Block of Endplate Channels by Permeant Cations in Frog Skeletal Muscle

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ABSTRACT Motor endplates of frog semitendinosus muscles were studied under voltage clamp. Current fluctuations induced by iontophoretic application of acetylcholine were analyzed to give the elementary conductance, γ, and mean open time, τ, of endplate channels. Total replacement of the external Na^+ ion by several other metal ions and by many permeant organic cations changed both γ and τ. Except with NH_4^+ ions, the γ values with foreign test ions were all smaller than expected from the independence relation and their previously measured permeability ratios. The more hydrophobic ions gave the smallest γ values. Foreign permeant cations also depress γ when mixed with Na^+ ions. These effects could be interpreted in terms of binding of ions to a saturable site within the endplate channel as they pass through. The site for organic ions would have a hydrophobic component. Similar evidence is given for a metal ion binding site on the cytoplasmic end of the channel accessible to internal ions. Most foreign cations also shortened τ when applied externally. The changes of gating did not seem to be correlated with changes in γ. Thus there is no evidence for control of τ by ions bound within the pore.

INTRODUCTION

Acetylcholine depolarizes the membrane of skeletal motor endplates by opening channels permeable to Na^+ ions. We began to study the ionic selectivity of these channels both because of the long-standing physiological interest in the nature of pores and because the endplate channel macromolecule will probably soon be characterized in considerable biochemical detail (Raftery et al., 1980). In agreement with much other literature, our first papers showed that the channel is relatively wide and poorly selective, being apparently permeable to at least 75 different cations (Dwyer et al., 1980; Adams et al., 1980). For organic cations, the permeability ratios measured by reversal potentials varied inversely with ionic size as expected for simple frictional drag. Larger size meant smaller permeability, and the apparent pore cross-section was ~6.5 Å × 6.5 Å.

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In the same study, we found that the amplitude of currents induced by acetylcholine was often much smaller than expected from the measured permeability ratios, as if factors other than ionic size determined conductance. However, as we measured only the flow of macroscopic current, the experiments could not distinguish low conductance at the single-channel level from other possible ionic effects on transmitter removal, transmitter-receptor interaction, mean channel open lifetime, etc. This paper involves measurements of single-channel parameters by fluctuation techniques with a variety of external test cations. The major result is that the independence relation (Hodgkin and Huxley, 1952) is not obeyed at the single-channel level, implying that many organic cations interact with the open channel while passing through.

A preliminary account of some of these results has been presented to the Biophysical Society (Nonner et al., 1980).

METHODS

Dissection and Solutions

The goal of our experiments was to measure acetylcholine-induced fluctuations of ionic current at motor endplates bathed in a variety of test solutions. Segments of single muscle fibers including the endplate region were dissected and voltage-clamped as previously described (Dwyer et al., 1980) with the following modifications. Fiber segments were taken from semitendinosus muscles of *Rana pipiens* and *Rana temporaria* that had been depolarized for 30–60 min in a solution containing 110 mM KCH_3SO_3, 2 mM $CaSO_4$, and 10 mM potassium morpholinopropanesulfonic acid buffer, pH 7.2. We hoped that long depolarization would decrease the later spontaneous release of quanta by the remaining nerve terminal as well as prevent twitching of fibers as axon branches were cut during the dissection. The preterminal axon was cut to a length of < 20 μm. The fiber fragment was then mounted in the plastic chamber containing a 120-mM CsF solution covering all compartments and partitions. Fine threads of the grease glisseal (Borer Chemie, Switzerland) were placed over the fiber at the partitions, and the ends were cut again to leave ~300 μm of fiber in each end pool.

The normal internal solution was 120 mM unbuffered CsF (pH $\simeq$ 7.2) and the external reference solution for most experiments contained 114 mM NaCl, 1 mM $CaCl_2$, and 10 mM histidine, pH 7.4. These solutions are expected to permit almost no current in K channels and to keep the internal Ca^{++} activity low. In the external test solutions, half or all of the NaCl was replaced by an osmotically equivalent amount of the test substance. When the test involved thallous ions, nitrate salts replaced chloride salts in the test and reference solutions and in the salt bridge leading to the reference electrode. Initial experiments tested the conditions needed for the lowest background noise in the absence of endplate activation. An internal solution with 120 mM CsF gave less background noise than 120 mM NaF or KF, and external tetrodotoxin was found to be unnecessary. External solution changes were accomplished by a relatively rapid (30 s) flushing through of 5 ml, followed by a slow, continuous flow during the whole period when records were taken. Exposures for more than a few minutes to external test solutions were avoided to reduce any possible ionic redistributions, and each test was followed by a longer exposure to the reference solution. The temperature was maintained at 12°C.

Recording Technique and Protocol

Fibers were voltage-clamped with some series resistance compensation (Hille and Campbell, 1976), and the membrane current was recorded as the voltage drop across a 1-MΩ resistor. Currents in endplate channels were activated by long iontophoretic pulses of acetylcholine (ACh) delivered from a pipette placed 20–50 μm from the fiber surface. Pipette resistances were typically 20 MΩ when filled with 2 M ACh chloride (0.1 M ACh was used in solutions with thallous ions). Between pulses, leakage of ACh was prevented by a backing current of −20 nA. In a typical experiment, we could make measurements in three to five external solutions, and in each solution we took at least one pair of current records, the first without and the second with ACh delivery. To avoid persistent desensitization of receptors, we waited several minutes between ACh pulses. During the experiment, the background noise would gradually increase as the resistance of the grease seal between the test pool and the current pool decreased. The best results were obtained while the seal resistance remained >2 MΩ. Therefore, it was convenient to monitor the background noise continuously with a digital rms voltmeter, and we terminated the experiment when it rose from a typical rms value of 40–60 pA to values >100 pA in the reference solution without ACh (measured at −73 mV from 1 to 800 Hz).

The basic experimental record consisted of 10.24 s of continuous digital recording of the endplate current with or without a 9.5-s period of continuous iontophoretic appliction of ACh. Two digital samples were taken every 0.5 ms; one was the direct-coupled current signal filtered through a four-pole Bessel low-pass filter with a cutoff at 200 Hz (half power frequency), and the other was an AC-coupled and more highly amplified current signal filtered through a one-pole, high-pass filter at 1 Hz (simple RC) and an eight-pole Butterworth low-pass filter at 800 Hz. The 12-bit samples for one record were stored directly on digital tape by our LM^2 computer as 20 continuous blocks of 1,024 samples of each channel. Fig. 1 shows the DC (*top*) and the AC (*bottom*) current records redrawn by the computer from an experiment with dimethylamine as the external test ion. The ACh-induced increase of current fluctuations is obvious in the AC-coupled records. As in this experiment, the ACh pulse was usually adjusted to give an ACh-induced endplate current of −15 to −80 nA.

Off-line Analysis

The object of the rest of the analysis, done with the computer after the experiment, was to calculate the mean ACh-induced current and the power spectral density of the ACh-induced current fluctuations. First, data blocks were selected for analysis if they showed no miniature endplate currents and no sharp transients near, for instance, the beginning or at the end of the response to ACh. The remaining AC blocks still showed some trend, since the DC current was never completely flat. Such trends were removed by least-squares fitting the entire AC record (minus discarded blocks) with a polynomial of order between one and five and subtracting the polynomial. This flattened the AC trace as is shown in Fig. 1 (*bottom*). Then one-sided, power-density spectra, including filter corrections and condensation of high-frequency points (Conti et al., 1976), were calculated on each block and averaged for all blocks of the record. Finally, the difference spectrum was formed by subtracting the background spectrum from that with ACh. The *triangles* in Fig. 2 represent the condensed background spectrum, and the *circles* are the difference spectrum calculated from the dimethylamine record in Fig. 1. Because of the lower recording impedance, our vaseline-gap method can give less background noise than microelectrode methods, especially in the range of 8–

100 Hz (cf. Anderson and Stevens [1973]), and therefore we have been able to study smaller single-channel currents than have previously been described. Thus, with dimethylamine, illustrated in Figs. 1 and 2, the single-channel currents and average ACh-induced variance were <30% of those with Na^+ and yet the extra fluctuations stand out clearly from the background noise.

In all, we obtained 800 averaged difference spectra in this study. They were analyzed in the conventional manner (Anderson and Stevens, 1973), assuming that

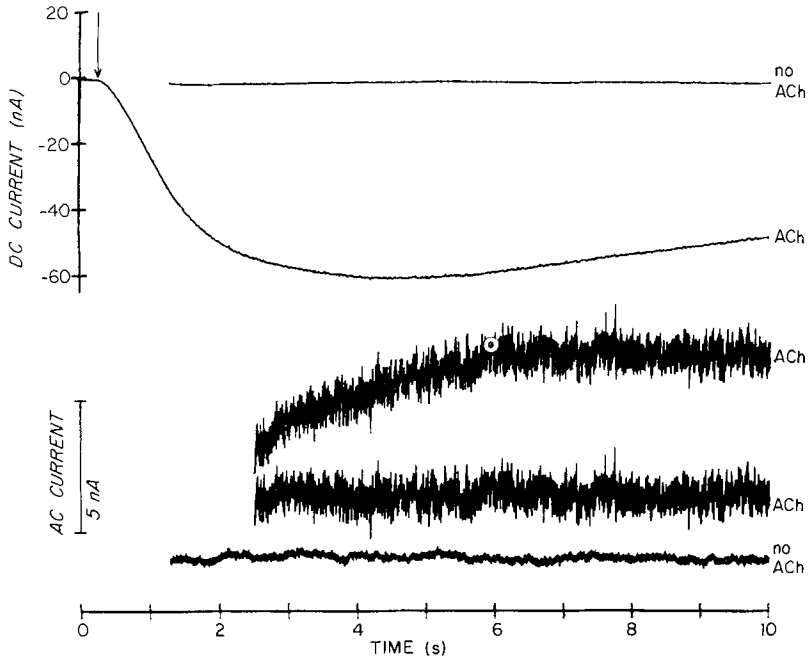


FIGURE 1. Voltage-clamp current recorded from an endplate bathed in dimethylamine test solution at −73 mV. *Top*: low-gain, DC-coupled records. One trace gives the baseline without ACh. The other shows net inward endplate current developing while ACh is applied iontophoretically beginning at $t = 0.25$ s (*arrow*). *Bottom*: high-gain, AC-coupled records of the same runs. The quietest trace is without ACh. The other two show a large increase in fluctuations during application of ACh. The *sloping trace* is the raw record, whereas the *straighter trace* is the same record after a polynomial fitted to its low frequency components has been subtracted.

one class of channels is opening randomly with a single time constant governing the lifetime of the open state. A nonlinear, weighted least-squares curve-fitting routine (Conti et al., 1976) was used to find the best-fitting Lorentzian curve (*smooth line*, Fig. 2). While recognizing that certain untested assumptions are implied, we will call the best-fitting time constant, τ, interchangeably by the names noise time constant and mean channel open time. These assumptions include that a single, first-order process adequately describes the result and that only a small fraction of the available channels are opened. The variance of σ_i^2 of the current fluctuations was obtained by integrating the original difference spectrum and was used to calculate the single-channel con-

ductance, γ, of endplate channels (Stevens, 1972):

$$\gamma = \frac{\sigma_i^2}{I_{ep}(E - E_r)} \quad (1)$$

where I_{ep} is the mean ACh-induced endplate current, E is the holding potential, and E_r is the reversal potential for current in endplate channels taken from Dwyer et al. (1980) and Adams et al. (1980). In both Eq. 1 and the identification of τ with the mean open time of the channel, we are assuming that the concentration of ACh at the receptors is well below that needed to open half the available channels. This assumption was tested for almost all cations, except a few that gave the smallest

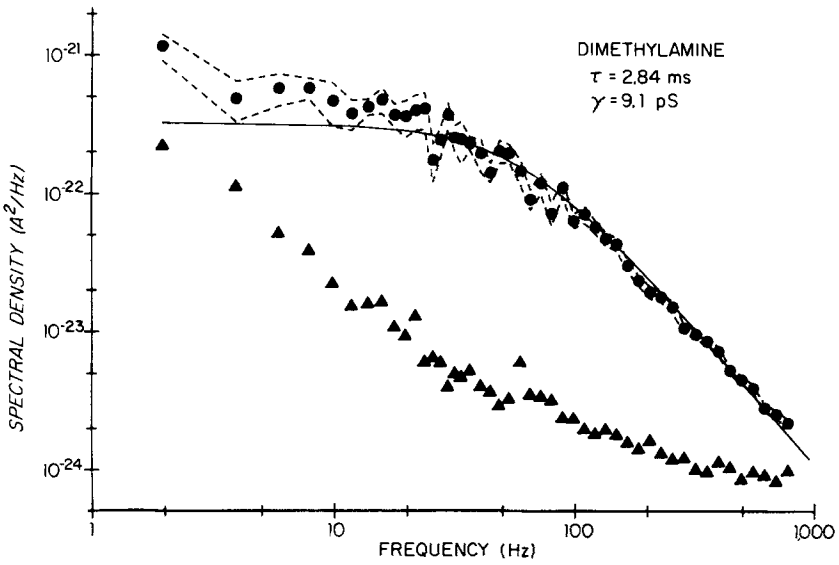
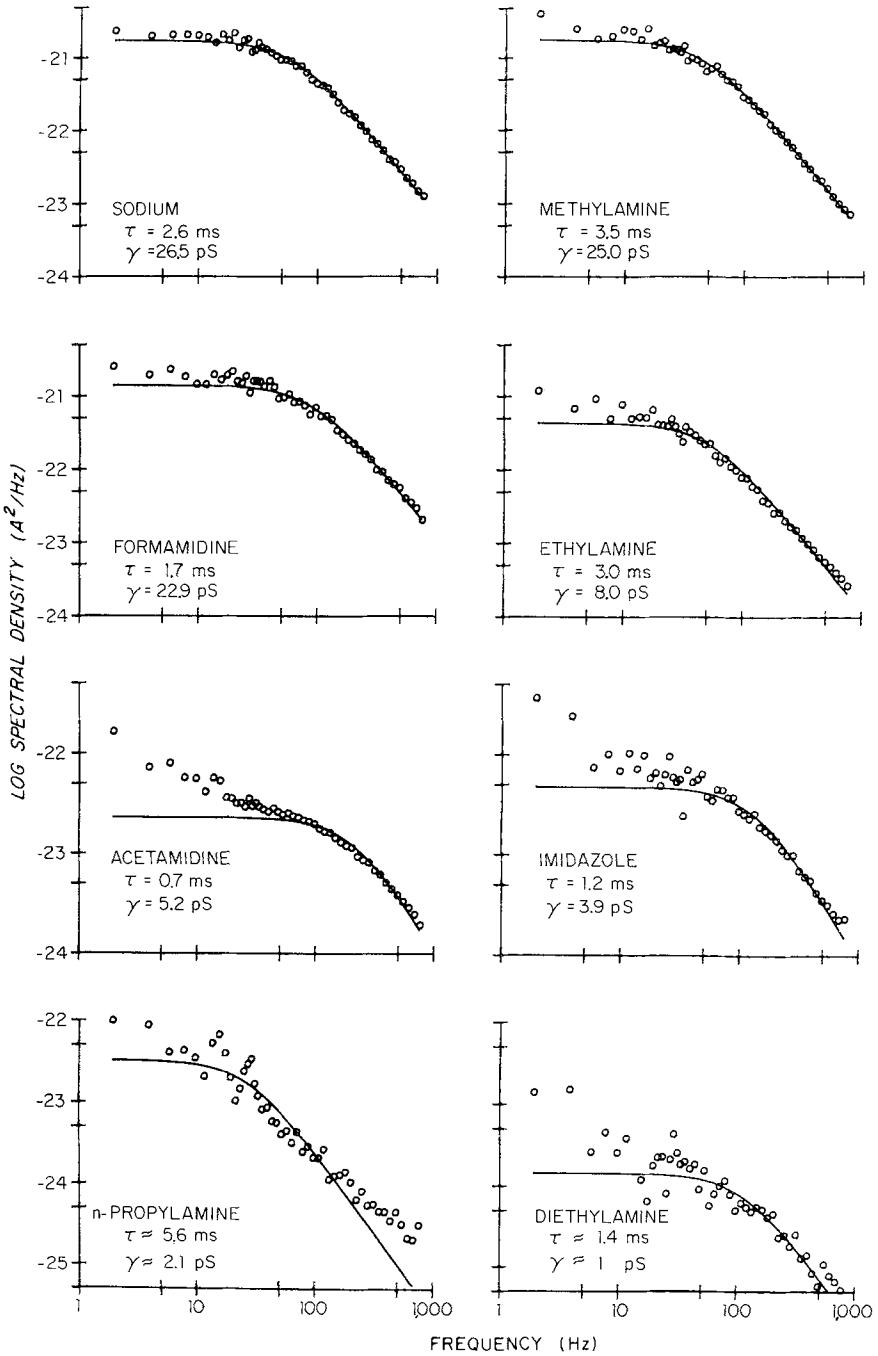


FIGURE 2. Power density spectra of current fluctuations in dimethylamine test solution. The spectra are calculated from the two AC-coupled records in Fig. 1. *Triangles*: background fluctuations without ACh, condensed to 47 points (Conti et al., 1976) below the lowpass filter frequency of 800 Hz. *Circles*: difference spectrum, fluctuations with ACh minus the background fluctuations. *Dashed lines* show ±1.0 SEM of all the values that were condensed and averaged into the final points. *Solid line*: a Lorentzian curve fitted by least squares to the difference spectrum with parameters τ = 2.84 ms and area (variance) = 0.030 $(nA)^2$. For this fiber, γ = 9.1 pS.

conductance, by making fluctuation measurements with several doses of iontophoresed ACh. For example, γ and τ might be compared for doses of ACh giving −20, −40, and −60 nA of endplate current. Increasing the current did not decrease γ and τ as it would when approaching saturation, so all measurements were lumped together in the final values given in the tables.

In the text and tables, different values are pooled and given as mean ± SEM. Independent difference spectral measurements on the same fibers and on different fibers were analyzed separately and counted as one observation. Figs. 1 and 2 show such a single measurement; however, for the purpose of illustration only, the spectra in Fig. 3 are the averages of several measurements.



RESULTS

Preliminary Experiments

Before examining the endplate permeability to foreign cations, we will consider a few control experiments.

Our first measurements of endplate current fluctuations were done with fibers of *Rana pipiens* bathed in the standard, Na-containing reference solution. The calculated power-density difference spectra were fitted well by a Lorentzian function with the frequent exception that the first few points at the lowest frequencies lay above the horizontal line. Fig. 3 (*top left*) shows an average of several different difference spectra, taken in the sodium reference solution, together with a fitted Lorentzian function. In 34 measurements at −73 mV, the single-channel conductance using Eq. 1 was 27.5 ± 0.6 pS, and the fitted noise time constant was 2.92 ± 0.08 ms at 12°C. These values are indistinguishable from those given by Colquhoun et al. (1975) for endplates of intact cutaneous pectoris fibers of *R. pipiens*: 25 pS and 3.2 ms at 10–15°C. In addition, the time constant lengthened to 8.4 ± 0.8 ms (n = 5) with hyperpolarization to −143 mV and shortened to 0.99 ± 0.07 ms (n = 8) with depolarization to +43 mV, while the single-channel conductance was unaffected (28.9 ± 0.9 and 27.5 ± 1.6 pS, respectively). Hence, the endplate channels of our fibers with cut ends bathed in 120 mM CsF have properties similar to those of intact fibers. On the other hand, as in our earlier experiments (Dwyer et al., 1980), the decay time constant of the miniature endplate currents in our fibers is close to 6 ms at −73 mV, or about twice the mean open lifetime of single channels. We suggest, therefore, that cholinesterase may have become inhibited by exposure to F^- ions (Froede and Wilson, 1971), so that ACh molecules released in a quantum may activate receptors several times before leaving the synaptic cleft.

Because of a problem with the supply of animals, a later series of experiments had to be done with fibers from *Rana temporaria*. To our surprise, the mean open time of the endplate channels of these fibers (1.71 ± 0.03 ms, n = 49) was consistently shorter than that with *R. pipiens*. Therefore, time-constant measurements with the two species are clearly separated in the tables that follow. On the other hand, the single-channel conductance was only margin-

FIGURE 3. (*Opposite*) Averaged difference spectral densities of ACh-induced fluctuations at −73 mV with eight different external test cations. The *circles* are experimental points and the *solid line* is the best-fitting Lorentzian curve. Numerous spectra, calculated and condensed as in Fig. 2 from different 10-s runs on the same and different fibers, were averaged together to give the points shown here. τ is the time constant of the curve drawn, but γ is the average of all measurements with the test cation (from Table I). The measurements with sodium methylamine and ethylamine were the *Rana pipiens*, and the others were with *R. temporaria*. The number of different runs averaged together is: sodium 5, methylamine 3, formamidine 2, ethylamine 4, acetamidine 10, imidazole 2, *n*-propylamine 4, and diethylamine 3.

ally smaller with *R. temporaria*, 25.9 ± 0.5 pS, so such measurements are not kept separate.

Further control experiments with *R. temporaria* fibers tested the influence of the buffer, the major anion, and the calcium concentration. To summarize, the single-channel conductance and time constants were: with 4 mM morpholinopropanesulfonic acid buffer, 24.6 ± 0.8 pS and 1.68 ± 0.04 ms (n = 23); with 10 mM histidine buffer, 27.0 ± 0.7 pS and 1.75 ± 0.05 ms (n = 26); with all chloride replaced by nitrate, 28.3 ± 1.0 pS and 1.74 ± 0.08 ms (n = 11); with all chloride replaced by glucuronate, 23.0 ± 2.6 pS and 1.4 ± 0.1 ms (n = 3); in 0 mM Ca, 28.6 ± 3.4 pS and 1.59 ± 0.04 ms (n = 3); and in 5 mM Ca, 19.7 ± 1.2 pS and 1.4 ± 0.1 ms (n = 6). Although some of these numbers may be significantly different, the differences are small in relation to those seen when Na^+ ions are replaced by other monovalent cations. Therefore, we consider in this paper that all effects we describe may be attributable to the monovalent cations rather than to the anions or to possible small changes in ionic strength or free divalent ion concentration.

Single-Channel Conductances Deviate from Independence

TOTAL REPLACEMENT OF SODIUM Acetylcholine-induced current fluctuations were measured with 19 different external test cations replacing all of the Na^+ ions of the reference solution. Fig. 3 shows averaged power-density difference spectra for some of the experiments together with the best-fitting Lorentzian function drawn as a smooth curve, and Table I lists the resulting single-channel properties. In this section we consider only the single-channel conductance and later we will return to the shape of the spectrum and the apparent mean open time of the channels.

Six relatively small cations, ammonium, hydrazinium, Cs^+, Rb^+, methylammonium, and formamidinium, give single-channel conductances at the endplate similar to or higher than that for Na^+ ions. According to our earlier measurements of reversal potentials (Dwyer et al., 1980; Adams et al., 1980), these same small cations also have a high permeability relative to Na^+, P_X/P_{Na} = 1.3–1.8. Methylamine was the organic cation whose properties at endplate channels seemed to approximate most closely those of Na^+. Ammonium, Rb^+, and Tl^+ increased the background fluctuations without acetylcholine, presumably because of their permeability in K channels.

Most test cations in Table I reduced the single-channel conductance of endplate channels, and almost half gave conductances of <10 pS. In this group are nine cations with three to five major atoms (neglecting hydrogens) and permeability ratios of 1.20–0.38. If the probability that an ion crosses an open endplate channel were independent of other ions, then the single-channel chord conductance with different external permeant ions would be a simple increasing function of the relative permeabilities of the ions. This independence relation (Hodgkin and Huxley, 1952) for conductances γ_A and γ_B seen with two different external ions A and B can be written in terms of the observed reversal potentials E_A and E_B and the permeability ratio P_A/P_B as

$$\gamma_A/\gamma_B = \frac{P_A[A]_0}{P_B[B]_0} \cdot \frac{1 - \exp\{(E - E_A)F/RT\}}{1 - \exp\{(E - E_B)F/RT\}} \cdot \frac{E - E_B}{E - E_A} \quad (2)$$

where R, T, and F are the usual thermodynamic quantities. Fig. 4 plots observed γ values vs. permeability ratio together with the predictions of independence (smooth curve) using the Na^+ ions as a reference. Independence definitely does not hold. All ions except NH_4^+ conduct less well than expected. The observations might be better understood by assuming that transport is a saturable process involving competition for at least one binding site in the channel and that the fraction of free sites is relatively small with most of the test solutions. Sodium and NH_4^+ ions would then be in the group giving the least saturation.

TABLE I

SINGLE-CHANNEL PROPERTIES AT −73 mV AND 12°C WITH DIFFERENT EXTERNAL CATIONS

$\gamma \pm$ SEM	n	P_X/P_{Na}	$\tau \pm$ SEM, *R. pipiens*	$\tau \pm$ SEM, *R. temporaria*	X (external)
pS			*ms*	*ms*	
43.7 ± 4.1	(6)	1.79	2.60 ± 0.29	—	Ammonium
30.9 ± 2.1	(7)	1.32	1.60 ± 0.12	—	Hydrazine (pH = 6.6)
28.9 ± 1.6	(7)	1.42	—	1.05 ± 0.06	Cs
28.5 ± 1.9	(5)	1.30	—	0.80 ± 0.02	Rb
26.5 ± 0.4	(83)	1.00	2.92 ± 0.08	1.71 ± 0.03	Na
25.0 ± 4.0	(16)	1.34	2.63 ± 0.10	—	Methylamine
22.9 ± 0.9	(11)	1.58	—	1.17 ± 0.08	Formamidine
16.9 ± 1.4	(7)	2.51	—	0.46 ± 0.06	Tl
13.7 ± 0.7	(7)	0.87	—	1.80 ± 0.05	Li
11.8 ± 1.4	(7)	0.72	—	1.67 ± 0.13	Ethanolamine
8.9 ± 0.2	(7)	0.87	2.38 ± 0.14	—	Dimethylamine
8.0 ± 0.3	(8)	1.13	2.65 ± 0.14	—	Ethylamine
6.8 ± 0.4	(11)	0.68	—	0.80 ± 0.05	Ethylenediamine (pH = 8.4)
5.2 ± 0.1	(11)	1.20	—	0.69 ± 0.02	Acetamidine
3.9 ± 0.2	(5)	0.95	—	(0.9 ± 0.1)	Imidazole (pH = 6.0)
2.1 ± 0.2	(3)	0.94	—	(1.4 ± 0.1)	Aminothanthiol
2.1 ± 0	(2)	0.68	—	(2.7 ± 0.1)	*n*-Propylamine
2.1 ± 0.1	(2)	0.82	—	(2.1 ± 0.2)	*i*-Propylamine
1	(4)	0.38	—	—	Diethylamine

External test solution: 114 mM test salt (XCl), 1 mM $CaCl_2$, 10 mM histidine.
Internal solution: 120 mM CsF. (For thallous ion, the solutions contained nitrate rather than chloride salt.)
Permeability ratios from Dwyer et al. (1980) and Adams et al. (1980).

MIXTURES OF SODIUM WITH TEST CATIONS The apparent saturation of channel sites by permeant ions was investigated further with 1:1 mixtures of the reference solution with test cations. Single-channel properties calculated from endplate fluctuations in the mixtures are listed in Table II, and the conductance values are compared with the predictions of independence in Fig. 5. According to independence (Eq. 2), in a 1:1 dilution of external sodium with an impermeant substance, γ should fall to ~16 pS. In fact, dilution with the sugar mannitol reduced γ to ~12 pS as if independence were not perfectly obeyed. Several poorly permeant cations, lysine, arginine, histidine, and glucosamine, reduced γ much more, and even more permeant cations, guanidine, acetamidine, Tris, and *n*-propylamine, also reduced γ strongly. The

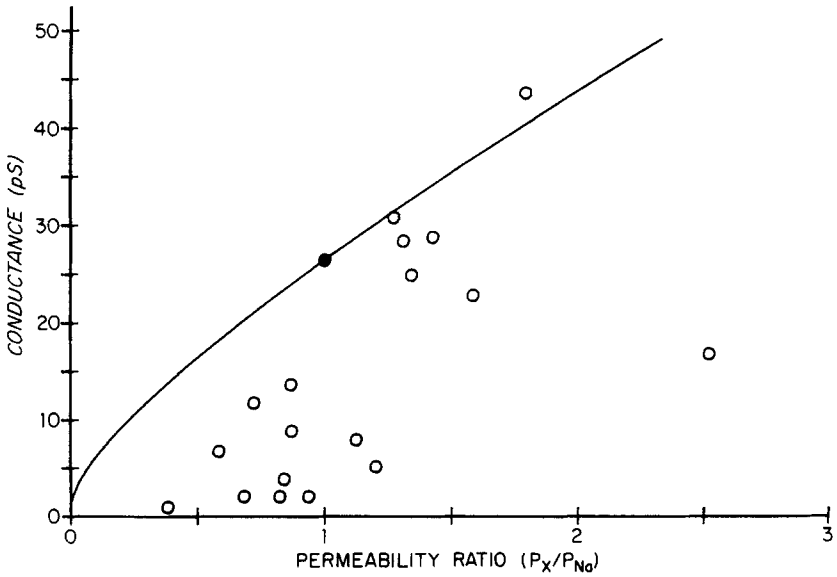


FIGURE 4. Test of the independence relation for total substitution of external Na^+ by test cations. *Circles* show the relation between single-channel conductance at −73 mV and permeability ratio (data of Table I), and the *smooth line* is the prediction of the independence relation (Eq. 2) using Na^+ ions as a reference. The filled point on the line is Na^+. Except for NH_4^+, all other ions give less conductance than predicted.

TABLE II

SINGLE-CHANNEL PROPERTIES AT −73 mV WITH SODIUM/ TEST-CATION MIXTURES

γ ± SEM	*n*	P_X/P_{Na}	τ ± SEM	X (mixed with Na)
pS			*ms*	
25.9 ± 0.5	(49)	1.00	1.71 ± 0.03	Na
22.1 ± 1.5	(11)	2.51	0.78 ± 0.10	Tl
12.9 ± 0.6	(5)	0.72	1.48 ± 0.04	Ethanolamine
11.7 ± 0.5	(15)	0	1.23 ± 0.05	Mannitol
7.3 ± 0.5	(7)	0.035	1.74 ± 0.13	Lysine
6.5 ± 0.4	(8)	1.59	0.48 ± 0.01	Guanidine
6.0 ± 0.1	(6)	1.20	0.94 ± 0.02	Acetamidine
5.9 ± 0.2	(8)	0.039	1.43 ± 0.06	Arginine
5.5 ± 0.6	(6)	0.043	1.81 ± 0.08	Histidine (pH = 6.1)
3.6 ± 0.1	(5)	0.034	3.20 ± 0.51	Glucosamine (pH = 6.2)
3.0 ± 0.1	(13)	0.18	4.0 ± 0.2	Tris (pH = 6.7)
2.1 ± 0.2	(3)	0.68	3.6 ± 0.1	*n*-Propylamine

All experiments were performed with *R. temporaria* at 12°C.
External test solution: 57 mM NaCl, 57 mM test salt (XCl), 1 mM $CaCl_2$, 10 mM histidine. (For thallous ion, the solutions contained nitrate rather than chloride salts.)
Internal solution: 120 mM CsF.
Permeability ratios from Dwyer et al. (1980) and Adams et al. (1980).

results show clearly that all cations tested block endplate channels partially even though most also have a high relative permeability by the reversal-potential criterion. For the few blocking cations studied both in pure form and in mixtures, i.e., ethanolamine, acetamidine, and *n*-propylamine, the conductance in the sodium mixture was, surprisingly, only slightly above that without sodium. Again, these effects can be interpreted in terms of competition within the channel for a saturable binding site that is also involved in ion transport.

REPLACEMENT OF THE INTERNAL CATION The major series of experiments was done with the cut ends of the muscle fragment bathed in 120 mM CsF. However, a few current fluctuation measurements were done on fibers cut in 120 mM NH_4F or in the mixture 12 mM CsF, 108 mM L-arginine-L-aspartate,

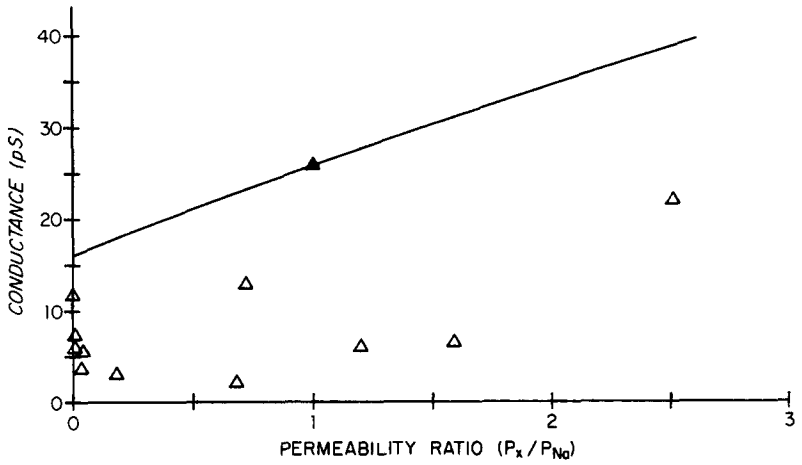


FIGURE 5. Test of the independence relation for mixtures of Na^+ with test cations. *Triangles* show the relation between single-channel conductance and permeability ratio to the test ion (data of Table II). The *smooth line* is the prediction of Eq. 2, taking E_A as the measured reversal potential in the mixture and P_A as the mean of the relative permeabilities for Na^+ and the test ion.

pH 6.9. The single-channel parameters at −73 mV are given in Table III. Without exception, dilution of the internal Cs^+ concentration increased γ as if internal CsF depresses γ in a way that NH_4F and arginine-aspartate do not. Switching from internal 120 mM CsF to 12 mM CsF increased γ by an average of 33%. Changing to internal NH_4F gave a similar increase, and when NH_4^+ ion was placed both inside and out, γ became as much as twice the control value with Cs^+ inside and Na^+ or Cs^+ outside. The conductance-enhancing effect of internal arginine contrasts markedly with the conductance-blocking effect already seen with external arginine (Table II).

VOLTAGE DEPENDENCE OF γ Although the majority of our experiments were done at −73 mV, we made a few measurements at +43 mV and at −130 or −143 mV. Table IV lists γ values for the most extensively studied solutions.

No *major* voltage dependence of the single channel conductance was observed with any of the test cations that gave γ values of >15 pS. This includes Cs^+, Na^+, methylamine, and formamidine. On the other hand, with ions of low conductance, γ was always larger at +43 mV than it was at −73 mV, showing

TABLE III

INCREASE OF γ WITH INTERNAL NH_4^+ OR LOW Cs^+

γ*	γ ± SEM	*n*	τ ± SEM	External cations
pS	*pS*		*ms*	
Ends cut in 120 mM NH_4F				
43.7	58.7 ± 0.1	(2)	1.8 ± 0.03	Ammonium
25.9	31.9 ± 0.2	(2)	1.4 ± 0.1	Na
Ends cut in 12 mM CsF±108 mM arginine aspartate				
25.9	29.2 ± 2.3	(9)	2.54 ± 0.18	Na
11.7	15.8 ± 1.6	(3)	1.76 ± 0.11	Mannitol/Na
6.5	8.0 ± 0.2	(5)	0.62 ± 0.03	Guanidine/Na
5.9	7.5 ± 1.1	(5)	2.56 ± 0.15	Arginine/Na
5.5	5.7 ± 1.3	(4)	7.0 ± 1.5	Glucosamine/Na
3.0	4.3 ± 0.3	(5)	5.1 ± 0.4	Tris/Na

All γ and τ values were measured at −73 mV and 12°C with *R. temporaria* fibers, except γ*, for ammonium which was measured for *R. pipiens*.
γ* values from Tables I and II for fibers with ends cut in 120 mM CsF.
External solutions denoted guanidine/Na^+, etc., are mixtures of 57 mM Na^+ plus the test substance as in Table II.

TABLE IV

VOLTAGE DEPENDENCE OF SINGLE-CHANNEL CONDUCTANCE

−130 or (*) −143 mV	γ ± SEM (*n*)		External ions
	−73 mV	43 mV	
pS	*pS*	*pS*	
30.9 ± 2.4 (3)	28.9 ± 1.6 (7)	—	Cs^+
28.9 ± 0.9* (5)	26.5 ± 0.4 (83)	27.5 ± 1.6 (8)	Na^+
26.0 ± 0.9* (6)	25.0 ± 4.0 (16)	20.1 ± 2.4 (3)	Methylamine
30.5 ± 0.7 (4)	22.5 ± 0.9 (11)	24.5 ± 2.4 (3)	Formamidine
14.0 ± 1.1 (3)	11.7 ± 0.5 (15)	17.0 ± 0.7 (2)	Na^+/mannitol
12.6 ± 1.3 (6)	13.7 ± 0.7 (7)	18.9 ± 0.1 (2)	Li^+
9.4 ± 0.5 (6)	6.5 ± 0.4 (8)	11.4 ± 3.2 (3)	Na^+/guanidine
6.3 ± 0.4 (2)	6.0 ± 0.1 (6)	—	Na^+/acetamidine
4.2 ± 0.1 (2)	5.6 ± 0.9 (10)	11.1 ± 0.6 (2)	Na^+/glucosamine
2.0 ± 0.2 (4)	3.0 ± 0.1 (13)	15.7 ± 0.2 (2)	Na^+/Tris
2.4 ± 0.2 (4)	2.10 ± 0.1 (4)	4.6 ± 0.2 (2)	Propylamines

An equal number of observations with 2-propyl and *n*-propylamine are included under propylamines.
Internal solution: 120 nM CsF except for half of the Na^+/glucosamine values, which are with 12 mM CsF and 108 mM arginine aspartate.

that outward currents carried by Cs^+ ions (from internal solution) flow more readily than inward currents carried by the poorly conducting ions. This effect was found with pure dimethylamine, ethylamine, and *n*- and *i*-propylamine, as well as with sodium mixtures of mannitol, guanidine, glucosamine, Tris,

and *n*-propylamine. Lumping all these observations together, the γ at +43 mV averaged 2.3 times the value at −73 mV. With sodium mixtures of Tris, histidine, glucosamine, or arginine, there was also a strong additional reduction of γ during hyperpolarizations from −73 mV, but with many other compounds this did not occur. The voltage dependence of γ suggests that the "blocking" site for permeant ions like the propylamines, histidine, or Tris is within the electric field of the membrane as expected for a site within the channel pathway itself.

Foreign Cations Change Channel Gating

As others have noted (Van Helden et al., 1977; Ascher et al., 1978; Gage and Van Helden, 1979; Marchais and Marty, 1979), the gating rate constants for ACh-activated channels depend on the permeant cations in the external solution. With many of the pure cations and cation mixtures we studied, the power spectral density of current fluctuations retained an approximately Lorentzian shape (Figs. 2 and 3) and the noise time constant was shorter than in the Na reference solution (Tables I–III). However, at frequencies <10 Hz, the spectral points always rose above the fitted Lorentzian to varying degrees, a deviation that was not very pronounced with ions giving γ values >6 pS.

For ions giving very low single-channel conductances, a single Lorentzian curve was obviously a poor fit to the fluctuation spectra (Fig. 3), as if more processes than a single open-close step were kinetically significant. The problem was worst with the propylamines (γ = 2.1 pS) and diethylamine ($\gamma \simeq 1$ pS). It was less obvious in Na^+ mixtures and in fibers depolarized to +44 mV, and it was more obvious in fibers hyperpolarized to −130 mV. In these experiments with ions of low γ, we often had to increase the iontophoretic dose of ACh considerably to bring the peak endplate current up to a useful range (10–40 nA). Nevertheless, the excess fluctuation power, proportional to $I \cdot \gamma$, was necessarily still small and more subject to systematic errors in the background correction. Probably even more importantly, the mean currents with these ions almost always decayed to 20–30% of their peak value during the ACh pulse, presumably by the development of desensitization and/or use-dependent block. Desensitization has numerous time constants, some of which could account for the low-frequency rise in our spectra (Sakmann et al., 1980). Whatever the cause, the big deviations from a Lorentzian curve prevent us from giving a reliable mean open lifetime for the last six cations in Table I.

Most external test cations shortened the mean channel open time. Of the highly permeant ions tested, only Li lengthened the open time, and that only slightly (Table I). Of the less permeant ions, the propylamines seemed to lengthen τ, but the non-Lorentzian spectral shape precludes a reliable estimate. In mixtures with Na, however, *n*-propylamine, glucosamine, and Tris cations clearly did lengthen the mean open time, whereas all the more permeant ions shortened it (Table II). Replacing the internal CsF by arginine aspartate also had a consistent effect on gating (Table III), lengthening the open time by an average of 58% in the six external solutions studied. As is the case for intact muscle fibers, hyperpolarization lengthened and depolarization

shortened the mean open time with all combinations of external and internal ions that we tested.

DISCUSSION

Comparison with Previous Work

As we have said, the single-channel properties measured in the Na reference solution agree well with those found by others using amphibian neuromuscular junctions (for a review, see Colquhoun [1979]). The only previous reports of single-channel properties with foreign monovalent cations at the motor endplate come from Gage and co-workers, who were the first to show that mean channel open time depends on the permeant ions. In qualitative agreement with most of our observations, they report for the ions Li^+, Na^+, Cs^+, and NH_4^+ single-channel conductances in the ratios 0.5:1.0:1.2:1.3, and mean open times in the ratios 1.3:1.0:0.9:1.6 (Van Helden et al., 1977; Gage and Van Helden, 1979; Takeda et al., 1980). The one appreciable difference is that they found a 60% lengthening of τ by NH_4^+, whereas we found an 11% decrease. Other earlier work showed that a wide variety of Na^+ substitutes alter the responses to iontophoretic ACh pulses and the amplitude and time-course of miniature endplate potentials or currents, but such experiments could not distinguish effects on γ and τ from the many other ways of altering amplitude and time-course (literature summarized in Dwyer et al. [1980], and Adams et al. [1980]).

Ascher et al. (1978) and Marchais and Marty (1979 and 1980) have shown that foreign monovalent cations also change the properties of ACh-activated channels of *Aplysia* neurons. In seawaters made with Li^+, Na^+, or Cs^+ ions, they reported single-channel currents at −80 mV in the ratios 0.5:1.0:1.7 and relaxation time constants in the ratios 1.3:1.0:1.9. They also found that Tris or glucosamine added to Na-containing solutions markedly depress γ, with no change of τ for Tris, and a strong lengthening of τ for glucosamine. In their work, all Na-free solutions lengthened. These results show that although there are remarkable similarities in conductance properties between the channels of *Aplysia* neurons and amphibian endplates, there are some clear differences in the gating processes. In particular, we find that most Na-free solutions, including Cs^+, shorten τ at the endplate.

Ions Bind While Crossing Endplate Channels

EVIDENCE FOR BINDING OF PERMEANT IONS Recent investigations of fluxes in a variety of biological ionic channels have revealed deviations from independence with different permeant ions. Acetylcholine-activated channels have been no exception. For example, Ca^{++} ions added to Ringer's solution bathing a frog endplate depress γ, and pure $CaCl_2$ solutions give a much smaller γ than would be predicted from P_{Ca}/P_{Na} measured by reversal potentials (Lewis, 1979). Lewis and Stevens (1979) modeled the deviation with a Ca^{++} binding site in the channels halfway across the membrane. Similar observations have inspired a similar model for cholinergic channels of *Aplysia* neurons (Marchais

and Marty, 1979 and 1980). In addition, single-channel currents in cholinergically activated channels of rat myotubes saturate when the Na^+ content of the medium bathing the myoplasmic face is increased (Horn and Patlak, 1980). The apparent dissociation constant for internal Na^+ was ~140 mM at +100 mV.

Our work also suggests that permeant ions bind within the channel. Most external Na^+ substitutes give a smaller γ than is predicted by independence from their relative permeabilities (Fig. 4). Many permeant cations even reduce γ when added to Na^+ solutions instead of increasing it (Fig. 5). The reduction of γ is partially relieved by depolarization. Even internal Cs^+ ions seem to block, for diluting them with arginine aspartate or replacing them with NH_4^+ increases γ (Table III). In our earlier experiments (Dwyer et al., 1980), we found increased *macroscopic* conductances when internal NaF was diluted with arginine aspartate. The increased current there was probably due in part to a combination of increased γ and longer τ, as was seen with dilution of internal CsF (Table III).

ESTIMATED DISSOCIATION CONSTANTS If we assume that all deviations from independence in Figs. 4 and 5 are due to partial saturation of a binding site within the channel, we can estimate the relative saturation under each test condition. The procedure is to divide the observed conductance ratios γ_X/γ_{Na} by the ratios expected from independence (Eq. 2), giving the quotients Q listed in Table IV. In a simple, one-ion model, the quotient Q is equal to the fraction of channels free (without a bound ion) in solution X divided by the fraction free in the Na reference solution. By definition, the reference solution gives a quotient of 1.0. All other ions except NH_4^+ give smaller values, meaning that they bind more strongly than Na^+, whereas NH_4^+ binds less. Lewis (1979) found no evidence for binding of Na^+ ions, but our finding of a $Q > 1.0$ with ammonium suggests that there may be some. To proceed, we assume arbitrarily that 80% of the channels are still free in the reference solution. Then the column labeled "0.8 Q" in Table V represents the absolute fraction of free channels in each solution. According to the calculation, several organic cations, both pure and in Na^+ mixtures, nearly saturate the assumed binding site and leave only 5–15% of the channels free. These numbers are then used with conventional binding theory to calculate the apparent dissociation constant K_{diss} at −73 mV for each ion in Table V.

The estimated dissociation constants decrease as carbon atoms are added to the organic cations, suggesting that there is a large hydrophobic component to the binding energy. Fig. 6 is a semilogarithmic plot of K_{diss} vs. the number of carbon atoms in a compound. The *filled circles* represent the homologous series of the simple alkylammonium ions derived from NH_4^+. For each carbon added, the apparent affinity increases 4.4-fold on the average, corresponding to a standard free energy increment of −840 kcal/mol per carbon. This relationship, shown as a straight line, corresponds closely to the increment of −884 kcal/mol per methylene for transfer of hydrocarbons from water to liquid hydrocarbon (Tanford, 1973). Comparisons of ions of similar size show that carbons do not increase affinity simply by increasing the ionic size. For

example, formamidine binds less than ethylamine, and ethylene diamine, ethanolamine, and acetamidine bind less than the propylamines. In each of these examples, polar groups decrease binding and methylene groups increase it. The calculations of Table V also suggest that Li^+ and Tl^+ ions are the only

TABLE V

DEVIATIONS FROM INDEPENDENCE AND APPARENT DISSOCIATION CONSTANTS FOR CHANNEL SATURATION

Q	0.8 Q	K_{diss}	Compound X
		mM	
Pure compounds:			
1.09	0.87	758	Ammonium
1.00	0.80	456	Na
0.98	0.79	422	Hydrazine
0.89	0.71	281	Rb
0.85	0.68	241	Cs
0.77	0.61	181	Methylamine
0.62	0.50	113	Formamidine
0.57	0.46	96	Li
0.56	0.45	92	Ethanolamine
0.37	0.30	48	Ethylenediamine
0.37	0.30	48	Dimethylamine
0.33	0.26	40	Tl
0.28	0.22	33	Ethylamine
0.17	0.14	18	Acetamidine
0.17	0.13	17	Imidazole
0.10	0.08	10	*n*-Propylamine
0.09	0.07	9	*i*-Propylamine
0.08	0.07	8	Aminoethanthiol
0.07	0.06	7	Diethylamine
Mixtures with Na^+:			
0.72	0.58	94	Mannitol
0.62	0.50	65	Ethanolamine
0.56	0.45	51	Tl
0.45	0.36	34	Lysine
0.36	0.29	24	Arginine
0.33	0.27	15	Histidine
0.22	0.17	12	Glucosamine
0.22	0.17	12	Acetamidine
0.21	0.17	12	Guanidine
0.17	0.13	9	Tris
0.09	0.07	5	*n*-Propylamine

Q is (γ_X/γ_{Na}) at −73 mV divided by the conductance ratio predicted by independence (Eq. 2, see also legend to Fig. 5).

K_{diss} is the dissociation constant needed to make the fraction of free channels equal to 0.8 Q (taking into account 67 mM Na^+ in mixtures).

monovalent metal ions that bind appreciably from the outside. This conclusion, however, rests on our assumption that the channel site is far from saturated in the Na^+ reference solution.

Two notes of caution should be sounded here. First, the K_{diss} values given in Table V have been determined by comparing P and γ values at only one

high concentration of the test ion. New measurements should now be done spanning the appropriate concentration range for each ion. Second, if, as we believe, the binding site is within the pore, the estimated K_{diss} values for permeant ions are not true equilibrium constants but rather, like a Michaelis constant, are kinetic constants that include passage into the cell as part of the "off" rate.

THE CHANNEL Earlier work has given an indication of the topography of the endplate channel. We found that ions up to the size of glucosamine are permeant and suggested that the narrowest region of the pore must have a

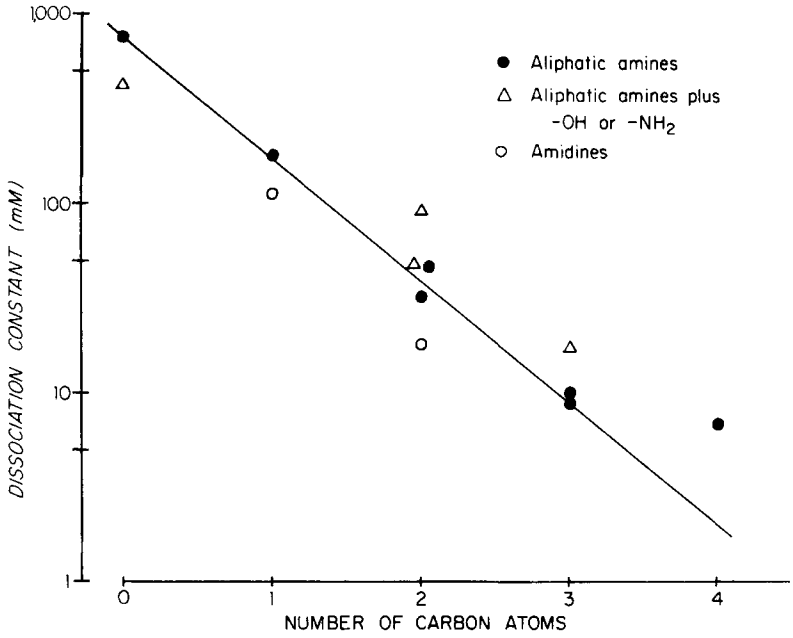


FIGURE 6. Evidence for a hydrophobic site in endplate channels. Semilogarithmic plot of the calculated dissociation constants for test cations vs. the number of carbon atoms in the compound. All values in Table IV for non-metal cations measured at full strength are included, except for aminoethanthiol, which has been omitted. The *line* represents a binding free-energy increment of −840 kcal/mol per carbon atom.

cross-section of at least 6.5 Å × 6.5 Å (Dwyer et al., 1980). Others have shown that the outer half of the pore is even wider than this. Thus, externally applied large hydrophobic ions, including procaine, quaternary lidocaine, decamethonium, tubocurarine, tetraethylammonium, and akylguanidines, all produce a block of channels that is intensified by hyperpolarization and weakened by depolarization (Adams, 1977; Neher and Steinbach, 1978; Adams and Sakmann, 1978; Colquhoun et al., 1979; Adler et al., 1979; Adams and Feltz, 1980; Farley et al., 1981). The voltage dependence is consistent with a binding site for these blocking ions halfway across the membrane and with an intrinsic dissociation constant of 0.1–200 μM for the different blocking ions. The site is

evidently not readily accessible to local anesthetic derivatives applied from the myoplasmic side of the channel (Horn et al., 1980).

We suggest that the hydrophobic site we have identified for permeant organic ions could be the same as this site for block by local anesthetics and their relatives. Extrapolation of the binding relation of Fig. 6 predicts dissociation constants of <300 nM for these blocking agents, even stronger binding than is observed. However, one could reasonably anticipate that the available hydrophobic pocket or pockets are limited in extent and not infintely adaptable to such large molecules. In line with this suggestion, dissociation constants for channel block by alkyl guanidines from ethyl to octyl guanidine (Farlay et al., 1981) fit fairly well onto our Fig. 6 with a slope that decreases for the higher homologues. Our observation that arginine cations block the channel readily when applied from the outside (Table II) and not from the inside (Table III) seems also to parallel the sidedness of action found by Horn et al. (1980) for local anesthetic derivatives. Many types of ionic channels have binding sites for hydrophobic drugs thought to act within the pore itself. The endplate channel apparently differs from the well-described Na and K channels in having its hydrophobic site more accessible to extracellular than to intracellular ligands.

Our earlier work with organic cations also demonstrated that permeability P measured by reversal potentials depends inversely on the size of the permeating cation as if friction is the major determinant of P (Dwyer et al., 1980). Thus, to simplify a bit, γ is determined by hydrophobicity and P is determined by size. Evidently, the channel as seen by organic cations may be represented as a sequence of one or several barriers, whose height (governing P) increases with the cation size, and one or several wells, whose depth (governing γ) increases with hydrophobicity, and at least for larger cations, the barriers in the inner half of the channel are higher than those in the outer half. (See Hille [1975] for a discussion of factors affecting P, γ, and independence.)

For the small metal cations, all of which are permeant (Adams et al., 1980), the channel is of course chemically the same but the chemistry might translate into different effective positions of barriers and wells. In marked contrast to the organic ions, metal ions seem to reach their strongest binding site more easily when applied from the inside than from the outside. Both Na^+ and Cs^+ depress conductance from the inside and enhance it from the outside. Only nonphysiological monovalent ions such as Li^+ and Tl^+ bind strongly from the outside. As Barry et al. (1979) already found, γ with external Li^+ increases toward the control value when the membrane is depolarized. The voltage dependence with Tl^+ was not studied. We have not tested whether such ions also block more strongly from the inside. The explicit two-barrier models already proposed for endplate channels (Lewis and Stevens, 1979; Horn and Brodwick, 1980) do not include a sidedness for metal ion binding and thus need to be modified either to have a lower inner barrier or, as seems more likely, to have a new metal binding site that does not bind arginine or ammonium and faces the inside. Further experiments are needed before we

can say whether the site for hydrohobic ions is also a binding site for metals and then whether it corresponds to the inner or the outer metal site.

A different style of theory of permeation in endplate channels has been introduced by Barry et al. (1979; Takeda et al., 1980). They solve the Nernst-Planck electrodiffusion equations for a homogeneous neutral membrane sufficiently thick that electroneutrality must be achieved by anions entering the channel. Each ion is characterized by a partition coefficient and mobility in the membrane. The thick-membrane assumption automatically differentiates their theory from the constant field and independent fluxes of the Goldman-Hodgkin-Katz theory (Goldman, 1943; Hodgkin and Katz, 1949), and the explicit assumption of homogeneity and lack of saturation differentiates it from binding theories. In our view, the channel has topographic variety and asymmetry, and interactions with small molecules are likely to take place at discrete sites. Thus, for example, Na^+ and Cs^+ ions block from the inside but not from the outside, whereas arginine and quaternary local anesthetic analogs block from the outside and not from the inside. Such discrete effects are more easily handled in a theory with binding sites than in one with a homogeneous membrane. In addition, the high resistance part of the channel may not be long enough for electroneutrality to hold.

Ions Affect Mean Open Time

We confirm and extend the observation of Van Helden et al. (1977) that channel gating depends on the external cation: with each test cation, the channel has a different mean open time. Two testable suggestions have been made to account for this effect. (*a*) Van Helden et al. (1977; Gage and Van Helden, 1979) noted a near constancy of the product $\gamma \cdot \tau$ with Li^+, Na^+, K^+, and Cs^+ solutions bathing toad endplates despite individual changes of γ and τ. They suggested that the channel might be designed to maintain a constant charge transfer so that γ and τ would automatically vary inversely. However, Takeda et al. (1980) noted that this idea is not consistent with their τ and γ values for NH_4^+ ions. Fig. 7 plots the product $\gamma \cdot \tau$ vs. γ from our work and shows, as Takeda et al. had suggested, that there is no general constant-charge relation. Indeed, most organic ions give both small γ's and short τ's. (*b*) Working with *Aplysia* neurons, Marchais and Marty (1979 and 1980) proposed a theory modeled after local anesthetic effects on endplate gating (Adams, 1977; Neher and Steinbach, 1978), namely that any permeant or impermeant ion bound to a site within the channel prevents the gate from closing. Thus, the more time a channel is free of ions the faster it closes. Their theory works well for *Aplysia* neurons and can even be used to explain the voltage dependence of normal gating there. Fig. 8 plots τ vs. the estimated fraction of free channels in different solutions for our experiments. Evidently, occupancy and gating are not so simply related in the motor endplate channel. Occupancy does retard gate closing with local anesthetic derivatives and large impermeant molecules (Adams, 1977; Neher and Steinbach, 1978; Colquhoun et al., 1979; Adams and Feltz, 1980), and it may slow gating with the largest permeant cations, but does not slow it with the smaller ones. The same conclusion is

reached when we consider replacement of *internal* CsF by arginine aspartate. A block is relieved by removing Cs^+ and yet τ becomes longer.

The hypothesis that permeant ions pause at the local anesthetic binding site while crossing the membrane does not require a complete similarity to the effects of local anesthetics. For example, the block with large, charged channel blockers is always intensified by hyperpolarization (Adams, 1977; Neher and Steinbach, 1978; Adams and Sackmann, 1978; Colquhoun et al., 1979; Adler et al., 1979; Adams and Feltz, 1980; Farley et al., 1981). However, as Woodhull

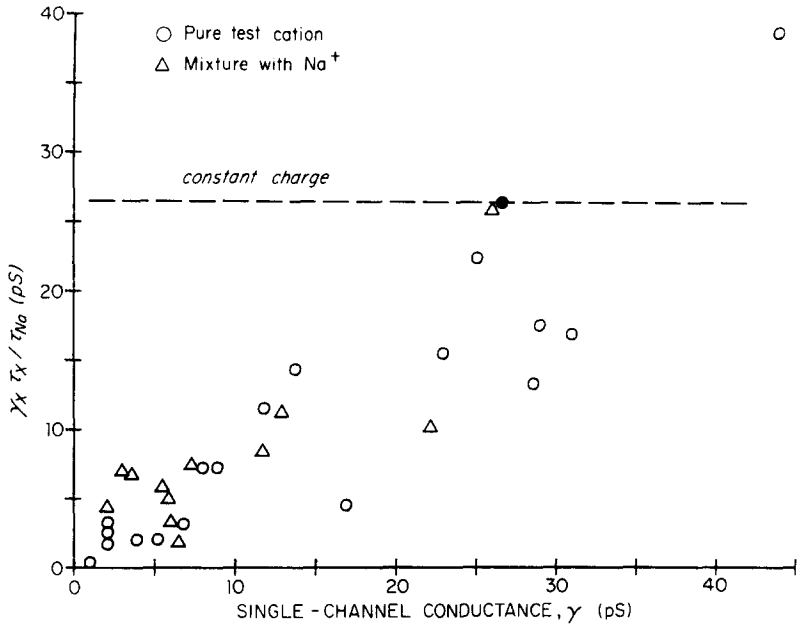


FIGURE 7. A test of the theory of constant charge transfer in endplate channels. The product $\gamma \cdot \tau$ is plotted against γ for measurements at −73 mV with pure test cations (*circles*) and with Na mixtures (*triangles*). The *dashed line* through the point for sodium represents approximately the expectation of the constant charge theory. A more rigorous presentation would use the product of τ times the single channel *current* rather than *conductance*, but the result would be similar as the electrical driving forces on the ions do not vary much from solution to solution. The *filled circle* is for Na reference solution.

(1973) showed, occupancy of a site can show quite a variety of voltage dependences if the blocking ion can leave its site by crossing the membrane (a permeant ion) in addition to returning to the compartment of origin. Indeed, our γ values for different ions do show a variety of voltage dependences (Table IV). The local-anesthetic analogy might also suggest that the blocking step should add another detectable relaxation to the power density spectrum, explaining why the spectra with ions of very low conductance could not be fitted by a single Lorentzian. If this were the correct explanation of the spectral shapes, then the rate constants would not fit well with our hypothesis

that the ions are simply pausing *en passant*. Even with the propylamines as test cations, the single-channel current at −73 mV is as high as 0.14 pA, corresponding to the transit of 875 propylamine cations/ms. If each cation pauses, the relaxation from that process would occur at 5 MHz, well outside our experimental range. The forward rate constants for block by the traditional endplate channel blockers are $\sim 10^7$ $M^{-1}s^{-1}$ and they too would show relaxations >1 MHz if studied at 100-mM concentrations. For this reason and because desensitization, background subtraction, and other errors could be the cause, we have avoided trying to interpret the smallest difference spectra in terms of multiple relaxations.

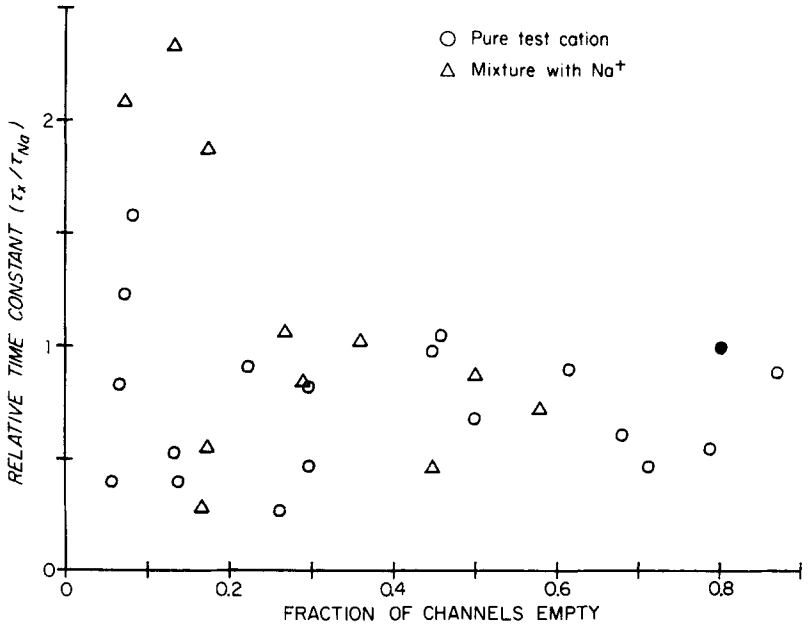


FIGURE 8. A test of the theory that occupancy by permeant ions slows closing of channels. Mean open time is plotted against the estimated fraction of empty channels (0.8 Q from Table V) for pure test cations (*circles*) and sodium mixtures (*triangles*). The *filled circle* is for Na reference solution.

Conclusions

The permeability of the endplate channel to organic cations is inversely related to size, whereas the conductance to these same cations is inversely related to hydrophobicity. Evidently there is a hydrophobic site within the channel that is readily saturated so that the overall rate of passage of ions is reduced. By contrast, there is little evidence for saturation with the physiological Na^+ ion. As might be expected to achieve efficient depolarizing action at the endplate, Na^+ ions probably bind little from the outside of the pore and thus establish a high single-channel conductance. The mean channel open time differs for each bathing solution but this change seems not to be related

to binding to the hydrophobic site. Furthermore, there is still no evidence that the voltage dependence of gating in endplate channels could be accounted for by voltage-dependent occupancy of an ion binding site within the channel. Finally, the available experiments do not prove that the ion affects on channel open time occur within the pore. There may be other sites of cation action at the external and internal faces of the channel macromolecule.

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