

Slow Permeation of Organic Cations in Acetylcholine Receptor Channels

JORGE A. SANCHEZ, JOHN A. DANI, DETLEF SIEMEN, and
BERTIL HILLE

From the Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle, Washington 98195

ABSTRACT Block, permeation, and agonist action of small organic amine compounds were studied in acetylcholine receptor (AChR) channels. Single channel conductances were calculated from fluctuation analysis at the frog neuromuscular junction and measured by patch clamp of cultured rat myotubes. The conductance was depressed by a few millimolar external dimethylammonium, arginine, dimethyldiethanolammonium, and Tris. Except with dimethylammonium, the block was intensified with hyperpolarization. A two-barrier Eyring model describes the slowed permeation and voltage dependence well for the three less permeant test cations. The cations were assumed to pause at a site halfway across the electric field of the channel while passing through it. For the voltage-independent action of highly permeant dimethylammonium, a more appropriate model might be a superficial binding site that did not prevent the flow of other ions, but depressed it. Solutions of several amine compounds were found to have agonist activity at millimolar concentrations, inducing brief openings of AChR channels on rat myotubes in the absence of ACh.

INTRODUCTION

The acetylcholine-activated channels of vertebrate neuromuscular junctions are wide, cation-selective, aqueous pores. Previous studies from this laboratory showed that at least 75 species of small cation can pass through the open pore, carrying readily measurable current (Dwyer et al., 1980; D. J. Adams et al., 1980). Reversal potentials for endplate currents with many of these cations showed that their permeability relative to Na^+ , P_x/P_{Na} , is often near 1.0. Despite this high permeability, the acetylcholine-induced endplate currents were frequently far smaller with the organic cations than for Na^+ .

In a subsequent study, we measured single channel conductances from current fluctuations induced by a steady iontophoretic application of acetylcholine (ACh)

Address reprint requests to Dr. Bertil Hille, Dept. of Physiology and Biophysics, SJ-40, University of Washington, Seattle, WA 98195. Dr. Sanchez's present address is Department of Pharmacology, CINVESTAV, Apartado Postal 14-740, Mexico, D.F. 07000, Mexico. Dr. Dani's present address is Section of Molecular Neurobiology, Yale University School of Medicine, New Haven, CT 06510. Dr. Siemen's present address is Physiologisches Institut, Justus-Liebig-Universität, Aulweg 129, D-6300 Giessen, Federal Republic of Germany.

(D. J. Adams et al., 1981). Many permeant organic cations, tested as pure solutions or mixed 1:1 with Na Ringer, greatly reduced the single channel conductance. We concluded that they might bind to a site within the channel with dissociation constants of only a few millimolar, pausing for a significant time before passing through. Hence, when tested at concentrations of 57 or 114 mM, they carry much less current than would be expected from their high reversal potentials. In addition, to our surprise, the power spectra of fluctuations were not of simple Lorentzian shape.

This paper aims to answer questions left open by our fluctuation measurements with permeant organic cations at high test ion concentrations. (*a*) Does the decrease of conductance follow the theoretical concentration dependence of a binding reaction when conductances are measured with low concentrations of organic cation added to Na solutions? (*b*) Does the reaction have a voltage dependence appropriate for binding taking place within the conducting pore and as a part of the permeation process? (*c*) Why did the previously measured power-density spectra of current fluctuations in the presence of many organic cations deviate strongly from the simple Lorentzian shape seen with the standard Na Ringer? To address these questions, we used both fluctuation analysis and the patch-clamp method for measuring unitary currents. Preliminary descriptions of this work have been presented at meetings (Sanchez et al., 1983; Hille et al., 1983).

METHODS

The major measured variable was the single channel current in ACh receptor (AChR) channels as a function of the cation composition of the extracellular bathing medium and of the membrane potential. We used two methods: fluctuation analysis of endplate current at the frog neuromuscular junction (during iontophoretic application of ACh), and patch-clamp measurements of unitary currents in cultured rat myotubes. The experiments were done in 1981–1983.

Fluctuation Experiments

Currents induced by 10-s, continuous iontophoretic applications of ACh were measured on cut semitendinosus muscle fibers of large adult *Rana pipiens* in a vaseline-gap voltage clamp. The quantities recorded were the net current with and without ACh and the current fluctuations with and without ACh. These were used to calculate difference fluctuation-density spectra and the single channel current, i , defined as difference variance divided by the macroscopic difference current. All procedures are as described by D. J. Adams et al. (1981) with the following minor exceptions: the fibers were dissected without prior depolarization and were used only if they could propagate a twitch through the endplate region; fibers were mounted using petroleum jelly rather than glisseal; current was recorded across a 2-M Ω resistor; a polynomial trend was not subtracted from the fluctuation record; and no filter correction was applied to the spectra. As before, the ends of the muscle fibers were cut in 120 mM CsF (Alfa Products, Danvers, MA). The recording chamber was cooled to 15°C.

Patch-Clamp Experiments

Unitary currents were measured from rat myotubes using the gigaseal pipette method of Hamill et al. (1981). Dissociated embryonic leg muscle cells were grown on collagen-

coated plastic coverslips according to the method of Lawrence and Catterall (1981) and treated with 10 μM D-arabinofuranosylcytosine after 60 h to inhibit the growth of fibroblasts. Cells were used 7–14 d after the initial plating. Before the recordings were made, the coverslip was rinsed several times with standard mammalian Ringer with the following composition (in mM): 150 NaCl, 5, KCl, 1.5 CaCl₂, 1 MgCl₂, 5 glucose, and 5 Na HEPES buffer, pH 7.4. Cells were observed under modulation-contrast illumination on the stage of an inverted microscope and currents were recorded across the 10-G Ω resistor of a homemade patch clamp with compensated frequency response extending to 5 kHz. The patch pipette was held at virtual ground. The current signal was filtered through a four-pole low-pass Bessel filter at 630 Hz (3 dB attenuation) and sampled digitally every 500 μs with an LM² minicomputer.

Patch pipettes were drawn in two stages from hard-glass capillary measuring pipettes, coated with a polystyrene plastic dope, and fire-polished. They were filled with the test solution, which usually included ACh to activate AChR channels at a low rate. Inside-out membrane patches were excised from the myotubes and were usually transferred through the air-water interface to a smaller pool containing the “intracellular” medium, which was 158 mM NaF and 10 mM histidine, pH 7.2 (both from Baker Chemical Co., Phillipsburg, NJ). Patch-clamp measurements were made at room temperatures ranging from 20 to 25°C.

In most experiments, there was a dominant unitary-current step size in the records. Occasional smaller current steps were observed but are not considered in this paper. Currents were measured manually from records played back after the experiment. Membrane potentials are reported according to the usual physiological convention of cytoplasmic side minus extracellular side, and outward currents are positive. Throughout this paper, the channel conductance, γ , is the chord conductance calculated from

$$\gamma = i/(E - E_r), \quad (1)$$

where i is the apparent single channel current, E is the membrane potential, and E_r is the zero-current potential or reversal potential for current in the channel. In fluctuation experiments where E_r was not determined directly, it was calculated from the Goldman-Hodgkin-Katz potential equation using ionic permeability ratios determined previously in the same preparation (Dwyer et al., 1980; D. J. Adams et al., 1981).

Several figures compare the concentration dependence of γ with the predictions of the independence principle of Hodgkin and Huxley (1952). Suppose γ and γ' are conductances measured under two conditions where the reversal potentials are E_r and E'_r . If only the external ion concentrations have been changed, the predicted conductance ratio is

$$\frac{\gamma}{\gamma'} = \frac{\sum P c_0}{\sum P' c'_0} \frac{1 - \exp[(E - E_r) F/RT]}{1 - \exp[(E - E'_r) F/RT]} \frac{E - E'_r}{E - E_r}, \quad (2)$$

where $\sum P c_0$ stands for the sum of products of permeability ratio and concentration for all external cations and RT/F is 25 mV. Other figures show the predictions of a barrier model, based on Eyring rate theory, for permeation of the pore. The absolute rates and energies are defined exactly as in Hille (1975), except that this model has only two energy barriers and one central energy well. The difference between activity and concentration is ignored throughout.

Extracellular Test Solutions

In our previous work, the principal extracellular solutions contained nearly isotonic amounts of NaCl or of the salt of the organic test cations. The new experiments required variable amounts of test cation to be combined with NaCl, and all solutions were made by

mixing variable proportions of test cation Ringer with Na Ringer. These mixtures are denoted in the text either by the percentage of test cation Ringer used or by the final test ion concentration. For fluctuation experiments, done with amphibian muscle, the Na Ringer contained: 114 mM NaCl, 1.0 mM CaCl₂, and 10 mM histidine, pH 7.4. For patch-clamp experiments, done with mammalian myotubes, the Na Ringer contained: 158 mM NaCl, 1.0 mM BaCl₂ or CaCl₂, and 10 mM histidine, pH 7.4. The patch pipette usually contained 200–400 nM ACh. By having no K ion on either side of the membrane (and by sometimes having Ba²⁺), we avoided seeing signals from K channels. In the pure test cation Ringer, all of the NaCl was replaced by an equivalent concentration of the test cation Cl salt. In some patch-clamp experiments, the buffer was Na phosphate. The main test substances and their abbreviation used in the text were L-arginine and dimethyldiethanolamine (DMDEA) (both from Eastman Organic Chemicals Division, Eastman Kodak Co., Rochester, NY), and dimethylamine (DMA) HCl and Tris (both from Sigma Chemical Co., St. Louis, MO). We also often use the name of the free amine to designate the cation. In the experiments on agonist action, we used triethylamine HBr (99%+, Eastman Kodak Co.), 4-aminopyridine (98%) and diethylamine HCl (both from Sigma Chemical Co.), and acetamidine HCl (98%+) and *n*-propylamine (98%, both from Aldrich Chemical Co., Milwaukee, WI).

RESULTS

Low Concentrations of Organic Cations Depress Conductance

We began with the protocol used in our previous work to determine the single channel conductances, γ , of the frog endplate channel by fluctuation analysis, but using low as well as high test cation concentrations. Fig. 1 shows γ , calculated from ACh-induced current noise at -135 (triangles) and -75 mV (circles), vs. the mole fraction of DMA or Tris cation in the external Na Ringer/test cation mixtures. Replacing Na ions with either of these permeant test cations reduced γ at the endplate.

The observed conductance changes can be compared with those expected for mixtures of ions of known permeability moving independently (Hodgkin and Huxley, 1952). Earlier biionic reversal potential measurements assigned permeability ratios P_X/P_{Na} of 0.87 to DMA and 0.18 to Tris cations in this preparation (Dwyer et al., 1980). The upper curves in Fig. 1 are the conductance variations predicted from independence, using these permeability ratios; the dashed curves are for -135 mV and the solid curves are for -75 mV. The observed conductances fell well below the predictions at all concentrations of DMA or Tris, which shows that these test cations, though permeant, retard the flow of other ions in the endplate channel. Only a few millimolar test cation sufficed to reduce γ appreciably. Thus, in control frog Ringer, the conductance was 32.6 pS at -135 mV, whereas with 1.1 mM added Tris, it had fallen to 20.8 pS. As others have noted (Lewis, 1979), Tris is therefore not the buffer of choice for routine studies of AChR channel physiology.

Additional fluctuation measurements on endplates are summarized in Fig. 2. The conductance at -135 and -75 mV is plotted vs. mole fraction for Na DMDEA and Na arginine mixtures. Again, the test cations depress channel conductance below that expected from independence. The largest effect occurs with DMDEA, a permeant ion with a permeability ratio of 0.09 relative to Na⁺

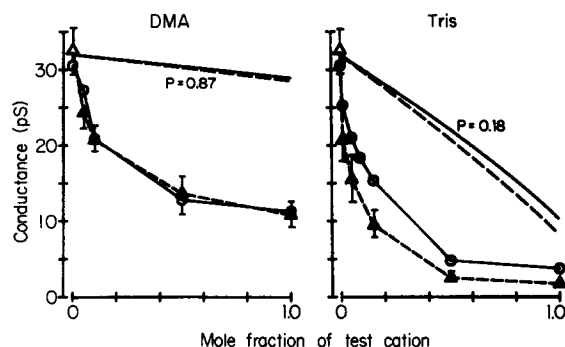


FIGURE 1. Reduction of frog endplate channel conductance by external DMA and Tris cations. Single channel conductances were measured by the fluctuation method as increasing fractions of the external Na ions were replaced by DMA or Tris. Triangles show the mean \pm 2 SEM ($N = 6-9$) of γ determined with Eq. 1 from measurements at -135 mV. Circles show the mean ($N = 6-9$) γ at -75 mV. For comparison, the upper smooth curves labeled $P = 0.87$ and $P = 0.18$ show the conductance variation predicted from independence (Eq. 2) using γ in pure frog Na Ringer as a reference.

(Dwyer et al., 1980). Single channel inward currents were not measurable with the fluctuation method in pure external solutions of these two least permeant cations, arginine and DMDEA.

Patch-clamp experiments on rat myotubes gave similar results. Fig. 3 shows single channel records made with patch pipettes containing 200–400 nM ACh and various cation mixtures. Each record is from a different patch with a different seal resistance and number of channels. The records with 80 mM DMA and with 16 and 80 mM arginine in the pipette show the activation of more than one

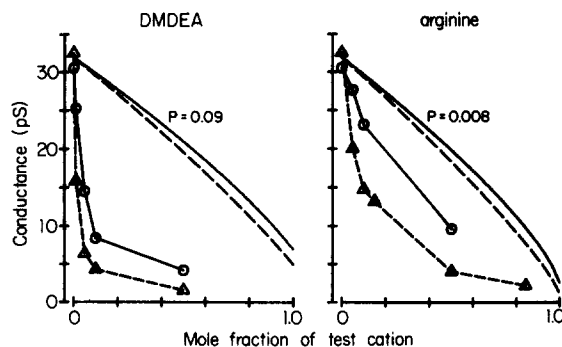


FIGURE 2. Mole fraction dependence of frog endplate channel conductance at two membrane potentials. Fluctuation measurements with DMDEA and L-arginine test cations are plotted as in Fig. 1 together with predictions from independence (upper curves). Triangles and dashed lines are at a holding potential of -135 mV, and the circles and solid lines are at -75 mV. On the average, each point is the mean of seven measurements, and the experimental errors are comparable to those given in Fig. 1.

channel at a time. The unitary current steps in the control patch correspond to an average conductance change of 47 pS in the rat myotube, significantly higher than the ~ 30 pS conductance we calculated from noise in frog endplates. (Note that the temperature is 10° warmer and the salt concentration 33% higher in the work with mammalian cells.) As some of the Na ions in the pipette are replaced by organic test ion, the single channel conductance is reduced. Quali-

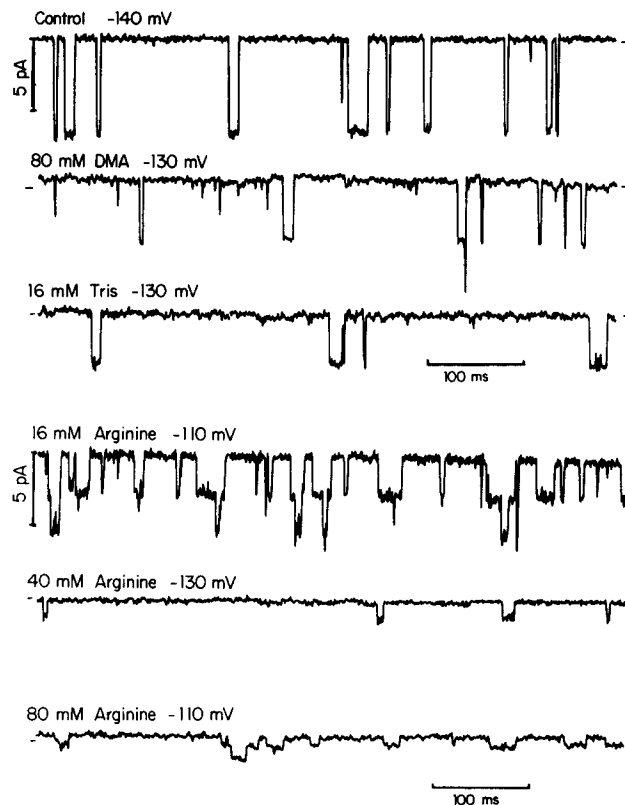


FIGURE 3. Patch-clamp unitary currents in rat myotube AChR channels. Excised inside-out patches were made with pipettes containing mammalian Na Ringer (control) or mixtures of test cations and Na Ringer plus 200–400 nM ACh. The membrane potential and the concentration of test cation are labeled on the records. The remainder of the cation is Na. By necessity, each record is from a different patch.

tatively, the organic cations do not induce an obvious excess of flickering of the open state, so if there is any block-unblock reaction, it would have to be occurring at frequencies above the 630-Hz recording bandwidth. Simulations with our filters show that the lifetime of hypothetical flickering blocked states would have to be $<400 \mu\text{s}$ to be consistent with the absence of detected extra noise.

Unitary conductance measurements at -75 and -135 mV with different

external (pipette) mole fractions of DMA, Tris, and arginine are summarized in Fig. 4. The upper lines are predictions of independence (using permeability ratios from frog endplates since we have not measured them for rat myotubes). The lower lines are the predictions of a barrier model described in the Discussion. Qualitatively, the mole-fraction dependence of γ for patch clamp of the embryonic rat cells is similar to that for fluctuation analysis of the adult frog endplate. In either preparation, low concentrations of organic cation sufficed to reduce the channel conductance, and Tris was more effective than arginine or DMA. While DMA does not reduce the conductance as much in the rat channels as in the frog channels, its effect appears to reach saturation at a lower concentration.

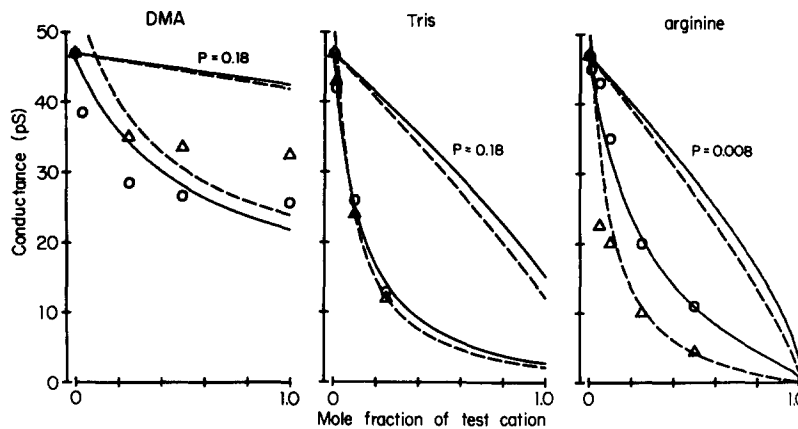


FIGURE 4. Mole fraction dependence of unitary conductances of embryonic rat AChR. Chord conductances at -135 mV (triangles) and -75 mV (circles) from patch-clamp records are compared with the predictions of independence (upper curves) and of a barrier model described in the Discussion (lower curves). On the average, each point represents measurements from three different patches. The dashed lines show theoretical curves for -135 mV and the solid lines are for -75 mV.

Binding in the Channel Is Voltage Dependent

The depression of conductance by low concentrations of external test cation requires a saturable binding process either on the outer portion or within the pore of the AChR channel. If the site is deep within the pore, the dissociation constant will be voltage dependent, reflecting the need to cross part of the membrane electric field to reach and leave the site (Woodhull, 1973). Thus, with the larger external test cations, arginine or DMDEA, hyperpolarization intensifies the depression of current, an effect seen qualitatively in the measurements of Figs. 2 and 4, where any concentration of arginine or DMDEA depressed γ more at -135 than at -75 mV. This is in the direction expected if the putative binding site is within the pore and is more accessible to the outside than to the inside. The effect was also seen with Tris on the amphibian preparation but not with the mammalian preparation. For the smallest test cation, DMA, little voltage dependence was observed with either preparation.

Voltage dependence is more easily analyzed from the single channel current-voltage relations determined in patch-clamp experiments (Fig. 5). In the control solution, the typical single channel i - E relation was nearly linear with a slope of 45–48 pS. Our measurements with 8, 16, 40, and 80 mM arginine are summarized (symbols) in Fig. 5D. Arginine converted the nearly linear control i - E

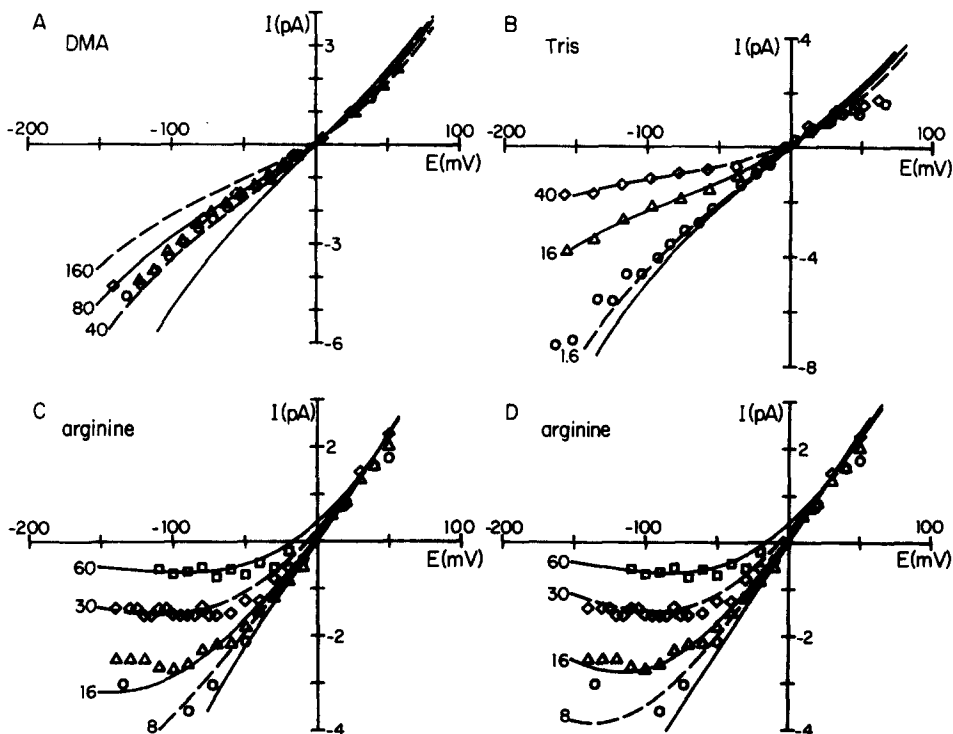


FIGURE 5. Current-voltage relations of rat myotube AChR channels with external DMA, Tris, or arginine mixed with Na^+ . The concentration of test ion is indicated. The pipette also contained 200–400 nM ACh. With DMA and arginine, the inside-out patches were excised into the NaF histidine intracellular medium. With Tris, patches were excised into the standard mammalian Ringer that bathed the cells. (A–C) Symbols give the averaged experimental observations from several patches tested at each concentration. The lines give predictions from a two-barrier model described in the Discussion. The theoretical line for pure Na Ringer is also given, although no data are shown for this condition. (D) Symbols are arginine data repeated from C. The lines are predictions of a Woodhull model with $\delta = 0.5$ and $K_{\text{diss}} = 210$ mM.

relation into curves with strongly depressed currents at hyperpolarized potentials but barely affected currents at very positive potentials.

As a first approximation, arginine can be treated as an impermeant blocking agent following the theory of Woodhull (1973), and the modified i - E relations can be used to extract an apparent dissociation constant and its voltage depend-

ence. The smooth curves in Fig. 5D were calculated from the Woodhull (1973) theory with the following simplifying assumptions. (a) The control *i-E* relation is a straight line with a slope of 47 pS that passes through the origin. (b) The unblocked *i-E* relation would follow the predictions of the independence relation (Hodgkin and Huxley, 1952) as the Na ion is diluted. (c) Arginine is impermeant and blocks at a site 50% of the way across the membrane field with an equilibrium dissociation constant of 210 mM at 0 mV. (d) There is no competition at the binding site from Na ions. These assumptions, based on a binding site within the pore, seem to describe the *i-E* relations well, but we recognize that assumptions *b* and *d* are already known to be incorrect. Fig. 5C shows the same arginine data with smooth lines derived from a barrier model described later, a model that does not require these assumptions.

Fig. 5 also shows the effects of DMA and Tris cations on the single channel *i-E* relations (DMDEA was not studied). Again, there was more reduction of inward current than outward current, but these cations are relatively permeant, so the Woodhull theory for an impermeant blocking agent is not appropriate. Any theory would need to include current carried by the test cation as well as by the Na ion and an unbinding step that can proceed toward the cytoplasm as well as toward the external solution. Such a theory is described later.

Two additional remarks are required on the data in Fig. 5. First, many points are the averages of several measurements made on different patches. Since among patches the interpolated zero-current potentials varied by a few millivolts, we shifted the data from each patch along the voltage axis to make its interpolated zero-current potential coincide with the theoretical value expected from the internal and external solutions before averaging and plotting. This adjustment removed scatter and simplified the comparison of the shape of these curves from many patches with theoretical predictions. Second, the patches with Tris as the test cation were excised into mammalian Ringer rather than NaF solution, so the former cytoplasmic face was exposed to 1.5 mM Ca²⁺ and 1 mM Mg²⁺. This difference probably accounts for the sag of the *i-E* relations at positive potentials in Fig. 5B—a block of outward Na⁺ flux by “internal” divalent ions.

Solutions of Some Test Cations Contain Agonist Activity

Two observations in our earlier work suggested that, in addition to actions in the pore, certain test cations might interact with the agonist binding sites on endplate channels. Both observations concerned possible desensitization processes. With solutions of some cations, notably ethanolamine, the macroscopic endplate current induced by puffs of ACh was very large during the first puff but faded to a vanishing size in subsequent puffs (Dwyer et al., 1980). It did not recover even without further puffs in ethanolamine but recovered quickly if control Ringer was perfused through the bath. This loss of responsiveness could be a desensitization-like process requiring the test cation alone or in combination with ACh. It forced us to change into and out of ethanolamine solution for each ACh puff when we were measuring reversal potentials for endplate currents. The second observation concerned the shape of the difference power spectrum

of current fluctuations induced by ACh. When the bathing solution was Na Ringer, the power spectrum had a nearly pure Lorentzian shape, indicative of a single, dominant open-closed kinetic step. By contrast, in 57 and 114 mM solutions of acetamidine, propylamine, diethylamine, and many other cations, the power spectra did not level out at low frequencies, which indicated a

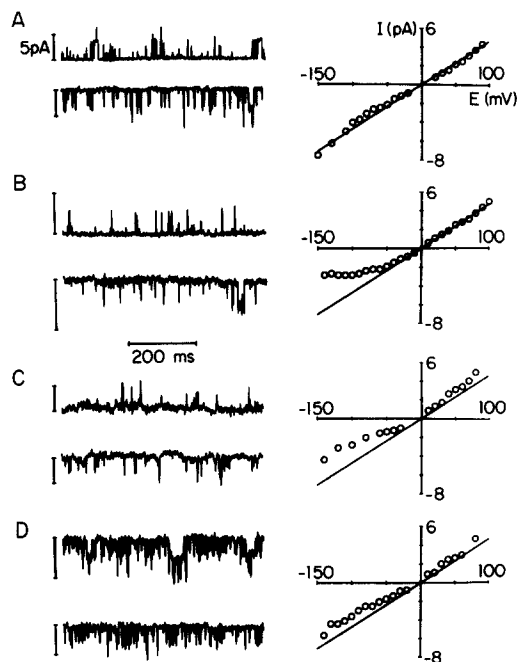


FIGURE 6. Channel openings induced by test cation solutions in the absence of ACh. Most panels show: a trace of unitary currents measured at a positive membrane potential, a trace at a negative potential, and the i - E relations of these events compared with a line of slope 47 pS. Calibration bars are all 5 pA. The pipette solution was NaCl plus the organic salt at a combined concentration of 158 mM plus 1 mM BaCl₂, 10 mM Na phosphate buffer, pH 7.4, no ACh; excised inside-out patches were transferred to 158 mM NaF. The i - E relations were created using only single channel events that lasted several digital sample intervals. Most events were too short to measure. The concentration of organic cation in the pipette bathing the external surface of the patch and the holding potential for the upper and lower single channel traces are: (A) 1.58 mM propylamine, +80 mV, -90 mV; (B) 0.8 mM triethylamine, +60 mV, -110 mV; (C) 1.6 mM diethylamine, +70 mV, -120 mV. (D) The upper insert and i - E relation are with 4.5 mM acetamidine at -60 mV; the lower insert is an on-cell patch with 3.2 mM 4-aminopyridine at -40 mV plus the cell resting potential (no i - E relation given).

contribution of a new slow process to the gating—possibly desensitization (see Fig. 3 in D. J. Adams et al., 1981). In addition, the log-log plots of the spectra often fell off at high frequencies with a slope shallower than the slope of 2.0 expected for a Lorentzian, which suggests a contribution of a new rapid gating process as well.

We now show that many organic cation solutions have weak agonist activity on rat myotube AChR channels at the high concentrations previously used for permeability measurements. Patch-clamp pipettes were filled with solutions containing a few millimolar test cation without added ACh. Some test cation solutions initiated a barrage of channel openings with far briefer open times than those activated by ACh (Fig. 6, left side). Most open durations were <1 ms and therefore could not be well resolved. The pairs of current traces show outward currents at positive voltages and inward currents at negative voltages induced by a few millimolar solutions of propylamine (A), triethylamine (B), and diethylamine (C), and inward currents at negative voltages induced by solutions of acetamidine and 4-aminopyridine (both in D). The i - E relations of these channels shown on the right side have a slope near 47 pS (solid line) at positive membrane potentials, and some fall off to lower conductances at negative potentials. The i - E relations would be indistinguishable from the i - E relations of ACh-activated channels if we suppose that diethyl- and triethylamine exert a voltage-dependent blocking effect like that found with Tris, arginine, and DMEA cations. Hence, several of the ions we studied may be agonists that open AChR channels only briefly. At the 57 and 114 mM concentrations tested in our previous paper, these cation solutions would not only open channels but they would undoubtedly also desensitize receptors. The list of definitely active solutions includes propyl-, diethyl-, and triethylammonium, acetamidine, and 4-aminopyridine (all shown in Fig. 6). With three other cations, we obtained some silent patches and a few active ones; the solutions tested were 16 mM ethanolamine, 8 mM glucosamine, 3.2 and 8 mM guanidine, and 24 mM histidine. If these solutions are agonists, they are far less effective at millimolar concentrations than the first group.

DISCUSSION

Agonist Actions

We have demonstrated the agonist action of solutions of primary, secondary, and tertiary amines at millimolar concentrations. Our experiments show that these solutions contain an agonist, but they do not prove that the major cation of our reagents is the agonist. The reagents we have used are specified as 98% or 99% pure. If by chance they contained a 0.1% impurity of an agonist as potent as ACh, then the agonist action could be due to the impurity, acting at micromolar concentration. In any case, the solutions of these commercial salts have agonist activity, and since there are probably hundreds of papers where, for example, 4-aminopyridine solutions have been applied to excitable cells, it is necessary to bear in mind that they contain a nicotinic agonist.

Previous reports of nicotinic agonists concern quaternary ammonium compounds studied at micromolar concentration. The simplest such quaternary compounds, with no ester groups, are all derivatives of tetramethylammonium ion (TMA), including TMA itself. They are methyl-, ethyl-, 2-hydroxyethyl-, 2-thioethyl, butyl-, pentyl-, 3-phenylpropyl-, and 3(hydroxyphenyl)propyl trimethylammonium and decamethonium (P. R. Adams, 1976; P. R. Adams and Sakmann, 1978*a, b*; Auerbach et al., 1983; Colquhoun et al., 1975; Gardner et al., 1984). Where measured, the unitary conductance steps elicited by micromolar

concentrations of these TMA derivatives are reported to be indistinguishable in amplitude but much shorter in duration than those elicited by ACh in the same preparations. These brief openings of normal amplitude with quaternary ions agree with those we found with millimolar concentrations of primary, secondary, and tertiary amines.

Simple quaternary molecules such as TMA and ethyltrimethylammonium are known to desensitize ACh receptors strongly at their agonist concentrations (P. R. Adams, 1976). The agonist activity of some of our solutions would explain the intense apparent desensitization seen earlier (Dwyer et al., 1980) and probably the surprising shape of the power spectra (D. J. Adams et al., 1981). The difference power spectrum would contain several fast time constants from the combined agonist actions of ACh plus test cation as well as several slow time constants from the combined desensitizing actions of ACh and test cation.

Block by Organic Cations

Our observations. The qualitative similarity of the patch-clamp results to those with fluctuations (*a*) suggest that embryonic mammalian and adult amphibian AChRs have similar permeability and block properties and (*b*) illustrate that fluctuation methods can be valid even when the underlying events have unsuspected kinetic complexity. We found that millimolar concentrations of organic cations lower the conductance of AChR channels qualitatively as was predicted from fluctuation measurements in 114 mM test cation solutions (D. J. Adams et al., 1981; see also Fiekers and Henderson, 1982). For some cations, the reduction is stronger at negative potentials than at positive potentials, as if the test cation bound to a site within the pore. Any block-unblock reaction occurs too rapidly to be visible as flickers in single channel openings or to contribute a detectable shoulder to current fluctuation spectra. The single channel currents simply appear smaller. The apparent short lifetime of any blocking complex is consistent with a single channel flux of 10^6 – 10^7 test ions/s, which can be calculated from the finite permeability of many of these ions.

Results from other laboratories. Many organic cations have been shown to block open AChR channels in a voltage-dependent manner from the outside. The block has frequently been interpreted in terms of reversible entry and plugging of the pore using the one-site model of Woodhull (1973). Some of the best analyzed examples are listed in Table I, which gives the equilibrium dissociation constant and the mean lifetime of the blocking complex at -80 mV, as well as the actual charge of the ion and the apparent valence z' of the blocking reaction. This apparent valence is defined so that, for example, a reaction moving three full charges to a site halfway across the electric field of the membrane would have $z' = 1.5$ (Hille, 1984). It is used here instead of the electrical distance δ because with multivalent ions like curare or decamethonium, the charges are so far apart on the molecule that each probably moves through a different fraction of the field during complex formation. The ratio z'/z would give the average electrical distance of movement of all the charges.

Most of the blockers studied before were larger, impermeant, or very poorly permeant molecules. In the studies listed in Table I, several results agreed well

with the concept of binding partway through the pore at a site easily accessible from the outside: the equilibrium dissociation constants all have a voltage dependence consistent with crossing 20–80% of the membrane electric field. Part of the voltage dependence comes from the on rate, and about an equal part from the off rate. The forward rate constants at 0 mV are reported to be in the range 5×10^6 – 5×10^7 $M^{-1}s^{-1}$ for all blockers, as might occur in aqueous diffusion into a constricted space. The dissociation constant is therefore determined largely by the residence time of the blocker on the blocking site—a high dissociation constant for short-lived complexes and a low one for long-lived complexes. Hence, our observations of rapid entry, weak binding with some voltage dependence, and a block time too short to resolve with a 630-Hz frequency response would be expected for the small, more permeant molecules we have studied.

TABLE I
Blockers of Open ACh Receptor Channels

Compound	Properties of drug complex at –80 mV				Molecular weight
	K_{dis}	Lifetime	z'	z	
Quinacrine*	50 nM	200 ms	0.45	2	437
D-Tubocurarine [‡]	100 nM	1 s	0.80	2	611
Procaine [§]	20 μ M	—	0.5	1	236
Gallamine [¶]	34 μ M	920 μ s	0.95	3	511
QX-222 [†]	100 μ M	850 μ s	0.78	1	276
Deca**	150 μ M	680 μ s	0.38	2	259
TEA ^{‡‡}	300 μ M	<50 μ s	0.20	1	130
Ethylguanidine ^{¶¶}	3 mM	—	—	1	87
Acetylcholine	4 mM	11 μ s	0.8	1	146
Arginine ^{¶¶¶}	33 mM	—	0.43	1	174

z , positive charge on molecule; z' , effective valence of blocking reaction.

References: *P. R. Adams and Feltz (1980); [‡]Colquhoun et al. (1979); [§]P. R. Adams (1977); [¶]Colquhoun and Sheridan (1981); [†]Neher and Steinbach (1978); **P. R. Adams and Sakmann (1978b); ^{‡‡}Adler et al. (1979); ^{¶¶}Farley et al. (1981); ^{||}Sine and Steinbach (1984); ^{¶¶¶}Dwyer and Farley (1984).

Most of the blockers previously studied do not act when applied on the cytoplasmic side of the membrane. Sidedness of action has been demonstrated for AChR channel block by tetraethylammonium, methylatropine, D-tubocurarine, octylguanidine, QX-314, and QX-222 (Aguayo et al., 1981; del Castillo and Katz, 1957; Farley and Narahashi, 1983; Horn et al., 1980). In the frog endplate, internal arginine apparently does not block (D. J. Adams et al., 1981). On the other hand, Dwyer and Farley (1984) recently reported that arginine blocks from either side in chick myotube patches, and they present a nearly symmetrical barrier model for the blocking action. However, as they point out, their symmetrical model does not describe the steady increase of block that we and they see as the externally treated membrane is hyperpolarized more and more (Fig. 5C).

Comparison with a simple barrier model. We wish to try more than a qualitative check that the reduction of single channel current by small organic cations can be due to binding within the pore. Therefore, we compared our data with predictions of a two-barrier, one-site model of permeation. For simplicity, the site was placed halfway through the electric field in the pore and the barriers were placed at electrical distances of 0.25 and 0.75. This left three free parameters, two barrier energies and one well energy, for each permeant ion. The energies were chosen to give absolute current-voltage relations and permeability ratios in agreement with the data and to keep the lifetime of the complex between any test ion and the site too short to resolve in our records. We neglect the possible effects of any bathing ion other than Na and the test cation. In particular, a more complete analysis would include the 1 mM Ca^{2+} or Ba^{2+} in the extracellular solution (Dani and Eisenman, 1985). The energies listed in Table II are those calculated for AChR channels of embryonic rat myotubes. Had we tried to model the adult amphibian channel, the results would not have been very different since the observations were similar.

TABLE II
Energies and Site Dissociation Constant of the Barrier Model

Ion	Outer barrier	Site	Inner barrier	K_{dis}
	RT	RT	RT	mM
Na	10.0	-2.5	10.2	82
DMA	9.4	-3.2	10.7	41
Tris	10.8	-4.2	12.5	15
Arginine	11.0	-2.9	16.6	55

Energies in RT units are appropriate for 1-M standard states of the permeant ions and assuming activity coefficients of 1.0 as in Hille (1975). To convert them for activity coefficients of 0.75 and a mole fraction = 1.0 standard state, as used by Dani (1986), subtract 4.3 from all values. K_{dis} is calculated at 0 mV with no competing ions.

The calculations from the model are shown as smooth curves in Figs. 4 and 5, A-C. For Tris, the agreement was excellent, giving an appropriate concentration and voltage dependence. According to the model, Tris is bound several times more strongly than Na^+ and the site becomes nearly saturated at the highest concentration (40 mM) tested. The conductance at negative potentials then falls well below that expected from independence because the Tris ion has to cross over a much higher total barrier ($12.5 + 4.2 = 16.7 RT$) to enter the cell than the Na ion ($10.2 + 2.5 = 12.7 RT$). The barrier profile for Na is nearly symmetrical and that for Tris has a small asymmetry (the inner barrier is 1.7 RT higher), which leads to a small voltage dependence of the "block."

The calculations for arginine are about as good as with the Woodhull model for an impermeant ion (Fig. 5D), even though the barrier model gives arginine a finite permeability, 0.008 relative to Na^+ . As expected, the inner barrier is now much higher than the external one and access to the site from the outside is not greatly impeded. This gives a large voltage dependence of binding. The barrier model gives a lower intrinsic dissociation constant for arginine binding

than the Woodhull model to compensate for competition with Na ions for the site.

The best fit of the model for DMA is poor. The shape of the current-voltage relation and the observation that the "block" is small and without voltage dependence are adequately described. However, the concentration dependence of the block cannot be described. The data show that the block is nearly complete at 40 mM DMA. Hence the binding site should have a dissociation constant much lower than 40 mM. When this is done by deepening the energy well at the site, the rate constants for leaving the site automatically become so slow that DMA could conduct far less current than was observed. This limitation is not overcome by adding more barriers and wells or by moving the positions of the existing ones; it is inherent in conventional one-ion saturable models. What is needed are saturable sites that when occupied do not prevent flow through the pore but rather just depress it. Thus, a site in the outer vestibule of the pore (before the voltage drop starts) with an effective dissociation constant of ~10 mM that depresses flux by 30% when occupied would account for the data. A detailed theoretical framework for describing such a situation in terms of molecular properties is given by Dani (1986). Alternative, more complex models that probably could be made to work would be one-ion models with barriers that fluctuate with thermal motions of the channel (Läuger, 1984; Eisenman and Dani, 1986) and multi-ion models (Hille and Schwarz, 1978).

The structural consequences of our observations can now be summarized. There is a region within the pore that is attractive for cations and lies near the middle of the membrane voltage drop in the channel. This region is accessible to small cations coming from either side of the membrane but not to larger cations. Cations of the size of Tris, arginine, and DMDEA have easy access from the outside but encounter a higher barrier in passing from there to the inside of the cell. Cations as large as gallamine, quinacrine, or D-tubocurarine probably reach the same region easily from the outside but can pass no further. According to our work (D. J. Adams et al., 1980) and that of others (Farley et al., 1981), binding in this region is favored when the test cation has more carbons. In short, the region provides hydrophobic attraction to passing cations.

Work from this laboratory (Dwyer et al., 1980) has suggested that the pore of the AChR channel reduces to a width of 0.65×0.65 nm at some point along its length. Three of the permeant cations studied here would make a relatively close fit in this part of the pore. From space-filling models, the minimum dimensions of a rectangular box built around the cations are: DMA, $0.38 \times 0.42 \times 0.61$ nm; Tris, $0.55 \times 0.56 \times 0.64$ nm; DMDEA, $0.52 \times 0.58 \times 0.85$ nm; and arginine, $0.47 \times 0.64 \times 1.12$ nm. For comparison, the corresponding dimensions of quinacrine are $0.6 \times 1.0 \times 1.6$ nm, and gallamine and D-tubocurarine are larger. It seems inescapable that the diameter of the external part of the pore up to the hydrophobic binding region in the middle of the membrane electric field must be larger than the minimum 0.65×0.65 -nm pore dimensions. The narrowest region then lies toward the axoplasmic side from this region. We also have evidence that there may be additional attractive regions out in the wide funnel of the channel, where, as suggested by Dani (1986), attracted ions do not totally obstruct the flow of other ions.

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