Effects of Ammonium Ions on Endplate Channels

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ABSTRACT Miniature endplate currents, recorded from voltage-clamped toad sartorius muscle fibers in solutions containing ammonium ions substituted for sodium ions, were increased in amplitude and decayed exponentially with a slower time constant than in control (Na) solution. The peak conductance of miniature endplate currents was also greater in ammonium solutions. The acetylcholine null potential was -2.8 ± 0.8 mV in control solution, and shifted to 0.9 ± 1.6 mV in solutions in which NH_4Cl replaced half the NaCl. In solutions containing NH_4Cl substituted for all the NaCl, the null potential was 6.5 ± 1.3 mV. Single channel conductance and average channel lifetime were both increased in solutions containing ammonium ions. The exponential relationship between the time constant of decay of miniature endplate currents or channel lifetime and membrane potential was unchanged in ammonium solutions. A slight but consistent increase in peak conductance during miniature endplate currents and single channel conductance was seen as membrane potential became more positive (depolarized) in both control and ammonium solutions. Net charge transfer was greater in ammonium solutions than in control solution, whether measured during a miniature endplate current or through a single channel. The results presented here are consistent with an endplate channel model containing high field strength, neutral sites.

INTRODUCTION

Recent studies on the acetylcholine-activated channel at the amphibian motor endplate have indicated that many simple chemicals and drugs can alter channel lifetime. Attempts to describe the physical nature of the ion permeation channel have revealed that channel lifetime is both voltage- and temperature-sensitive (Anderson and Stevens, 1973), and that the nature of permeant cations affects both channel lifetime and conductance (Van Helden et al., 1977; Gage and Van Helden, 1979). Raising the divalent cation concentration prolongs the decay of miniature endplate currents (Cohen and Van der Kloot, 1978), while lowering the extracellular pH has been reported to lengthen the decay of endplate (Scuka, 1975) and miniature endplate currents (Mallart and Molgo, 1978). Both of these effects have been attributed to a reduction in surface potential arising from screening of negative fixed surface charge (Van

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der Kloot and Cohen, 1979), although other divalent cations (e.g., zinc) also prolong channel lifetime in concentrations at which magnesium ions have no effect (Takeda et al., 1978).

Earlier workers have shown that the acetylcholine channel at the endplate is permeable not only to sodium, potassium, and calcium ions (Takeuchi and Takeuchi, 1960; Takeuchi, 1963*a*,*b*), but also to a wide range of organic cations (Furukawa and Furukawa, 1959; Koketsu and Nishi, 1959; Nastuk, 1959; more recently, see also Maeno et al., 1977; Bregestovski et al., 1979; Dwyer et al., 1979). Classical approaches to the description of endplate channel ion selectivity and concentration-dependent behavior have been shown to be largely unsatisfactory (Linder and Quastel, 1978; Gage and Van Helden, 1979; Lewis, 1979). The observation of the voltage dependence of single channel conductance and the effects of the monovalent alkali cations on channel characteristics (Gage and Van Helden, 1979; Van Helden et al., 1979) have led to the proposal of a "neutral site" channel model for the endplate (Barry et al., 1979*a*,*b*). We were interested firstly, in obtaining more information about the molecular mechanisms determining effects of cations on channel conductance and lifetime, and secondly, in further testing the applicability of the neutral site channel model. Ammonium ions were investigated because they have a limiting equivalent conductivity in free solution similar to that of potassium ions, have the ability to form hydrogen bonds, unlike the alkali cations, and have the unusual property, like thallium ions, of being the only cations measurably permeant in both sodium and potassium channels of frog node of Ranvier and squid axon (for review, see Hille, 1975). We have found that substitution of NH_4Cl for extracellular NaCl increases channel conductance and lifetime.

Some of these results have appeared elsewhere in preliminary form (Barry et al., 1979; Takeda et al., 1979).

METHODS

Sartorius muscles from the toad, *Bufo marinus*, were used in all experiments. Muscles were pinned out at ~ 1.2 times their rest length on a Sylgard (Dow Corning Corp., Midland, Mich.) base in a Perspex bath (School of Physiology Workshop) and were fine-dissected under a Wild stereomicroscope (Wild Heerbrugg Instruments, Inc., Farmingdale, N.Y.). The bath was fitted into a Perspex jacket through which a methanol-water mixture at constant temperature (Lauda K2 RD, Lauda Div., Brinkmann Instruments, Inc., Westbury, N.Y.) was circulated. Temperature was monitored with a thermistor placed close to the muscle. Solutions were aerated and flowed continuously through the bath (volume 3–4 ml) at a rate of 2–3 ml/min. Normal Ringer's solution (Na solution) contained (millimolar): Na^+, 115.8; K^+, 2.5; Ca^{2+}, 1.8; Cl^-, 121.1; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 1.8, and had a pH of 7.1–7.2. Solutions in which ammonium ions were substituted for sodium ions were made by equimolar substitution of NH_4Cl for all the NaCl (NH_4 solution), or for half the NaCl (0.5 Na/0.5 NH_4Cl solution). The pH of these solutions was 6.9–7.1. Muscles were glycerol-treated (Gage and Eisenberg, 1967; Howell and Jenden, 1967) using 400–680 mM glycerol for 60–70 min, followed by 20 min in Na solution containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$ (Eisenberg et al., 1971). In

experiments where the pH was altered, HCl or NaOH (in Na solution) or NH_4OH (in NH_4 solution) were added to the HEPES-buffered solutions.

Voltage-recording microelectrodes were filled with 2 M KCl; current-passing microelectrodes were filled with a 2 M K citrate–0.8 M KCl mixture and had resistances of 3–8 MΩ. The bath was grounded with an electrode made from a Ag/AgCl wire in a 2 M KCl- 3% agar mixture. Potentials were measured differentially using high-input impedance, capacity-compensated amplifiers (1026, Teledyne-Philbrick, Dedham, Mass.). A conventional two-microelectrode voltage clamp was used. Clamp current was monitored differentially across 100 kΩ in series with the current-passing electrode and was appropriately filtered to match the current electrode RC characteristics. A grounded metal shield was placed between the electrodes and care was taken to minimize the solution depth (< 3 mm) over the endplate region. Poor spatial control of voltage resulted in a voltage trace "kick" coincident with the peak of a miniature endplate current (MEPC). Poorly clamped MEPCs "caught" with a transient recorder (Neurograph N-3, Transidyne General Corp., Ann Arbor, Mich.) had a distinct overshoot and a nonexponential decay (see Gage and McBurney, 1972). Acetylcholine (ACh) was applied iontophoretically from high resistance (30–50 MΩ) electrodes filled with 2 M AChCl, using a constant current generator. A reverse (bucking) current of 5–15 nA was usually applied, and iontophoretic current was monitored differentially across 100 kΩ in series with the electrode. Iontophoretic currents of 5–20 nA gave mean endplate currents of 30–70 nA. At different voltages in one cell, the iontophoretic current was always constant. Endplate current and membrane potential were amplified (AC-coupled with a 1-s time constant), frequency-modulated (Electrodata Associates, Sydney, Australia), and recorded on a Sony TC377 tape deck (Sony Corp. of America, Long Island City, N.Y.) (overall effective bandwidth of system: 2 kHz 3 dB point). Endplate current and ACh-iontophoretic current were recorded (DC-coupled) on a pen recorder (7402A, Hewlett-Packard Co., Palo Alto, Calif.).

MEPCs were analyzed as previously described in detail (see Gage et al., 1978). Noise records were filtered (2-pole, cascaded Butterworth filter, 40 dB/decade roll-off, 800 Hz cutoff), sampled at 2 kHz, scanned visually in 250 ms blocks on a video-display unit in order to exclude MEPCs and obvious artifacts, and were stored on RKO5 disks using a PDP 11/34 computer (Digital Equipment Corp., Marlboro, Mass.). Noise analysis of 8,192 data points in 512 point blocks was then carried out as reported previously (see Van Helden et al., 1977; Gage et al., 1978). Analysis was based on the model and theory presented by Anderson and Stevens (1973). Using least squares fitting of the log $G(f)$ values, single-sided noise power spectra were obtained, which were well described by the Lorentzian

$$G(f) = \frac{G(0)}{1 + (2\pi f \tau_N)^2}$$

from which the zero frequency asymptote $G(0)$ and the average open channel lifetime τ_N were determined. Single channel conductance was calculated from power spectra [γ(psd)] using the relationship

$$\gamma(\text{psd}) = \frac{G(0)}{4\mu_i(V_m - \epsilon_0)\tau_N},$$

where μ_i is the mean ACh-induced endplate current, V_m is membrane potential, and ϵ_0 is the null (zero current) potential. Also, measurement of the variance of current

fluctuations, σ_i^2, allowed another estimate of single channel conductance [γ(var)] from the relationship

$$\gamma(\text{var}) = \frac{\sigma_i^2}{\mu_i(V_m - \epsilon_0)}.$$

Values of γ(var) were corrected for high-frequency loss using the correction factor $(2/\pi)\tan^{-1}(2\pi F\tau_N)$ where F is the filter cutoff frequency (see Colquhoun et al., 1977).

Measurements of single channel conductance (γ) were often made over a wide range of potentials in one cell, as illustrated in Fig. 1. It is true that, as the driving

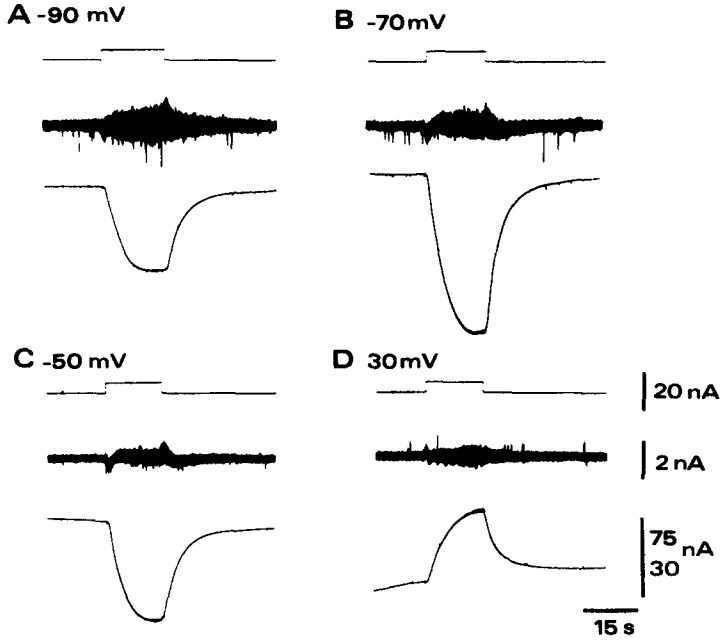

FIGURE 1. Endplate current fluctuations arising from iontophoresis of ACh in NH_4 solution, at −90, −70, −50, and 30 mV (A, B, C, and D, respectively) in the same cell. In each of the four panels, the upper trace is the iontophoretic current through the ACh electrode, the middle trace is the high-gain, AC-coupled record of the ACh-induced current fluctuations, and the lower trace is the DC-coupled mean endplate current. Note that the amplitude of the iontophoretic current was unchanged at each potential. Single channel conductance was 30.3, 34.9, 37.5, and 42.1 pS in A, B, C, and D, respectively. Temperature: 15°C. Horizontal calibration: 15 s. Vertical calibration: 20 nA for ACh iontophoretic current; 2 nA for AC-coupled noise; 75 nA (in A) and 30 nA (in B, C and D) for the DC-coupled mean endplate current.

force decreases, the mean endplate current also decreases (Fig. 1), so that the instrument plus background noise would represent a greater proportion of the total signal noise. However, this is very unlikely to lead to spuriously high estimates of γ close to the null potential because the base line was always subtracted before the agonist-induced noise was analyzed, and large endplate currents could be recorded on both sides of the null potential (Fig. 1).

To minimize cell to cell variability, conductance data were normalized relative to

one potential (−50 mV, or in the case of the theoretical analyses, −70 mV) and the normalized data averaged. This resulted in two sets of errors arising from the raw data: relative standard error of the mean (SEM) for relative conductance values and absolute SEM for the averaged absolute value of the conductance (measured experimentally at −50 mV). If, for example, the relative conductance in Na solution at −70 mV with respect to the −50 mV value was 0.915 ± 0.044 and the absolute value of conductance at −50 mV was 28.5 ± 1.63 pS, then the absolute conductance at −70 mV was calculated to be 28.5 × 0.915 = 26.1 pS with relative SEM given by 26.1 × 0.044/0.915 = 1.26. When the values normalized to −50 mV were now renormalized to −70 mV, for comparison with theoretical predictions, the relative SEM of the conductance G_v, at another potential V, now became $G_v \times [(\bar{\sigma}_v/G_v)^2 + (1.26/26.1)^2]^{1/2}$ where $G_v \pm \bar{\sigma}_v$ is the value of the conductance (at that potential) ± the old relative SEM (i.e., normalized to −50 mV). In the case of the −50 mV value, the old $\bar{\sigma}_v$ was zero. The errors considered so far have been for relative conductances in one particular cation solution. However, in order to compare conductance values in different solutions, it was necessary to correct for absolute errors. Since comparisons were done at −70 mV, the absolute error, $\bar{\sigma}_\gamma$, was calculated from the proportionate relative error at −70 mV (1.26/26.1) and the absolute proportionate error at −50 mV (1.63/28.5) to give $\bar{\sigma}_\gamma = 26.1\ [(1.26/26.1)^2 + (1.63/28.5)^2]^{1/2} = 2.0$, and thus, an absolute conductance value of 26.1 ± 2.0 pS. The effect of the SEM of the null potential measurement was also allowed for in both the relative and absolute values to give the total SEM, $\bar{\sigma}_T$. In the case of the absolute conductance values for example, $\bar{\sigma}_T$ was calculated from

$$\bar{\sigma}_T = [(\bar{\sigma}_\gamma/G_v)^2 + (\bar{\sigma}_{\epsilon_0}/(V - \epsilon_0))^2]^{1/2},$$

where $V = -70$ mV and $\bar{\sigma}_{\epsilon_0}$ is the SEM of the null potential ϵ_0. In order to normalize conductances in NH_4 and 0.5 Na/0.5 NH_4 solutions with respect to the conductance in Na solution, a similar procedure was used to give corrected proportionate SEMs, calculated as the square root of the sum of the squares of proportionate total absolute SEMs (see also Barry et al., 1979 *b*).

No explicit correction was made for changes in junction potentials occurring when ammonium solutions were introduced, as the calculated changes were small (≤0.8 mV). A more serious complication involves the liquid junction potential between the microelectrode and the inside of the cell. This potential is not easily measurable and is difficult to calculate due to many unknown variables (e.g., intracellular anion conductance, mobility and activity of intracellular ions, nature of fixed charges in the cytoplasm, and microelectrode tip potential; see also Cole and Moore, 1960; Barry and Diamond, 1970). However, preliminary calculations suggest that this junction potential ≤2 mV, and would tend to make all the measured null potentials more negative. As no correction was made here, the absolute accuracy of the quantitative data presented in this study could be affected slightly. For example, making the null potentials 2 mV more negative would result in the equilibrium constants relative to potassium being reduced by < 9%, but the relative mobilities determined from the voltage sensitivity curves would remain essentially unchanged.

RESULTS

Endplate regions of muscle fibers were localized by determining where a rapid depolarization was caused by iontophoretically applied ACh and by the presence of miniature endplate potentials having fast rise times (≤1 ms) and amplitudes of at least 0.5 mV. In normal solution MEPCs and ACh-induced

current fluctuations (noise) were usually recorded at two or more potentials before changing to test solutions.

MEPCs

When ammonium ions were substituted for sodium ions, the amplitude of MEPCs increased and their decay became slower. These effects were evident within 5 min of changing solutions. (The bath changeover time was kept slow so that cells could be readily held through solution changes). As previously noted (Furukawa et al., 1957), MEPC frequency increased greatly and, after 40–50 min, declined to almost normal levels (presumably because of depletion of transmitter). In Fig. 2A, an average of 20 MEPCs in the normal solution

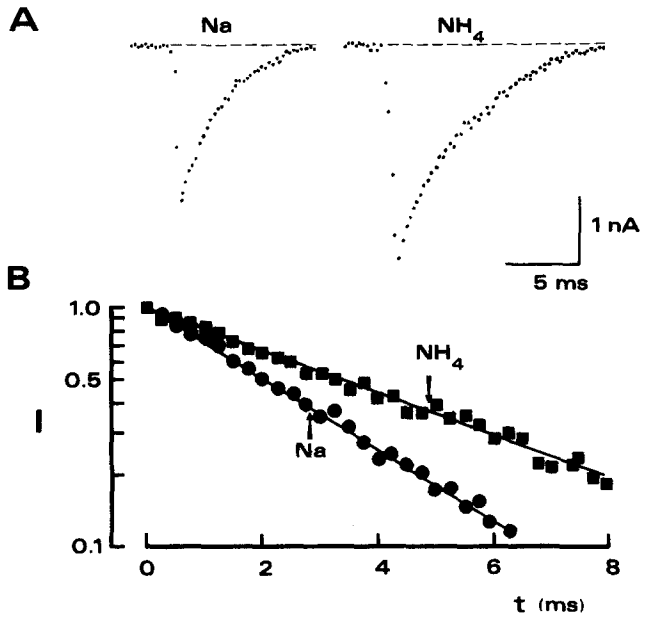

FIGURE 2. (A) Averaged MEPCs (n = 20) recorded at the same endplate in Na solution and 45 min later in NH_4 solution. Clamp potential: −50 mV. Temperature: 15°C. Horizontal calibration: 5 ms. Vertical calibration: 1 nA. (B) The averaged MEPCs shown in A were normalized and their decay was plotted semilogarithmically against time. MEPCs decayed exponentially with a single time constant (τ_D). In Na solution (●) τ_D was 2.8 ms. In NH_4 solution (■) τ_D was 5.0 ms (arrows).

(Na solution) is shown on the left. The mean peak MEPC amplitude was 2.3 nA and the decay was exponential with a time constant of decay of 2.8 ms (clamp potential −50 mV, temperature 15°C). After 45 min in a solution containing NH_4Cl substituted for NaCl (NH_4 solution), the average peak amplitude of 20 MEPCs (recorded in the same cell at the same clamp potential and temperature) was 3.2 nA. The decay of the average MEPC remained exponential as shown in the semilog plot of normalized current vs. time in Fig. 2B (■) and the time constant of decay increased to 5.0 ms.

Null Potential

An increase in MEPC amplitude could be caused by a shift in the acetylcholine null potential to a more positive value. In fact, a positive shift was seen, but it was insufficient to account for more than a small fraction of the increase in MEPC amplitude. This is illustrated in Fig. 3 A in which mean peak MEPC amplitude (I_p) is plotted against membrane potential. It can be seen that in

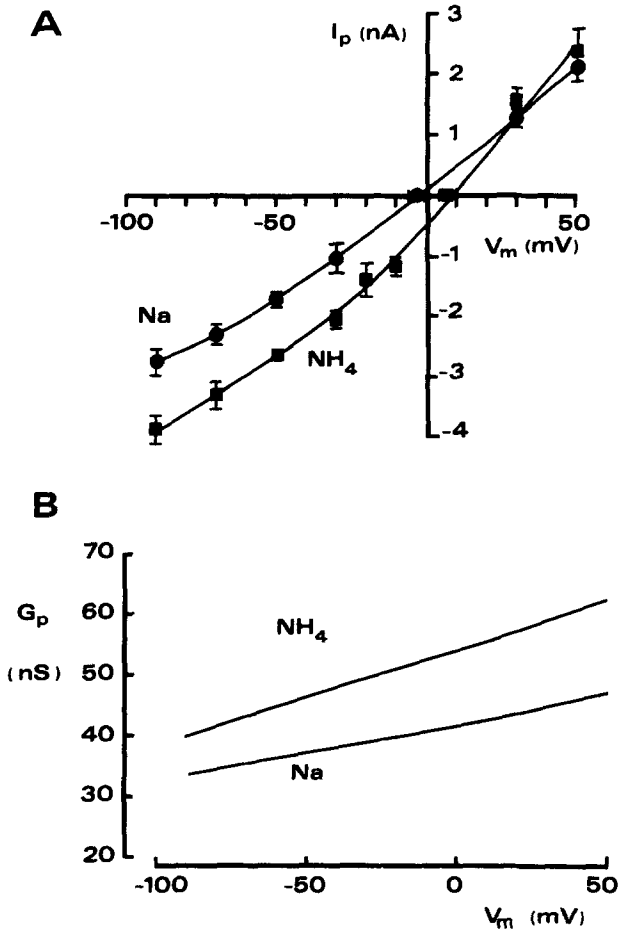

FIGURE 3. The effect of membrane potential on the peak amplitude (I_p) and peak conductance (G_p) of MEPCs. (A) Averaged peak MEPC amplitudes plotted as a function of clamp potential for Na solution (●, 12 fibers) and for NH_4 solution (■, 8 fibers). Error bars indicate ± 1 SEM. Curves were drawn by eye through the data points. Zero current potentials plotted on the abscissa were determined from ACh iontophoresis for Na solution, and from both ACh iontophoresis and MEPC reversal for NH_4 solution. Temperature: 15°C. (B) Peak MEPC conductance-voltage relationship in Na (lower curve) and in NH_4 (upper curve) solutions. The curves were obtained from the I_p-voltage curves shown in A.

Na solution (results from 12 fibers) the null potential was −3 mV whereas the null potential in NH_4 solution was 7 mV (results from 8 fibers). The estimate of null potential for MEPCs suffers from the limitation of a deteriorating signal-to-noise ratio at potentials close to the null potential. Therefore the null potential was also measured by determining the potential at which iontophoretic application of ACh generated no change in clamp current. The null potential determined in this way was −2.8 ± 0.8 mV (mean ± 1 SEM) in Na solution (six fibers) and 6.5 ± 1.3 mV in NH_4 solution (three fibers).

However, this shift in null potential is obviously insufficient to account for the increase in MEPC amplitude in NH_4 solution. When peak MEPC amplitude was divided by $(V_m\text{-}\epsilon_0)$, where V_m is the membrane potential and ϵ_0 is the null potential, to give the peak conductance G_p, the value in Na solution was less than in NH_4 solution over a wide range of potentials (Fig. 3 B; see also Table I). As $G_p = n\gamma$, where n is the number of channels open at the peak of an MEPC and γ is the conductance of each channel, it seemed that either γ was increased or that the number of channels activated by a quantum of ACh was increased in NH_4 solution. In order to distinguish between these two possibilities, noise analysis (Katz and Miledi, 1970; Anderson and Stevens, 1973) was used to measure γ.

Channel Characteristics

Mean endplate current (μ_i) and current fluctuations in response to iontophoresis of ACh were increased in NH_4 solution. Noise data were usually obtained after 45–50 min in NH_4 solution. The increase in current fluctuations can be seen in Fig. 4 A. The trace on the left shows the response to an iontophoretic current of 19 nA through the ACh electrode in Na solution; the trace on the right shows the response to an iontophoretic current of 9 nA in NH_4 solution in the same cell. The traces also show the characteristic increase in MEPC frequency in NH_4 solution (Furukawa et al., 1957).

Power spectral density curves of such current fluctuations in Na (●) and in NH_4 solution (■) were both well fitted by single Lorentzians as shown in Fig. 4 B. It can be seen that the half-power frequency (arrows) shifted to a lower frequency in NH_4 solution indicating that average channel lifetime was increased. Single channel conductance, whether calculated from the variance-to-mean ratio of endplate current [γ(var)], or from power spectra [γ(psd)] was found to be increased in NH_4 solution. Values of γ measured in several experiments are given in Table I.

Half Sodium/Half Ammonium Solution

Characteristics of MEPCs and single channels were measured in solutions in which half the NaCl had been replaced with NH_4Cl (0.5 Na/0.5 NH_4 solution) in order to examine any possible sodium-ammonium ion interactions. Peak MEPC amplitude and τ_D were increased in this solution, but not as much as in NH_4 solution. The null potential determined using ACh iontophoresis shifted to 0.9 ± 1.6 mV ($n = 3$), whereas the interpolated value using averaged MEPC data was 1.5 mV. Single channel conductance data are given in Table I (see also Fig. 6).

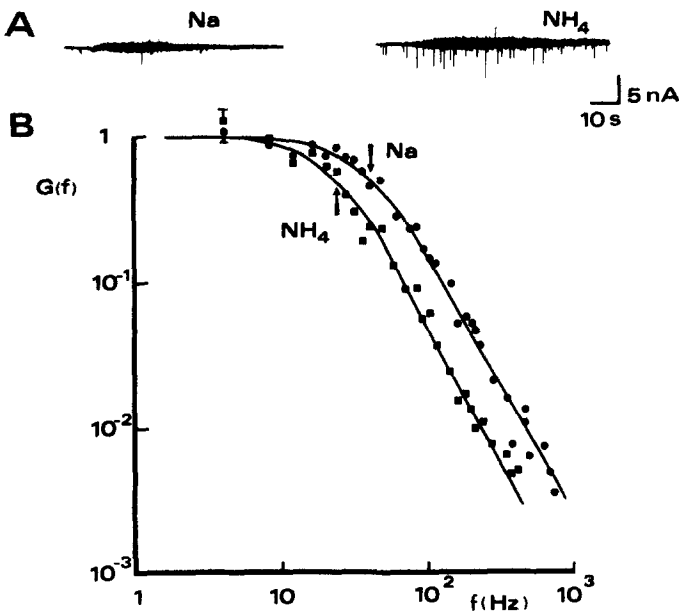

FIGURE 4. (A) Endplate current fluctuations evoked by ACh iontophoresis in Na solution, and 50 min later in NH_4 solution at the same endplate. Inward current is downwards The large upward deflection is an artifact caused by switching off ACh iontophoretic current, which was 19 nA in Na and 9 nA in NH_4 solution (backing current 5 nA). Note increase in MEPC frequency in NH_4 solution. Pen recorder bandwidth was 1.6–125 Hz. Membrane potential: −70 mV. Temperature: 15°C. Horizontal calibration: 10 s. Vertical calibration: 5 nA. (B) Normalized power spectra of the ACh-induced current fluctuations. The lines show the best (least squares) fits to single Lorentzian curves. Half-power frequencies (arrows) of 40 Hz in Na solution (●) and 22.5 Hz in NH_4 solution (■) gave mean channel lifetimes of 3.98 and 7.04 ms, respectively. Asymptotic spectral densities were 2.05×10^{-21} and 4.04×10^{-21} A^2s for corresponding steady endplate currents of 68 nA in Na solution and 55 nA in NH_4 solution. Single channel conductances determined in this case from power spectra [γ(psd)] were 28.3 and 33.0 pS, respectively, while channel conductances determined from the current variance-to-mean ratio [γ(var)] were 27.4 and 34.4 pS, respectively, for Na and NH_4 solutions. Note logarithmic axes. Error bars at 4 Hz indicate 1 SEM.

Effect of Membrane Potential

Measurements of the time contant of decay of MEPCs (τ_D) recorded over a range of membrane potentials in a number of experiments are shown in Fig. 5A. In NH_4 solution (■), a consistent increase in τ_D was seen. The slope of the exponential relationship between τ_D and membrane potential was not changed in NH_4 solution. The regression lines shown are described (Magleby and Stevens, 1972; Anderson and Stevens, 1973; Gage and McBurney, 1975) by the equation

$$\tau(V) = \tau(0) \exp(-V/H),$$

where $\tau(V)$ is the average channel lifetime at membrane potential V, $\tau(0)$ is the average channel lifetime at zero membrane potential and H is the membrane potential change required for an e-fold change in τ. As for τ_D, average channel lifetimes measured from power spectra of ACh-induced current fluctuations (τ_N) were greater in NH_4 solution (■) than in Na solution (●) (Fig. 5 B). It was also clear that the voltage sensitivity of τ_N had not changed significantly in NH_4 solution. In 0.5 Na/0.5 NH_4 solution, both τ_N and τ_D were slightly greater than in Na solution, whereas the voltage sensitivity was again unchanged. Values of $\tau(0)$ and H obtained from MEPCs and from noise analysis in Na, NH_4, and 0.5 Na/0.5 NH_4 solutions are listed in Table II.

TABLE I

MEAN PEAK CONDUCTANCE DURING MEPCS (G_p) AND MEAN CHANNEL CONDUCTANCE (γ) IN Na, NH_4, AND 0.5 Na/0.5 NH_4 SOLUTIONS

	G_p		γ(psd)	
Solution	−90 mV	50 mV	−90 mV	30 mV
	nS		*pS*	
Na	33±3 (6)	45±4 (5)	25.0±2.3 (8)	35.1±3.4 (4)*
NH_4	40±2 (8)	54±3 (4)	32.2±2.1 (3)	40.9±3.9 (3)‡
0.5 Na/0.5 NH_4	35±2 (9)	47±3 (4)	31.5±2.1 (9)	42.0±4.0 (7)§

Results are shown as mean ± SEM appropriately corrected for the normalization procedure, with the number of fibers in parentheses. Values of γ(psd) were not significantly different from γ(var) estimates, following correction for filtering. Temperature: 15°C. The statistical significance of the increase in channel conductance (γ) in each of the solutions over the two voltages was calculated using the relative SEM (e.g., Table V for NH_4 values) rather than the absolute SEM.

* $P < 0.025$, Student's t test.

‡ $P < 0.1$, Student's t test.

§ $P < 0.005$, Student's t test.

In NH_4 solution, the conductance change at the peak of MEPCs (G_p) was always larger than in Na solution. It was also apparent that G_p increased at depolarized potentials both in Na solution and in NH_4 solution (see Fig. 3 B). Similarly, single channel conductance (γ) obtained from noise analysis was greater in NH_4 solution than in Na solution, and a slight increase in γ with membrane depolarization was observed (Fig. 6). This potential-dependent change in γ, which has been previously reported for Na solution (Gage and Van Helden, 1979; Van Helden et al., 1979) was also quite clear in NH_4 solution. In 0.5 Na/0.5 NH_4 solution, γ values (Fig. 6, ▲) lay between those in Na and in NH_4 solution and had similar voltage sensitivity (see also Table I).

Net Ion Movements

In previous experiments in which monovalent alkali cations were substituted for sodium ions, it was found that changes in both channel lifetime and the time constant of decay of MEPCs were accompanied by reciprocal changes in channel conductance and peak MEPC conductance respectively, so that charge transfer through a channel or during a MEPC tended to remain

constant (Van Helden et al., 1977; Gage and Van Helden, 1979). Clearly this was not so with ammonium ions. Graphs of charge transfer during MEPCs ($Q_m = I_p \cdot \tau_D$) and through a single channel ($Q_c = \tau_N \cdot \gamma \cdot (V_m - \epsilon_0)$) are shown over a range of potentials for Na solution (●) and NH_4 solution (■) in Fig. 7. Charge transfer both during a MEPC and through a single channel was greater in NH_4 solution than in Na solution. Values of Q_m and Q_c in 0.5 Na/

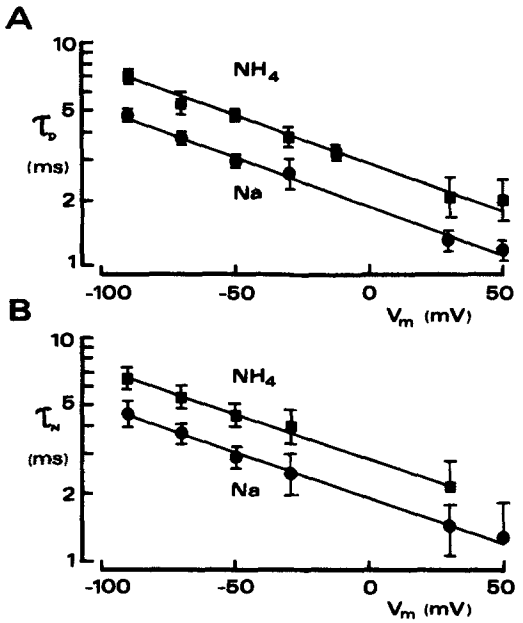

FIGURE 5. The effect of membrane potential on MEPC time constant of decay (τ_D) and on channel lifetime (τ_N). (A) Voltage sensitivity of τ_D in Na solution (●, 12 experiments) and in NH_4 solution (■, 8 experiments). Each data point is the mean ± 1 SEM of at least 45 MEPCs measured from a minimum of three cells. From the relationship $\tau(V) = \tau(0) \exp(-V/H)$. H values determined by linear regression were 102 mV for Na solution and 115 mV for NH_4 solution. (B) Voltage sensitivity of τ_N in Na solution (●, 12 experiments) and in NH_4 solution (■, 7 experiments). Data points are mean ± 1 SEM from at least three cells. H values for τ_N were 110 mV in Na solution and 128 mV in NH_4 solution. Temperature: 15°C.

0.5 NH_4 solution were greater than in Na solution but were less than those measured in NH_4 solution (see Table III).

Possible Effects of pH

In ammonium solutions at pH ~ 6.9–7.1, a small proportion of ammonia will be present, assuming that the pK_a of ammonium ions ≃ 9.5 (Boron and De Weer, 1976). In squid axons and snail neurons, low concentrations (~10 mM) of ammonium ions cause rapid and reversible intracellular pH transients and these alkalinizations have been attributed to high ammonia fluxes followed by protonation to ammonium ions (Boron and De Weer, 1976; Thomas,

1976). Since pH effects on surface charge potentials are well known (see e.g., Hille et al., 1975; Van der Kloot and Cohen, 1979), and lowering the pH has been reported to lengthen the decay of EPCs and MEPCs at frog endplate (Scuka, 1975; Mallart and Molgo, 1978), we examined the effects of changing pH in Na solution. In a preliminary series of experiments, no large effects were found on the time constant of decay of MEPCs over the pH range 4–10. In four cells at pH 4, τ_D was 1.11 ± 0.06 times the value at pH 7.1, while in three cells at pH 10, τ_D was 0.95 ± 0.04 times the control value. As reported by Mallart and Molgo (1978), MEPC amplitude was unchanged over the pH range 4–10. Recently, similar observations have been made independently.[1] In one experiment, channel conductance and lifetime at −50 mV were obtained from noise analysis in Na solution at pH 5, and were not significantly different from values measured at pH 7. Attempts to modify the proportion of ammonia in NH_4 solution were made by raising the pH to 8.0, and MEPCs

TABLE II

VOLTAGE SENSITIVITY OF MEPC TIME CONSTANT OF DECAY (τ_D) AND OF CHANNEL LIFETIME (τ_N) IN SODIUM AND AMMONIUM SOLUTIONS

	H		$\tau(0)$		$\bar{r}$	
Solution	MEPC	Channel	MEPC	Channel	MEPC	Channel
	mV		*ms*			
Na	103±9 (12)	110±7 (12)	1.9±0.2	2.0±0.2	−0.99	−0.98
NH_4	115±14 (8)	128±16 (7)	3.0±0.3	3.1±0.3	−0.98	−0.99
0.5 Na/0.5 NH_4	109±10 (11)	117±12 (12)	2.7±0.2	2.5±0.3	−0.98	−0.99

From linear regression lines fitted to the ln τ values as a function of V, $\tau(0)$ and H values were obtained using the equation $\tau(V) = \tau(0) \exp(-V/H)$. Results are presented as mean ± SEM with the number of fibers in parentheses, (correlation coefficient, $\bar{r}$). Temperature: 15°C.

were not different from those seen in NH_4 solution at pH 7. We conclude that any long term effects of intracellular pH changes in ammonium solutions probably did not contribute greatly to the observations of increased channel conductance and lifetime. Furthermore, it is unlikely that significant accumulation of ammonium ions had occurred, because even after long periods (>4 h) in NH_4 solution, MEPC amplitude was always greater than in Na solution.

Replacement of $CaCl_2$ with $BaCl_2$

Activation of postsynaptic channels by ACh in an aplysia neuron can result in the development of late potassium current, presumably turned on by an influx of calcium ions (Ascher et al., 1978). The presence of a secondary calcium-activated potassium current could lead to overestimates of not only the null potential but also of single channel conductance. Replacement of extracellular calcium with cobalt or barium ions has been reported to block outward potassium currents in aplysia (Ascher et al., 1978; Adams, 1978). In

[1] Hamill, O. P. Personal communication.

two experiments, we found no significant change in the null potential in Na solution, when extracellular $CaCl_2$ was replaced with $BaCl_2$.

DISCUSSION

It is clear from the results that when sodium ions are replaced by ammonium ions, the peak amplitude and time constant of decay of MEPCs are increased. The greater amplitude of MEPCs could not be explained by the shift in null potential seen in ammonium solutions, but is best accounted for by the observation of an increased single channel conductance. Increases in endplate channel conductance have been recently reported when sodium ions are replaced by both cesium and potassium ions (Gage and Van Helden, 1979).

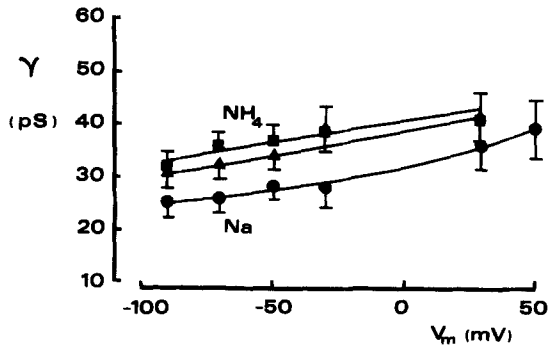

FIGURE 6. Voltage sensitivity of single channel conductance γ. Curves were drawn by eye through the data points. Error bars for −50 mV data indicate 1 SEM; in the other cases they represent the SEM of the normalized conductance relative to the −50 mV value (see Methods). Each data point represents 3–12 estimates of γ(psd); Na solution (●, 12 fibers), NH_4 solution (■, 7 fibers), 0.5 Na/0.5 NH_4 solution (▲, 12 fibers). Temperature: 15°C.

Similarly, when sodium ions are replaced with cesium ions in an aplysia neuron, ACh-activated single channel conductance is increased, as is channel lifetime (Ascher et al., 1978). In lipid bilayers, gramicidin A channel conductance is also increased when cesium replaces sodium ions (Kolb and Bamberg, 1977). The increased channel conductance in ammonium solutions (compared to Na solution) is consistent with the observation that $P_{NH_4} > P_{Na}$, as is evident from the null potential measurements. For channel conductances estimated from noise analysis, we have assumed that all the current flowed through ACh-activated endplate channels, and that secondary currents like the late calcium-activated potassium currents seen in aplysia (Ascher et al., 1978) were not present. Ammonium ions passing through open endplate channels may turn on other currents through the muscle membrane, but the increased peak MEPC amplitude (assuming the number of channels activated is constant) in ammonium solutions and the high resting ammonium permeability of the muscle membrane (judging from the depolarizing action of ammonium ions) argue against this possibility.

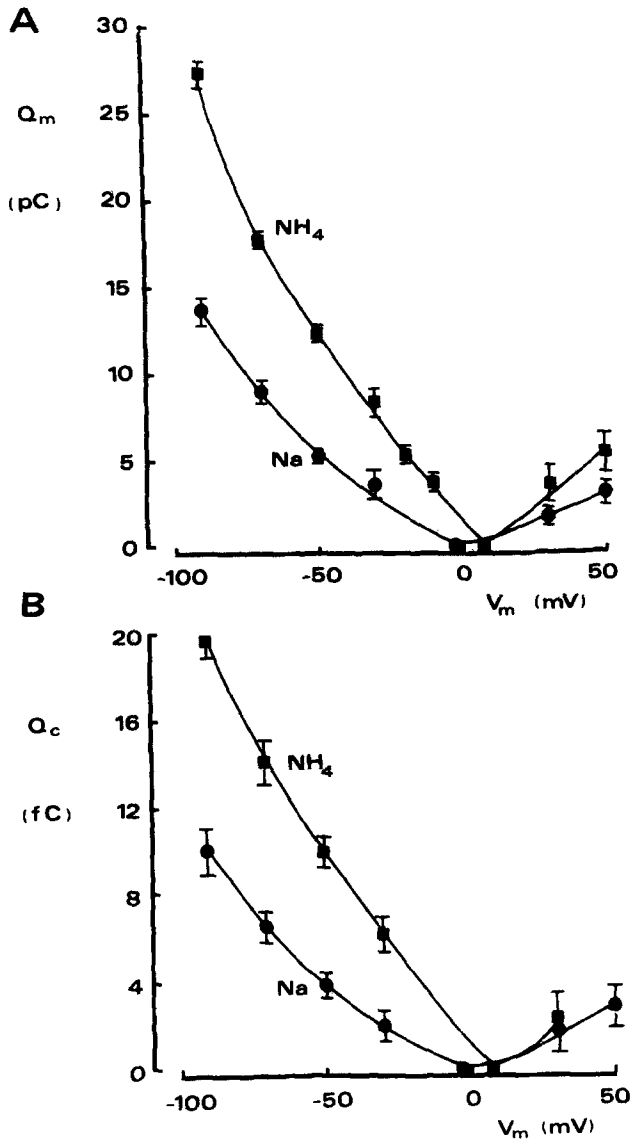

FIGURE 7. The effect of membrane potential on charge transfer during MEPCs (Q_m) and through a single channel (Q_c). (A) Net ion movement for MEPCs in NH_4 solution (■, 8 fibers) was greater than in Na solution (●, 12 fibers). The curves were drawn by eye. Error bars indicate ± 1 SEM. (B) Similarly net ion movement through a single channel in NH_4 solution (■, 7 fibers) was consistently greater than in Na solution (●, 12 fibers). Temperature: 15°C.

Previously, it has been reported (Van Helden et al., 1977; Gage and Van Helden, 1979) that, for the alkali cations, channel lifetime and conductance appeared to be reciprocally related, so that charge transfer through a channel or during a MEPC tended to remain constant. Clearly, this was not so for

ammonium solutions. Neither the voltage dependence of channel conductance nor the voltage dependence of channel lifetime was greatly different in ammonium solutions. If channel lifetime is determined by "electrostatic stabilization" of the open conformation while an ion is bound to a site, then ions having high site affinities might tend to keep the channel open longer (see Gage and Van Helden, 1979; Kolb and Bamberg, 1977). Alternatively, ammonium ions hydrogen bonding to intersite ligands may make the open conformation more favored, and thus prolong channel lifetime.

From the known permeability of several large organic cations, the acetylcholine-activated channel at the endplate could be thought of as a large, aqueous pore. However, the apparent absence of chloride permeability (Takeuchi and Takeuchi, 1960) strongly suggests the presence of at least one high resistance barrier to anions. There have been several recent studies on endplate ion selectivity. For example, selectivity sequences for single channel conductance and null potential have been obtained for the alkali cations lithium, sodium, potassium, and cesium (Van Helden et al., 1977; Gage and Van Helden, 1979). P_{NH_4}/P_{Na} values of 1.79 (Dwyer et al., 1979), ~1.1 (Linder and

TABLE III

NET CHARGE TRANSFER DURING A MEPC (Q_m) OR THROUGH A SINGLE CHANNEL (Q_c) IN SODIUM AND AMMONIUM SOLUTIONS AT 15°C

	Q_m		Q_c	
Solution	−90 mV	50 mV	−90 mV	30 mV
	pC		*fC*	
Na	13.5±1.8 (6)	2.7±1.0 (5)	10.5±1.6 (8)	1.6±.6 (4)
NH_4	27.5±2.7 (8)	5.1±1.3 (3)	19.9±1.9 (3)	2.0±.4 (3)
0.5 Na/0.5 NH_4	17.9±2.5 (9)	3.3±1.2 (4)	15.4±1.8 (9)	2.1±.3 (7)

Results are shown as mean ± SEM with the number of fibers in parentheses.

Quastel, 1978), and ~1.0 (Maeno et al., 1977) have been reported, which compare with the value 1.47 found in this study. Apart from the possible species difference, it should be noted that dissimilar techniques have been used to obtain the results. The relative permeability ratios obtained from null potential shifts can be predicted by not only the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949), but also by both the neutral site and the charged site models (Barry et al., 1979*b*). However, it has been previously shown that single channel conductance is voltage dependent and that the nature of the alkali cation influences the voltage sensitivity of conductance (Van Helden et al., 1977, 1979; Gage and Van Helden, 1979). These observations together with the concentration dependence of conductance can be predicted adequately only by the neutral site channel model (Barry et al., 1979*a*, *b*).

Neutral Site Channel Model

The model was based on the assumption that the channel length was much greater than the Debye length within it, and that there was at least one high

resistance barrier for anions. The sites lining the channel were considered to be represented by the negative ends of polar groups, and the analysis suggested that these sites were high field strength sites (Barry et al., 1979*b*).

We were interested in seeing whether single channel conductance measured in the presence of an "organic" cation such as ammonium also fitted the predictions of the neutral site channel model or whether the hydrogen-bonding capacity of the ammonium ion caused deviations from the model. The same technique of fitting the data was used here as reported before in detail (Barry et al., 1979*b*). The relative permeabilities P_{NH_4}/P_K and P_{Na}/P_K were obtained from measurements of the null potential ϵ_0, which was given by:

$$\epsilon_0 = \frac{RT}{F} \ln\left\{\frac{[K]_o + (P_{Na}/P_K)[Na]_o + (P_{NH_4}/P_K)[NH_4]_o}{[K]_i + (P_{Na}/P_K)[Na]_i + (P_{NH_4}/P_K)[NH_4]_i}\right\}. \quad (1)$$

In the absence of any significant anion permeation, this equation is very similar in form to the Goldman-Hodgkin-Katz equation, where R, T, and F have their usual significance. However, in the equation for the neutral site channel, the relative permeability terms can be separated into two components: relative partition coefficients (K_{NH_4}/K_K) or equilibrium constants and relative mobilities (u_{NH_4}/u_K), which are related by:

$$\frac{P_{NH_4}}{P_K} = \frac{u_{NH_4}}{u_K} \cdot \frac{K_{NH_4}}{K_K}. \quad (2)$$

Relative permeabilities were calculated from the null potential measurements using Eq. 1, but the individual relative mobility and partition coefficient terms could not be determined separately from the null potentials alone. Nevertheless, different combinations of relative mobilities and partition coefficients predicted by both neutral and charged site models did give rise to very different degrees of voltage sensitivity of conductance. Hence, a given degree of voltage sensitivity implies a unique combination of relative mobilities and partition coefficients. The equation for single channel conductance γ is

$$\gamma = i/(V_m - \epsilon_0), \quad (3)$$

where the single channel current i, is given by

$$i = B_1 \frac{\xi U'' - U'}{\xi C'' - C'} (V_m - \epsilon^*), \quad (4)$$

and

$$\xi = \exp(V_m F/RT); \quad (5)$$

$$U' = \sum_j u_j K_j a_j'; \quad (6)$$

$$C' = \sum_j K_j a_j'. \quad (7)$$

The summation is overall j, where j represents each of the cations sodium, potassium, and ammonium. Superscript refers to the external solution and

a is the activity of the cation. A similar set of equations arises for U'' and C'' where the superscript $''$ now refers to the internal solution. ϵ^* is defined by

$$\epsilon^* = \frac{RT}{F} \ln(C'/C'').$$

B_1 is a factor defined elsewhere (Barry et al., 1979*b*) and is dependent on both cation and anion concentrations and their partition coefficients, but is independent of their mobilities and the membrane potential.

The above procedure for obtaining permeation parameters is illustrated for NH_4 solution in Fig. 8A in which experimental values of single channel conductance plotted as a function of membrane potential are compared with neutral site channel predictions. Since a small concentration of sodium ions was present in NH_4 solution (see Methods) and within the cell (assumed to be about 10 mM), it was necessary to use sodium parameters in these calculations. Initially, values of $K_{Na}/K_K = 2$ and $u_{Na}/u_K = 0.57$ (Barry et al., 1979*b*) based on the null potential and the voltage sensitivity measurements of Gage and Van Helden (1979) were used, although, with so little sodium present, the curves were very insensitive to the value of K_{Na}/K_K. A K_{NH_4}/K_K value of about 4 (with $u_{NH_4}/u_K = 0.42$) was considered to provide the best fit to the experimental data, although the value of 3 (with $u_{NH_4}/u_K = 0.56$) gave only a marginally worse fit.

From the null potential measurements in NH_4 and Na solutions, the predicted null potential in 0.5 Na/0.5 NH_4 solution is $2.3^{+0.4}_{-0.6}$ mV. This value is independent of the particular combination of mobilities and partition coefficients used and agrees within experimental error with the measured null potential of 0.9 ± 1.6 mV. Using the parameters that provided the best fit to the data in Fig. 8A, relative single channel conductance values were predicted and compared with those measured experimentally in 0.5 Na/0.5 NH_4 solution and the results are shown in Fig. 8B. In addition, the predicted curve for a higher K_{Na}/K_K of 4 is shown. This value was slightly more compatible with the Na solution data obtained here, although, in view of the standard errors, it was not significantly different from the value obtained previously (Barry et al., 1979*b*). Using these parameters and allowing for a range of uncertainty in K_{Na}/K_K, the predicted absolute single channel conductances in NH_4 and 0.5 Na/0.5 NH_4 solutions at −70 mV (relative to the value in Na solution) were calculated and are compared with the experimental values in Table IV. Overall, a value of K_{NH_4}/K_K between 3 and 4 provided the best fit for both the absolute conductance data and for the voltage sensitivity data and suggests that $K_{NH_4} \gtrsim K_{Na} > K_K$.

For the four alkali cations in the previous study (Barry et al., 1979*a*, *b*), the product of the relative partition coefficient and mobility for an individual cation was approximately constant, being equal to 1.07 ± 0.14 (SD). In contrast, for ammonium ions, the product $(K_{NH_4}/K_K) \times (u_{NH_4}/u_K)$ equals 1.68 ± 0.05 (SEM). This implies a much higher mobility for ammonium ions than would have been predicted for an alkali cation having the same partition coefficient. Possibly this higher mobility arises from the ability of the ammonium ion to form hydrogen bonds with other intersite ligands lining the

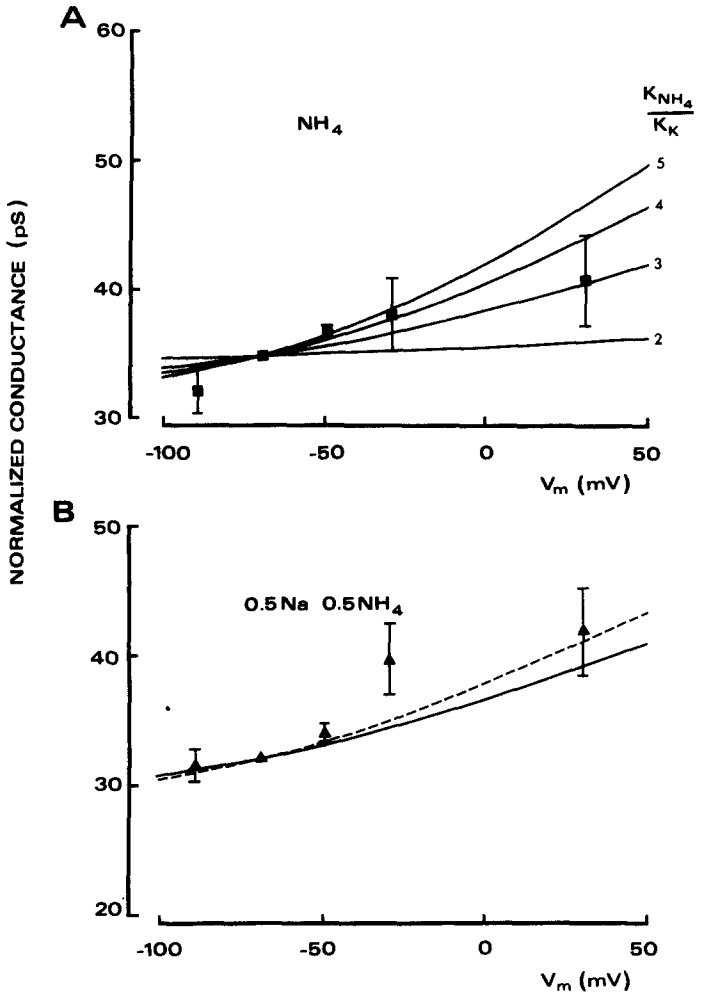

FIGURE 8. Single channel conductance [γ(psd)] measured as a function of membrane potential and compared with theoretical predictions. The experimental values (see Fig. 6) have now been normalized to the average value of conductance obtained at −70 mV. In each case the errors shown are the SEM of the average conductance corrected for the normalization procedure and for the SEM of the null potential measurement (see Methods). (A) Results for NH_4 solution are compared with the theoretical curves predicted using the neutral site channel model. The values of K_{NH_4}/K_K used in each case are shown adjacent to each of the curves. Although a value of $K_{Na}/K_K = 2$ was used, the curves are very insensitive to the particular value chosen, since there is very little sodium present. A K_{NH_4}/K_K value of ~ 4 was considered to be the best fit, although the value of 3 is only marginally worse. (B) Experimental results obtained in a 0.5 Na/0.5 NH_4 solution are compared with the theoretical curves predicted using $K_{NH_4}/K_K = 4$. The full curve is that obtained for a $K_{Na}/K_K = 2$, whereas the broken curve is for $K_{Na}/K_K = 4$.

channel. Since conductance is a function of both the equilibrium concentration and the mobility of cations within the channel, there may also be a contribution to the conductance from "proton jumping" (Grotthus conductivity; e.g., Moore, 1972), when the sites are occupied by ammonium ions, inasmuch as this may appear as an increased u_{NH_4}/u_K.

From the data in this paper and the earlier data already mentioned (see Barry et al., 1979*b*), the equilibrium constant sequence appears to be $K_{Li} > K_{NH_4} \gtrsim K_{Na} > K_K > K_{Cs}$. This represents a high field strength sequence (Eisenman, 1965). The position of ammonium in the sequence would at first appear to be somewhat anomalous since it would suggest that the radius of the ammonium ion $\lesssim$ the radius of the sodium ion. In fact, the ammonium ion has an ionic radius 1.12 times that of the potassium ion and an almost

TABLE IV

EXPERIMENTAL AND PREDICTED VALUES OF SINGLE CHANNEL CONDUCTANCE IN SODIUM AND AMMONIUM SOLUTIONS

Solution	Experimental γ(psd)	Theoretical predictions for γ $K_{Na}/K_K = 3 \pm 1$		
		$K_{NH_4}/K_K = 3$	$K_{NH_4}/K_K = 4$	$K_{NH_4}/K_K = 5$
	pS		*pS*	
Na	26.1	26.1	26.1	26.1
NH_4	34.9±3.3	$34.1^{+4.3}_{-4.6}$	$30.3^{+3.8}_{-4.6}$	$27.5^{+3.5}_{-4.1}$
0.5 Na/0.5 NH_4	32.1±3.3	$30.2^{+1.7}_{-2.6}$	$28.3^{+1.9}_{-2.7}$	$26.8^{+2.0}_{-2.8}$

The experimental results in each case were based on the raw data obtained at −70 mV and used in Fig. 8. However, they now take into account absolute errors in each solution and, in the case of NH_4 and 0.5 Na/0.5 NH_4 solutions, were normalized to the experimental values in Na solution. The errors were appropriately corrected for the normalization procedure (see Methods). The theoretical conductance predictions were based on the neutral-site channel model, and the +/− range given represents the upper and lower limit of K_{Na}/K_K, respectively, listed above.

identical free solution mobility (Robinson and Stokes, 1959). However, in other biological situations, ammonium ions behave as if they had a much smaller radius, and thus bind more strongly than potassium ions. This is true for the sodium channel in the frog node of Ranvier, where $P_{NH_4}/P_K \simeq 2$ (Hille, 1972) and is also true for the high field-strength lithium aluminosilicate glass (LAS11-188) where both ammonium and sodium are more selected than potassium ions (Eisenman, 1967). In some cases, this enhanced binding of ammonium ions probably results from hydrogen bonding to suitably spaced oxygen ligands at the sites, because ammonium ion binding is very sensitive to the spatial configuration of the site ligands (Szabo et al., 1973). In gallbladder, some of the lower field-strength ion selectivity data also indicate

that ammonium ions bind more strongly than potassium and sometimes, even sodium ions (Moreno and Diamond, 1975). Ammonium, although not being as polarizable as thallium, is much more polarizable than the potassium ion, although the energy contribution due to polarization effects may not be significant in comparison to the contribution from hydrogen bonding.

Voltage Sensitivity of Conductance

The voltage sensitivity of single channel conductance in NH_4 solution is shown in Table V (see also Fig. 6) and is compared with Goldman-Hodgkin-Katz and neutral site predictions. Note that a reversed voltage dependence of conductance is predicted by the Goldman-Hodgkin-Katz equation. Other workers have indicated that single channel conductance is not very voltage-sensitive in normal solutions (Anderson and Stevens, 1973; Lewis, 1979).

TABLE V

VOLTAGE SENSITIVITY OF SINGLE CHANNEL CONDUCTANCE IN NH_4 SOLUTION

V_m	Experimental	Neutral site	Goldman-Hodgkin-Katz
mV	*pS*	*pS*	*pS*
−90	32.2±1.1	34.3	35.3
30	40.9±3.2	40.0	32.2

Note that the Goldman-Hodgkin-Katz equation predicts the opposite voltage sensitivity compared to the experimental results and the neutral site predictions. The permeability ratios, $P_{NH_4}/P_K = 1.68$ and $P_{Na}/P_K = 1.15$, calculated from the observed null potentials, were used in the Goldman-Hodgkin-Katz predictions. For the neutral-site channel model, these permeability ratios were separated into equilibrium constant and relative mobility terms (using the conductance voltage sensitivity data) and the following values were used: $K_{NH_4}/K_K = 3$, $u_{NH_4}/u_K = 0.56$; $K_{Na}/K_K = 3$, $u_{Na}/u_K = 0.38$. Temperature: 15°C.

However, the consistent increase in conductance with membrane depolarization is best seen at a single endplate over a wide voltage range, as is illustrated in Fig. 1. Furthermore, variability in the conductance data obtained from different endplates and measurement over a more limited voltage range make the voltage dependence difficult to detect. A linear relationship between variance and mean endplate current and a more pronounced voltage dependence of conductance have been recently reported in denervated toad sartorius muscle fibers (Gage and Hamill, 1980).

Fig. 9 illustrates the physical principles underlying the voltage sensitivity of conductance. The curve of single channel conductance has been replotted for the same parameters as used in Fig. 8A but now over an extended voltage range. The voltage sensitivity arises from the difference in mobility within the channel (and to some extent, differences in relative partition coefficients) for the different cations and from the asymmetrical nature of the external and internal solution composition (as depicted in the two schematic insets). At extreme hyperpolarizing potentials, the neutral sites in the channel would be

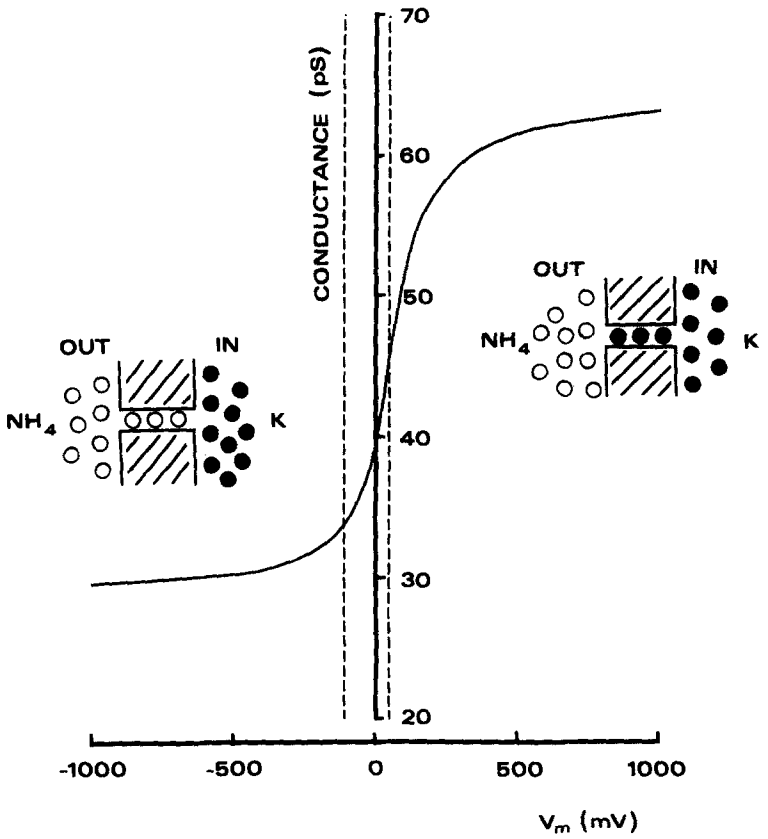

FIGURE 9. Single channel conductance predicted as a function of membrane potential for a neutral site channel model in NH_4 solution. The parameters used were the same as those used for the $K_{NH_4}/K_K = 4$ curve in Fig. 8A and normalized for the experimental value of conductance at −70 mV. The range from −110 to 50 mV considered in Fig. 8 is indicated by the two vertical broken lines. The extended voltage range shown here illustrates the physical principles underlying the voltage sensitivity of conductance predicted by the model. At extreme hyperpolarizing potentials, as illustrated schematically by the *left inset*, the neutral sites in the channel are predominantly occupied by ammonium ions; thus the conductance arises essentially from them alone. Because the ammonium ion has lower relative mobility ($u_{NH_4}/u_K = 0.42$, estimated from the equilibrium constants and null potential measurements), the conductance at such hyperpolarizing voltages is relatively small. In contrast at extreme depolarizing potentials, as illustrated schematically by the *right inset*, the neutral sites in the channel are predominantly occupied by potassium ions; thus the conductance arises essentially from potassium ions alone. Since the potassium ion mobility is considerably greater than that of the ammonium ion, the conductance at such depolarizing voltages will be much larger.

predominantly occupied by ammonium rather than by potassium ions and hence, because ammonium ions have the lower mobility, the channel conductance would be minimal. In contrast, at extreme depolarizing potentials, the neutral sites in the channel would be predominantly occupied by potassium ions and the conductance would be maximal. Although the ratio of conductances at the two voltage extremes is determined essentially by the relative mobilities alone, the actual shape of the curve elsewhere is also dependent on the relative partition coefficients. It is interesting to note that the experimental voltage range occurs over part of the steepest section of the curve.

Two further points should be made about the applicability of the neutral site channel model. First of all, the mathematical model was derived earlier (Barry et al., 1979*b*) assuming that cation concentrations in the channel were well below site saturation levels. Allowing for the possibility of site saturation has shown that this was indeed a reasonable assumption (Barry et al., 1979*c*). Secondly, the model was originally derived using diffusion theory. However, virtually identical predictions can be made using a rate theory approach provided there are enough energy barriers (Barry et al., 1979*c*). Thus, single channel conductance and null potential measurements in NH_4 and 0.5 Na/0.5 NH_4 solutions appear to be compatible with an endplate channel containing high field strength, neutral sites, and perhaps one or more high resistance barriers to anions. Furthermore, the ammonium ion permeation parameters would seem to imply some hydrogen bonding effects within these aqueous channels.

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