

# Effect of Acetylcholine on Postjunctional Membrane Permeability in Eel Electroplaque

N. L. LASSIGNAL and A. R. MARTIN

From the Department of Physiology, University of Colorado Medical Center, Denver, Colorado 80262

**ABSTRACT** Acetylcholine (ACh) was applied iontophoretically to the innervated face of isolated eel electroplaques while the membrane potential was being recorded intracellularly. At the resting potential (about  $-85$  mV) application of the drug produced depolarizations (ACh potentials) of 20 mV or more which became smaller when the membrane was depolarized and reversed in polarity at about zero membrane potential. The reversal potential shifted in the negative direction when external  $\text{Na}^+$  was partially replaced by glucosamine. Increasing external  $\text{K}^+$  caused a shift of reversal potential in the positive direction. It was concluded that ACh increased the permeability of the postjunctional membrane to both ions. Replacement of  $\text{Cl}^-$  by propionate had no effect on the reversal potential. In  $\text{Na}^+$ -free solution containing glucosamine the reversal potential was positive to the resting potential, suggesting that ACh increased the permeability to glucosamine. Addition of  $\text{Ca}^{++}$  resulted in a still more positive reversal potential, indicating an increased permeability to  $\text{Ca}^{++}$  as well. Analysis of the results indicated that the increases in permeability of the postjunctional membrane to  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Ca}^{++}$ , and glucosamine were in the ratios of approximately 1.0:0.9:0.7:0.2, respectively. With these permeability ratios, all of the observed shifts in reversal potential with changes in external ionic composition were predicted accurately by the constant field equation.

## INTRODUCTION

The major effect of acetylcholine (ACh) at the end-plate of vertebrate skeletal muscle is to produce an increase in membrane permeability to  $\text{Na}^+$  and  $\text{K}^+$  (Takeuchi and Takeuchi, 1960). At the resting membrane potential the main consequence of these permeability increases is an inward flux of  $\text{Na}^+$ , as the membrane is at or near the equilibrium potential for  $\text{K}^+$ . At more depolarized membrane potentials the inward movement of  $\text{Na}^+$  is accompanied by outward movement of  $\text{K}^+$ , the two ionic currents becoming equal and opposite at the so-called reversal potential, where the net ACh-induced current is zero. Estimates of the reversal potential at the motor end-plate range from 0 to about  $-15$  mV (delCastillo and Katz, 1954; Takeuchi and Takeuchi, 1960; Takeuchi, 1963*a, b*; Kordaš, 1969; Gage and McBurney, 1972; Magleby and Stevens, 1972; Dionne and Stevens, 1975). Although the postjunctional membrane of the eel electroplaque is analogous to the motor end-plate and has been used as a source of

purified ACh receptors (Olsen et al., 1972; Klett et al., 1972; Patrick and Lindstrom, 1973), its electrophysiological properties are much less well defined. In particular, the reversal potential for ACh-induced membrane currents has not been determined accurately, nor have the permeability changes underlying such currents. With bath-applied agonists the postjunctional current does not appear to reverse at any potential (Lester et al., 1975), apparently because the overall conductance of the drug-activated channels is reduced to zero by extreme membrane depolarization. Because of this it has been necessary to estimate the reversal potential by extrapolation of the relation between agonist-induced current and membrane potential, obtained in the region of membrane potential where the channels are still open. Similarly, extrapolation has been used to estimate the reversal potential of junctional potentials evoked by nerve stimulation (Ruiz-Manresa and Grundfest, 1971).

In the experiments to be reported here, ACh was applied iontophoretically to the postjunctional membrane of the electroplaque (delCastillo et al., 1972) at various membrane potentials. Using this technique, we were able to reverse the ACh potential at about zero membrane potential. The reversal potential was altered by changes in  $\text{Na}^+$ ,  $\text{K}^+$ , or  $\text{Ca}^{++}$  concentrations in the bathing solution, but not by changes in extracellular  $\text{Cl}^-$ , suggesting that the permeability changes produced by ACh were similar to those at the motor end-plate. Preliminary reports of the present results have appeared elsewhere (Lassignal and Martin, 1976*a, b*).

#### MATERIALS AND METHODS

Single electroplaques were dissected by the method of Schoffeniels and Nachmansohn (1957) and mounted horizontally between two chambers (Fig. 1). The innervated face was exposed to the upper chamber through a small ( $0.43 \text{ mm}^2$ ) hole, while the entire noninnervated face was exposed to the lower chamber. A thin layer of cured Sylgard resin (Dow Corning Corp., Midland, Mich.) was applied to the bottom of the upper chamber and uncured resin was used as a seal between this surface and the innervated face of the cell. All experiments were done at  $22^\circ\text{C}$ .

Recording micropipettes were filled with 4 M K-acetate and had resistances between 15 and 40  $\text{M}\Omega$ . Tip potentials ranged from  $-6$  to  $-14$  mV in the various experimental bathing solutions. The membrane potential of the innervated face was measured by two micropipettes glued together so that their tips were staggered by about  $80 \mu\text{m}$ . The cell was impaled by the leading electrode tip while the other remained just outside; the difference in potential between the two tips was then taken as the membrane potential. The membrane potential was altered by passing current between the two chambers through calomel half-cells. Current was monitored by a current-to-voltage converter between the calomel electrodes in the upper chamber and ground. Either voltage clamp or current clamp techniques could be used, but only the latter was employed in the experiments to be reported here. As the resistance of the innervated membrane was quite low (approximately  $8 \Omega \text{ cm}^2$ ), the potential across the fluid resistance between the two recording pipette tips could contribute significantly to the apparent change in membrane potential when current was passed through the cell. This was compensated for by adjusting the slider on the 5K potentiometer connected to point A (Fig. 1) so that no voltage was produced by applied currents when the leading electrode tip was just outside the cell (cf. Hodgkin et al., 1952).

The normal saline solution had the following composition (mM): NaCl, 160; KCl, 2.5; CaCl<sub>2</sub>, 2.0; MgCl<sub>2</sub>, 2.0, buffered to pH 7.4 with the Na<sup>+</sup> phosphate (1.5 mM). In addition, all solutions contained 0.5 μg/ml tetrodotoxin to block action potential initiation. Na-deficient solutions were made by replacing NaCl with glucosamine-HCl in equiosmolar quantities. The pH of such solutions was adjusted by addition of NaOH. In preliminary experiments, replacement of Na by Tris hydroxymethyl aminomethane (Tris) or by methylamine was found to be unsuitable, as both appeared to permeate the ACh-activated channels. In experiments in which K<sup>+</sup> concentration was changed, sufficient glucosamine-HCl was substituted for NaCl in the control solution so that increases in KCl

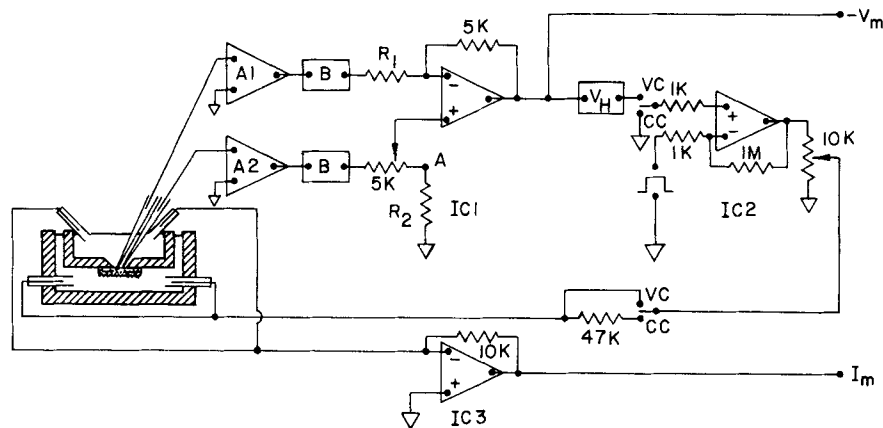


FIGURE 1. Experimental arrangement. Single electroplaque was mounted between two chambers and membrane potential measured by intracellular electrode (connected to amplifier A1) with reference to second microelectrode just outside the membrane (A2). Output from IC2 used to pass current across the cell for depolarizing cell membrane. Current was measured by current-to-voltage converter (IC3). B, bucking potentials to set inputs of IC1 to zero.  $R_1$  and  $R_2$  adjusted to provide unity gain at output of IC1 for signals from A1 and A2 with slider at point A. Moving slider away from point A compensated for voltage drop between microelectrode tips when current was passed through cell. All experiments reported here were with current clamp (switches in CC position).  $-V_m$ : membrane potential output;  $I_m$ : membrane current output.

could be accompanied by equiosmolar decreases in glucosamine-HCl, thus keeping the Na concentration constant. Cl concentration was reduced by substituting Na propionate for NaCl.

After the cell had been impaled with the recording electrode, ACh was applied iontophoretically from a micropipette filled with 4 M ACh (Nastuk, 1953), by using current pulses of 0.5–1.0 μA and 50–60 ms duration. Although visual observation was obscured by the dense network of blood vessels and nerves over the innervated face of the cell, it was usually possible to place the ACh pipette within about 20 μm of the recording electrodes. Under these conditions variations in the responses to ACh application from one position to the next were more likely to be related to the relative success in penetrating the overlying tissue with the ACh pipette than to any intrinsic variations in sensitivity of the cell membrane.

When a suitable ACh potential was obtained it was superimposed on a succession of

membrane depolarizations of 600 ms duration (Fig. 2) to obtain the reversal potential ( $E_r$ ). In a given cell,  $E_r$  for each solution tested was obtained by averaging the results of from one to five impalements. The solution was then removed and replaced after the chamber had been washed three times with the new solution. An equilibration time of 20 min was allowed in each solution before records were taken. The final  $E_r$  for a particular solution was taken as the average of the results from all cells tested in that solution.

## RESULTS

### *Normal Solution*

In the normal saline solution the average resting potential of the innervated membrane was  $-88.2 \pm 6.1$  mV (mean  $\pm$  SD, 18 cells). Iontophoretic application of ACh produced a depolarization which rose to a peak in 75–200 ms. Such a response is shown in Fig. 2 at a resting potential of  $-94$  mV and at various levels of depolarization. As the membrane was depolarized the response became

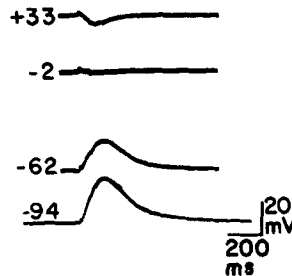


FIGURE 2. Responses to iontophoretically applied ACh at resting membrane potential ( $-94$  mV) and various levels of membrane depolarization. Response reverses at about  $-2$  mV. Duration of ACh pulse indicated by artifact at beginning of response.

smaller, disappearing at about zero membrane potential and reversing with reversed membrane polarization. The results from the experiment of Fig. 2 are plotted in Fig. 3, which shows the relation between ACh potential amplitude and membrane potential. In this particular experiment the ACh potential reversed at a membrane potential of about  $-3$  mV. In all experiments the relation became markedly nonlinear at membrane potentials positive to the reversal potential. At positive membrane potentials the amplitude of the reversed ACh potential became almost independent of membrane potential and sometimes even decreased rather than increased with increasing polarization beyond  $+30$  mV. This behavior was not due to any rectifying properties of the extrajunctional membrane as a similar relation was observed when ACh was applied under voltage clamp conditions: i.e. the junctional current was not linearly related to membrane potential (Lassignol and Martin, 1976*a*). In many of the current clamp experiments the amplitude of the reversed ACh potential was small (1–4 mV) and it was necessary to obtain at least a 20-mV ACh potential at the resting membrane potential in order to observe reversal. In the 18 experiments in normal solution the average reversal potential was  $+1.9 \pm 5.6$  mV.

*Effect of Na<sup>+</sup> on Reversal Potential*

In order to investigate the role of Na<sup>+</sup> in the response to ACh, extracellular Na<sup>+</sup> was replaced by glucosamine. This ion was chosen because it is relatively impermeant at the frog motor end-plate (Maeno et al., 1977). In addition, the effects of changes in external Na were tested in solutions in which divalent cations (Ca<sup>++</sup> and Mg<sup>++</sup>) were eliminated, as Ca<sup>++</sup> is known to penetrate ACh-activated channels at the motor end-plate (Takeuchi, 1963*b*) and the contribution of such Ca<sup>++</sup> current becomes relatively more important as external Na<sup>+</sup> is reduced. The control solution, then, consisted of 160 mM NaCl, 2.5 mM KCl, 1.5 mM Na<sup>+</sup> phosphate buffer (pH 7.4). The low Na<sup>+</sup> test solution contained 32 mM NaOH,

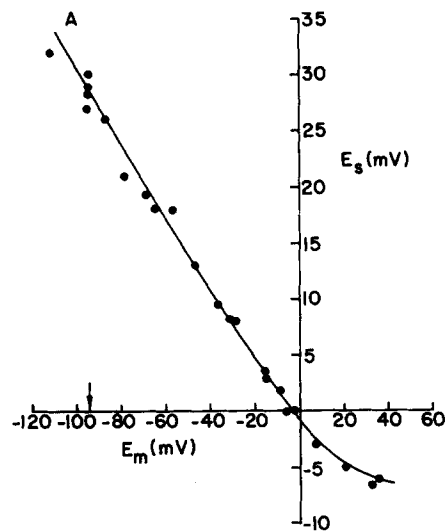


FIGURE 3. Amplitude of ACh response ( $E_s$ ) as a function of membrane potential ( $E_m$ ) in normal bathing solution. Arrow indicates resting membrane potential. Response reverses at about  $-3$  mV membrane potential.

128 mM glucosamine-HCl, 2.5 mM KCl, 1.5 mM Na<sup>+</sup> phosphate buffer (pH 7.1). Changes from control to test solutions and back were made in both chambers. The results of one such experiment are presented in Fig. 4. In the control solution (filled circles) the reversal potential was  $+4$  mV. Reducing extracellular Na<sup>+</sup> concentration by a factor of 5 shifted the reversal potential in the negative direction to  $-19$  mV. In a total of 18 experiments the average reversal potential in the control solution was  $+4.7 \pm 2.9$  mV, a value not significantly different from that obtained from the experiments in the solution containing Ca<sup>++</sup> and Mg<sup>++</sup>, and the average resting potential  $-88.7 \pm 5.0$  mV. In the low Na<sup>+</sup> solution (24 cells) the average reversal potential was  $-18.9 \pm 4.7$  mV and the average resting potential  $-78.6 \pm 5.0$  mV. Thus a fivefold reduction in extracellular Na produced an average shift of  $-23.6$  mV in reversal potential.

*Effect of K<sup>+</sup> on Reversal Potential*

To test the possibility that K<sup>+</sup> current might contribute to the ACh potential,

reversal potentials were measured in normal (2.5 mM)  $K^+$  and 32 mM  $K^+$ . Again, divalent cations were eliminated from the control and test solutions and all solution changes were made in both the upper and lower compartments. The control solution contained 120 mM NaCl, 9.4 mM NaOH, 30 mM glucosamine-HCl, 2.5 mM KCl, 1.5 mM  $Na^+$  phosphate buffer (pH 7.1), and the test solution 130 mM NaCl, 32 mM KCl, and 1.5 mM  $Na^+$  phosphate (pH 7.1). In 12 experiments the average reversal potential in the control solution was  $-1.5 \pm 2.9$  mV with an average resting potential of  $-83.7 \pm 3.9$  mV. In 10 experiments in the high  $K^+$  solution the average resting potential was reduced to  $-31.9 \pm 3.2$  mV and the reversal potential increased to  $+3.0 \pm 3.4$ , not significantly different

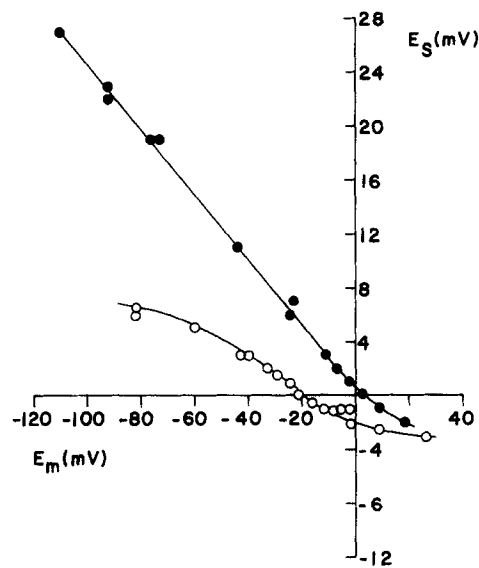


FIGURE 4. Effect of reducing extracellular  $Na^+$  on reversal potential. Closed circles: ACh response as a function of membrane potential in normal (161.5 mM)  $Na^+$ . Reversal potential about +3 mV. Open circles: responses in reduced (33.5 mM)  $Na^+$ . Reversal potential is shifted to about -19 mV.  $Na^+$  was replaced by glucosamine.

from that in the control solution. From this result it could not be determined whether or not  $K^+$  flux contributed to the ACh potential. The lack of a shift in reversal potential is not necessarily an indication that  $K^+$  is not involved in the response. If one assumes that the reversal potential is determined by the constant field equation (Goldman, 1943), then little shift in the reversal potential might be expected (Anwyl and Usherwood, 1975). This may be appreciated intuitively by considering that increasing external  $K^+$  not only shifts the  $K^+$  equilibrium potential to a more positive value but also increases  $K^+$  conductance (assuming constant  $K^+$  permeability). Thus, when the membrane is depolarized the two effects tend to cancel, and the outward  $K^+$  current (and hence the reversal potential) may be similar to that in the control solution. However, the Goldman equation predicts that the effects of changes in  $K^+$  concentration

should be more apparent in low  $\text{Na}^+$  solutions. Consequently reversal potentials were measured at the same two  $\text{K}^+$  concentrations in solutions containing 33.5 mM  $\text{Na}^+$  rather than the normal 161.5 mM. The control solutions contained 32 mM  $\text{NaOH}$ , 2.5 mM  $\text{KCl}$ , 128 mM glucosamine-HCl, 1.5 mM  $\text{Na}^+$  phosphate buffer, and the test solution contained 32 mM  $\text{NaOH}$ , 32 mM  $\text{KCl}$ , 100 mM glucosamine-HCl, 1.5 mM  $\text{Na}^+$  phosphate. Both solutions were at pH 7.1. Results obtained in one experiment are shown in Fig. 5. In the solution containing normal  $\text{K}^+$  the average resting potential was  $-78.6 \pm 5.0$  mV and the reversal potential  $-18.9 \pm 4.7$  mV (24 cells). In the high  $\text{K}^+$  solution (18 cells) the average membrane potential was reduced to  $-29.3 \pm 2.4$  mV and the average reversal potential shifted in the positive direction to  $-10.3 \pm 2.6$  mV.

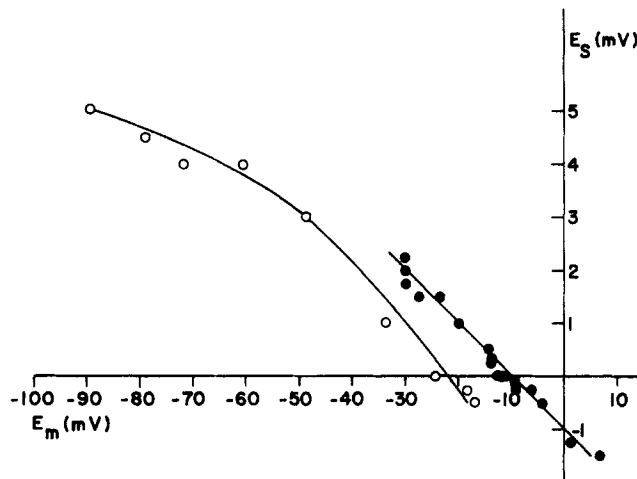


FIGURE 5. Effect of  $\text{K}^+$  on reversal potential. Open circles: amplitudes of responses in low-Na, glucosamine solution with normal (2.5 mM)  $\text{K}^+$ . Closed circles: responses in high (32 mM)  $\text{K}^+$ . Reversal potential is shifted from about  $-23$  mV to  $-10$  mV by increasing extracellular  $\text{K}^+$ .

#### *Effect of $\text{Cl}^-$ on Reversal Potential*

The possibility that  $\text{Cl}^-$  might permeate the ACh-activated channels was tested by comparing responses in the control solution (160 mM  $\text{NaCl}$ , 2.5 mM  $\text{KCl}$ , 1.5 mM  $\text{Na}^+$  phosphate) with those in a test solution in which the  $\text{NaCl}$  was replaced by  $\text{Na}^+$  propionate. In four experiments in which the test solution was present in the upper chamber the average resting potential was  $-80.6 \pm 1.5$  mV and the reversal potential  $+5.0 \pm 3.4$  mV. The reversal potential was not significantly different from that obtained in the control solution ( $+4.7 \pm 2.9$  mV, see section on  $\text{Na}^+$ ), indicating that  $\text{Cl}^-$  did not contribute to the ACh response.

#### *Glucosamine and $\text{Ca}^{++}$*

In order to analyze the results obtained with  $\text{Na}^+$  and  $\text{K}^+$  it was necessary to determine to what extent, if any, glucosamine contributed to the ACh response. To investigate this point the cell was bathed on both sides with a solution

containing 140 mM glucosamine-HCl, 32 mM KOH, 1.5 mM  $K^+$  phosphate buffer (pH 7.1). The results of one such experiment are plotted in Fig. 6 (open circles). At the resting potential of  $-30$  mV a small ACh potential was obtained which became reduced in amplitude on depolarization and reversed at a membrane potential of about  $-21$  mV. In 13 such experiments the average resting potential was  $-28.3 \pm 5.0$  mV and the average reversal potential  $-22.2 \pm 5.1$  mV. Thus the reversal potential was about 6 mV positive to the resting potential. If we assume that the resting membrane potential was at or near the  $K^+$  equilibrium potential, then the only ion present which would be expected to

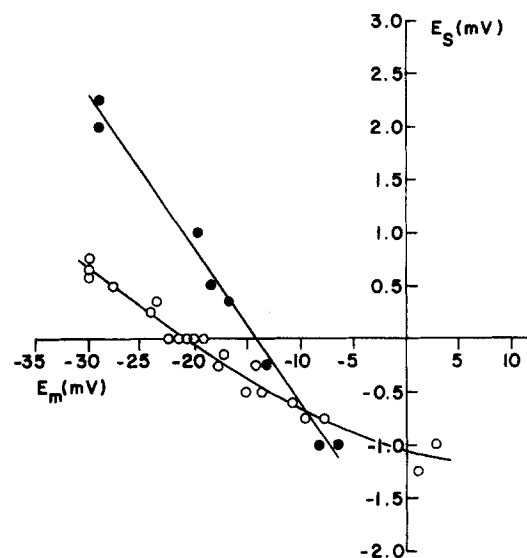


FIGURE 6. Open circles: amplitudes of responses in  $Na^+$ -free glucosamine solution containing 32 mM  $K^+$ . Reversal potential ( $-22$  mV) is positive to resting membrane potential ( $-30$  mV), suggesting that ACh increases membrane permeability to glucosamine. Closed circles: 20 mM  $CaCl_2$  added to bathing solution shifts reversal potential to about  $-14$  mV, indicating increased permeability to  $Ca^{++}$  as well.

carry any significant amount of inward current was glucosamine. It was concluded that the ACh-activated channels were slightly permeable to this ion.

In 5 of the 13 experiments part of the glucosamine in the  $Na^+$ -free solution was replaced by  $Ca^{++}$  to determine the effect of the latter on the reversal potential. The test solution contained 20 mM  $CaCl_2$ , 105 mM glucosamine-HCl, 32 mM KOH, buffered to pH 7.1 with 2.0 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES). Fig. 6 illustrates the effect of adding the test solution to the upper chamber in one experiment. The resting potential ( $-29$  mV) was essentially unchanged; the reversal potential (closed circles) shifted in the positive direction by about 7 mV to  $-14$  mV. In the five experiments the average resting potential was  $-34.5 \pm 3.4$  mV and the average reversal potential  $-12.9 \pm 3.3$  mV. Addition of 20 mM Ca, then, shifted the reversal potential by an average of about +9 mV.



## ANALYSIS

The results indicate that the effect of ACh on the postjunctional membrane was to increase its permeability to  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Ca}^{++}$ , and, to a lesser extent, glucosamine. In the following analysis it will be assumed that the reversal potential  $E_r$  is determined by the relative permeabilities of the ACh-activated channels to the ions present according to the constant field equation (Goldman, 1943). Another possible treatment of the results will be considered subsequently. In  $\text{Ca}^{++}$ -free solution, then,

$$E_r = \frac{RT}{F} \ln \frac{K_0 + aGA_0 + b\text{Na}_0}{K_1 + aGA_1 + b\text{Na}_1} \quad (1)$$

where  $R$ ,  $T$ , and  $F$  have their usual meanings,  $K_0$ ,  $K_1$ , etc. represent the concentrations (or more strictly, the activities) of the various ions outside and inside the cell, respectively. It should be noted that glucosamine is not completely ionized at neutral pH;  $GA_0$ , then, represents the ionic concentration, calculated by assuming that the ionized fraction exists as a monovalent cation and considering the solution to be electrically neutral. The constants  $a$  and  $b$  represent  $\Delta p_{GA}/\Delta p_K$  and  $\Delta p_{Na}/\Delta p_K$ , respectively; i.e. the ratios of the increases in permeability produced by ACh. In using Eq. (1) it was assumed that  $aGa_1$  was small with respect to the other terms in the denominator and this term was ignored in the subsequent calculations.

*Internal Sodium and Potassium*

The main problem encountered in applying Eq. (1) to the results is that accurate values of  $\text{Na}_1$  and  $K_1$  are not available. For the purposes of the following analysis it was assumed that  $\text{Na}_1$  was 15 mM, which would mean that in normal bathing solution the  $\text{Na}^+$  equilibrium potential was +60 mV (see Discussion; Lassignal and Martin, 1976a). This value will be used in all of the calculations presented here. Similarly, all subsequent calculations (except those involving glucosamine and  $\text{Ca}^{++}$  permeabilities) were done by using an estimated value for  $K_1$ . This value was calculated by assuming that in high  $\text{K}^+$  solutions the resting membrane potential was equal to the  $\text{K}^+$  equilibrium potential. The average resting potential in high (32 mM)  $\text{K}^+$  was about -30 mV, equivalent to an internal  $\text{K}^+$  concentration of 105 mM. In normal (2.5 mM)  $\text{K}^+$ , this internal concentration results in a calculated  $\text{K}^+$  equilibrium potential of -95 mV. The overall average resting potential in normal  $\text{K}^+$  was -84.5 mV. If it is assumed that the resting potential is determined by the concentrations of  $\text{Na}^+$  and  $\text{K}^+$  on either side of the membrane and the relative permeability of the resting membrane to the two ions according to the constant field equation, then the average resting potential is consistent with a resting  $\text{Na}^+$  to  $\text{K}^+$  permeability ratio of 0.008, a value which is not unreasonable (Karlin, 1967).

*Permeability to Glucosamine*

Experiments like that shown in Fig. 6 (open circles) were used to determine the constant  $a$ . In using Eq. (1) the assumptions already mentioned were made, namely  $aGA_1$  negligible,  $\text{Na}_1 = 15$  mM, and  $K_1 = K_0 \exp(-E_m F/RT)$ , where  $E_m$  is the resting membrane potential in the high  $\text{K}^+$  solution. In the experiments under consideration  $\text{Na}_0$  was zero. Eq. (1) then becomes

$$E_r = \frac{RT}{F} \ln \frac{K_0 + aGA_0}{K_0 e^{-E_m F/RT} + b(15)}$$

where the ionic concentrations are in mM. On rearranging we obtain

$$a = \frac{K_0(e^{(E_r - E_m)F/RT} - 1) + b(15)e^{E_r F/RT}}{GA_0}$$

Assuming  $b = 0.9$  (see section on  $\text{Na}^+$  below) and using average values for  $E_m$  and  $E_r$  from 13 experiments gave a value of 0.21 for  $a$ .

#### *Effect of External $\text{Na}^+$ on Reversal Potential*

The average reversal potential in the control solution was +4.7 mV. With the assumptions outlined above, the reversal potential should be given by

$$E_r = \frac{RT}{F} \ln \frac{K_0 + (0.21)GA_0 + b\text{Na}_0}{105 + b(15)} \quad (2)$$

It follows that

$$b = \frac{105 e^{E_r F/RT} - K_0 - (0.21)GA_0}{\text{Na}_0 - 15 e^{E_r F/RT}} \quad (3)$$

In the control solution the ratio of  $\text{Na}^+$  to  $\text{K}^+$  permeability changes calculated from Eq. (3) was 0.86. By using this value for  $b$  in Eq. (2), it was predicted that lowering the external  $\text{Na}^+$  concentration from 161.5 mM to 33.5 mM would shift the reversal potential in the negative direction by about 26 mV; i.e. to -21.0 mV. The reversal potential observed experimentally in the low- $\text{Na}^+$  solution was  $-18.9 \pm 4.7$  mV, in good agreement with the prediction.

#### *Effect of External $\text{K}^+$ on Reversal Potential*

When reversal potentials were compared in normal (2.5 mM) and high (32 mM)  $\text{K}^+$ , in the presence of 131 mM  $\text{Na}^+$ , little difference was observed. Such a lack of effect of changes in external  $\text{K}^+$  on reversal potential is predicted by Eq. (2). In the control solution  $E_r$  was -1.5 mV, resulting in a calculated value for  $b$  of 0.79. When one used this value, the predicted reversal potential in high  $\text{K}^+$  was 3.8 mV. The observed reversal potential was  $+3.0 \pm 3.4$  mV, not significantly different from the predicted.

Somewhat larger changes in reversal potential with changes in external  $\text{K}^+$  are predicted if the external  $\text{Na}^+$  concentration is reduced. In the low (33 mM)  $\text{Na}^+$  solution the reversal potential in normal  $\text{K}^+$  was -18.9 mV, giving a value of 1.0 for  $b$ . This gave a predicted  $E_r$  in high  $\text{K}^+$  of -10.2 mV, in close agreement with the observed value of  $-10.3 \pm 2.6$  mV.

#### *Permeability to $\text{Ca}^{++}$*

In the experiment illustrated in Fig. 6 it was found that adding 20 mM  $\text{CaCl}_2$  to the external solution resulted in a shift of the reversal potential in the positive direction. If we assume, at least for the moment, that the shift reflected a contribution of  $\text{Ca}^{++}$  current to the ACh response rather than some indirect effect of  $\text{Ca}^{++}$  on  $a$ , then we can calculate a value for the increase in  $\text{Ca}^{++}$

permeability produced by ACh as a fraction of the increase in  $K^+$  permeability; i.e., we can calculate  $c = \Delta p_{Ca} / \Delta p_K$ . At the reversal potential the sum of all the ionic currents due to ACh application is zero or, in other words,

$$I_K + I_{Na} + I_{GA} + I_{Ca} = 0. \quad (4)$$

If we use the assumptions inherent in the derivation of the constant field equation (Goldman, 1943; Hodgkin et al., 1952), then for any ion  $X$  with valence  $z$ , change in permeability  $\Delta p_X$  and internal and external concentrations  $X_i$  and  $X_o$ , respectively, the ionic current due to ACh application at the reversal potential is given by

$$I_X = \frac{\Delta p_X z^2 F^2 E_r}{RT} \frac{X_i e^{zFE_r/RT} - X_o}{e^{zFE_r/RT} - 1}. \quad (5)$$

This expression can be substituted in Eq. (4) for each of the ionic currents. In the experiments  $Na_o$  was zero, and the same assumptions were made as previously, namely,  $Na_i = 15$  mM,  $GA_i$ , and  $Ca_i$  negligible and  $K_i = K_o \exp(-E_m F/RT)$ . Making these substitutions and rearranging, we arrive at an expression for  $c$ :

$$c = \frac{\Delta p_{Ca}}{\Delta p_K} = \frac{e^{E_r F/RT} + 1}{4CA_o} [K_o(e^{(E_r - E_m)F/RT} - 1) - aGA_o + b(15)e^{E_r F/RT}].$$

Values of 0.21 and 0.9 were used for  $a$  and  $b$ , respectively. In the five experiments the average values for  $E_m$  and  $E_r$  were  $-34.5$  and  $-12.9$  mV and  $c$  was calculated to be 0.70.

#### DISCUSSION

All of the shifts in reversal potential with changes in extracellular  $Na^+$ ,  $K^+$ , and glucosamine agreed well with those predicted by the constant field equation. Given that use of the equation is then appropriate, a value for  $b$  can be calculated for each of the experimental conditions. The average of such calculations for the four basic extracellular solutions (normal and low  $Na^+$  combined with normal and high  $K^+$ ) was  $0.88 \pm 0.11$ , assuming  $a = 0.21$ . The overall results can be summarized then, by saying that in the presence of ACh the postjunctional membrane behaves in a manner consistent with the constant field equation with increases in permeability to  $K^+$ ,  $Na^+$ ,  $Ca^{++}$ , and glucosamine being in the ratios of 1.0:0.9:0.7:0.2, respectively.

The good agreement between the shifts in reversal potential observed experimentally and those predicted theoretically depends on the values used for internal  $Na^+$  and  $K^+$  concentrations. Both values ( $Na_i = 15$  mM;  $K_i = 105$  mM) are consistent with previous estimates for electroplaques. Higman et al. (1964) found that the membrane potential of the innervated face followed the Nernst relation for  $K^+$  with increasing external concentrations above 20 mM, so the assumption made here that the membrane potential was near the  $K^+$  equilibrium potential in 32 mM  $K^+$  seems reasonable. Previous estimates of internal  $Na^+$  have ranged from 10 mM to 30 mM (Schoffeniels, 1959; Karlin, 1967; but see Ruiz-Manresa and Grundfest, 1971), and  $p_{Na}/p_K$  for the resting membrane has been estimated at about 0.01 (Karlin, 1967), in agreement with the present estimate of 0.008.

The results reported here differ in one important respect from those reported previously with a variety of muscle preparations (e.g., Takeuchi, 1963*a, b*; Ritchie and Fambrough, 1975). In general, shifts in reversal potential with changes in external  $\text{Na}^+$  have not been consistent with those expected from the constant field equation. Instead such shifts have been in agreement with those predicted by the conductance equation proposed by Takeuchi and Takeuchi (1960) derived from an electrical model of the postjunctional membrane. The equation states that the reversal potential should be given by

$$E_r = \frac{E_K + rE_{\text{Na}}}{1 + r}, \quad (6)$$

where  $E_K$  and  $E_{\text{Na}}$  are the  $\text{K}^+$  and  $\text{Na}^+$  equilibrium potentials and  $r = \Delta G_{\text{Na}}/\Delta G_K$ , the ratio of the changes in conductance produced by ACh. It follows that

$$r = \frac{E_r - E_K}{E_{\text{Na}} - E_r}. \quad (7)$$

The analytical procedure is to determine the conductance ratio from Eq. (7) and then use this value in Eq. (6) to predict shifts in reversal potential with changes in external  $\text{Na}^+$  and  $\text{K}^+$ . With changes in external  $\text{K}^+$ , it has been necessary to allow for the fact that  $\text{K}^+$  conductance increases (and  $r$  decreases) with increasing concentration, a treatment equivalent to using the constant field equation. In contrast, shifts in reversal potential with changes in external  $\text{Na}^+$  have been predicted accurately by Eq. (6), with a constant value for  $r$ , implying that  $\text{Na}^+$  conductance is independent of concentration. This, in turn, suggests that  $\text{Na}^+$  permeability increases as the external concentration is decreased. Various explanations for this apparent anomaly have been proposed (for summaries see: Ritchie and Fambrough, 1975; Gage, 1976). The problem does not arise in the present experiments, where the results obtained by changing external  $\text{Na}^+$  were consistent with the constant field equation and not with the conductance equation.

If we apply the conductance equation to the present results, then in the control ( $\text{Ca}^{++}$ -free) solution, assuming  $E_{\text{Na}} = +60$  mV and  $E_K = -95$  mV, a reversal potential of zero results in a value of  $r$  of 1.58 (Eq. [7]). Using this value in Eq. (6) predicts a shift of  $-24.6$  mV in  $E_r$  when going from the control solution to the low- $\text{Na}^+$  solution, in apparent agreement with the observed shift of  $-25.2$  mV. However, in using Eq. (6), no account is taken of the contribution of the glucosamine in the low- $\text{Na}^+$  solution to the inward current associated with the response. This cannot be included in the conductance equation as the equilibrium potential for glucosamine is unknown, but from Eq. (5) the glucosamine current would be expected to be approximately 87% of the  $\text{Na}^+$  current, in which case the conductance equation would predict a shift in  $E_r$  of only 9.1 mV when changing from normal to low  $\text{Na}^+$ .

In summary, then, the present results imply that  $\text{Na}^+$  permeability in the ACh-activated channels is independent of  $\text{Na}^+$  concentration, while previous experiments on muscle preparations suggest that  $\text{Na}^+$  permeability increases when external concentration is reduced. The difference seems most likely to be

related to the fact that glucosamine was used here as an  $\text{Na}^+$  substitute while in previous experiments  $\text{Na}^+$  concentration in the bathing solution was reduced by substituting sucrose for  $\text{NaCl}$ , thus reducing the ionic strength of the solution. This might alter the permeability characteristics of the postjunctional membrane, for example by unmasking fixed surface charges, in such a way as to increase  $\text{Na}^+$  permeability (relative to that of  $\text{K}^+$ ) and thereby make  $\text{Na}^+$  conductance appear independent of concentration.

In the present experiments the ratio of  $\text{Ca}^{++}$  to  $\text{K}^+$  permeabilities was about 0.7; the  $\text{Ca}^{++}$  to  $\text{Na}^+$  permeability ratio was therefore about 0.77. In experiments on the motor end-plate (Takeuchi, 1963*b*) the  $\text{Ca}^{++}$  to  $\text{Na}^+$  conductance ratio was found to be about 0.08. If, in Eq. (5), we replace  $E_r$  by  $E_m$  and  $I_x$  by  $\Delta G_x(E_x - E_m)$ , where  $\Delta G_x$  is the increase in conductance for ion  $X$  and  $E_x$  is its equilibrium potential, then we have an expression for the relation between permeability and conductance for ion  $X$  at the resting membrane potential. Using this relation for  $\text{Ca}^{++}$  and  $\text{Na}^+$  and the same assumptions about  $\text{Ca}^{++}$  distribution as those made for the motor end-plate ( $\text{Ca}_0 = 2 \text{ mM}$ ;  $\text{Ca}_i = 1.18 \text{ mM}$ ;  $E_{\text{Ca}} = 6.7 \text{ mV}$ ), we arrive at  $\Delta G_{\text{Ca}}/\Delta G_{\text{Na}} = 0.06$ , a value similar to that reported previously. With a somewhat more realistic assumption that  $\text{Ca}_i = 1 \mu\text{M}$  ( $E_{\text{Ca}} = 100 \text{ mV}$ ), the conductance ratio is reduced to 0.03.

Finally, the results reported here differ from those obtained previously with bath application of ACh agonists to the electroplaque (e.g., Lester et al., 1975) in that reversal of the ACh response was readily obtained. Difficulty in obtaining reversal potentials with bath applications seems likely to be due not only to inaccuracies associated with the small magnitudes of the permeability changes at extreme membrane depolarization but also to changes in internal ionic concentrations during the period of application of the agonist (Karlin, 1967).

This work was supported by research grant no. NS 09660 from the National Institutes of Health, Bethesda, Md.

Received for publication 21 March 1977.

#### REFERENCES

- ANWYL, R., and P. N. R. USHERWOOD. 1975. Ionic permeability changes occurring at excitatory receptor membranes of chemical synapses. *Nature (Lond.)*. **257**:410-412.
- DELCASTILLO, J., E. BARTELS, and J. A. SOBRINO. 1972. Microelectrophoretic application of cholinergic compounds, protein oxidizing agents and mercurials to the chemically excitable membrane of the electroplax. *Proc. Natl. Acad. Sci. U. S. A.* **69**:2081-2085.
- DELCASTILLO, J., and B. KATZ. 1954. The membrane change produced by the neuromuscular transmitter. *J. Physiol. (Lond.)*. **125**:546-565.
- 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.
- GAGE, P. W. 1976. Generation of end-plate potentials. *Physiol. Rev.* **56**:177-247.
- GAGE, P. W., and R. N. MCBURNEY. 1972. Miniature end-plate currents and potentials generated by quanta of acetylcholine in glycerol-treated toad sartorius fibres. *J. Physiol. (Lond.)*. **226**:79-94.

- GOLDMAN, D. E. 1943. Potential, impedance, and rectification in membranes. *J. Gen. Physiol.* **27**:37-60.
- HIGMAN, H. B., T. R. PODLESKI, and E. BARTELS. 1964. Correlation of membrane potential and potassium flux in the electroplax of electrophorus. *Biochim. Biophys. Acta.* **79**:138-150.
- HODGKIN, A. L., A. F. HUXLEY, and B. KATZ. 1952. Measurements of current-voltage relations in the membrane of the giant axon of *Loligo*. *J. Physiol. (Lond.)*. **116**:424-448.
- KARLIN, A. 1967. Permeability and internal concentration of ions during depolarization of the electroplax. *Proc. Natl. Acad. Sci. U. S. A.* **58**:1162-1167.
- KLETT, R. P., B. W. FULPIUS, D. COOPER, M. SMITH, E. REICH, and L. D. POSSANI. 1973. The acetylcholine receptor. I. Purification and characterization of a macromolecule isolated from electrophorus electricus. *J. Biol. Chem.* **248**:6841-6853.
- KORDAŠ, M. 1969. The effect of membrane polarization on the time course of the end-plate current in frog sartorius muscle. *J. Physiol. (Lond.)*. **204**:493-502.
- LASSIGNAL, N. L., and A. R. MARTIN. 1976a. Reversal of acetylcholine potentials in eel electroplaque. *Science (Wash. D. C.)*. **191**:464-466.
- LASSIGNAL, N. L., and A. R. MARTIN. 1976b. Conductance changes produced by acetylcholine in eel electroplaque. *Neurosci. Abstr.* **2**:413.
- LESTER, H. A., J.-P. CHANGEUX, and R. SHERIDAN. 1975. Conductance increases produced by bath application of cholinergic agonists in *Electrophorus* electroplaques. *J. Gen. Physiol.* **65**:797-816.
- MAENO, T., C. EDWARDS, and M. ANRAKU. 1977. Permeability of the end-plate membrane activated by acetylcholine to some organic cations. *J. Neurobiol.* **8**:173-184.
- MAGLEBY, K. L., and C. F. STEVENS. 1972. The effect of voltage on the time course of end-plate currents. *J. Physiol. (Lond.)*. **223**:151-171.
- NASTUK, W. L. 1953. Membrane changes at a single muscle end-plate produced by transitory application of acetylcholine with an electrically-controlled microjet. *Fed. Proc.* **12**:102.
- OLSEN, R., J.-C. MEUNIER, and J.-P. CHANGEUX. 1972. Progress in the purification of the cholinergic receptor protein from *electrophorus electricus* by affinity chromatography. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **28**:96-100.
- PATRICK, J., and J. LINDSTROM. 1973. Autoimmune response to acetylcholine receptor. *Science (Wash. D. C.)*. **180**:871-872.
- RITCHIE, A., and D. M. FAMBROUGH. 1975. Ionic properties of the acetylcholine receptor in cultured rat myotubes. *J. Gen. Physiol.* **65**:751-767.
- RUIZ-MANRESA, F., and H. GRUNDFEST. 1971. Synaptic electrogenesis in eel electroplaques. *J. Gen. Physiol.* **57**:71-92.
- SCHOFFENIELS, E. 1969. Ion movements studied with single isolated electroplax. *Ann. N. Y. Acad. Sci.* **81**:285-306.
- SCHOFFENIELS, E., and D. NACHMANSOHN. 1957. An isolated single electroplax preparation. I. New data on the effect of acetylcholine and related compounds. *Biochim. Biophys. Acta.* **26**:1-15.
- TAKEUCHI, A., and N. TAKEUCHI. 1960. On the permeability of the end-plate membrane during the action of transmitter. *J. Physiol. (Lond.)*. **154**:52-67.
- TAKEUCHI, N. 1963a. Some properties of conductance changes at the end-plate membrane during the action of acetylcholine. *J. Physiol. (Lond.)*. **167**:128-140.
- TAKEUCHI, N. 1963b. Effects of calcium on the conductance changes at the end-plate membrane during the action of transmitter. *J. Physiol. (Lond.)*. **167**:141-155.