It appears that the sodium pore in myelinated nerve is generally less permeable than the pore in squid axons. This may represent a species difference, or may reflect the different ionic concentrations of these preparations.

An efflux of radiolabeled guanidinium across squid axon membranes was observed by Tasaki and Spyropoulos (1961). An increase in efflux over resting levels was found when the axon was stimulated to produce action potentials at a high rate and when the axon was depolarized by potassium concentration. These types of experiments cannot yield a quantitative permeability measurement nor can they even identify the channel through which guanidinium passes since either potassium or sodium channels could be involved. Considering the very few cations that are able to permeable potassium channels (Hille, 1975*b*), these results are most likely due to guanidinium permeating sodium channels.

Formamidinium seems to be slightly less permeant through sodium channels than guanidinium when measured with the same methods. The external solution change and biionic potential method (with Na SIS I) yield values of P_{Form}/P_{Na} of 0.19–0.20, a bit smaller than the corresponding P_{Guan}/P_{Na} values of 0.30–0.32. Higher values of about 0.3 for formamidinium are obtained with the biionic potential method with different internal and external solutions (*see* Table 4). Smaller values near 0.14 are found when the internal solution change method is used. The relative formamidinium permeability depends not only on the method used for its measurements but also depends on the solutions used for any particular method.

We find no evidence that any of the small electrolytes tested (formamide, urea, thiourea, and acetone) can permeate sodium channels in squid axons. If urea permeates sodium channels, it does so 7–50

times less well than Na ions. The resting axon membrane is sufficiently permeable to the other nonelectrolytes that a meaningful upper limit to their permeabilities through sodium channels cannot be determined.

These results are consistent with experiments in which urea (Tasaki & Spyropoulos, 1961; Hidalgo & Latorre, 1970*b*) and thiourea (Tasaki & Spyropoulos, 1961) fluxes were measured in squid axons. No increase in these fluxes were detected when the axons were stimulated to produce a high rate of action potentials.

In contrast, Huang, Catterall and Ehrenstein (1979) were able to detect both formamide and urea fluxes through batrachotoxin-treated sodium channels in two neuroblastoma cell lines. The urea: sodium permeability ratios were 0.04 and 0.03 and for formamide values of 0.11 and 0.05 were found for the two cell lines. Since batrachotoxin increases sodium channel permeabilities, the permeability ratios for untreated channels are, no doubt, smaller. Such permeabilities are probably too small to have been resolved by our techniques.

Hidalgo and Latorre (1970*a*) measured resting fluxes of several nonelectrolytes across squid membranes. They obtained resting permeabilities for urea and thiourea of about 0.5 and 3×10^{-6} cm/sec, very similar to those shown in Table 3.

Mechanism of Nonelectrolyte Permeability in Squid Axons

Our results demonstrate that the small nonelectrolytes tested permeate the sodium channel poorly, if at all. The action potential experiments of Tasaki and Spyropoulos (1961) and Hidalgo and Latorre (1970*b*) suggest that these compounds are also very impermeant to potassium channels which are opened during an action potential. We also showed that the nonelectrolytes do not readily pass through the pathway that provides the ionic leak conductance. We therefore conclude that these molecules cross membranes primarily through the lipid bilayer portion. Finkelstein (1976) found urea and formamide permeabilities through lecithin/cholesterol membranes of 0.6 and 27×10^{-6} cm/sec and even larger values for pure lecithin membranes. Our values are well within this range.

Except for formamide the permeabilities of the nonelectrolytes are in the same order as the oil/water partition coefficients. However, the nonelectrolytes need not cross the bilayer via the "classic" solubility-diffusion mechanism. This may contribute, but these molecules (especially formamide) are small enough to also cross the bilayer through "kinks" in the hydrocarbon chains of the phospholipids (Träuble, 1971). Finkelstein (1976) suggests that this may account for the large water and formamide permeability in artificial bilayers.

Sodium Channel Permeability

We have shown that the permeability of formamidinium relative to that of Na depends on the method and on the types of solutions used. The data presented here combined with results from Cahalan and Begenisich (1976) indicate the same is true for guanidinium. This dependence of permeability ratio on method and on ionic concentration is expected for pores that can be simultaneously occupied by more than one ion (Hille & Schwarz, 1978; Begenisich & Cahalan, 1980*a*,*b*). The permeation properties will, in general, be different for a pore with one Na ion, or one with two Na ions, or a pore with one Na ion and one guanidinium ion, etc.

Permeation through the sodium pore is potentiated if the permeating species is charged. We find that urea is at least 15 times less permeant than guanidinium ions. This corresponds to the charge lowering an energy barrier for permeation by about 1.6 kcal/mole. It may be that the molecular groups in the pore composing the energy barrier contain a negative charge. The interaction of a positive charge with this site results in a smaller net energy. Thus it would be easier for an ion to cross the pore than a neutral molecule.

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References

Begenisich, T., Busath, D. 1981. Sodium flux ratio in voltage-clamped squid giant axons. *J. Gen. Physiol.* **77**:489–502

Begenisich, T., Cahalan, M. 1980*a*. Sodium channel permeation in squid axons. I. Reversal potential experiments. *J. Physiol. (London)* **307**:217–242

Begenisich, T., Cahalan, M. 1980*b*. Sodium channel permeation in squid axons: II. Non-independence and current-voltage relations. *J. Physiol. (London)* **307**:243–257

Begenisich, T., Lynch, C. 1974. Effects of internal divalent cations on voltage-clamped squid axons. *J. Gen. Physiol.* **63**:675–689

Bockris, J.O'M., Reddy, A.K.N. 1970. Modern Electrochemistry. Vol. 1. Plenum, New York

Busath, D., Begenisich, T. 1982. Unidirectional sodium and potassium fluxes through the sodium channel of squid giant axons. *Biophys. J.* **40:**41–49

Cahalan, M., Begenisich, T. 1976. Sodium channel selectivity: Dependence on internal permeant ion concentration. *J. Gen. Physiol.* **68:**111–125

Finkelstein, A. 1976. Water and nonelectrolyte permeability of lipid bilayer membranes. *J. Gen. Physiol.* **68:**127–135

Hidalgo, C., Latorre, R. 1970*a*. Temperature dependence of non-electrolyte and sodium permeability in giant axon of squid. *J. Physiol. (London)* **211:**173–191

Hidalgo, C., Latorre, R. 1970*b*. Effect of stimulation and hyperpolarization on non-electrolyte and sodium permeability in perfused axons of squid. *J. Physiol. (London)* **211:**193–202

Hille, B. 1971. The permeability of the sodium channel to organic cations in myelinated nerve. *J. Gen. Physiol.* **58:**599–619

Hille, B. 1975*a*. Ionic selectivity, saturation, and block in sodium channels. A four-barrier model. *J. Gen. Physiol.* **66:**535–560

Hille, B. 1975*b*. Ionic selectivity of Na and K channels of nerve membranes. *In:* Membranes. Vol. 3: Lipid Bilayers and Biological Membranes: Dynamic Properties. G. Eisenman, editor. pp. 255–322. Marcel Dekker, New York

Hille, B., Schwarz, W. 1978. Potassium channels as multiion single-file pores. *J. Gen. Physiol.* **72:**409–442

Hironaka, T., Narahashi, T. 1977. Cation permeability ratio of sodium channels in normal and Grayanotoxin-treated squid axon membranes. *J. Membrane Biol.* **31:**359–381

Hodgkin, A.L., Huxley, A.F. 1952. Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J. Physiol. (London)* **116:**449–472

Huang, L.M., Catterall, W.A., Ehrenstein, G. 1979. Comparison of ionic selectivity of batrachotoxin-activated channels with different tetrodotoxin dissociation constants. *J. Gen. Physiol.* **73:**839–854

Meves, H. 1970. The ionic permeability of nerve membranes. *In:* Permeability and Function of Biological Membranes. L. Bolis, editor. p. 261. North Holland, Amsterdam

Tasaki, I., Spyropoulos, C.S. 1961. Permeability of the squid axon membrane to several organic molecules. *Am. J. Physiol.* **201:**413–419

Träuble, H. 1971. The movement of molecules across lipid membranes: A molecular theory. *J. Membrane Biol.* **4:**193–208

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