Comparison of Ionic Selectivity of Batrachotoxin-Activated Channels with Different Tetrodotoxin Dissociation Constants

LI-YEN MAE HUANG, WILLIAM A. CATTERALL, and GERALD EHRENSTEIN

From the Laboratory of Biophysics, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20014; and from the Department of Pharmacology, University of Washington, Seattle, Washington 98105. Dr. Huang's present address is Laboratory of Neurophysiology, National Institutes of Health, Bethesda, Maryland 20014.

ABSTRACT The purpose of these experiments is to test whether the differences between normal and tetrodotoxin-resistant Na^+ channels reside in the selectivity filter. To do this, we have compared the selectivity of batrachotoxin-activated channels for alkali cations, organic cations, and nonelectrolytes in two neuroblastoma clonal cell lines: N18, which has normal tetrodotoxin (TTX) sensitivity, and C9, which is relatively TTX-resistant. We have also studied the effect of H^+ on Na^+ permeability and on the interaction between TTX and its receptor site in both cell lines. There is no qualitative difference between the two cell lines in any of these properties. In both cell lines the batrachotoxin-activated Na^+ channels have a selectivity sequence of $Tl^+ > Na^+ > K^+$, guanidinium $> Rb^+ > Cs^+$, methylamine. Also, in both cell lines H^+ blocks Na^+ channels with a pK_a of 5.5 and inhibits the action of TTX with the same pK_a. These observations indicate that the selectivity filters of the Na^+ channels in C9 and N18 do not differ significantly despite the 100-fold difference in TTX-affinity. Our selectivity studies of batrachotoxin-activated Na^+ channels for both cell lines suggest that these toxin-activated Na^+ channels have a limiting pore size of 3.8×6.0Å, as compared to a pore size of 3.0×5.0 Å for potential-activated Na^+ channels.

INTRODUCTION

Tetrodotoxin (TTX) specifically blocks Na^+ channels of various nerve and muscle membranes (Narahashi et al., 1964; Kao, 1966). Because TTX action is highly specific, it has become an important marker for chemical and physiological identification of Na^+ channels. Based on chemical structure of this toxin (Woodward, 1964), Kao and Nishiyama (1965) proposed that the guanidinium group of TTX enters the Na^+ channel and lodges in the pore because of its large size. The transport of Na^+ ions is then impeded by the presence of this large molecule in the channel mouth. From analysis of permeability of various organic and metal alkali cations, Hille (1971, 1972) proposed a molecular

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structure for Na^+ channels and a specific site in the channel where TTX binds (Hille, 1975). He suggested that the Na^+ channel is an oxygen-lined pore with a narrow region (3 × 5Å cross section) called the selectivity filter, which acts as a sieve to exclude large molecules. A carboxyl group which is suggested to be part of the selectivity filter may be important for binding of TTX as well as monovalent cations. This hypothesis is supported by the finding that H^+, Tl^+, and Ca^+ do compete with TTX for the binding site (Henderson et al., 1974).

Although most Na^+ channels are sensitive to TTX, there are many exceptions. Nerves of molluscs (Twarog and Yamaguchi, 1975), denervated mammalian muscle (Redfern and Thesleff, 1971), embryonic rat muscle (Harris and Marshall, 1973), and both nerves and muscle of puffer fish (Kao and Fuhrman, 1967) and American newts (Grinnel, 1975) have a sodium-dependent action potential, but are relatively resistant to TTX. A possible explanation for these observations is that the anionic site of Na^+ channels is modified in such a way as to reduce the affinity for TTX. Alternatively, it may be that the portion of the channel that binds to the uncharged parts of TTX is modified. If the anionic site that is postulated to be in the selectivity filter is significantly modified, it would be expected that the selectivity and certain other properties of TTX-resistant Na^+ channels would be quite different from those of TTX-sensitive Na^+ channels. In contrast, if other parts of the channel are modified, little selectivity difference would be expected. To resolve the source of TTX sensitivity differences, we have studied the selectivity of Na^+ channels for various alkali cations and organic molecules, the effect of H^+ on Na^+ permeability, and the interaction between H^+ and TTX in two neuroblastoma clonal cell lines which have very different TTX sensitivity. The ion selectivity of Na^+ channels in these cell lines was determined by ion flux methods in batrachotoxin-activated Na^+ channels (Catterall and Nirenberg, 1973; Catterall, 1975, 1977). Batrachotoxin, a toxin produced by the Columbian frog, *Phyllobates aurotaenia*, causes a persistent activation of Na^+ channels in nerve (Albuquerque et al., 1971; Narahashi et al., 1971; Khodorov, 1978), and in neuroblastoma cells (Catterall, 1975, 1977). As a result, the Na^+ channels are open long enough to allow the flux measurement. Preliminary results of this work have been presented (Huang et al., 1978 *a*).

METHODS

Materials

Chemicals and media used were obtained from the following sources: Dulbecco-Vogt modification of Eagle's minimal essential medium (DMEM) and Dulbecco's phosphate-buffered saline from the Media Unit of the National Institutes of Health; fetal calf serum from Grand Island Biological Co., Grand Island, N. Y.; recrystallized trypsin from Worthington Biochemical Corp., Freehold, N. J.; tetrodotoxin from Calbiochem Behring Corp., San Diego, Calif.; ouabain from Sigma Chemical Co., St. Louis, Mo.; ^{22}NaCl, [^{14}C]urea, and [^{14}C]methylamine from New England Nuclear, Boston, Mass.; [^{14}C]guanidine and [^{14}C] formamide from ICN Pharmaceuticals, Inc., Irvine, Calif. Batrachotoxin was kindly provided by Doctors J. Daly and B. Witkop, Laboratory of Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases, National

Institutes of Health. This toxin was stored in ethanol at 250 μM concentration and was added to the preincubation media immediately before use.

Cell Culture

Cell lines N18 of mouse neuroblastoma, C1300, and C9 of rat brain neuroblastoma (West et al., 1977) were used in this work. C9 was kindly supplied by G. West and H. Herschman, Laboratory of Nuclear Medicine and Radiation Biology, School of Medicine, University of California at Los Angeles. The cells were grown as described (Catterall and Nirenberg, 1973). Briefly, cells were propagated in growth medium consisting of 5% fetal calf serum, 95% DMEM in humidified atmosphere of 10% CO_2, 90% air. For experiments, cells were suspended from stock cultures with Ca^{++}, Mg^{++}-free Dulbecco's phosphate-buffered saline and 0.02% (wt/vol) trypsin; and were then seeded with growth medium at a density of 15,000 cells/cm^2 in multiwell plates (16 mm diameter, Costar Co., Cambridge, Mass.). Growth medium was changed on days 3 and 5, and cultures were ready for use on day 6 or 7. [^{3}H]Leucine (0.2 μCi/ml) was added to the growth medium 1 d before use so that the protein recovery in the experiments can be measured from ^{3}H counts. The results are expressed as nanomoles of permeant species influx per minute per milligram cell protein.

Measurement of Influx Rate

Cells were first preincubated with batrachotoxin (BTX) at 36°C for 60 min in a sodium-free medium consisting of 135 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$, and 50 mM Hepes (pH adjusted to 7.4 with Tris base). The initial rate of passive $^{22}Na^+$ influx was then measured for 30 s in uptake medium containing 130.0 mM choline chloride, 2–10 mM test permeant molecules (as indicated in figure Legends), 5.4 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.0 mM ouabain, 1 μCi/ml ^{22}NaCl, or other labelled compounds. Inasmuch as the action of BTX is only slowly reversible (Catterall, 1975; this paper), it was not necessary to include BTX (which is only available in small quantities) in the uptake medium. The uptake was terminated by washing the cells four times with 3 ml of 0°C wash medium which was composed of 135 mM choline chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5.0 mM Hepes (adjusted to pH 7.4 with Tris base). The concentration of permeant ions was kept below 20 mM. Under these conditions, the increase in ion permeability caused by batrachotoxin had a very small effect on membrane potential. The dependence of permeant molecule influx on external concentration conformed to the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949) indicating that under the experimental conditions used, the measured influx of test molecules is directly proportional to permeability. In experiments where the dependence of ^{22}Na uptake on pH was studied, 50 mM Hepes was replaced in the assay medium by a mixture of 10 mM glycylglycine (pK_a = 3.08, 8.08), 10 mM 2(*N*-morpholino) ethane sulfonic acid (MES, pK_a = 6.2), 10 mM *N*-hydroxyethyl-piperazine-*N*'-ethanesulfonic acid (Hepes, pK_a = 7.5), 10 mM tris (hydroxymethyl) methylamino-propane sulfonic acid (TAPS, pK_a = 8.4), 10 mM cyclohexylamino propane sulfonic acid (CAPS, pK_a = 10.4).

Cells were then removed from culture dish by incubating them in 0.6 ml of 0.4 N Na OH or 1% sodium lauryl sulphate and were transferred to scintillation vials containing 1 ml 1 M Tris-HCl (pH = 7.4), and 10 ml of scintillation mixture (5.53% [vol/vol] Research Products International Corp. [Elk Grove Village, Ill.] scintillator fluid, 61.14% Toluene, and 33.33% Triton X-100 [Rohm & Haas Co., Philadelphia]). The cell protein was determined by a modification of the Lowry method (Lowry et al., 1951).

RESULTS

Activation of $^{22}Na^+$ Uptake by Batrachotoxin

Batrachotoxin (1 μM) causes a 25-fold increase in the initial rate of $^{22}Na^+$ uptake in clone N18 of mouse neuroblastoma C1300 (Catterall, 1975). The dissociation constant of activation by BTX is 0.4–0.7 μM in this cell line (Catterall, 1975, 1977). The variant neuroblastoma cell line C9 responds to batrachotoxin in a similar fashion as do N18 cells. The activation of Na^+ uptake by BTX in C9 is slow and reversible. Activation is complete in 40 min at 1 μM BTX. Activation is reversed when BTX is washed out of the cells with a half-time of 35 min. Therefore, cells were incubated for 60 min with BTX to allow the toxin to equilibrate with its receptor sites. The dependence of Na^+ uptake on BTX concentration for C9 and N18 is given in Fig. 1. The data can be well fit by the modified Michaelis-Menton equation (solid line):

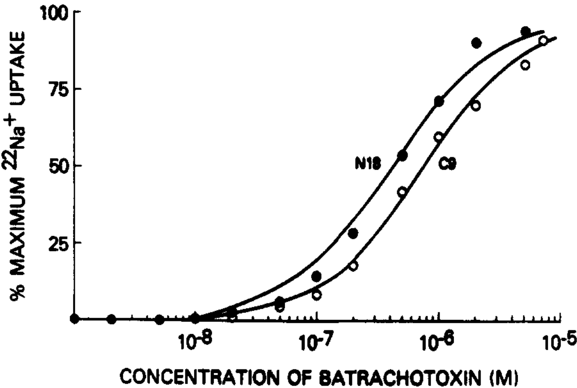

FIGURE 1. Effect of BTX concentration on $^{22}Na^+$ uptake in C9 (○) and N18 (●). Cells were incubated at 37°C with indicated concentration of BTX in high-K^+ medium for 60 min. After incubation, Na^+ uptake was measured for 30 s in assay medium.

$$V = V_{\max}(B/(K_{0.5} + B),$$

where $K_{0.5}$ is the apparent dissociation constant, B the BTX concentration, and $V_{\max}$ the maximum uptake at infinite activation concentration. The average values of $K_{0.5}$ and $V_{\max}$ for C9 obtained from such dose-response curves are $K_{0.5} = 0.8 \pm 0.2$ μM, $V_{\max} = 100 \pm 21$ nmol/min per mg compared to values of $V_{\max} = 150$ nmol/min per mg, and $K_{0.5} = 0.4$–0.7 μM for N18 (Catterall, 1975, 1977). Thus in C9, batrachotoxin activates Na^+ uptake at a similar concentration and yields similar maximum uptake as in N18.

Inhibition by Tetrodotoxin

BTX-dependent uptake of $^{22}Na^+$ in N18 cells is completely abolished by low concentration ($K_I = 11$ nM) of tetrodotoxin (Catterall, 1975). The inhibition by tetrodotoxin is noncompetitive (Catterall, 1975). We have repeated tetrodotoxin titration curves for these cells under the conditions of our present experiments

and find a K_I of 18 nM (Fig. 2), in good agreement with the earlier work. This, together with other lines of evidence considered in the Discussion, suggests that BTX specifically activates normal sodium channels in neuroblastoma cells.

In C9, the increase in Na^+ permeability by BTX is very resistant to inhibition by TTX (Fig. 2). The apparent dissociation constant for TTX is 1.5 ± .3 μM which is about 100-fold higher than K_I value for TTX in N18. Thus, the Na^+ channel in C9 has a low affinity for TTX, in agreement with the work of West et al. (1977).

Selectivity for Alkali Metal Cations

Previous studies showed that Na^+ influx induced by BTX measured the changes in Na^+ permeability due to activation of Na^+ channels (Catterall, 1977). The ionic influx (J_i) depends on ion permeability, external ion concentration, and membrane potential according to the Goldman-Hodgkin-Katz equation (Gold-

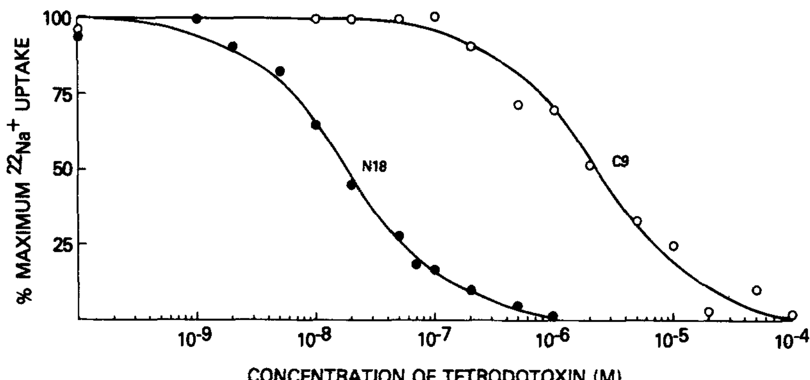

FIGURE 2. Inhibition of BTX-dependent ^{22}Na uptake by TTX in C9 (○) and N18 (●). Cells were preincubated with 1 μM BTX and indicated concentration of TTX for 60 min. The uptake was measured for 30 s in the uptake medium containing same amount of BTX and TTX as in the preincubation medium.

man, 1943; Hodgkin and Katz, 1949). To prevent entry of Na^+ into cells and loss of intracellular K^+ during preincubation, cells were incubated with toxins in 135 mM K^+, 0 mM Na^+ preincubation medium. The influx was subsequently measured in an assay medium containing a high concentration of choline chloride and a low concentration of test molecules (<20 mM). Under these experimental conditions, the membrane potential is unaffected by the increased sodium conductance caused by BTX treatment (Catterall, 1977). The uptake of test molecules (J_i) during the first 30 s is then simply a linear function of permeability (P_i) and of external concentration. To determine P_i, the uptake was measured for several external concentrations of each test molecule. Therefore, the slope (S_i) of the linear plot of influx vs. external concentration is directly proportional to P_i. As a control, Na^+ influx was measured in each experiment. The data are presented as the ratio R_i of the permeability of test molecules to that of Na^+. R_i can be obtained directly from the ratio of the slopes

S_i/S_{Na} in that $R_i = P_i/P_{Na} = S_i/S_{Na}$. In all the results presented, the influx of test molecules in the absence of BTX has been subtracted.

The dependence of the uptake of alkali cations on their external concentration is given in Fig. 3. The linear relationship observed between J_i and concentration verifies that there is constant membrane potential and negligible flux coupling under these experimental conditions as shown previously (Catterall, 1977). The selectivity sequence for alkali metal cations in BTX-activated channels is $Tl^+ \gtrsim Na^+ > K^+ > Rb \gtrsim Cs$ for both N18 and C9 (Table I). For all

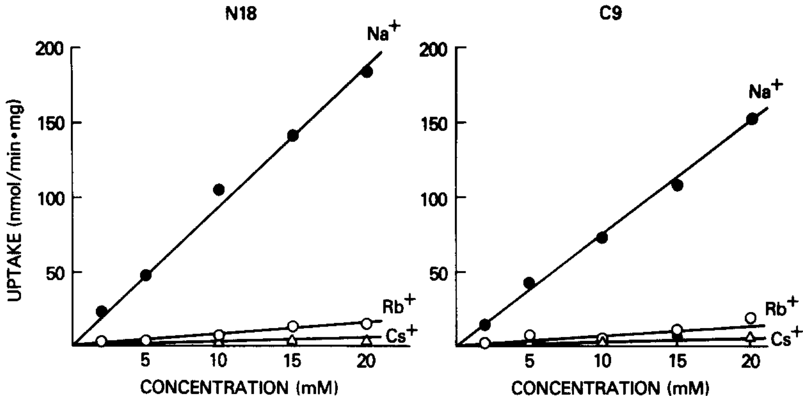

FIGURE 3. Effect of concentration on alkali cations uptake in C9 and N18. Two identical sets of cells were preincubated for 60 min at 37°C in preincubation medium containing 0 and 1 μM BTX. The uptake of Na^+ (●), Rb (○), and Cs (△) were measured for 30 s at various indicated concentrations. The uptake of alkali ion in the absence of BTX was subtracted from uptake data obtained from cells that were preincubated with BTX.

TABLE I

PERMEABILITY RATIO OF ALKALI CATIONS AND ORGANIC MOLECULES

Transported molecules	Molecular formula	$R \left(\frac{\text{permeability of test molecules}}{\text{permeability of Na}^+}\right) \pm SD$	
		N18	C9
Alkali cation			
Sodium	Na^+	1.0	1.0
Potassium	K^+	0.17±0.06	0.39±0.07
Rubidium	Rb^+	0.08±0.01	0.11±0.02
Cesium	Cs^+	0.04±0.02	0.06±0.02
Thalium	Tl^+	1.06±0.25	1.10±0.10
Organic cations			
Guanidinium	$NH{=}C(NH_3^+)(NH_2)$	0.38±0.09	0.37±0.06
Methylamine	$NH_3^+{-}CH_3$	0.06±0.02	0.10±0.02

these ions except potassium, the permeability ratios for the two cell lines are the same within experimental error. For potassium ions, the permeability ratios for the two cell lines differ by somewhat more than experimental error, but show qualitative similarity. For all ions tested, the increase in uptake caused by BTX can be inhibited by TTX. Thus, the permeability increases were in the sodium channels.

Selectivity for Organic Cations

Hille (1971) showed that a number of organic cations were transported by the sodium channel. Of the cations studied by Hille, only methylamine and guanidinium are available in labelled form. In potential-activated sodium channels, methylammonium has been shown to be nonpermeant and guanidinium to be permeant electrophysiologically (Hille, 1971). We found that batrachotoxin-activated sodium channels are highly permeable to guanidinium (Fig. 4

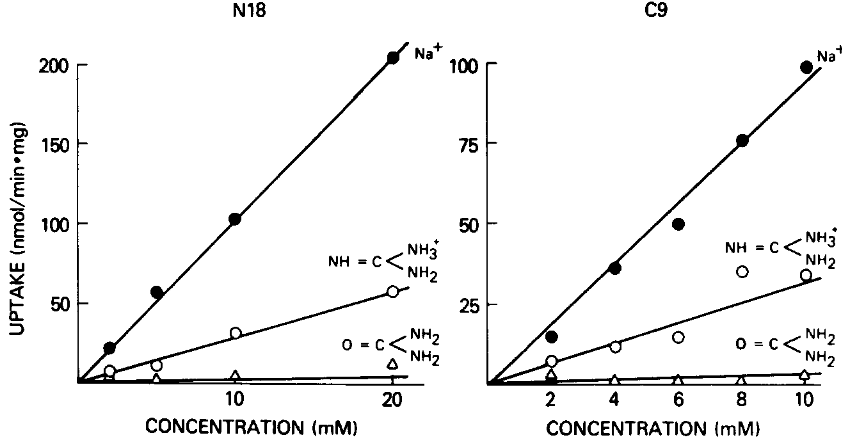

FIGURE 4. Effect of concentration on the uptake of Na^+ (●), guanidinium (○), and urea (△). Two sets of cultures were incubated for 60 min in preincubation medium containing 0–1 μM BTX. The uptakes of various compounds were measured for 30 s in assay medium containing indicated concentration of test molecules. The uptake of these molecules in the absence of BTX was subtracted.

and Table I) in both N18 (R = 0.38) and C9 (R = 0.37), and are much less permeable to methylamine (R = 0.06–0.10, Table I). The batrachotoxin-dependent increase in permeability in both guanidinium and methylamine is blocked by TTX. Therefore, BTX-activated sodium channels in C9 and N18 have similar permeability to guanidinium and methylamine.

Selectivity for Nonelectrolytes

The data of Hille (1971) are consistent with a selectivity filter region of 3.0 × 5.0Å. A structure of these dimensions should allow passage of small nonelectrolytes such as urea and formamide in addition to cations. Inasmuch as our isotopic flux methods are suitable for measurement of nonelectrolyte permeabilities, we have studied urea and formamide permeability. These molecules,

like guanidinium, are planar in shape due to the partial double bond character of the C-N bonds. Thus, on steric grounds it would be anticipated that their permeability would be similar to that of guanidinium.

The rates of uptake of urea are illustrated in Fig. 4. As for the cations studied, the influx is linearly proportional to extracellular concentration. The flux ratios for urea and formamide in N18 are 0.02 and 0.055, respectively (Table II). However, in that membrane potential affects influx of cations, but not the influx of nonelectrolytes, a correction for the membrane potential driving force must be made in calculating the ratio of nonelectrolyte permeability to Na^+ permeability. Under our experimental conditions, the membrane potential of neuroblastoma cells is about −41 mV. According to the Goldman-Hodgkin-Katz equation, this potential will result in a twofold increase in measured flux of charged molecules. Thus, the permeability ratio for nonelectrolytes relative to Na^+ is double the value of the flux ratios. The permeability ratios for urea and formamide in N18 are therefore 0.04 and 0.11 (Table II). The large uncertainty

TABLE II

PERMEABILITY RATIOS OF NONELECTROLYTES

Transported molecules	Molecular formula	N18		C9	
		J_i/J_{Na}	P_i/P_{Na}	J_i/J_{Na}	P_i/P_{Na}
Sodium	Na^+	1.00	1.00	1.00	1.00
Urea	$O{=}C(NH_2)_2$	0.02±.01	0.04±0.02	0.015±.01	0.03±0.02
Formamide	$O{=}C(NH_2)H$	0.055±.02	0.11±0.05	0.025±.01	0.05±0.02

in these values is due to the greater resting permeability of the cells to these nonelectrolytes as compared to cations and the low permeabilities of these nonelectrolytes. The batrachotoxin-dependent increase in permeability to these nonelectrolytes is blocked by tetrodotoxin indicating that they do go through Na^+ channels. Table II compares nonelectrolyte permeability in N18 and C9 cells. For urea, the permeabilities of the two cell lines are equal within experimental error, but for formamide, N18 sodium channels seem to be somewhat more permeable than C9 sodium channels. Because of the relatively small permeability of these nonelectrolytes and the relatively large uncertainty in these values, we only conclude that there is no qualitative difference in formamide permeability between cell lines.

pH Dependence of BTX-Activated Na^+ Channel

Protons block sodium channels in myelinated nerve by protonating a group with a pK_a of 5.2 (Hille, 1968; Woodhull, 1973). The effect of pH on BTX-activated Na^+ channels was examined to determine whether protons would block drug-activated Na^+ channels. These experiments were carried out by incubating 60 min with 1 μM BTX at pH 7.4, then incubating at various pH for 2 min, and finally measuring $^{22}Na^+$ uptake at various pH for 30 s. This procedure allows

BTX to saturate its receptor sites at pH 7.4. Then, because the action of BTX is only slowly reversible (Catterall, 1975), the inhibition of the BTX-activated sodium channels by changes in pH can be studied. We found that the BTX-dependent Na^+ uptake was markedly reduced with increasing H^+ concentration (Fig. 5). The permeability increase is completely blocked when pH is lower than 3.5. On the other hand, Na^+ uptake remains constant as we raised pH of the solution above 7.4 (Fig. 5). The pH effect occurs fast (<1 min) and is reversible at all pH studied if the cells are not incubated at extreme pH for more than 5 min. Background $^{22}Na^+$ uptake in the absence of BTX or in the presence of saturating concentrations of TTX is unaffected by pH. This result indicates that protons specifically block Na^+ channels and have little effect on the resting Na^+ permeability. The data are well fit by a theoretical titration curve with a single pK_a of 5.5 (solid line in Fig. 5). We have obtained an almost identical pK_a of BTX-activated Na^+ channels for both N18 and C9 (Fig. 5).

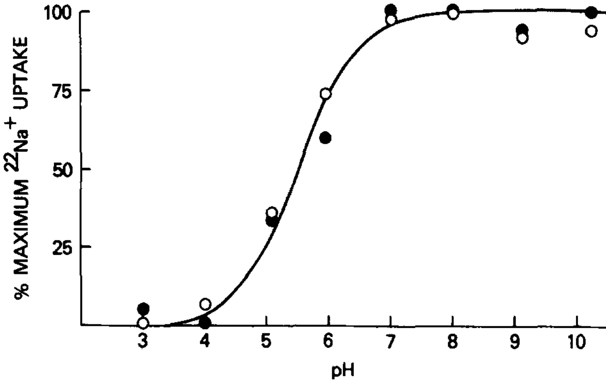

FIGURE 5. Effect of pH on $^{22}Na^+$ uptake in C9 (○) and N18 (●). Cells were incubated in preincubation medium containing 1 μM BTX for 60 min. The medium was subsequently removed and replaced by a medium containing a mixture of good buffers (see Methods). Cells were equilibrated in this medium which was buffered at the indicated pH, for 2–3 min. The Na^+ uptake was measured at the same pH in the assay medium for 30 s. Solid line is the least squares fit to the theoretical dissociation equation with a pK at 5.5. The data were represented as ratio of $^{22}Na^+$ uptake relative to the $^{22}Na^+$ uptake at pH 7.

pH Dependence of Tetrodotoxin Action

It has been shown for intact nerve fibers and nerve homogenates that binding of TTX is reduced in acidic solution (Henderson et al., 1974; Reed and Raftery, 1976). There is also agreement between the concentration of H^+ and Tl^+ required to block Na^+ current and the concentration needed to displace TTX binding (Woodhull, 1973; Henderson et al., 1974; Hille, 1975). These observations are consistent with the proposal that TTX and H^+ or Tl^+ bind to a common site which is important for the transport of cations in Na^+ channels (Hille, 1975). We have studied the inhibition of BTX-activated Na^+ uptake by TTX at several different pH in N18 and C9. The experiments were designed to

test whether TTX interacts with H^+ in similar fashion in Na^+ channels that have different TTX affinity. The same experimental procedure was used as described in the previous section except that the increasing concentrations of TTX were added to the assay medium as shown in Fig. 6 *a*. The data obtained from N18 cells are shown in Fig. 6 *a*. The apparent dissociation constant (K_{app}) for TTX increases with decreasing external pH. The K_{app} is a linear function of proton concentration (Fig. 6 *a*). The straight line (Segal, 1975) can be fit by

$$K_{app} = \frac{K_I}{K_H}[H^+] + K_I,$$

where K_H and K_I are dissociation constants for H^+ and TTX, and $[H^+]$ is the concentration of protons. The intercept on the $[H^+]$ axis gives $K_H = 10^{-5.5}$ which corresponds to the same pK_a as was found for inhibition of Na^+ uptake (Fig. 5). We conclude that H^+ blocks binding of TTX completely. The same kind of experiments were repeated in C9. The results (Fig. 6 *b*) are identical to those for N18. Thus, the binding site for TTX in C9 has the same pK_a as that in N18, even though the Na^+ channel in C9 is TTX-resistant. This pK_a is similar to the pK_a for inhibition of sodium channels by H^+ in both of these cell lines.

DISCUSSION

Specificity of Batrachotoxin Action

In comparing the selectivity of BTX-activated Na^+ channels in N18 and C9, it is important to consider the evidence supporting the view that BTX acts specifically on normal Na^+ channels. There are three lines of evidence suggesting strongly that BTX activates normal Na^+ channels. (*a*) TTX, a specific blocker of normal Na^+ channels, inhibits BTX-activated Na^+ uptake at the same concentration range as required to block normal Na^+ channels (Kao, 1966; Albuquerque et al., 1971; Cuervo and Adelman, 1970; Catterall, 1975). (*b*) Studying the time-course of the action of BTX in node of Ranvier, Khodorov et al. (1975) and Khodorov (1978) have observed that under the influence of BTX, a new component of Na^+ current of different voltage dependency gradually develops concurrently with the disappearance of normal Na^+ current. (*c*) Neuroblastoma cell lines specifically lacking Na^+ channels (Minna et al., 1971) do not respond to treatment with BTX (Catterall and Nirenberg, 1973; Catterall, 1975, 1977). In the discussion to follow, we therefore assume that BTX specifically acts on Na^+ channels.

Selectivity of Batrachotoxin-Activated Sodium Channels in Mammalian Neuroblastoma Cells

Khodorov (1978) has shown electrophysiologically that batrachotoxin alters the ion selectivity of sodium channels in the frog node of Ranvier. Normal channels have a selectivity sequence of $Na^+ > Tl^+ >$ guanidinium $> K^+ \gg Rb^+, Cs^+$, methylamine (Table III; Chandler and Meves, 1965; Hille, 1971, 1972; Khodorov, 1978) whereas batrachotoxin-activated sodium channels have a selectivity sequence of $Tl^+ > Na^+ > K^+ =$ guanidinium $> Rb^+ > Cs^+ \cong$ methylamine

(Table III; Khodorov, 1978). Our results show that batrachotoxin-activated sodium channels in mammalian neuroblastoma cells have a selectivity sequence similar to that of batrachotoxin-activated channels in frog node. In both cases, Tl^+, K^+, Rb^+, Cs^+, and methylamine are relatively more permeant than in

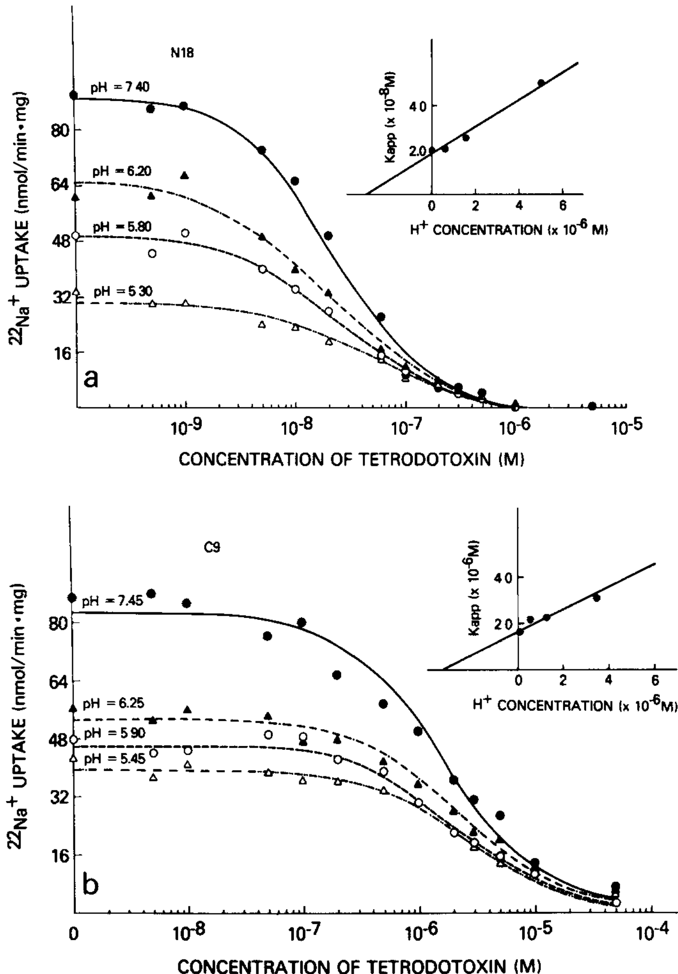

FIGURE 6. (*a*) Effect of pH on TTX inhibition of Na^+ uptake in N18. Cells were incubated for 60 min in preincubation medium containing 1 μM BTX and indicated concentration of TTX. The medium was then removed and replaced by a medium which contains a mixture of good buffers and buffered at indicated pH. After 2–3 min, the Na^+ uptake was measured at the same pH in the assay medium for 30 s. The solid line is the least squares fit to the equation

$$V = V_{\max}\left(1 - \frac{[\mathrm{TTX}]}{K_{\mathrm{app}} + [\mathrm{TTX}]}\right).$$

The K_{app}'s obtained at different pH were replotted as a function of pH (*inset*). The data can be fitted by a straight line. (*b*) Same experiment as in (*a*) was done on C9 cell line.

untreated frog node. Thus, our results support the conclusion of Khodorov (1978) that batrachotoxin alters the structure of the selectivity filter such that its minimum dimensions are increased to 3.8 × 6.0 Å to allow passage of Cs^+ and methylamine.

In a normal Na^+ channel in frog node, Tl^+ is one-third as permeable as Na^+ (Hille, 1972). After BTX treatment, the permeability ratio for Tl^+ increases about fourfold. This selectivity sequence change between Tl^+ and Na^+ in BTX-activated channels cannot be explained by the sieving action of the pore, for Na^+ has a much smaller crystal radius (r = 0.95 Å) than Tl^+ (r = 1.40 Å). Chemical interactions between cation and pore structure which would lower the energy barrier for Tl^+ must be taken into consideration. Therefore, BTX may both increase the minimum dimensions of the selectivity filter and also alter the chemical properties of groups in the vicinity of the selectivity filter.

TABLE III

PERMEABILITY RELATIVE TO Na^+

Ion	Normal frog* node	BTX-treated‡ frog node	BTX-treated neuroblastoma N18	BTX-treated neuroblastoma C9
Na^+	1.00	1.00	1.00	1.00
Tl^+	0.33	1.50	1.06	1.10
Guanidinium	0.13	0.40	0.38	0.37
K^+	0.09	0.45	0.17	0.39
Rb^+	<0.01	0.25	0.08	0.11
Cs^+	<0.01	0.15	0.04	0.06
Methylamine	<0.01	0.10	0.06	0.10

* From Hille (1971, 1972).

‡ From Khodorov (1978).

Permeability of Nonelectrolytes in Sodium Channels

The studies of sodium channel permeability to organic cations carried out by Hille (1971) led to the conclusion that the selectivity filter region was ~3.0 × 5.0 Å. Our results and those of Khodorov (1978) suggest a somewhat larger pore size for batrachotoxin-activated Na^+ channels. In either case, the pore should be permeable to small nonelectrolytes like formamide and urea. Our results provide the first estimates of the permeability of these nonelectrolytes in batrachotoxin-activated sodium channels. We find permeability ratios of 0.4, 0.3 for urea and 0.11, 0.05 for formamide. These results demonstrate that a positive charge is not absolutely required for passage through the Na^+ channel. Guanidinium, urea, and formamide have similar molecular structure and shape and are generally similar in size. Cationic guanidinium is far more permeant than neutral urea or formamide. This result indicates that positive charge has a strong enhancing effect on the permeability of molecules of similar size and shape, an effect that has also been found in acetylcholine-activated channels (Huang et al., 1978 *b*).

Comparison of Ion Selectivity of TTX-Sensitive and TTX-Resistant Sodium Channels

We chose clone N18 of C1300 mouse neuroblastoma and clone C9 derived from an induced rat brain tumor (West et al., 1977) for studying the selectivity of TTX-sensitive and relatively TTX-resistant Na^+ channels. These two cell lines respond to BTX with a large increase in Na^+ permeability at comparable concentrations. The increase in Na^+ permeability in both cell lines is inhibited by TTX. BTX-activated Na^+ uptake in N18 has normal sensitivity to TTX (K_I = 20 nM); in contrast, the Na^+ uptake in C9 is much more resistant to TTX (K_I = 1.5–2 μM). Thus, in C9 the Na^+ channel or its immediate region is modified such that the binding of TTX becomes less favorable. To examine whether a conformational change of the selectivity filter is involved, we have studied the permeability of monovalent alkali cations and organic molecules, the effect of H^+ on Na^+ permeability, and the interactions between H^+ and TTX in both cell lines.

There are two related questions we want to address. The first is whether there is a TTX-binding site at the selectivity filter and the second is whether that selectivity filter is significantly different in N18 and C9.

Regarding the first question, evidence supporting the view that there is a TTX-binding site at the selectivity filter has been provided by Henderson et al. (1974), who showed that the ability of lithium to reduce TTX binding is comparable to its ability to reduce the flux through the sodium channel. In this paper we have provided additional support for this view by showing that the pK_a for preventing TTX inhibition of the flux through sodium channels is the same as the pK_a for blocking of the sodium channel.

It should be pointed out, however, that there is recent evidence for a carboxyl group that is important for TTX binding that is not at the selectivity filter. Reed and Raftery (1976) showed that methylation of a carboxyl group blocks TTX binding. If this carboxyl group were located at the selectivity filter, methylation should block the sodium channel or markedly alter its ionic selectivity. In fact, the methylated (and thus TTX-insensitive) sodium channel in frog muscle is functional, has normal ion selectivity, and is blocked by H^+ with a pK_a of 5.2 (Spalding, 1978). This implies that the carboxyl group(s) outside the selectivity filter is (are) important for TTX binding. This does not support the view described above that there is a TTX-binding site at the selectivity filter, but it does not necessarily contradict that view.

Regarding the question concerning the reason for the difference between the binding constants of TTX to N18 and C9, our results indicate that although the potassium permeability is somewhat higher in C9, there is no qualitative difference between the two cell lines in the selectivity properties we studied. This suggests that the selectivity filter region of the sodium channel is similar in N18 and C9. This suggestion is further supported by our observation that the pK_a values for blocking sodium channels and for preventing TTX binding are the same in N18 and C9. A likely possibility is that N18 and C9 are significantly different in a portion of the sodium channel that binds to the uncharged part of the TTX molecule.

As indicated above, the hypothesis that a TTX receptor site is located at the selectivity filter is probable, but by no means certain. However, this uncertainty does not affect the conclusion that the structural difference between N18 and C9 sodium channels that accounts for their different TTX affinity is not located at the selectivity filter. This conclusion is based on our finding that Na^+ channels with widely different affinity for TTX have nearly identical ion selectivities and pK_a for blockage of Na^+ channels by H^+.

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