pH Dependence of the Acetylcholine Receptor Channel

A Species Variation

E. M. LANDAU, B. GAVISH, D. A. NACHSHEN, and I. LOTAN

From the Department of Physiology, Yale University School of Medicine, New Haven, Connecticut 06510, the Physics Department, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, and the Department of Physiology and Pharmacology, Tel Aviv University Sackler School of Medicine, Tel Aviv, Israel. E. M. Landau's present address is Department of Physiology and Pharmacology, Tel Aviv University Sackler School of Medicine, Tel Aviv, Israel. D. A. Nachshen's present address is Department of Physiology and Biophysics, University of Maryland, Baltimore, Maryland 21201.

ABSTRACT The effects of pH changes on the miniature endplate current (mepc) and on endplate current fluctuations (acetylcholine [ACh] noise) were examined at the neuromuscular junction in vitro in two species of frogs. In *Rana pipiens* the relationship between the decay time constant of the mepc (τ') and pH had a symmetrical bell shape; the value of τ' being largest at pH 7 and decreasing at more acid or more alkaline pH. In acid pH the mepc amplitude (A) decreased relative to its value at pH 7, and in alkaline pH A increased. In *Rana ridibunda* a narrower and asymmetric bell-shaped dependence of τ' on pH, having a maximum of pH 5.5, was found. The mepc amplitude was again reduced in acid pH but had a peak at pH 5.5. Also, its value at pH 9 was larger than at pH 7. These results were obtained with a number of different buffers and were not found to be sensitive to the nature of the buffer chosen. By performing ACh-noise analysis we found that in *Rana pipiens* at acid pH (5.5–5.0), the single channel conductance (γ) and the single channel open time (τ) were significantly reduced relative to their value at pH 7. However, in *Rana ridibunda* at acid pH (5.4) γ was unchanged and τ was markedly increased relative to their values at pH 7. The results can be explained quantitatively by electrostatic interaction between two fixed and titratable ionic groups and a mobile charge in the receptor molecule. The model fits the data for groups having pKs ~4.8 and ~9.8 for *Rana pipiens* and ~4.6 and ~6.3 for *Rana ridibunda*. The groups can be tentatively identified as amino acid residues; glutamic or aspartic and lysine or tyrosine for *Rana pipiens*; glutamic or aspartic and histidine for *Rana ridibunda*. The difference in the fitted values of the other model parameters for these two species can be attributed to differences in the spatial configuration of the charged groups.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/81/06/0647/20 $1.00 647
Volume 77 June 1981 647–666

INTRODUCTION

pH changes can be employed to titrate fixed charges in membranes. Fixed charges can contribute to a membrane surface potential (Gilbert and Ehrenstein, 1969; McLaughlin et al., 1971; Hille et al., 1975; Campbell and Hille, 1976) or can be located within an ionic channel (Woodhull, 1973). Also, such charges may bind positively charged transmitter molecules such as acetylcholine (ACh) and may regulate the rate of enzymatic reactions. A number of studies have been devoted to the effects of H^+ ions on the neuromuscular ACh receptor. Thus, working on the frog, Scuka (1975) found that acidifying the medium prolonged the duration of the endplate current (epc), whereas making it more alkaline shortened the duration. Mallart and Molgó (1978) confirmed this result for miniature epcs (mepcs). Landau and Nachshen (1979) measured ACh-induced epc fluctuations (ACh noise) in *Rana ridibunda* and found that reducing the bathing pH from 7.4 to 5.4 increased the open time (τ) but did not affect the single channel conductance (γ). On the other hand, we obtained a conflicting result while working on the American frog *Rana pipiens*. We found that in acid pH the mepc decay time constant decreases instead of increasing. To clarify this discrepancy, a detailed analysis of the effects of pH on the mepc and single channel parameters was undertaken for both the American frog *Rana pipiens* and the Israeli frog *Rana ridibunda*. We found that in both species the pH dependence of the mepc decay time constant exhibits a clear peak. However, the curves of the two species differ in some details, which explain the discrepancy that led to this study. We suggest a quantitative model that explains these results by pH-modulated electrostatic interaction between charged amino acid residues occurring during the conformational changes attending the closing of the ACh-induced ionic channel. The species variation can be explained by a simple amino acid replacement in the ACh receptor molecule and some variations in the geometry of the charged groups.

MATERIALS AND METHODS

Preparation

All experiments were performed in vitro on the frog cutaneus pectoris preparation, pared down to a single layer of cells (Peper and McMahan, 1972). Endplates were visualized with the help of Nomarksi optics (McMahan et al., 1972). For most of the experiments we used *Rana pipiens* (northern variety). Some experiments were done on the Israeli frog *Rana ridibunda*. The muscle was mounted in a Perspex bath and was cooled with a Peltier-type cooler. Temperature was monitored with a thermistor.

Solutions and Reagents

All solutions contained (millimolar): NaCl, 115; KCl, 2.5; $CaCl_2$, 0.1; $MgCl_2$, 1; and tetrodotoxin, 10^{-7} g/ml. The low calcium ion concentration was used to reduce the frequency of mepcs (Cohen and Van der Kloot, 1976). Different buffers were employed to verify that the pH effects were not buffer dependent. The solutions used are shown in Table I. The buffers were from Sigma Chemical Co., St. Louis, Mo., the salts (reagent grade) from Baker Co., Inc., Sanford, Maine, and the tetrodotoxin from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif. The pH was adjusted by adding NaOH or HCl.

Recording and Iontophoresis

For voltage clamping we used conventional glass micropipettes filled with 3 M KCl and having a resistance of 2—8 MΩ. The pipettes and the bath were connected to the apparatus through AgCl-coated silver wires. The voltage clamp apparatus was similar to that of Anderson and Stevens (1973). The current flowing into the cell was measured over a 1 MΩ resistor and was further amplified and filtered before being led to the oscilloscope and to a computer terminal. The micropipettes used for iontophoresis were filled with acetylcholine chloride (Sigma) at an approximate concentration of 3 M, and their resistance was 40–60 MΩ. Backing currents of 25–35 nA were used. The constant current source used for iontophoresis was similar to that used by Dionne and Stevens (1975).

TABLE I

BUFFER SOLUTIONS USED

Solution	Buffer	Concentration	Useful range*	Buffer‡ capacity
		mM	*pH*	*mM*
H	HEPES	4	6.8–8.2	—
HP	HEPES-propionate	4	3.7–8.2	2–3
HPh	HEPES-phthalate	4	2.8–8.2	—
TM	TRIS-maleate	2	5.2–8.6	0.8–1
B	CAPS, TAPS, HEPES, MES, propionate, fumarate	2 each	2–11	1.3–2.6
GM	MOPS, glycine	4 each	6.5–10.5	—
CH	CAPS, HEPES	4 each	11–9 7–6	—

HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid
TRIS, tris(hydroxymethyl)aminomethane
CAPS, cyclohexylaminopropane sulfonic acid
TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropane sulfonic acid
MES, 2-(*N*-morpholino)ethane sulfonic acid
MOPS, 3-(*N*-morpholino)propane sulfonic acid
* See Fasman(1976) and Martell and Smith (1977).
‡ Buffer capacity for useful range, millimoles of NaOH added per pH change of a 1-liter buffer solution. Temperature range, 11°–27°C. Buffer capacity did not appreciably change over this temperature range.

Measurement of mepc

Two voltage clamp micropipettes were inserted ~20–50 μm apart into a muscle fiber in the endplate region. The cell was clamped to −70 mV, and the mepc signal was amplified 200–1,000 times and passed through a band-pass filter (0.5–1,000 Hz). The mepcs were displayed on the screen of an oscilloscope and were led to the A to D converter of a PDP 11/34 minicomputer (Digital Equipment Corp., Marlboro, Mass.). A computer program that could sample and store individual mepcs was employed. For each experimental condition, 20–40 mepcs were obtained. The sampling interval was 0.1 ms. Only focal mepcs with a rise time of 0.4 ms or less were used. Later, the mepcs were averaged and an exponential curve was fitted to the decaying phase of the average mepc. A typical recording is shown in Fig. 1. Fig. 1 *A* shows a signal derived from averaging 114 mepcs with the superimposed fitted exponential curve.

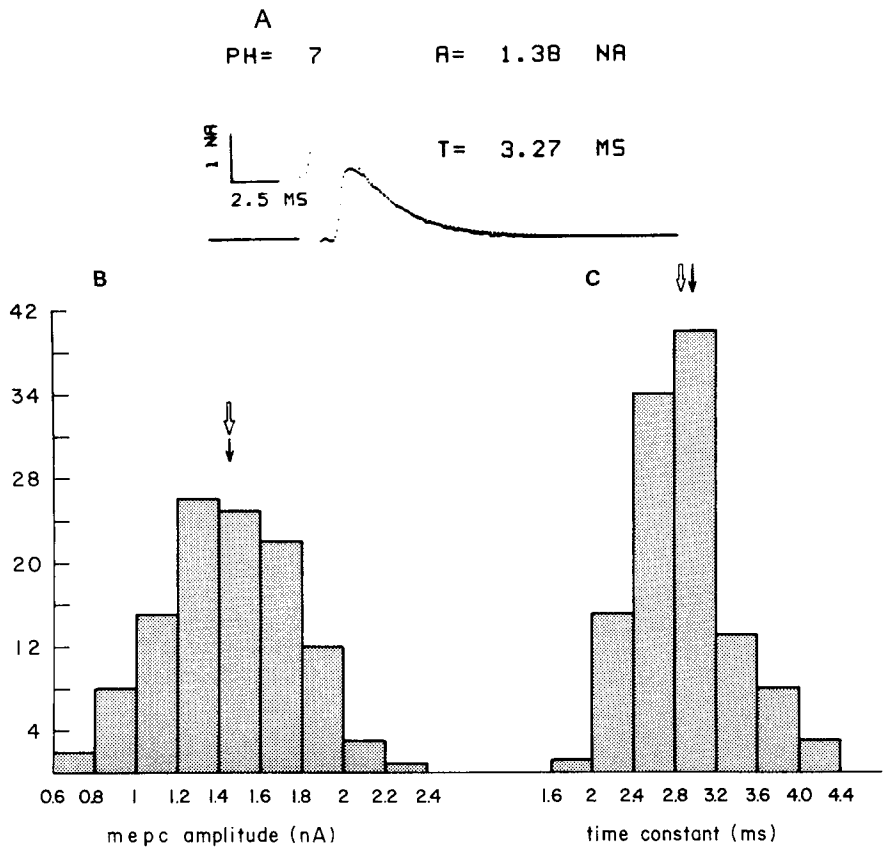

FIGURE 1. The analysis of mepc properties (*Rana pipiens*). (*A*) 114 mepcs were averaged to produce this sample mepc. A theoretical single exponential is fitted to the decaying phase of the mepc (dotted line). The fitted amplitude (A) and time constant of decay (T) are shown above the figure. The Figure also contains current (nA) and time (ms) calibrations. The computer program also calculated an amplitude histogram of the mepc amplitudes, which indicated a normal distribution (see text). mepc record is inverted from the conventional display. (*B*) The distribution of mepc amplitudes. Each bar indicates the number of mepcs (ordinate) falling within a given amplitude bin (in nA, abscissa). The white arrow indicates the median, and the black arrow the mean. (*C*) The distribution of mepc time constants of decay. Each bar indicates the number of mepcs (ordinate) falling within a given time range (in ms, abscissa). Arrows indicate mean and median as in *B*.

The fitted amplitude (A) and time constant (τ') values are also displayed. Fig. 1 *B* and *C* shows the distribution of amplitudes and time constants of these 114 mepcs, each measured separately with the same fitting program. In this particular instance, the distributions were nearly normal, with a coincidence of the median and the mean. Such a detailed analysis was not usually performed. However, the program routinely estimated the amplitude of individual mepcs, computed the average amplitude (A)

and the standard deviation (SD), and constructed a histogram with the following bin boundaries; $A - 3$ SD, $A - 2$ SD, $A -$ SD, A,$A +$ SD, $A + 2$ SD, and $A + 3$ SD.

For the cell shown in Fig. 1, the six respective bins contained 3, 16, 38, 40, 15, and 2 mepcs. The number of mepcs smaller than A (57) was equal to the number of mepcs larger than A (57), which again points to a normal distribution. This relatively crude method of estimating the amplitude distribution thus gave results similar to those of the more detailed analysis of Fig. 1 *B*. The automatic distribution analysis was done for each average mepc. It was found that when the mepc became small the distribution became somewhat skewed, the number of mepcs smaller than A ($N1$) exceeding the number of mepcs larger than A ($N2$). A skew index, which is the ratio $N1$:$N2$, was computed. Skew indices as high as 1.7 were obtained at low pH, indicating that some mepcs were lost in the background noise and that the mepc amplitude was somewhat overestimated. However, such high skew values were exceptional, and the usual range for the skew index was 1–1.3.

Measurement of Endplate Current Fluctuations (ACh Noise)

The fiber was voltage clamped, as in the mepc experiments. In addition, an iontophoretic micropipette was positioned so as to give a maximal response to a standard pulse of current (usually +30 nA with a backing current of −25 nA; duration, 0.5 s). The iontophoretic micropipette was then slightly elevated from the muscle surface to reduce the rate of desensitization (Anderson and Stevens, 1973). The epc signal was recorded at a low amplification (× 20; bandwidth, 0–500 Hz) and at a high amplification (× 1,000; bandwidth, 0.5–450 Hz). To measure ACh noise, ACh was released from the micropipette, usually by simply reducing the backing current. The method used for analyzing the ACh noise was similar to that described by Anderson and Stevens (1973) and Ruff (1977). 8 s of noise were sampled at 1,024/s and analyzed over the frequency range of 4 to 512 Hz. Decline of epc due to desensitization was generally <50% over this time period. 8 s of control noise (no ACh application) were also taken. The control spectrum was subtracted from the ACh-noise spectrum to correct for instrumental contributions to the spectrum (Anderson and Stevens, 1973). Each current trace was displayed before the spectrum was computed, and traces with mepcs or large DC drifts were discarded. Each noise spectrum usually represented from 4,096 to 8,192 data points. To estimate the single channel parameters (conductance, γ; open time, τ), the following equation was fitted by eye to the spectral points (cf. Anderson and Stevens [1973]):

$$S(f) = \frac{S(0)}{1 + (f/f_c)^2}, \tag{1}$$

where (f) is the power spectrum in A^2 s), $S(0)$ the zero frequency asymptote for a two-sided spectrum, and f_c the corner frequency. The value of γ was computed from the following equation:

$$\gamma = \frac{S(0)\pi f_c}{2\mu_I(V - V_0)}, \tag{2}$$

where μ_I is the mean endplate current (measured from the low-gain recording), V the holding potential, and V_0 the ACh null potential, which was taken to be −3 mV for both *Rana pipiens* and *Rana ridibunda* (see Results; Landau and Nachshen, unpublished results). τ was computed as follows:

$$\tau = \frac{1}{2\pi f_c}. \tag{3}$$

Measurement of the Null Potential

Clamping and iontophoresis were as described above. Iontophoretic pulses were given (+30 to +50 nA with a backing current of −25 nA; duration, 0.5 s), and the epc was recorded on the screen of a storage oscilloscope. The cell was slowly depolarized, and epcs were obtained at different holding potentials. In each experiment, the epc was actually inverted, and the null potential was obtained by interpolation (cf. Fig. 6).

Experimental Procedure

For each pH change, recordings were made from a few fibers in the control solution (pH 7). The microelectrodes were then withdrawn from the cell, and the old solution was replaced at least seven times with a new solution. An equilibration time of 10 min was allowed to elapse before a fiber was penetrated and measurements were made in the new solution. Very often the same cells could be repenetrated in the test solution. The test solution was removed within 1 h, the preparation returned to the control pH, and a few control measurements were again made. Sometimes, a second test solution was applied to the same preparation. Cells were often tested in three to four different solutions so as to have measurements from the same cell under different conditions. The temperature in the tissue bath was controlled by changing the current through the Peltier device and varied by <1°C in a given experiment. The general temperature range was 8°–14°C in *Rana pipiens* and 10°–18°C in *Rana ridibunda*. When the time constant of decay of the mepc (τ') was computed, the experimental values were corrected for the variations in temperature. The correction was based on the decay rate constant having a Q_{10} value of 2.8 (Anderson and Stevens, 1973), and all τ' values were referred to 10°C. A similar correction was employed for τ, the single channel open time, derived from ACh-noise measurements, except for the experiments in *Rana ridibunda*, for which the results were corrected to 15°C.

RESULTS

mepc Amplitude and Decay Time Constant are Decreased at Low pH in Rana pipiens

In experiments on *Rana pipiens* muscles, when the bathing pH was reduced from 7 to 5.5 or less, the mepcs diminished in amplitude and in duration. These effects were fully established after exposure to low pH for 10 min and were completely reversible, provided the exposure to the test solution did not exceed 1 h. The mepc wave form at low pH was accurately fitted by a single exponential curve, and there was no indication of additional decay rate constants.

A full description of the effects of pH on the mepc amplitude A and on the mepc time constant τ' for *Rana pipiens* is given in Figs. 2 and 3. From Fig. 2 it is clear that in acid pH (5.3–5.0) the value of A decreases sharply, until at pH 4.5 mepcs can no longer be seen above the noise level (Fig. 2, filled circle). In three cells in which A was apparently zero at pH 4.5, it recovered fully after return to pH 7. A increases when the pH is increased to 9.5. It is important to note the effect of the choice of buffer. There was no significant difference in A values at pH 7 in B, TM, CH, H, HP, or HPh buffer (see Table I). Also, A at pH 5.5 was checked with buffers B, TM, and HP; at pH 5.13 with buffers HP and HPh; and at pH 9.5 with buffers B and CH. The choice of buffer did

not affect A. From this it can be concluded that the observed change in A cannot be attributed to a protonated form of the buffer blocking the ACh-dependent ionic channel. On the other hand, when the buffer TM was applied at pH 4.5, which is outside its useful range and where its capacity is only 0.2 mM, the decrease in A was much less than with HP. It is thus clear that the synaptic cleft has some buffering capacity and that an effective buffer must

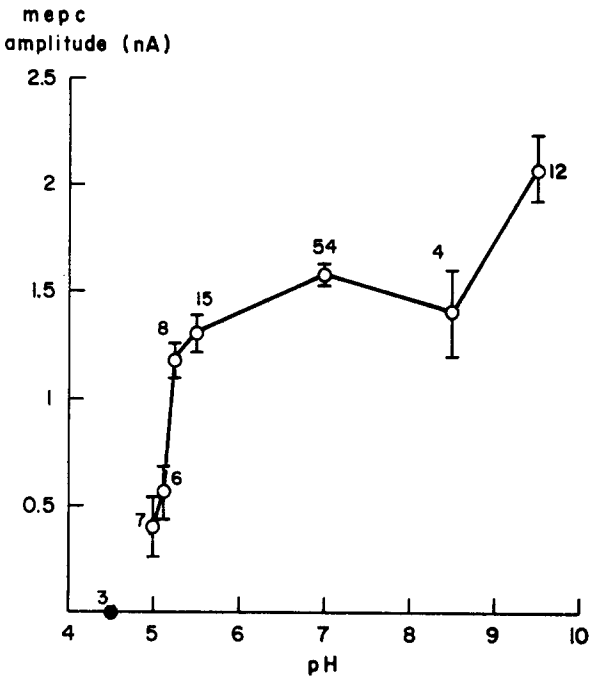

FIGURE 2. The pH dependence of the mepc amplitude (A) in *Rana pipiens*. The relationship between A (nA, ordinate) and pH (abscissa). Bars indicate ±1 SEM. The numbers near each point indicate the number of cells averaged. The following buffers (see Table I for key to buffers) and frogs were used. pH 9.5: B and CH (winter frogs [WF]); pH 8.5 TM, (WF); pH 7: pooled from B, TM, H, and CH, (WF) and H, HP, and HPh (summer frogs [SF]). pH 5.5: B, TM (WF), and HP (SF). pH 5.25: HP(SF); pH 5.13: HP and HPh (SF); pH 5: HP (SF); pH 4.5, filled circle: HP (WF). In none of these cases was there a difference between results obtained in the different buffer solutions and in winter or summer frogs.

be used to modify the pH in the cleft. A summary of all experiments that deal with the effects of pH on τ' is shown in Fig. 3. It is clear that the largest values of τ' are found at pH 7 and that τ' becomes smaller whenever pH is raised or lowered from that level. When winter and summer frogs were compared regarding their τ' value, the τ' at pH 7 was somewhat smaller for summer frogs (t test, $P < 0.02$). However, in the summer frogs, as in the winter frogs, the τ' values decrease in acid pH. Here again, the choice of buffer did not affect the value of τ' obtained at various pH levels.

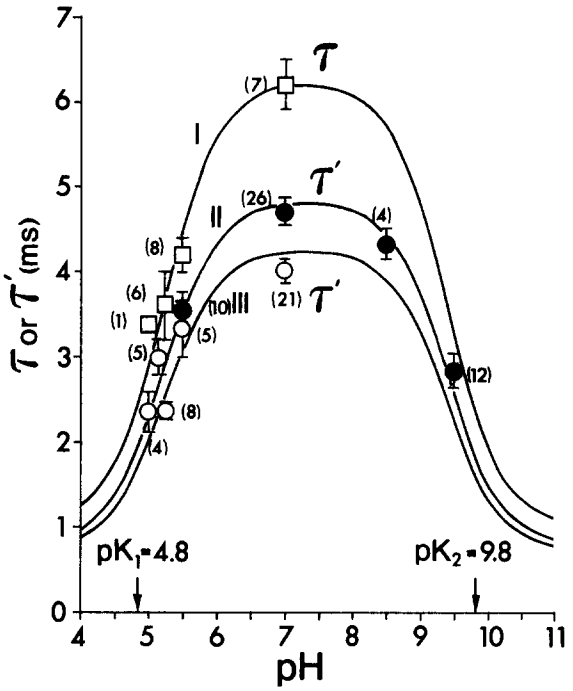

FIGURE 3. The dependence of the mepc decay time constant (τ') and the channel open-time (τ) on pH. The data are corrected to 10°C. In *Rana pipiens* τ and τ' (ms, ordinate) plotted vs. pH (abscissa). Open circles and squares: summer frogs; filled circles: winter frogs. The squares represent single channel values obtained from Table II. The number of experiments is indicated in parentheses. The effect is interpreted by the electrostatic interaction between two fixed and titratable charged groups and a constant charge which moves during the transition open→closed of the ACh channel. The curves are the best fit of Eq. T4 to the data (see Theory). For curves I–III $A_1 = -1.9$, $A_2 = 1.9$, $pK_1 = 4.8$, and $pK_2 = 9.8$. If pK_1 and pK_2 belong to an acidic and a basic group, respectively, the τ_0 values are 0.141 ms (I), 0.109 ms (II), and 0.096 ms (III). However, if both groups are taken to be acidic, we find 0.947 ms (I), 0.727 ms (II), and 0.642 ms (III). A reasonable fit can be obtained as well for pKs and As differing by as much as 0.1 units from the values given above. The Z_1 group is tentatively identified as Glu or Asp (acidic), and the Z_2 as Lys (basic) or Tyr (acidic). A possible arrangement of such groups is shown in Fig. 9.

Effect of pH on τ' and A in Rana ridibunda

Our findings with *Rana pipiens* are at variance with those of other workers, who found A to be invariant with pH and τ' to increase as pH was reduced below 7 (Scuka, 1975; Mallart and Molgó, 1978; Landau and Nachshen, 1979). This discrepancy was not due to the lower temperature at which the present experiments were done. An experiment was conducted at 20°C and τ' was 2.4 ± 0.2 ms at pH 7 (SEM, $n = 6$, buffer B) and 1.9 ± 0.04 ms at pH 5.5 (SEM, $n = 4$, buffer B). We therefore performed a detailed study of the

pH dependence of A and decay time constants in the frog *Rana ridibunda* and the results are shown in Figs. 4 and 5. Here again the results were insensitive to the buffer chosen. (See legend of Fig. 4.) As other workers had shown, τ' indeed increased when the pH was reduced down to 5.5. However, below pH 5.2, we observed a reduction in both A (Fig. 4) and τ' (Fig. 5). The following conclusions can be drawn from these experiments. First, the pH profile of τ' (Fig. 5) has a peak, as did the pH profile in *Rana pipiens* (Fig. 3). However, the

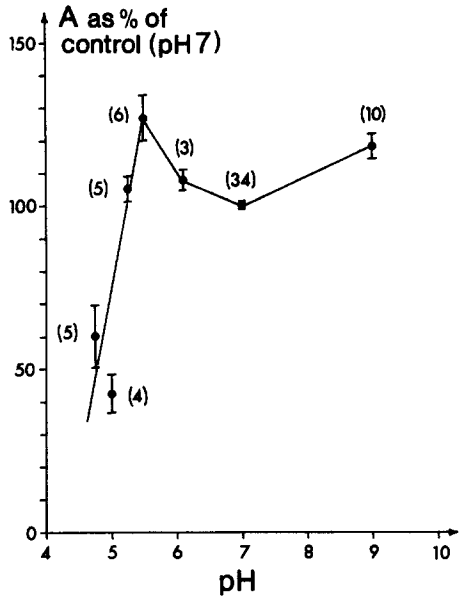

FIGURE 4. The dependence of the mepc amplitude on pH in *Rana ridibunda*. The relative increase in the mepc amplitude, compared to control at pH 7 (ordinate), is plotted vs. the pH (abscissa). In each cell we had a reading both in control and in a test solution, and each result was compared to its control. The number of experiments and SEM values are given. HP buffer was used for all experiments, except for those with pH 9. Here we used either the CH buffer or the GM buffer for both test and control. No difference between the results in these two buffers was found, and they were therefore pooled to give one experimental point.

two profiles differ in shape and in the location of their peak. In *Rana ridibunda* the pH dependence of τ' is no longer symmetric around the maximum and its shape is narrower. The maximum is displaced from a pH of 7 to 6. The pH profile of A in *Rana ridibunda* (Fig. 4) is generally similar to that of *Rana pipiens* (Fig. 5). Thus, A decreases sharply in the acid pH range below 5.5 and increases above control level at pH 9. However, the profile in *Rana ridibunda* has a peak with a maximum at pH 5.5, a feature that was absent in *Rana pipiens*.

H^+ Ions Decrease Postsynaptic Sensitivity to ACh but Do Not Shift the Null Potential

To elucidate the mechanism of the decrease in A, we applied ACh iontophoretically (see Methods) and varied the pH. A representative experiment is shown in Fig. 6. Here a constant pulse of ACh was applied to an endplate,

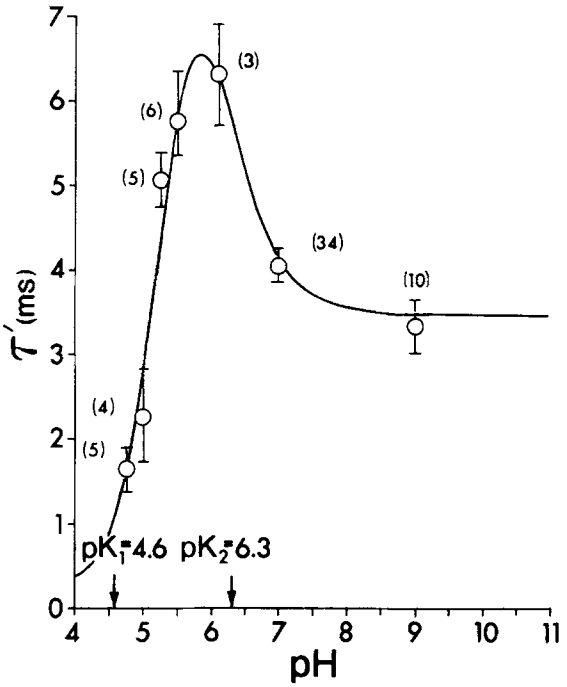

FIGURE 5. The relationship between the mepc time constant of decay and pH in *Rana ridibunda*. The time constant, (τ' [ms], ordinate) plotted vs. the pH (abscissa). The number of experiments and SEM values shown. The data are corrected to 10°C. The curve represents the best fit of Eq. T4 to the data, assuming the existence of an acidic group having $pK_1 = 4.6$, tentatively identified as Asp or Glu and a basic group having $pK_2 = 6.3$, which may be His. $\tau_0 = 0.0385$ ms, $A_1 = -4.5$, and $A_2 = 1.2$ are the best fit values of these parameters. A reasonable fit is obtained for pK_1 in the range 4.6–4.8 and for $pK_2 = 6.3$–6.5, with $A_1 = -3.0$ to -4.5 and $A_2 = 1.0$–1.2. A possible arrangement of such groups is shown in Fig. 9.

and the holding potential was varied between −40 and +40 mV. The curve denoted with open circles was obtained at pH 7, with a null potential of −4 mV. When the pH was 5.25, the synaptic response was reduced, but the null potential remained unaltered. When the pH was 5, no response could be obtained from this endplate. The constancy of the null potential was confirmed in a few more experiments. Thus, the average null potential at pH 7 was −3.4 ± 0.5 mV (SEM, $n = 10$), whereas at pH 5.25 it was −3.5 ± 0.4 mV (SEM,

$n = 6$). The reduction in sensitivity was not a constant finding at pH 5.25. Of four cells examined both at pH 7 and at pH 5.25, sensitivity decreased in two, remained the same in one, and increased in one. This variation may be due to some inaccuracy in relocating the iontophoretic pipette after changing the bathing medium, inasmuch as no attempt was made to fully map the endplate. In all cells tested, however, at pH 5 the sensitivity was unequivocally reduced. Five cells at pH 5 gave no response to ACh, even though good sensitivity was found at pH 7, both before and after exposure to the test solution. Two other cells at pH 5 gave minute responses only. Also, three cells at pH 4.5 showed a reversible disappearance of ACh sensitivity. In essence, these findings confirm those of Scuka (1977) concerning the unaltered null potential and decreased ACh sensitivity found in low-pH solutions.

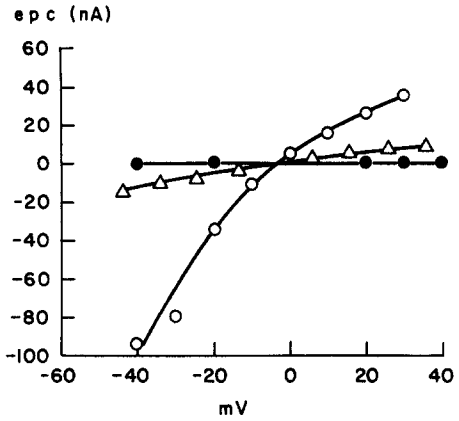

FIGURE 6. The effect of pH on the endplate current (epc) obtained by iontophoretic application of ACh. (*Rana pipiens*) A pulse of current was passed through the ACh micropipette (+30 nA against a holding current of −25 nA (duration, 0.5 s). The resulting epc values (nA, ordinate) are plotted against the holding potential (mV, abscissa). Open circles: pH 7; open triangles: pH 5.25; closed circles: pH 5. The order of trials was pH 5, pH 7, pH 5.25.

H^+ Ions Affect the Conductance and Open Time of the ACh Receptor Channel

To further elucidate the mechanism by which postsynaptic sensitivity is reduced by H^+ ions, we measured ACh-induced endplate current fluctuations in *Rana pipiens* (ACh noise; see Methods). Three ACh-noise spectra, in the control solution (pH 7), at low pH (pH 5.5), and after washout (pH 7), are shown in Fig. 7. The spectra can be fitted by a Lorentzian function (Eq. 1), and at low pH the power decreases and the curve is shifted to the right, i.e., toward higher frequencies. At pH 5.5 the single channel conductance (γ) decreases from 26 to 17.1 pS, and the single channel open time (τ) decreases from 5 to 3.3 ms. Both parameters recover after washout ($\gamma = 23$ pS, $\tau = 6.1$ ms).

A similar reversible reduction in γ and τ was found in four experiments at pH 5.5, in four experiments at pH 5.25, and in one experiment at pH 5. In one experiment at pH 5.5 no decrease in γ, but a decrease in τ, was found. We also measured γ in five cells with a control reading either before or after exposure to low-pH conditions. Reducing the pH causes γ to decrease significantly and washing back to pH 7 causes γ to recover (Table II), τ also decreases at low pH and recovers completely in control solutions (Fig. 3).

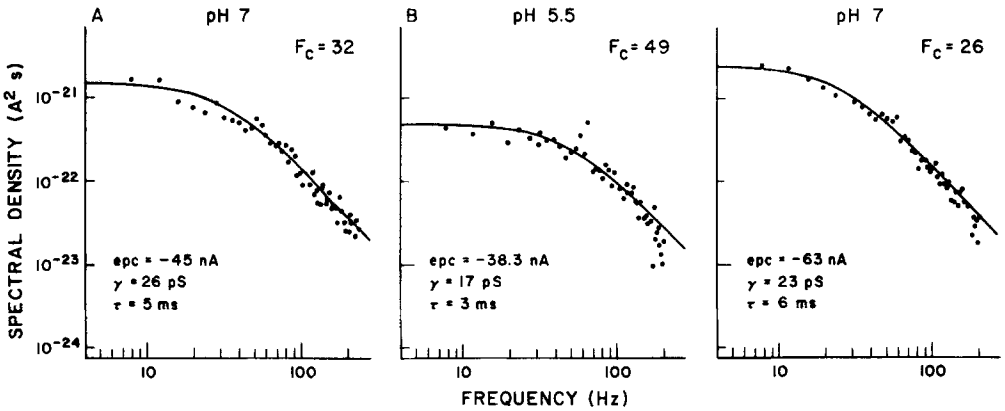

FIGURE 7. The effect of acid pH on the ACh-noise power spectrum (*Rana pipiens*). Three spectra are shown, obtained at pH 7, 5.5, and 7, consecutively. In each spectrum the power spectral density (in A^2 s, ordinate) is plotted against the spectral frequency (in Hz, abscissa). Note logarithmic coordinates. Points at frequencies >200 Hz have been omitted because here the filter started reducing the signal amplitude. The line is plotted according to Eq. 1, and γ and τ are calculated with Eqs. 2 and 3, respectively. In the control spectrum the value of $S(0)$ was 1.6×10^{-21} A^2 s, μ_I was −44.9 nA, f_c was 32 Hz, V was −72 mV, and V_0 was taken as −3 mV. The resting potential before clamping (RP) was −75 mV. γ was calculated to be 26 pS, and τ to be 5 ms. The temperature was 10.6°C. In the spectrum obtained at pH 5.5, 50 min after change-over, the respective values were $S(0) = 5.3 \times 10^{-22}$ A^2 s; $\mu_I = 31.3$ nA; $f_c = 49$ Hz; $V = -65$ mV; $V_0 = -3$mV; RP = −75 mV; temperature, 10.1°C; $\gamma = 17.1$ pS; and $\tau = 3.3$ ms. In the spectrum obtained 20 min after returning to pH 7, the respective values were $S(0) = 2.7 \times 10^{-21}$ A^2 s; $\mu_I = -62.8$ nA; $f_c = 26$ Hz; $V = -78$ mV; $V_0 = -3$mV; RP = −80 mV; temperature, 10.3°C; $\gamma = 23.2$ pS; and $\tau = 6.1$ ms.

A few additional points deserve comment. First, the small difference between the control γ for the first and second group of experiments in Table II is not statistically significant, and the *pooled* control γ was 24.1 ± 1.6 pS (SEM, $n = 13$), close to the published γ value for this species of frogs (Lewis, 1979). The *pooled* τ value was 5.7 ± 0.3 ms (SEM, $n = 13$), which is larger than the mepc τ' value for the same type of frog (*R. pipiens*, summer frogs, $\tau' = 4.02$ ± 0.14, SEM, $n = 21$). We have no explanation for the difference between τ and τ'; but the estimation of τ from noise is generally considered less accurate than the determination of τ' from mepcs.

Next, we performed a few noise experiments in *Rana ridibunda.* Going from pH 7 to 5.4, we found no significant change in γ, which contrasts with the increase in the mepc amplitude seen at the pH (Fig. 4). On the other hand, the channel open time increased significantly in pH 5.4, and the percent increase (+42%) was the same as found at the same pH for the mepc decay time constraint (+44%).

Finally, we examined the dependence of γ on the holding membrane potential at pH 5.25 in *Rana pipiens.* In two cells we obtained the γ at −30 and −80 mV and in a third cell γ was read at −30 and −130 mV. In all those experiments, γ decreased slightly, by about 10%, for a 50-mV change. This

TABLE II

VALUES OF THE SINGLE CHANNEL CONDUCTANCE (γ) AND OPEN TIME (τ) UNDER VARIOUS PH CONDITIONS IN THE INDICATED FROG SPECIES

	γ(*pS*)	*% of control*	τ(*ms*)	*% of control*	*n*
Rana pipiens (10°C)					
Control at pH 7	21.4±1.7*	100	6.2±0.4	100	7
pH 5.5	14.1±1.0‡	65.9	4.2±0.2	67.7	8
Washout	17.8±1.5§	83.2	6.2±0.3	100	6
Control	26.4±3.3	100	5.2±0.4	100	5
pH 5.25	12.6±3.0‖	47.7	3.6±0.4	69	6
Washout	24.1±2.6§	91.3	5.9±0.5	113.5	5
Control at pH 7.4	31.9	100	5.5	100	1
pH 5	8.9	27.9	3.4	62	1
Washout	18.7	58.6	6.8	123.6	1
Rana ridibunda (15°C)					
Control at pH 7.4	25.3±3.8*	100	1.9±0.14	100	4
pH 5.4	22.1±1.0¶	87.4	2.7±0.08**	142	3

* SEM

‡ γ at pH 5.5, significantly smaller than control, *t* test $P < 0.01$

§ γ washout, not significantly different from control

‖ γ at pH. 5.25, significantly smaller than control, *t* test $P < 0.02$

¶ γ at pH 5.4 not significantly smaller than at pH 7.4

** τ at pH 5.4 significantly larger than at pH 7.4, *t* test $P < 0.01$

slight change cannot be taken as significant, indicating the pH effect on the channel conductance is independent of the membrane voltage, or at most weakly affected by it.

THEORY

We would like to present a simple physical theory to account for the observed effects of H^+ ions on the open time (τ) of the single ACh receptor channel. By their very nature, pH changes will affect the average electric charge on acidic and basic groups, which are likely to be found in the active site of the receptor molecule and near the channel entrance. If the conformational change that accompanies the closing process of the channel involves variations of the

distances between charged groups, part of the energy barrier for this process will be dominated by electrostatic interaction. Therefore, pH-dependence of the electric charge of these groups will modify this energy barrier and will affect τ. The simplest arrangement of charges that can explain our observation is shown in Fig. 8 *A*: Two electric charges Z_1e and Z_2e have fixed locations (Z_1 and Z_2 can be either positive or negative; e is the electron charge). A third

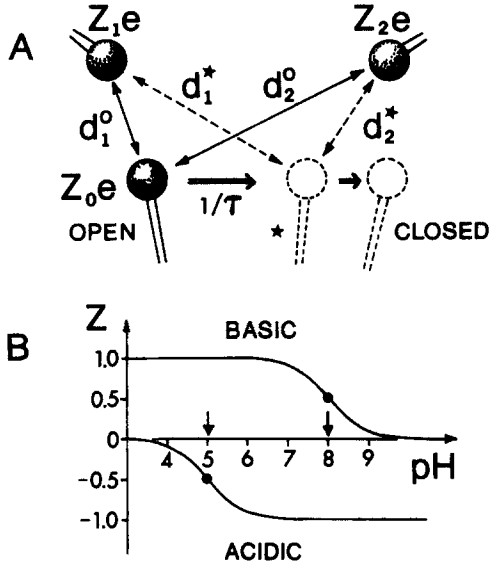

FIGURE 8. (*A*) The basic features of the model are as follows. Two fixed charges Z_1e and Z_2e, which represent titratable groups, interact electrostatically with a third mobile charge Z_0e. The latter is displaced during the conformational change that accompanies the open→closed transition of the channel at rate $1/\tau$. The dotted forms represents intermediate states along the trajectory of this charge, which can be identified with the "reaction coordinate" of the transition. The star denotes the location of the top of the energy barrier. Thus, the contribution of the electrostatic interaction to the height of the energy barrier is determined by the value of the interaction at distances d_1^o and d_2^o at the "open" state and d_1^* and d_2^* at the barrier-top state (Eq. T1). (*B*) The charge (in electron charge units) of acidic and basic groups at a function of the pH as given by Eqs. T6 and T7. The pK values for this specific example are denoted by arrows.

charge Z_0e is displaced along some trajectory in space, during the open→close transition, which can be identified with the "reaction coordinate." Obviously, the distances between Z_0e and Z_1e or Z_2e vary during this transition. Let ΔG_0^* be the height of the potential energy barrier for the transition in the open→close direction in the absence of electrostatic interaction, and ΔG^* the corresponding quantity in the presence of this interaction. Thus, $\Delta U = \Delta G^*$

$-\Delta G^*_0$ is the difference in the electrostatic interaction between the barrier-top location (*) and the open-state location (O) of Z_0 denoted in Fig. 8 *A*. We find

$$\Delta U = \frac{Z_0 Z_1 e^2}{\epsilon}\left(\frac{1}{d_1^*} - \frac{1}{d_1^O}\right) + \frac{Z_0 Z_2 e^2}{\epsilon}\left(\frac{1}{d_2^*} - \frac{1}{d_2^O}\right), \tag{T1}$$

where ϵ is the local dielectric constant. The temperature dependence of the rate of the receptor inactivation process has been shown to follow an Arrhenius-type law (Mallart and Molgó, 1978). This justifies the assumption of energy barrier-mediated process for which

$$\tau \alpha e^{\Delta G^*/kT}. \tag{T2}$$

Thus

$$\tau = \tau_0 e^{(\Delta G^* - \Delta G_0^*)/kT} = \tau_0 e^{\Delta U/kT}. \tag{T3}$$

Substituting expression T1 for ΔU in T3 we find

$$\tau = \tau_0^{(A_1 Z_1 + A_2 Z_2)}, \tag{T4}$$

where

$$A_1 = \frac{Z_0 e^2}{\epsilon k T}\left(\frac{1}{d_1^*} - \frac{1}{d_1^O}\right) \qquad A_2 = \frac{Z_0 e^2}{\epsilon k T}\left(\frac{1}{d_2^*} - \frac{1}{d_2^O}\right). \tag{T5}$$

We shall further assume for simplicity that Z_0 is not affected by the pH in the range for which Z_1 and Z_2 vary. Thus, A_1 and A_2 are independent of the pH. Let us roughly estimate the values of A for reasonable values of the parameters: $Z_0 = 1$, $e = 4.8 \cdot 10^{-10}$ esu, $k = 1.47 \cdot 10^{-16}$ erg/degree, $T = 300°$K, $d^* = 3$ Å, $d^O = 5$ Å, and $\epsilon = 3$. The latter is a conventional value for ϵ near low-dielectric-constant media like membranes ($\epsilon \sim 2$ for hydrocarbons) and proteins (see, for example, Takashima [1962]) or in the vicinity of another charge which lowers ϵ by ordering the water molecules (Laidler, 1950). We find for these values $|A| = 4.7$. The pH dependence of Z for acidic group A is the following: By definition, $AH \overset{K_A}{\rightleftarrows} A^- + H^+$, where $K_A = [A^-][H^+]/[AH]$. Since $Z_A = -[A^-]/([AH] + [A^-])$, we find

$$Z_A = -1/[1 + 10^{(pK_A - pH)}] < 0. \tag{T6}$$

Similarly, for a basic group, satisfying $B + H^+ \overset{K_B}{\rightleftarrows} BH^+$, we obtain

$$Z_B = 1/[1 + 10^{(pH - pK_B)}] > 0. \tag{T7}$$

Z_A and Z_B are plotted vs. the pH at Fig. 8 *B*. Eqs. T6 and T7 suggest that if the mobile group is titratable and Z_0 reaches a nonzero constant value over the pH range, in which Z_A and Z_B vary, then $|Z_0| = 1$. Choosing $Z_0 = +1$, for convenience, we find from Eq. T5 that $A_i > 0$ when $d_i^* < d_i^O$ ($i = 1,2$). Geometrically, it means that the mobile charge $Z_0 e$ approaches the fixed charge $Z_i e$ during the transition, as demonstrated by the example shown in

Fig. 8 *A* for i = 2. Similarly, $A_i < 0$ when $d_i^* > d_i^0$; e.g., see Fig. 8 *A* for i = 1. It can be seen from Fig. 8 *B* that for $A_i > 0$, the quantity A_iZ_i is a decreasing function of the pH for *both* acidic (negatively charged) and basic (positively charged) groups. Analyzing the various possible pH profiles of the quantity $A_1Z_1 + A_2Z_2$, we find through Eq. T4 that the observed bell-shaped dependence of τ on pH can be obtained only for $A_1 < 0$ and $A_2 > 0$, provided $pK_1 < pK_2$ and $Z_0 = +1$, *irrespective of the sign of* Z_1 *and* Z_2. The relation

$$Z_B - Z_A = 1 \qquad (pK_A = pK_B) \tag{T8}$$

that can be derived from Eqs. T6 and T7, enables us to interpret a fitted τ vs. pH curve by four combinations of acidic group A and basic group B, which establish the charges Z_1 and Z_2 and possess the same pK_1 and pK_2 values, i.e., A and A, A and B, B and A, B and B. These combinations only differ by the value of τ_0. The number of amino acid residues, which are likely to be identified with A or B, having a given pK value is highly restricted (Cohn and Edsall, 1943). This is helpful in eliminating improbable combinations and in identifying the amino acids most probably accounted for by the observations. Eq. T4 fits the data for *Rana pipiens* (Fig. 3) for a group with $pK_1 \sim 4.8$, which could be glutamic or aspartic acid, and for a group with $pK_2 = 9.8$, which may be lysine or tyrosine with $A_1 = -1.9$ and $A_2 = 1.9$, respectively, as shown. The identification of the residues is based on a study of titratable groups in many proteins, which has been summarized by Cohn and Edsall, (1943); the most probable pK range of some residues is: Asp, 3.0–4.7; Glu ~4.4; His, 5.6–7.0; ϵ-Lys, 9.4–10.6; Tyr, 9.8–10.4; and Arg, 11.6–12.6. Eq. T4 fits the data of *Rana ridibunda* (Fig. 5) for a group having $pK_1 = 4.6$, which could be glutamic or aspartic acid, as for *Rana pipiens*, and a group at $pK_2 = 6.3$, which may be histidine with $A_1 = -4.5$ and $A_2 = 1.2$. The results are compatible with our predicted magnitude of *A*. The mobile charge, if positive, should have $pK_0 > 11$, which is satisfied only by arginine. However, pK_0 might not be restricted if Z_0 is not exposed to the solvent; in such a case, the mobile group cannot be identified by this method. The qualitative and the quantitative differences in the pH profile for these two frogs stimulate the question, how small can differences in the configuration of the charges be and still establish the observed effects? Fig. 9 demonstrates that a 2-Å displacement of the initial position of the mobile charge can transform A_1 and A_2 of *Rana pipiens* to those of *Rana ridibunda*. We may conclude that the pH dependence of τ is a sensitive probe for the pK values and the spatial configuration of the charged groups, which are involved in the open→close transition and are believed to reflect some structural features of the receptor molecule.

DISCUSSION

Our results highlight the role of fixed charges in regulating the function of the ACh receptor. Titrating the charges by changing the pH affects both the open time and the conductance of the synaptic channels (see Fig. 7 and Table II) and modifies dramatically the shape of the mepc (Figs. 2–5).

The amplitude of the mepcs (A) depends on a number of factors, the single channel conductance being only one of these (see Landau [1978]). It is thus not surprising that changes in A do not always follow the changes in γ. Thus in *Rana ridibunda*, A at pH 5.5 is larger than at pH 7, whereas γ at that pH is not different from the control. For this reason, it is difficult at present to discuss the dependence of A on pH, except in the very acid range, where a decrease in γ may partly explain the decrease in A. This reduction in γ resembles the decreased single channel conductance found by Woodhull (1973) and Sigworth (1977) at the frog node of Ranvier in acid pH. However,

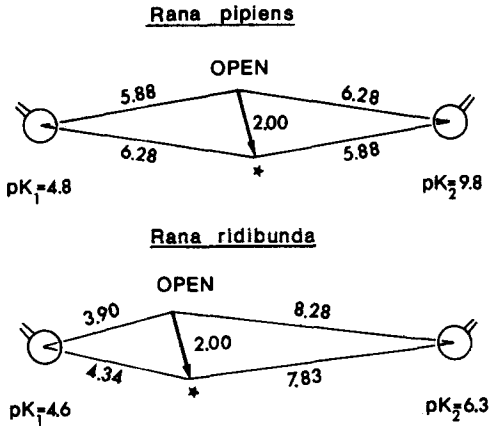

FIGURE 9. A possible geometry of the model charged groups that demonstrates how a slight geometrical change can account for the observed differences between the two species of frogs and the given values of the fitted parameters A_1 and A_2 (see values in Figs. 3 and 5). The distance between the fixed groups and the magnitude of the mobile-group displacement are taken to be 12 and 2 Å, respectively. All the distances are in ångström. A tentative identification of the titratable groups with amino acid residues relates the values of pK_1 to Glu or Asp for both species, pK_2 of *Rana pipiens* to Lys or Tyr, and pK_2 of *Rana ridibunda* to His. The expressions for A_1 and A_2 are given by Eq. T5, in which we take $Z_0e^2/\epsilon kT$ = 174 Å. This is obtained by assuming $Z_0 = 1$ and $\epsilon = 3$ at T = 300°K.

in contradistinction to the nerve channel, the ACh-receptor channel does not exhibit a clear voltage dependence for the pH effect. If the reduction in γ is caused by H^+ ions titrating a group within the channel, as suggested by Woodhull (1973), then this lack of voltage sensitivity might indicate that the relevant group is situated very superficially, close to the outer surface or the membrane, where only a small fraction of the membrane potential may be felt.

Other explanations for the effects of pH on γ are also possible. H^+ ions may reduce γ by affecting the energy barrier controlling the passage of ions through

the channel (Lewis and Stevens, 1979) by titrating surface charges, which in turn affect the surface concentration of permanent ions, or even by inducing a conformational change in the receptor, producing a channel with a smaller permeability. In the absence of more detailed information about the pH dependence of γ, we feel that this question must be left open. The increase in A found at pH 9 in both *Rana pipiens* and *Rana ridibunda* also remains unexplained. It is interesting that in a recent study alkaline pH was found to markedly increase γ in chick muscle in culture (Goldberg et al., 1979). It is thus possible that the increase in A is caused by an increase in γ. Further work is required to investigate this possibility.

Our most interesting finding is that the mepc decay time constant (τ') has a bell-shaped dependence on pH and that there is a species difference in this dependence. The reduction of τ' in the alkaline range has already been observed by other workers (Scuka, 1975; Mallart and Molgó, 1978). The decrease in τ' in acid pH is a new finding. Other workers have probably missed it by not trying low enough pH values, inasmuch as we have seen that reduction in τ' occurs at different pH ranges for different species of frogs. The observed changes in τ' are believed to reflect the true channel open time (τ), because the effects of acid pH on τ were the same as on τ' in both species of frogs (see Table II and Figs. 3 and 5). A similar conclusion was reached by Mallart and Molgó (1978) after studying the pH effects on τ' in the presence of acetylcholine esterase blockers.

In Theory we attribute the bell-shaped dependence to the pH-modulated electrostatic interaction between an ionic group within the receptor that moves when the channel closes and two other fixed and titratable ionic groups. The distances between the groups are in the Ångström range. The fixed acid group was tentatively identified as asparatic or glutamic acid in both species of frogs, and the basic group as lysine or tyrosine for *Rana pipiens* and as histidine for *Rana ridibunda.* That the codons for tyrosine UAU and UAC differ by only one nucleic acid from that of histidine CAU and CAC supports the speculation that the observed species difference is mainly due to a simple amino replacement which arises from a point mutation in the gene coding for the ACh receptor.

Other explanations of the bell-shaped dependence are also possible. For instance, the lengthening of τ' may be due to a titration of external surface changes, as suggested by Mallart and Molgó (1978), whereas the subsequent decrease in τ' may be due to a titration of internal surface charges. We do not favor this explanation, because increasing the bathing calcium concentration does not mimic the effects of acid pH, as it should according to surface charge theory (Bregestovski et al., 1979; Magleby and Weinstock, 1980; Landau and Nachshen, 1979). However, it would require selectively changing the intracellular pH (see Turin and Warner [1980], Thomas [1974], and Boron and De Weer [1976]) to rule out this possibility. An additional explanation is that H^+ ions bind to the phospholipids, thus inducing conformational changes in the membrane and modifying its fluidity. However, the finding that large changes in calcium concentration do not show effects similar to that of acid pH also

militates against this interpretation. If our model turns out to be correct, it opens up an interesting perspective; this would be, to the best of our knowledge, the first observation that indicates a species difference in the molecular structure of a receptor for a neurotransmitter. Such species variation is well known for other proteins, such as hemoglobin (Lehninger, 1975), and is known to underly a few major clinical disorders. It is tempting to speculate that the existence of structurally dissimilar receptor molecules may underlie some of the yet unexplained disorders of the nervous system.

We wish to thank Dr. C. F. Stevens for making his equipment available to E. M. Landau and for many helpful discussions. Help from Ms. J. Strong is gratefully acknowledged.
This work was supported by National Institutes of Health Neuroscience grant 12962 to C. F. Stevens and by National Science Foundation grants PCM79-05072 and HEW-18051 to B. Gavish.

Received for publication 19 July 1979.

REFERENCES

ANDERSON, C. R., and C. F. STEVENS. 1973. Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol. (Lond.)*. **235:**655–691.

BORON, W. F., and P. DE WEER. 1976. Intracellular pH transients in squid giant axons caused by CO_2, NH_3, and metabolic inhibitors, *J. Gen. Physiol.* **67:**91–112.

BREGESTOVSKI, P. D., R. MILEDI, and I. PARKER. 1979. Calcium conductance of acetylcholine-induced endplate channels. *Nature (Lond.)*. **279:**638–639.

CAMPBELL, D. T., and B. HILLE. 1976. Kinetic and pharmacological properties of the sodium channel of frog skeletal muscle. *J. Gen. Physiol.* **67:**309–323.

COHEN, I., and W. VAN DER KLOOT. 1976. The effect of pH change on the frequency of miniature end-plate potentials at the frog neuromuscular junction. *J. Physiol. (Lond.)*. **262:** 401–414.

COHN, E. J., and J. T. EDSALL. 1943. Proteins, Amino Acids and Peptides. Reinhold Publishing Corp., New York. Chapt. 20.

DIONNE, V. E., and C. F. STEVENS. 1975. Voltage dependence of agonist effectiveness at the frog neuromuscular junction: resolution of a paradox. *J. Physiol. (Lond.)*. **251:**245–270.

FASMAN, G. D. 1976. Handbook of Biochemistry and Molecular Biology. CRC Press, Inc., Cleveland. *1:*362.

GILBERT, D. L., and G. EHRENSTEIN. 1969. Effects of divalent cations on potassium conductance of squid axons. Determination of surface charge. *Biophys. J.* **9:**447–463.

GOLDBERG, G., Y. LASS, and E. M. LANDAU. 1979. Increased acetylcholine-induced channel conductance in cultured chick myoballs at alkali pH. *Isr. J. Med. Sci.* **15:**955.

HILLE, B., A. M. WOODHULL, and B. I. SHAPIRO. 1975. Negative surface charge near sodium channels of nerve: divalent ions, monovalent ions, and pH. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **270:**301–318.

LAIDLER, K. J. 1950. Chemical Kinetics. Tata-McGraw Hill, Bombay.

LANDAU, E. M. 1978. The relation between function and structure of the ACh receptor at the muscle end-plate. *Prog. Neurobiol.* **10:**253–288.

LANDAU, E. M., and D. A. NACHSHEN. 1979. Divalent cations and protons do not have the same effect on the frog end-plate. *Isr. J. Med. Sci.* **15:**954.

LEHNINGER, A. L. 1975. Biochemistry. Worth Publishers, Inc., New York.

LEWIS, C. A. 1979. Ion-concentration dependence of the reversal potential and the single channel conductance of ion channels at the frog neuromuscular junction. *J. Physiol. (Lond.).* **286:**417–445.

LEWIS, C. A., and C. F. STEVENS. 1979. Mechanism of ion permeation through channels in a postsynaptic membrane. *In* Membrane Transport Processes. Raven Press, New York. **3:**133–151.

MAGLEBY, K. L., and M. M. WEINSTOCK. 1980. Nickel and calcium modify the characteristic of the acetylcholine receptor-channel complex at the frog neuromuscular junction. *J. Physiol. (Lond.).* **299:**203–218.

MALLART, A., and J. MOLGÓ. 1978. The effects of pH and curare on the time course of end-plate currents at the neuromuscular junction of the frog. *J. Physiol. (Lond.).* **276:**343–352.

MARTELL, A. E., and R. M. SMITH. 1977. Critical stability constants, Vol. 3. Plenum Press, New York.

MCLAUGHLIN, S. G. A., G. SZABO, and G. EISENMAN. 1971. Divalent ions and the surface potential of phospholipid membranes. *J. Gen. Physiol.* **58:**667–687.

MCMAHAN, U. J., N. C. SPITZER, and K. PEPER. 1972. Visual identification of nerve terminals in living isolated skeletal muscle. *Proc. R. Soc. Lond. B Biol. Sci.* **181:**421–430.

PEPER, K., and U. J. MCMAHAN. 1972. Distribution of acetylcholine receptors in the vicinity of nerve terminals on skeletal muscle of the frog. *Proc. Roy. Soc. Lond. B Biol. Sci.* **181:**431–440.

RUFF, R. L. 1977. A quantitative analysis of local anaesthetic alteration of miniature end-plate currents and end-plate current fluctuations. *J. Physiol. (Lond.).* **264:**89–124.

SCUKA, M. 1975. The amplitude and the time course of the end-plate current at various pH levels in the frog sartorius muscle. *J. Physiol. (Lond.).* **249:**183–195.

SCUKA, M. 1977. The effects of pH on the conductance change evoked by iontophoresis in the frog neuromuscular junction. *Pfluegers Arch. Eur. J. Physiol.* **369:**239–244.

SIGWORTH, F. J. 1977. Sodium channels in nerve apparently have two conductance states. *Nature (Lond.).* **270:**265–267.

TAKASHIMA, S. 1962. Dielectric properties of water of adsorption on protein crystals. *J. Polym. Sci.* **62:**233–240.

THOMAS, R. C. 1974. Intracellular pH of snail neurones measured with a new pH-sensitive glass micro-electrode. *J. Physiol. (Lond.).* **238:**159–180.

TURIN, L. and A. F. WARNER. 1980. Intracellular pH in early *Xenopus* embryos, its effect on current flow between blastomeres. *J. Physiol. (Lond.).* **300:**489–504.

WOODHULL, A. 1973. Ionic blockage of sodium channels in nerve. *J. Gen. Physiol.* **61:**687–708.