Sodium Flux through the Sodium Channels of Axon Membrane Fragments Isolated from Lobster Nerves

FLOR V. BARNOLA and RAIMUNDO VILLEGAS

From the Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas (IVIC), Caracas 101, Venezuela

ABSTRACT The efflux of ^{22}Na from vesicles formed by axolemma fragments isolated from lobster nerves was studied in the presence and in the absence of drugs having well-known action on the sodium channels. The vesicles were equilibrated 12–14 h at 4°C with ^{22}Na in lobster solution containing 1 mM ouabain. Afterwards the suspension was divided: one portion was used as control and the others were treated with veratrine (0.025–0.50 mg/ml), tetrodotoxin (1–2,000 nM) in the presence of veratrine, or tetrodotoxin alone. After 3 h at 20–22°C, the suspensions were diluted into nonradioactive solutions and the ^{22}Na efflux followed by a rapid filtration technique. The results revealed that veratrine increases the efflux rate and the additional application of tetrodotoxin abolishes it, e.g., 0.50 mg of veratrine/ml increases the rate, expressed in 10^{-2} min^{-1}, from 0.59 ± 0.04 (mean ± SEM; n = 13) to 0.86 ± 0.05 (n = 13), and the addition of 100 nM tetrodotoxin diminishes it to 0.48 ± 0.07 (n = 4). This increase and diminution are statistically significant ($P < 0.005$), but this is not the case between the control and the veratrine plus tetrodotoxin values ($P > 0.05$). 50% of the diminution is produced by 11.9 ± 2.4 nM tetrodotoxin. Tetrodotoxin alone produces a slight diminution of the ^{22}Na efflux. Batrachotoxin (0.50 μM) has an action similar to veratrine's. These findings are considered evidence of the presence of functioning sodium channels in the isolated axolemma fragments.

INTRODUCTION

The isolation of the axon plasma membrane (axolemma) from squid (1–3), lobster (4, 5), and garfish (6) nerves has been accomplished in recent years. In some of these axolemma preparations, as well as in some axolemma-enriched total nerve plasma membrane preparations, specific tetrodotoxin receptors have been demonstrated (5, 7, 8). However, the presence of functioning ionic channels remains to be shown since the precise structural and topological relationship between channels and receptors is unknown.

The purpose of this work was to investigate the presence of functioning sodium channels in the fragments of axolemma isolated from lobster walking-leg nerves. These axolemma fragments, whose tetrodotoxin receptors had been previously characterized (5), form vesicles of a rather small size. To search for

the sodium channels in the vesicles, we essentially followed the methodology developed by Kasai and Changeux (9) for studying the efflux of ^{22}Na from vesicles formed by isolated electroplaque plasma membranes, and the approach of Henderson and Strichartz (10) for measuring the tetrodotoxin action in blocking the sodium flux through the channels of intact garfish axons. As Henderson and Strichartz did, we used veratrine or batrachotoxin to open otherwise inactivated sodium channels (11–15). To identify the channels used by ^{22}Na to cross the axolemma, we have also relied on the specificity of tetrodotoxin for blocking the sodium channels (16, 17). A preliminary report of this work has already been made (18).

EXPERIMENTAL METHODS

Isolation of the Axonal Plasma Membrane

The axolemma preparations used in the present work were obtained from the walking-leg nerves of living lobsters *Panulirus argus*. The axolemma was isolated following the method described for the squid nerves (1) as adapted to lobster nerves (5). A detailed description of the method is given in reference 5. At the end of the isolation procedure, the axolemma fragments (membrane fraction I of reference 5) were suspended in the appropriate solution. When they were observed at the electron microscope, they appeared as closed vesicles approximately spherical in shape with a diameter of about 0.24 μm, bound by a single membrane (Gloria M. Villegas and the authors of the present article, unpublished results).

Measurement of the ^{22}Na Efflux from the Axolemma Vesicles

The method used to determine the sodium efflux from the vesicles was essentially that described by Kasai and Changeux (9). The vesicles were first loaded with ^{22}Na by incubating them during 12–14 h at 4°C in lobster physiological solution containing 1 mM ouabain (Sigma Chemical Co., St. Louis, Mo.) and a tracer amount of $^{22}NaCl$ (Radiochemical Centre, Amersham, England). Ouabain was used to inhibit any remaining residue of the sodium pump. The composition of the lobster solution is NaCl 465 mM; KCl 10 mM; $MgCl_2$ 8 mM; $CaCl_2$ 25 mM; and Tris-HCl 10 mM. The pH was adjusted to 7.5.The final specific activity of ^{22}Na in the loading solution was about 20 μCi/mmol of sodium. The protein content of the membrane suspension, measured by the method of Lowry et al. (19), was adjusted to 2.5–5.0 mg of membrane protein per milliliter for the present experiments.

At the end of the 12–14-h loading period, the axolemma suspension was divided into different samples: one for control and the others to be treated with veratrine (Sigma Chemical Co., St. Louis, Mo.), with tetrodotoxin (Sankyo Chemical Co., Tokyo, Japan), or with tetrodotoxin in the presence of veratrine. Veratrine was directly dissolved in the lobster physiological solution, while tetrodotoxin was added from a 0.2 or a 2 mM stock solution in 10 mM acetic acid. The same volume of acid was added to the control and the veratrine-treated samples. The pH remained at 7.5 due to the presence of 10 mM Tris-HCl. The final veratrine concentrations were between 0.025 and 0.50 mg/ml, and those of tetrodotoxin were between 1 and 2,000 nM. Batrachotoxin (a kind gift from Dr. Bernhard Witkop) or batrachotoxin together with tetrodotoxin were used in some of the experiments. Batrachotoxin was added to a final 0.50 μM concentration from a 20 μM stock solution in 10% ethanol. The same volume of 10% ethanol was added to the other samples, the final concentration being 0.25%.

Once the drugs were added, all the samples, including controls, were kept 3 h at 20–22°C. At the end of this term, the measurement of the efflux was started by a 50-fold dilution of 200 or 250 μl of the respective sample into a nonradioactive solution, which was otherwise identical to the loading solution. At given times, 1.0-ml samples were quickly filtered on Millipore filters (HAW P 025 00, 25 ea. HA 0.45 μ plain white, 25 mm; Millipore Filter Co., Bedford, Mass.) and washed three times with 3 ml of lobster physiological solution. In close agreement with the observation of Kasai and Changeux (9), we found that under these conditions 95% or more of the vesicles remains on the Millipore. The filters were then dried and soaked in 10 ml of Instagel counting solution (Packard Instruments Co., Downers Grove, Ill.). The samples were counted in a liquid scintillation spectrometer (Tricarb, model 3320, Packard). The standard deviation in net counting was about 2.5%. The ^{22}Na content of the vesicles was estimated by subtracting from the total counts in the Millipore, first, the background, and second, the counts due to the finite dilution of the suspension of vesicles, i.e., the net number of counts in the filter divided by 50, the dilution factor. The background is the radioactivity retained by the filter in the absence of vesicles, which depends on the batch of Millipores.

RESULTS AND DISCUSSION

Efflux of ^{22}Na from the Axolemma Vesicles

The results obtained in 18 preparations of axolemma were used to characterize the kinetics of efflux of ^{22}Na from control vesicles treated with veratrine (0.50 mg/ml), and vesicles treated with tetrodotoxin (100 nM) in the presence of veratrine (0.50 mg/ml). As described under Methods, the samples of vesicles previously loaded with ^{22}Na were diluted into nonradioactive solutions that were otherwise identical to the loading media, and the ^{22}Na content of the vesicles, as function of time, was followed. The first measurement of the ^{22}Na was always made 1 min after dilution, and then either every 2.5 min up to 10 min, or every 10 min up to 1 h.

Note that at the end of the loading term, assuming that the ^{22}Na influx is equal to the measured efflux, at least 99% of the intravesicular sodium should be equilibrated with that present in the external solution because the highest half-time of efflux for the control vesicles (Table I, control efflux rate, 0.59×10^{-2} min^{-1}) was 1.96 h and the total loading period was always 15–17 h (see Methods). Due to the absence of active transport and to the ionic axolemma permeability, in all likelihood, the membrane potential was zero and the sodium potassium concentrations were the same inside and outside the vesicles at the time the efflux was measured. This is supported by results obtained in live garfish axons under similar experimental conditions (10).

Fig. 1 shows the ^{22}Na contents of the vesicles plotted as a function of time on a semilogarithmic scale. The time-course of the ^{22}Na efflux appears to be well fitted by two exponentials: a fast one, corresponding approximately to the 1–10-min period, and a slow one, to the 10–60-min term. The continuous lines are least-squares fits to the data. The experimental values that form the slow phases (which are mean ± SEM of 13 samples, except for the experiments with tetrodotoxin which are mean ± SEM of 4 samples), were always found to lie on a straight line. The rates of efflux of ^{22}Na calculated from these slow phase

data, expressed in 10^{-2} min^{-1}, are: 0.59 ± 0.04 for the controls, 0.86 ± 0.05 for the veratrine-treated vesicles, and 0.48 ± 0.07 for the veratrine plus tetrodotoxin-treated vesicles. The rates of efflux of the fast phases were calculated subtracting the contribution of the slow phase from the initial part of the curve (whose values are the mean ± SEM of five samples). The following rates in min^{-1} were obtained: 0.20 ± 0.05 for the controls, 0.30 ± 0.07 for the veratrine-treated vesicles, and 0.19 ± 0.08 for the veratrine plus tetrodotoxin-treated vesicles. All the values given above are summarized in Table I. Since the difference between the fast and slow rates is greater than one order of magnitude, the fast component should not appreciably affect the slow rate.

As will be dealt with in further detail later on, these results reveal that veratrine increases the efflux rate of the slow component and tetrodotoxin brings it back to a value similar to the control's. The veratrine-induced increase

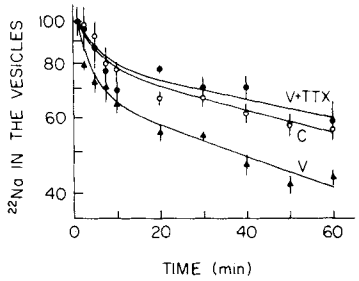

FIGURE 1. Efflux of ^{22}Na from axolemma vesicles. C corresponds to controls, V to vesicles treated with veratrine (0.50 mg/ml), and V + TTX to those treated with tetrodotoxin (100 nM) in the presence of veratrine (0.50 mg/ml). The vesicles were previously equilibrated with ^{22}Na lobster physiological solution containing 1 mM ouabain, pH 7.5, and the efflux was carried out in a nonradioactive solution which otherwise was identical to the loading solution. Values are relative to the 1-min measurement. Efflux measured at 20–22°C. For further details see text.

and the diminution caused by the additional application of tetrodotoxin are both statistically significant ($P < 0.005$), while the control and the veratrine plus tetrodotoxin values are not statistically different ($P > 0.05$). The fastest component follows the same tendency, but its changes are not statistically significant. There is, therefore, little doubt that the slow phase is related to the outward movement of ^{22}Na from inside the vesicles that have well-preserved sodium channels and receptors accessible to the drugs. Similar effects to those caused by veratrine and tetrodotoxin on the slow phase were produced by batrachotoxin and the additional application of tetrodotoxin, i.e, 0.50 μM batrachotoxin increases the rate, expressed in 10^{-2} min^{-1}, from 0.60 to 0.98, and tetrodotoxin diminishes it from 0.98 to 0.72 (see Methods). Since these results are from two axolemma preparations (four experiments), no statistical analysis was made, and they should be considered to indicate only that the isolated axolemma behaves towards batrachotoxin qualitatively like that of intact axons. A larger amount of batrachotoxin, not available at present, would be required for more detailed

study. Therefore, only different concentrations of veratrine and tetrodotoxin on the slow phase were assayed in order to quantify their effects on the vesicles' channels. This will be dealt with below.

Effect of Different Veratrine Concentrations on the ^{22}Na Efflux

The effect of different veratrine concentrations on the slow phase of the efflux curve of ^{22}Na from the vesicles was analyzed in 19 preparations of axolemma. The number of experiments and the rate of efflux obtained (mean ± SEM) are

TABLE I

CHANGES CAUSED BY VERATRINE AND TETRODOTOXIN ON THE ^{22}Na EFFLUX RATES FROM AXOLEMMA VESICLES

	Rates of ^{22}Na efflux	
	Fast phase	Slow phase
	min^{-1}	$10^{-2}\ min^{-1}$
Control	0.20±0.05 (5)	0.59±0.04 (13)
Veratrine (0.50 mg/ml)	0.30±0.07 (5)	0.86±0.05 (13)
Veratrine (0.50 mg/ml) plus tetrodotoxin (100 nM)	0.19±0.08 (5)	0.48±0.07 (4)

Values are mean ± SEM. The number of experiments is given in parentheses. Fast and slow phases of curves shown in Fig. 1. Efflux measured at 20–22°C. For details see text.

TABLE II

CHANGES CAUSED BY DIFFERENT VERATRINE CONCENTRATIONS ON THE RATE OF ^{22}Na EFFLUX FROM AXOLEMMA VESICLES

Veratrine concentration	Number of experiments	Rate of ^{22}Na efflux (slow phase)
mg/ml		$10^{-2}\ min^{-1}$
0 (controls)	19	0.63±0.02
0.025	7	0.86±0.05
0.10	4	0.85±0.03
0.25	8	0.90±0.07
0.50	13	0.86±0.05

Rate values are mean ± SEM.

summarized in Table II. These results are also shown in Figure 2, where the rates of efflux are plotted as a function of the veratrine concentrations. The continuous line corresponds to a least-squares fit to the data calculated on the assumption that the increment of the efflux rate is a rectangular hyperbolic function of the veratrine concentration. Veratrine was used, even though it is a mixture of alkaloid with different mechanisms of action (20), so that our results could be compared with those of Henderson and Strichartz (10). The control efflux rate is $0.63 \pm 0.02 \times 10^{-2}\ min^{-1}$, and the maximum rate of veratrine-treated vesicles that can be calculated from the curve is $0.87 \times 10^{-2}\ min^{-1}$

(plateau of the curve). This is about 40% higher than the control value. This percent increment, though lower, is comparable to that found in veratrine-treated garfish axons (average 54%, reference 10), loaded and equilibrated like the vesicles with ^{22}Na in physiological solution containing ouabain. However, note that the maximum net increase induced by veratrine is only 0.24×10^{-2} min^{-1}. Assuming that the efflux of ^{22}Na is balanced by the influx of nonradioactive sodium only, this increase of the efflux rate, for an average vesicle 0.24 μm in diameter, corresponds to a unidirectional sodium flux of 0.08 pmol/cm^2 s. This value is five times smaller than that found in similarly treated garfish axons (0.4 pmol/cm^2 s, reference 10). These results suggest that some of the functioning sodium channels are lost during the isolation of the axolemma from lobster

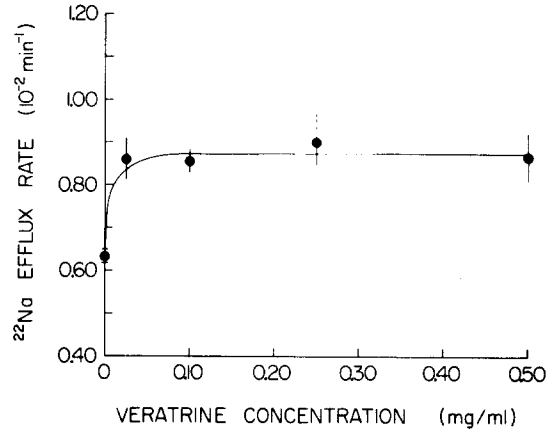

FIGURE 2. Rates of ^{22}Na efflux from axolemma vesicles treated with different veratrine concentrations. The rates correspond to the slow phase (10–60-min period) of the efflux curves. The continuous line is a least-squares fit to the data assuming that the increment of the rate is a rectangular hyperbolic function of the veratrine concentration. The number of experiments and the represented efflux rates (mean ± SEM) are given in Table II. For further details see text.

nerves, something that could be expected since the isolation procedure causes unavoidable damage to the axolemma.

Effect of Different Tetrodotoxin Concentrations on the ^{22}Na Efflux

The effect of different tetrodotoxin concentrations on the slow phase of the ^{22}Na efflux curved in the presence of 0.50 mg of veratrine per milliliter was studied in 13 preparations of axolemma vesicles. The number of experiments and the results obtained are summarized in Table III. These results are also shown in Fig. 3 where the efflux rates of ^{22}Na in the presence of veratrine are plotted as a function of tetrodotoxin concentrations. The continuous line is a least-squares fit to the data, calculated on the assumption that the diminution by tetrodotoxin of the veratrine-induced increment of the rate is a rectangular hyperbolic function of the tetrodotoxin concentration, as described by the following equation:

$$F = F_v - [(F_v - F_{TTX\infty})/(1 + K/[TTX])],$$

were F is the rate of ^{22}Na efflux in the presence of veratrine (0.50 mg/ml) at different tetrodotoxin concentrations, F_v is the rate in the presence of veratrine at zero tetrodotoxin concentration, $F_{TTX\infty}$ is the rate in the presence of veratrine as the tetrodotoxin concentration tends to infinity (plateau of the curve), [TTX] is the tetrodotoxin concentration, and K is the concentration of tetrodotoxin required to produce 50% of the maximum blocking effect. This equation corresponds to a model in which one tetrodotoxin molecule binds to a single specific site in the axolemma and blocks the sodium flux through the channel associated with that receptor. Complete blocking of the tetrodotoxin-sensitive sodium flux occurs when all receptors are occupied.

By means of the equation given above, it is calculated that the rate of sodium

TABLE III

CHANGES CAUSED BY DIFFERENT TETRODOTOXIN CONCENTRATIONS ON THE ^{22}Na EFFLUX RATE FROM VERATRINE-TREATED AXOLEMMA VESICLES

Veratrine	Tetrodotoxin concentration	Number of experiments	Rate of ^{22}Na efflux (slow phase)
mg/ml	*nM*		*10^{-2} min^{-1}*
0 (controls)	0 (controls)	13	0.59±0.04
0.50	0	13	0.86±0.05
0.50	1	3	0.83±0.08
0.50	10	4	0.71±0.06
0.50	100	4	0.48±0.07
0.50	200	2	0.46±0.07
0.50	1000	5	0.53±0.09
0.50	2000	6	0.50±0.02
0 (controls)	0 (controls)	3	0.65±0.05
0	100	3	0.50±0.04

Rate values are mean ± SEM.

efflux, equal to 0.86 ± 0.05 × 10^{-2} min^{-1} in the presence of veratrine (0.50 mg/ml) at zero tetrodotoxin concentration, can be diminished by the addition of tetrodotoxin to a value as low as 0.48 × 10^{-2} min^{-1}, i.e., a diminution of approximately 56%. Note that the control value (veratrine-free and tetrodotoxin-free vesicles) is 0.59 ± 0.04 × 10^{-4} min^{-1}. Observe in the same Table II that the rate of efflux in the presence of tetrodotoxin alone also tends to be lower than its control. These findings resemble those obtained by Henderson and Strichartz (10) in similarly treated intact garfish axons. As they point out, the result obtained in the absence of veratrine, suggests that even in a depolarized axolemma, a very small fraction of the sodium channels remains open.

Using the same equation, K, as defined above, was calculated as 11.9 ± 2.4 nM tetrodotoxin. This value is about equal to that which has been found (12 nM) in similarly treated intact garfish axons (10). These values of K are also closely similar to those determined by Colquhoun et al. (21) as the apparent dissociation constants for the specific binding of [^{3}H]tetrodotoxin to intact nerves of *Homarus*

americanus lobster (10.2 nM) and garfish (10.1 nM). In *P. argus* lobster intact axons, C. Sevcik and the authors of the present article (unpublished results), have found that 5 nM tetrodotoxin reduced by 50% the maximum rate of rise of the action potential, thus suggesting that the dissociation constant should be close to 5 nM. The values given above are comparable to those obtained by Barnola et al. (5), Benzer and Raftery (7), and Chacko et al. (8) for the specific binding of [^{3}H]tetrodotoxin to membrane fragments isolated from *P. argus* lobster and garfish nerves. The close similarities of the K values indicate that the sodium channels of the vesicles blocked by tetrodotoxin behave like those characteristic of intact axons, thus suggesting that they are the same.

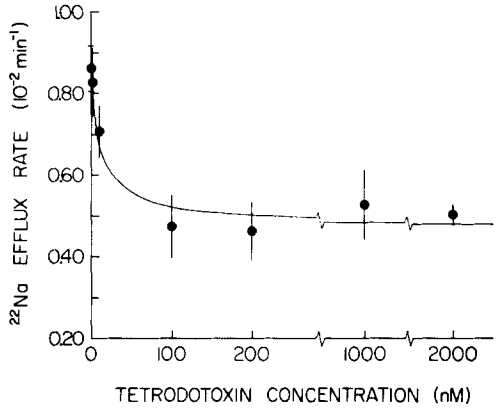

FIGURE 3. Rates of ^{22}Na efflux from axolemma vesicles treated with different tetrodotoxin concentrations in the presence of 0.50 mg of veratrine per milliliter. The rates correspond to the slow phase (10–60-min period) of the efflux curves. The continuous line is a least-squares fit to the data assuming that the diminution by tetrodotoxin of the rate in the presence of veratrine is a rectangular hyperbolic function of the toxin concentration. The number of experiments and the represented efflux rates (mean ± SEM) are given in Table III. For further details see text.

Sodium Channels in the Axolemma Vesicles

The main purpose of the present work was to investigate the presence of functioning sodium channels in the vesicles formed by axolemma fragments isolated from lobster nerves. Due to the knowledge about the pharmacological effects of veratrine, batrachotoxin, and tetrodotoxin on excitable cells, the results obtained are good evidence that the vesicles have functioning sodium channels. The availability of these axolemma fragments with such structures may be considered a further step towards the molecular characterization of the excitable membrane sodium channels.

The authors are deeply indebted to Dr. Bernhard Witkop for his kind gift of batrachotoxin, to Drs. Carlos Sevcik and Leopoldo Villegas for their suggestions and discussions; to Dr. Gloria M. Villegas for the electron microscopic study of the axolemma vesicles; to Dr. Guillermo Whittembury for his

critical reading of the manuscript, to Messrs. Paul Freund, Roberto Bischoff, and Enrique Arciniegas for their collaboration; to Messrs. N. Mujica, E. Pelaez, and K. Kutzniar for the lobster supply; and to Mrs. Olga Agosto for her assistance in the preparation of the manuscript.

Received for publication 9 June 1975.

REFERENCES

1. CAMEJO, G., G. M. VILLEGAS, F. V. BARNOLA, and R. VILLEGAS. 1969. Characterization of two different membrane fractions isolated from the first stellar nerves of the squid *Dosidicus gigas*. *Biochim. Biophys. Acta.* **193**:247.
2. FISCHER, S., M. CELLINO, F. ZAMBRANO, G. ZAMPIGHI, M. T. NAGEL, D. MARCUS, and M. CANESSA-FISCHER. 1970. The molecular organization of nerve membranes. I. Isolation and characterization of plasma membranes from the retinal axons of the squid: an axolemma-rich preparation. *Arch. Biochem. Biophys.* **138**:1.
3. MARCUS, D., M. CANESSA-FISCHER, G. ZAMPIGHI, and S. FISCHER. 1972. The molecular organization of nerve membrane. VI. The separation of axolemma from Schwann cell membrane of giant retinal squid axons by density gradient centrifugation. *J. Membr. Biol.* **9**:209.
4. DENBURG, J. L. 1972. An axon plasma membrane preparation from the walking legs of the lobster *Homarus americanus*. *Biochim. Biophys. Acta.* **282**:453.
5. BARNOLA, F. V., R. VILLEGAS, and G. CAMEJO. 1973. Tetrodotoxin receptors in plasma membranes isolated from lobster nerve fibers. *Biochim. Biophys. Acta.* **298**:84.
6. CHACKO, G. K., D. E. GOLDMAN, H. C. MALHOTRA, and M. M. DEWEY. 1974. Isolation and characterization of plasma membrane fractions from garfish *Lipisosteus osseus* olfactory nerve. *J. Cell Biol.* **62**:831.
7. BENZER, T. I., and M. A. RAFTERY. 1972. Partial characterization of a tetrodotoxin-binding component from nerve membrane. *Proc. Natl. Acad. Sci. U.S.A.* **69**:3634.
8. CHACKO, G. K., F. V. BARNOLA, R. VILLEGAS, and D. E. GOLDMAN. 1974. The binding of tetrodotoxin to axonal membrane fraction isolated from garfish olfactory nerve. *Biochim. Biophys. Acta.* **373**:308.
9. KASAI, M., and J. P. CHANGEUX. 1971. *In vitro* excitation of purified membrane fragments by cholinergic agonists. I. Pharmacological properties of the excitable membrane fragments. *J. Membr. Biol.* **6**:1.
10. HENDERSON, R., and G. STRICHARTZ. 1974. Ion fluxes through the sodium channels of garfish olfactory nerve membranes. *J. Physiol. (Lond.).* **238**:329.
11. ULBRICHT, W. 1965. Voltage clamp studies of veratrinized frog nodes. *J. Cell. Comp. Physiol.* **66** (6, Pt. 2):91.
12. ULBRICHT, W. 1969. The effect of veratridine on the excitable membranes of nerve and muscle. *Ergeb. Physiol. Biol. Chem. Exp. Pharmakol.* **61**:18.
13. HILLE, B. 1968. Pharmacological modifications of the sodium channel of frog nerve. *J. Gen. Physiol.* **51**:199.
14. NARAHASHI, T., E. X. ALBUQUERQUE, and T. DEGUCHI. 1971. Effects of batrachotoxin on membrane potential and conductance of squid giant axons. *J. Gen. Physiol.* **58**:54.
15. ALBUQUERQUE, E. X. 1972. The mode of action of batrachotoxin. *Fed. Proc.* **31**:1133.
16. NARAHASHI, T., J. W. MOORE, and W. R. SCOTT. 1964. Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J. Gen. Physiol.* **47**:965.
17. HILLE, B. 1966. Common mode of action of three agents that decrease the transient

change in sodium permeability in nerves. *Nature (Lond.)* **210:**1220.

18. VILLEGAS, R., F. V. BARNOLA, and P. FREUND. 1975. Ion flux through sodium channels of axon membrane fragments isolated from lobster nerves. *Biophys. J.* **15** (2, Pt. 2):44a.
19. LOWRY, O. H., N. J. ROSENBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193:**265.
20. OHTA, M., T. NARAHASHI, and R. F. KEELER. 1973. Effects of veratrum alkaloids on membrane potential and conductance of squid and crayfish giant axons. *J. Pharmacol. Exp. Ther.* **184:**143.
21. COLQUHOUN, D., R. HENDERSON, and J. M. RITCHIE. 1972. The binding of labelled tetrodotoxin to non-myelinated nerve fibres. *J. Physiol. (Lond.)* **227:**95.