Ionic Properties of the Acetylcholine Receptor in Cultured Rat Myotubes

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ABSTRACT The acetylcholine reversal potential (E_r) of cultured rat myotubes is −3mV. When activated, the receptor is permeable to K^+ and Na^+, but not to Cl^- ions. Measurement of E_r in $Tris^+$-substituted, Na-free medium also indicated a permeability to $Tris^+$ ions. Unlike adult frog muscle the magnitude of E_r was insensitive to change in external Ca^{++} (up to 30 mM) or to changes in external pH (between 6.4 and 8.9). The equivalent circuit equation describing the electrical circuit composed of two parallel ionic batteries (E_K and E_{Na}) and their respective conductances (g_K and g_{Na}), which has been generally useful in describing the E_r of adult rat and frog muscle, could also be applied to rat myotubes when E_r was measured over a wide range of external Na^+ concentrations. The equivalent circuit equation could not be applied to myotubes bathed in media of different external K^+ concentrations. In this case, the E_r was more closely described by the Goldman constant field equation. Under certain circumstances, it is known that the receptor in adult rat and frog muscle can be induced to reversibly shift from behavior described by the equivalent circuit equation to that described by the Goldman equation. Attempts to similarly manipulate the responses of cultured rat myotubes were unsuccessful. These trials included a reduction in temperature (15°C), partial alpha-bungarotoxin blockade, and activation of receptors with the cholinergic agonist, decamethonium.

INTRODUCTION

Myoblasts obtained from embryonic skeletal muscle and maintained in culture develop into multinucleated, striated myotubes and exhibit many of the properties of the mature skeletal muscle fiber in vivo (Yaffe, 1969; Fischbach et al., 1971; Powell and Fambrough, 1973). Such cultures acquire sensitivity to acetylcholine (ACh) within a few days after plating and coincident with the onset of cell fusion (Fambrough and Rash, 1971). As the myotubes mature, the number and density of ACh receptors increase (Hartzell and Fambrough, 1973). Such receptors have been shown to be similar to ACh receptors of adult muscle in that they depolarize in response to ACh and are

blocked by curare (Fambrough and Rash, 1971). The ACh receptors of our cultured rat myotubes are located over the entire muscle surface (Hartzell and Fambrough, 1973), and are thus similar in distribution to ACh receptors of rat fetal muscle before innervation (Diamond and Miledi, 1962), or of the adult skeletal muscle fiber after denervation (Axelsson and Thesleff, 1959).

A number of laboratories have found no difference in the ACh reversal potential of mammalian denervated and innervated skeletal muscle (Axelsson and Thesleff, 1957; Magazanik and Potapova, 1969; Beranek and Vyskocil, 1967). Recently, however, one laboratory has reported a substantial difference in the reversal potential of the frog end-plate receptor as compared to the extrajunctional receptors of the denervated muscle, and have thus raised the possibility that either the receptors of the denervated frog muscle differed in structure or that a change in the immediate environment of the receptor had influenced its ionic properties (Feltz and Mallart, 1971). Interestingly, the large shift in reversal potential found in denervated frog muscle could be reproduced in the innervated frog end plate by increasing the pH (Mallart and Trautmann, 1973).

In this study we examine the response of the ACh reversal potential in cultured rat myotubes to changes in external ion composition and compare our results to those found by others in innervated and denervated skeletal muscle. We have also attempted to modify the ionic properties of the receptor by changes in temperature, pH, and choice of agonist. It is hoped that such information will provide some insight into the mechanism of ion permeation. A preliminary report was presented at the Fourth Annual Meeting of the Society of Neuroscience, St. Louis, Missouri, October, 1974 (Ritchie and Fambrough, 1974).

METHODS

Tissue Culture

Embryonic rat muscle was grown in cell culture as described by Fambrough and Rash (1971). Rat fore and hind limbs were isolated from 17- to 18-day old rat embryos. The trypsin-dissociated cells were grown in collagen-coated 35-mm Falcon petri dishes at 36°C in modified Ham's F12 culture medium containing 2% embryo extract and 15% horse serum and gassed with 5% CO_2 in air. Cells were plated at 5×10^4 per culture dish.

Medium

Experiments were generally performed in a medium containing Hank's salt solution, 0.5% bovine serum albumin, 2% glucose, and 18 mM Na HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid) buffer, pH 7.4. The Hank's salt solution contained 5.3 mM KCl, 136 mM NaCl, 0.41 mM $MgSO_4$, 0.49 mM $MgCl_2$, 0.18 mM Na_2HPO_4, 0.44 mM KH_2PO_4, and 1.26 mM $CaCl_2$. All media also contained

penicillin, streptomycin, and fungizone. Changes in the composition of the media are noted in the text.

Electrophysiology

Cultures were mounted on an inverted phase microscope with a heating stage and maintained at 37 or 15°C by varying the temperature of the circulating water. The temperature of the solution in the culture dish was monitored with a thermocouple.

Membrane potentials were measured with an intracellular recording electrode. The electrode was filled by the method of Tasaki et al. (1968) with 2 M KCl. Electrodes were selected which had resistances between 20 and 150 MΩ and time constants no greater than 300 μs. The higher resistance electrodes were most often used with the younger myotubes which were smaller and more readily damaged. The recording electrode was connected to a negative capacitance feedback ELSA-4 Bak amplifier (Electronics for Life Sciences, Rockville, Md.) through a Ag-AgCl wire. The bath electrode was a 150 mM NaCl agar bridge which was connected to ground via a Ag-AgCl wire immersed in 2 M KCl. Recordings were displayed on a Tektronix 5103/D13 storage oscilloscope (Tektronix, Inc., Beaverton, Ore.) and photographed with a Polaroid camera (Polaroid Corp., Cambridge, Mass).

ACh reversal potentials were measured by an intracellular recording electrode during iontophoretic application of ACh. The steady-state membrane potential was altered by passing a 200- to 500-ms current through a second intracellular electrode prepared like the recording electrode, however, with the Ag-AgCl wire connected to a pulse generator via a stimulus isolation unit. Microelectrodes for iontophoresis were filled with 2 M AChCl or a saturated solution of decamethonium bromide (less than 2 M) and connected to a pulse generator via a stimulus isolation unit and a source of biasing voltage. The ACh pulse varied between 2- and 20-ms duration depending on the sensitivity of the myotube. The recording electrode, stimulating electrode, and iontophoretic pipette were usually placed within 20 μm of each other and well within the space constant of the fiber. In older cultures, 1 μg/ml of tetrodotoxin (Calbiochem, San Diego, Calif.) was added to the incubation medium to suppress action potential generation.

Hypertonic glycerol, often used on skeletal muscle to disrupt the tubular system, was used on myotubes (where noted in the text) to exactly reproduce conditions used in an earlier study on the effect of pH on the reversal potential of frog muscle (Mallart and Trautmann, 1973). These myotubes were bathed in HEPES buffered Hank's salt solution containing 400 mM glycerol for 1 h at room temperature.

Partial alpha-bungarotoxin blockade of ACh receptors was achieved by treatment of myotubes for 5 min with 0.2 μg/ml of the purified toxin (Hartzell and Fambrough, 1973). Excess toxin was then removed by washing the culture dish eight times with fresh medium over a 20-min period. This treatment resulted in a 70% decrease in ACh sensitivity compared to untreated controls.

RESULTS

Activation of ACh receptors in adult skeletal muscle results in a transient local depolarization due to the opening of channels in the membrane which

are permeable to Na^+ and K^+ ions, but not Cl^- ions (Takeuchi and Takeuchi, 1960). The amplitude of the ACh response is linearly related to the membrane potential. At the reversal potential (E_r), the current due to the net K^+ movement is exactly balanced by the current due to an opposite net movement of Na^+, thus no potential change is observed. Takeuchi and Takeuchi (1960) have studied the influence of ionic environment on the reversal potential and have shown that the magnitude of the reversal potential is dependent on the Nernst equilibrium potentials for Na^+ (E_{Na}) and K^+ (E_K) and the ratio of conductance changes to Na^+ and K^+ ($\Delta g_{Na}/\Delta g_K$). This relationship is described by the following equation:

$$E_r = \frac{E_K + (\Delta g_{Na}/\Delta g_K)E_{Na}}{1 + \Delta g_{Na}/\Delta g_K}. \quad (1)$$

A measure of the ionic selectivity of the ACh receptor, given by $\Delta g_{Na}/\Delta g_K$, can thus be estimated from E_r provided that E_K and E_{Na} are known. This equation describes an equivalent circuit composed of two parallel ionic batteries (E_K and E_{Na}) and their respective conductances (g_K and g_{Na}).

The oscilloscope traces during the determination of an ACh reversal potential in control medium are shown in Fig. 1 *a*. The ACh sensitivity of this myotube was 110 mV/nC at the resting transmembrane potential (E_m) of −55 mV. As in most cases, a plot of E_m vs. ACh response (Fig. 1 *c*, closed circles) was linear and actual reversal of the ACh response was obtained. The membrane potential at which the straight line (determined visually) intercepted the abscissa was taken as the reversal potential. The E_r of the myotube shown in Fig. 1 *a* was 0 mV. When reversal of the ACh response could not be obtained, or when the response was not linear, the data were discarded. Such infrequent instances can be attributed to voltage-dependent changes in membrane resistance or to changes in the size of the iontophoretic pulse of ACh. Large depolarizations, hence reversal of the ACh response, were particularly difficult to obtain in older cultures due to delayed rectification of the membrane.

The E_r measured in rat myotubes is independent of the age of the culture even though the resting transmembrane potential increases approximately five-fold during cell development (to be published). For these studies myotubes of all ages were used. However, the most convenient age was about 4–5 days in culture. Such myotubes had resting potentials near −40 mV and delayed rectification of the membrane was not pronounced. The mean reversal potential in standard BSA medium was -2.6 ± 1.7 mV ($N = 13$). All values are reported as the mean ± the standard deviation except where otherwise noted. The E_{Na} and E_K of the myotubes in this medium were +50 and −87 mV, respectively, and did not change appreciably during cell development in culture. These values were calculated from internal Na^+

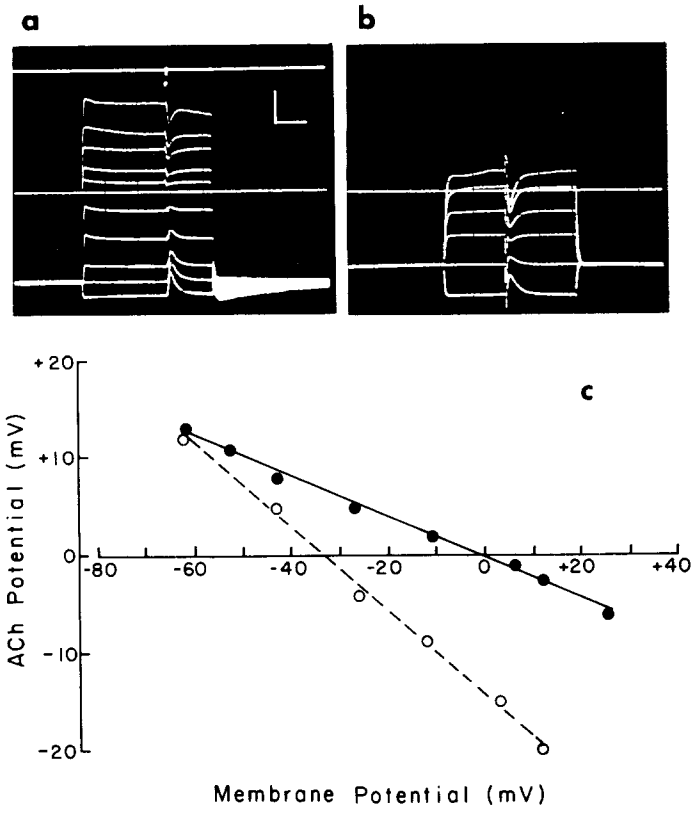

FIGURE 1. (*a*) Oscilloscope traces for measurement of the ACh reversal potential of a cultured rat myotube in standard medium. The ACh potential was recorded through an intracellular microelectrode as the membrane potential was displaced with a second intracellular current-passing electrode. The duration of the polarizing current was 400 ms. The duration of the iontophoretic pulse was 5 ms. The vertical calibration represents a 20-mV displacement for the recording electrode (hyperpolarizations in the downward direction) and a current of 5×10^{-8} A for the iontophoretic pipette (top trace, outward current in the downward direction). The horizontal calibration represents 100 ms. The middle trace is zero potential obtained at the end of the experiment by removing the recording electrode from the cell. The temperature was 37°C. (*b*) Oscilloscope traces for the measurement of an ACh reversal potential in medium containing 20 mM Na^+ and 5.3 mM K^+. The current from the iontophoretic pipette was not recorded in this trace. All other details are the same as in *a*. However, these measurements were not recorded from the same myotube shown in *a*. (*c*) A plot of the ACh response vs. membrane potential for the traces shown in 1 *a* (closed circles) and 1 *b* (open circles). The reversal potentials obtained from this plot were 0 mV and −33 mV for myotubes bathed in the control medium (136 mM Na^+, 5.3 mM K^+) and in the medium containing low Na^+ (20 mM Na^+, 5.3 mM K^+). In the latter case, isotonicity was maintained by replacing NaCl with sucrose.

(13 ± 1 mM; mean ± SEM, $N = 6$) and K^+ (153 ± 5 mM; mean ± SEM, $N = 6$) concentrations which were determined in rat myotubes by flame photometry (to be published). Using Eq. 1 the calculated value of the $\Delta g_{Na}/\Delta g_K$ ratio was 1.46.

Effect of External Ions on the ACh Reversal Potential

The reversal potential can also be considered to be an equilibrium potential whose magnitude might be expected to obey the Goldman constant field equation (Goldman, 1943) which has been successfully applied to describe the relative permeability of the resting membrane to Na^+ and K^+ ions (Hodgkin, 1951). This relationship is given in Eq. 2:

$$E_r = \frac{RT}{F} \ln \frac{K_o + P_{Na}/P_K(Na_o)}{K_i + P_{Na}/P_K(Na_i)}, \quad (2)$$

where P_{Na}/P_K is the relative permeability ratio of Na^+ to K^+ ions and the subscripts i and o refer to the concentrations of the respective ions inside and outside the cell. R, T, and F have their usual meaning. The Goldman equation was derived from thermodynamic flux equations, which, if translated into electrical terms, would predict large changes in channel conductance proportional to changes in external Na^+ or K^+ concentrations. Takeuchi and Takeuchi (1960), however, have shown that the ratio of Na^+ to K^+ conductance changes remains constant over a wide range of external Na^+ and K^+ concentrations (Eq. 1). Under ordinary circumstances, the Goldman equation cannot be used to describe the ACh reversal potential in adult frog muscle. The E_r of cultured rat myotubes was measured over a range of external ion concentrations and compared to values predicted by either Eq. 1 or 2.

Chloride Ions

Since the ACh receptor in adult frog muscle is impermeable to chloride ions an expression for the contribution of chloride to the E_r is absent in both Eqs. 1 and 2. Our results indicate that the receptor in cultured myotubes is also impermeable to Cl^- ions. When the Cl^- concentration in the test solution was completely replaced by sulfate ions the mean E_r was -3.0 ± 2.0 mV ($N = 6$) and did not differ from control medium ($-2.6 + 1.7$ mV, $N = 13$) containing 144 mM Cl^-. Isotonicity was preserved in the Cl^--free, $SO_4^=$-substituted medium by the addition of sucrose.

Sodium Ions

A decrease in the external Na^+ concentration to 20 mM produced a marked shift in the E_r (Fig. 1 *b* and *c*, open circles) to −33mV. In this example, iso-

tonicity was preserved by replacing NaCl with sucrose. Results are summarized in Fig. 2 in which E_r is plotted against the log of the external Na^+ concentration. The external Na^+ concentration was varied between 10 and 136 mM. Each solution was made isotonic by replacing NaCl with sucrose (closed circles) or Tris chloride (open circles). Reversal potentials were measured as soon as possible after each new test solution and within 15 min

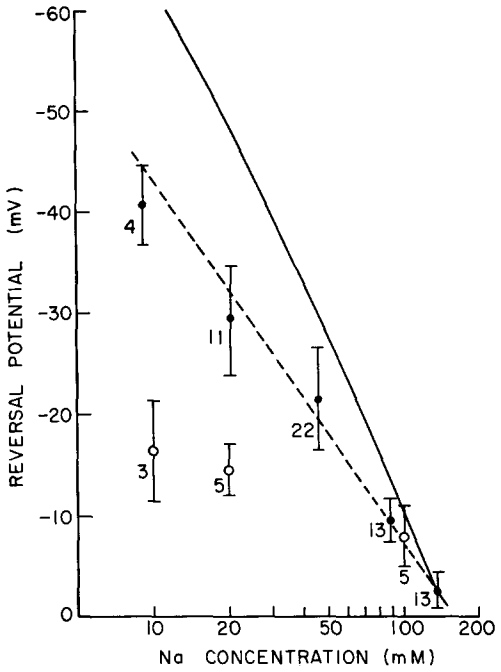

FIGURE 2. ACh reversal potential vs. external Na^+ concentration. The closed circles represent instances where NaCl in the medium was replaced by sucrose, and the open circles represent replacement of NaCl with Tris chloride. Theoretical lines are drawn for the E_r predicted at each Na^+ concentration by Eq. 1 (dashed line) or Eq. 2 (solid line). The temperature was 37°C. The number of myotubes examined at each concentration is provided in parentheses. Each point represents the mean ± SD.

of the change. There was no general trend between the first and the last reversal potential measured. On occasions when myotubes were returned to their normal control solutions, they gave reversal potentials identical to those obtained before equilibration in a test solution. It is thus unlikely that substantial changes in internal ions had occurred.

The calculated responses predicted by Eq. 1 (dashed line) and Eq. 2 (solid line) are also drawn in Fig. 2. In Eq. 1 a value of 1.46, determined in control medium, was inserted for the conductance ratio. The line for Eq. 2 was generated by using a pNa/pK ratio of 1.097 also calculated from the E_r

in control medium. When NaCl is replaced by sucrose, the change in reversal potential conforms reasonably well to Eq. 1 and is thus similar to results observed in the adult frog muscle (Takeuchi and Takeuchi, 1960). If NaCl is replaced by Tris chloride the reversal potentials are less negative than the values predicted by either equation. Either there is a finite conductance due to $Tris^+$ ions or $Tris^+$ ions inhibit the K^+ conductance change. The former explanation is more likely since the E_r in medium in which Na^+ is completely replaced by $Tris^+$ was -13.8 ± 2.2 mV ($N = 5$), i.e., far from the K^+ equilibrium potential.

Potassium Ions

The external K^+ concentration was varied between 0.44 and 50 mM at two different concentrations of Na^+ ions. These results are presented in Fig. 3 and are plotted as the reversal potential vs. log of external K^+ concentration. When Na_o was 136 mM (closed circles) the reversal potential changed very little between 0.44 and 10 mM K^+ and thus appeared to approximate the E_r predicted by Eq. 2 (solid line). In order to elicit a change in response to external K^+ and also avoid the problems of hypertonicity, the experiments were repeated in 46 mM Na_o (Fig. 3, open circles). In this case, K^+ was varied between 5.3 and 50 mM, and isotonicity was preserved by the addition of sucrose to the medium. The dashed line, predicted by Eq. 1, was generated as before by using the $\Delta g_{Na}/\Delta g_K$ ratio of 1.46 obtained in control medium. Since the pNa/pK ratio of Eq. 2 is a variable function of the external Na^+

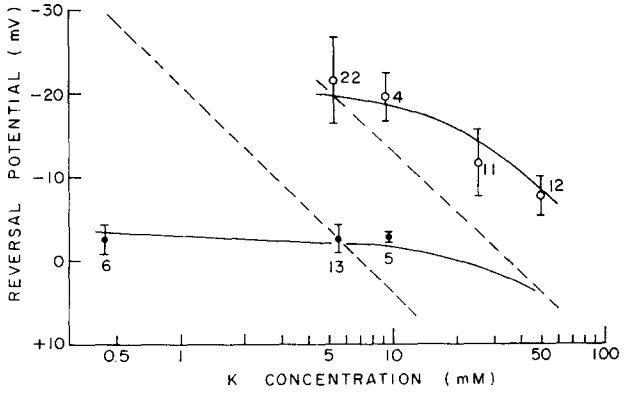

FIGURE 3. ACh reversal potential vs. external K^+ concentration. The K^+ concentration was varied at two different concentrations of external Na^+. The Na^+ concentration was either 136 mM (closed circles) or 46 mM (open circles). Isotonicity was maintained by varying the amount of sucrose in the medium. The theoretical lines are drawn for the E_r predicted at each K^+ concentration by Eq. 1 (dashed lines) or Eq. 2 (solid lines). The temperature was 37°C. The number of myotubes examined at each concentration is provided in parentheses. Each point represents the mean ± SD.

concentration (i.e., Eq. 2 which assumes constant pNa/pK ratios, does not describe the change in E_r in response to external Na concentration), the change in Na_o from 136 mM (closed circles) to 46 mM (open circles) required the selection of a different pNa/pK ratio than was observed in control medium. The pNa/pK value calculated from Eq. 2 when using the E_r value predicted by Eq. 1 for this concentration of external Na^+ was 1.75. This was also the pNa/pK value, which, when inserted into Eq. 2, produced the line of least squares deviation from the experimental points. The solid line in Fig. 2 was generated by using this pNa/pK value. Thus, in contrast to the results observed for Na^+, the response of the E_r to external K^+ concentrations is best predicted by Eq. 2 and not at all by Eq. 1.

Effect of Ca^{++} Ions

The ACh receptor of adult frog muscle is slightly permeable to Ca^{++} ions (Takeuchi, 1963a; Jenkinson and Nicholls, 1961). Furthermore, increases in external Ca^{++} concentration tends to shift the E_r of frog muscle towards slightly more negative values (Takeuchi, 1963 *b*). Rat myotubes which were bathed in media containing 30 mM Ca^{++} exhibited E_r similar to those obtained in 1.26 mM Ca^{++} (Table I).

Effect of pH

Mallart and Trautmann (1973) have shown that an increase in pH from 7 to 8 produced a change in the reversal potential of frog end-plate receptors from −15 to −42 mV. A similar change was not found in cultured rat myotubes when the pH was varied between 6 and 9 in 18 mM HEPES buffered medium at 37°C (Table II). Since Mallart and Trautmann used frog muscle which had been pretreated with hypertonic glycerol, we repeated the experiment with rat myotubes which had been similarly treated. The subsequent measure of E_r in Tris-buffered medium (18 mM) between 7.3 and 9.5 also failed to produce any substantial changes.

TABLE I

EFFECT OF EXTERNAL Ca^{++} CONCENTRATION ON ACETYLCHOLINE REVERSAL POTENTIALS

K^+	Na^+	ACh E_r 1.3 mM Ca^{++}	ACh E_r 30 mM Ca^{++}
mM	*mM*	*mV*	*mV*
5.3	87	−9.5±2.2 (13)	−11.8±1.3 (5)
5.3	9	−40.7±3.9 (4)	−39, −39, −30
50	46	−7.7±2.3 (12)	−10.7±1.2 (5)

The data are presented as the mean ± SD, where indicated, with the number of observations given in parentheses. The values for the E_r in normal Ca^{++} concentrations (1.3 mM) are also plotted on the graphs shown in Figs. 2 and 3.

TABLE II
EFFECT OF pH ON ACETYLCHOLINE REVERSAL POTENTIALS

pH	E_r
	mV
6.4	−1.2±2.2 (5)
7.0	−3.0±1.7 (5)
7.4	−2.6±1.7 (13)
8.3	−1.8±2.5 (5)
8.9	+1.9±2.2 (5)

The data are presented as the mean ± SD with the number of observations given in parentheses.

Attempts to Alter the Ionic Properties of the ACh Receptors by Various Conditions

Recently, conditions have been reported, such as low temperature, partial alpha-bungarotoxin blockade, and use of cholinergic agonist, which induce reversible shifts in E_r causing the receptor in adult frog and rat muscle to obey Eq. 2 rather than Eq. 1. The magnitude of the reversal potential was thus investigated under a variety of different conditions.

Reduction in the bathing temperature from 37 to 15°C failed to alter the response of the receptor to either Na_o or K_o (Table III). Partial alpha-bungarotoxin blockade (α-BuTx) of the ACh receptors likewise failed to alter E_r appreciably (Table III).

When receptors in adult frog and rat muscle are activated with the cholinergic agonist suberyldicholine a shift to Goldman equation behavior is also observed in varying external potassium concentrations (Dunin-Barkovski et al., 1969; Magazanik and Potapova, 1969). Its effectiveness was attributed to its ability to hold individual channels open for a longer period of time as compared to ACh. Since decamethonium produces markedly shorter channel open times in rat diaphragm muscle when compared to ACh and suberyldicholine (Katz and Miledi, 1973 *a*), we attempted to shift the Goldman-type response already observed in rat myotubes for external K^+ ions to the Eq. 1 response typical of adult muscle. Decamethonium, however, produced responses identical to those observed with ACh as agonist (Table III).

DISCUSSION

Ionic Specificity of ACh-Induced Conductance

The ionic properties of the ACh receptor have been studied in cultured rat myotubes under a variety of conditions and compared with similar studies on the adult skeletal muscle fiber, in vivo. The mean reversal potential observed in standard medium at 37°C was −2.6 mV. This is within the range of −10 to +10 mV reported for the cultured chick myotube (Fischbach, 1972; Harris et al., 1973) and myotubes of the rat myoblast L6 clonal cell

TABLE III

EFFECT OF TEMPERATURE, ALPHA-BUNGAROTOXIN, AND DECAMETHONIUM ON THE REVERSAL POTENTIAL IN MEDIA OF VARYING K^+ AND NA^+ CONCENTRATIONS

Condition	K_o	Na_o	E_r
	mM	*mM*	*mV*
37°C (control)	5.3	136	−2.6±1.7 (13)
15°C			−3.0±1.1 (5)
37°C (control)	5.3	20	−29.3±5.5 (11)
15°C			−29, −33
37°C (control)	5.3	9	−40.7±3.9 (4)
15°C			−40, −41
37°C (control)	50	46	−7.7±2.3 (12)
15°C			−10, −10, −9.5
ACh alone (control)	5.3	136	−2.6±1.7 (13)
ACh + partial α-BuTx block			−0.4±0.9 (5)
ACh alone (control)	5.3	46	−21.4±5.1 (22)
ACh + partial α-BuTx block			−20, −16
ACh alone (control)	50	46	−7.7±2.3 (12)
ACh + partial α-BuTx block			−8, −9.5
ACh (control)	0.44	136	−2.4±1.8 (6)
Decamethonium			−1, −1, −4
ACh (control)	5.3	136	−2.6±1.7 (13)
Decamethonium			−4, 0
ACh (control)	9	136	−2, −1
Decamethonium			−2, −1

The data are presented as the mean ± SD, where indicated, with the number of observations given in parentheses. The control values are also plotted on the graphs shown in Figs. 2 and 3.

line (Steinbach, 1973). These values are also near the −7- to −20-mV reversal potential found in vivo for innervated and denervated muscle of the rat diaphragm (Magazanik and Potapova, 1969), in several muscles of the denervated rat leg (Thesleff and Albuquerque, 1967), and in the denervated cat tenissimus muscle (Axelsson and Thesleff, 1959). The calculated value of the $\Delta g_{Na}/\Delta g_K$ ratio in rat myotubes was 1.46 for all ages studied. This is similar to the value of 1.29 calculated from the −15 mV reversal potential found in the end-plate receptors of innervated frog muscle (Takeuchi and Takeuchi, 1960; Feltz and Mallart, 1971). These values are in contrast to the ratio of 0.60 reported for the extrajunctional receptors of innervated frog muscle (Feltz and Mallart, 1971) and of the end-plate region of denervated

frog muscle (Mallart and Trautmann, 1973). The reversal potential in these instances was −42 mV.

Mallart and Trautmann (1973) also found that the E_r of both innervated and denervated frog muscle could be shifted reversibly between −15 and −42 mV by acid-base titration of the external medium. At pH 9 both muscle states gave an E_r of −42 mV. Cultured rat myotubes, on the other hand, gave reversal potentials which were insensitive to changes in pH between 6 and 9.

The rat myotubes were found to differ from adult frog muscle in their response to external calcium ions. In frog muscle an increase in external Ca^{++} concentration (up to 30 mM) produced a slight shift in E_r towards more negative values (Takeuchi, 1963 *b*) while no change in E_r was observed in rat myotubes.

The magnitude of the reversal potential in rat myotubes was also unaffected by complete removal of Cl^- ions from the medium. The cultured myotubes are thus similar in this respect to the adult muscle where more rigorous studies indicate that the receptor is impermeable to Cl^- ions (Takeuchi, 1963 *a*; Jenkinson and Nicholls, 1961).

Dunin-Barkovskii et al. (1969) have found that activation of frog end-plate receptors with suberyldicholine, but not decamethonium, renders the receptor permeable to $Tris^+$ ions. In cultured rat myotubes we find that when Tris chloride is used to replace NaCl in the medium there is a much smaller dependence of E_r on Na_o than is observed with sucrose substitution. Since complete replacement of Na^+ ions with $Tris^+$ in our medium still resulted in an E_r of −14 mV, rather than the K^+ equilibrium potential, we have concluded that the ACh-activated receptor in rat myotubes is also permeable to $Tris^+$ ions. Tris is a relatively large ion which has been shown to be impermeant to the Na^+ channel of the myelinated frog nerve (Hille, 1971). On the other hand, $Tris^+$ can apparently replace Na^+ ions in producing a generator potential at the crayfish stretch receptor (Grundfest, 1967).

Additional similarities and several differences between cultured rat myotubes and adult muscle were observed when the E_r was examined in media of different external Na^+ and K^+ concentrations. Reduction in Na_o, by replacement of NaCl with sucrose, produced a linear dependence of E_r on log Na_o which conformed reasonably well to Eq. 1, first described by Takeuchi and Takeuchi (1960) for adult frog muscle. The reversal potentials of myotubes which were bathed in media of varying K_o were not predicted by Eq. 1, but agreed reasonably well with values predicted by the Goldman constant field equation (Eq. 2). Although Takeuchi (1963 *a*) did find that external concentrations of K^+ greater than 10mM tended to produce more negative reversal potentials in adult frog muscle than those predicted by Eq. 1, this effect was not nearly as pronounced as the responses which we have ob-

served in rat myotubes, and which conform to Eq. 2. Previous observations on adult frog and rat skeletal muscle also indicate that the equation describing the dependence of E_r on K_o can, under certain circumstances, go from Eq. 1 to Eq. 2. Our attempts to similarly manipulate the Na^+ and K^+ dependence of the receptor between the responses predicted by the two equations were unsuccessful but in rat myotubes the K^+ dependence is already described by Eq. 2. These attempts included lowering the temperature to 15°C, partially blocking ACh receptors with alpha-bungarotoxin, and using the agonist decamethonium rather than ACh. These results, along with information found in the literature, are summarized in Table IV for ease of comparison.

Thus, the ionic behavior of the ACh receptor of cultured rat myotubes re-

TABLE IV

SUMMARY ON THE RESPONSE OF THE REVERSAL POTENTIAL TO EXTERNAL Na^+ K^+ AND $TRIS^+$ IONS

Species	Agonist*	Temperature	E_r‡	Response to Na^+_o	Response to K^+_o	Response to $Tris^+$	References
		°C	mV				
Rat myotubes	ACh	37	−2.6	Eq. 1	Eq. 2	Permeable	This study
	ACh	15	−3.0	Eq. 1	Eq. 2	—	
	C10	37	−2.0	—	Eq. 2	—	
	ACh (partial α-BuTx block)	37	−0.4	Eq. 1	Eq. 2	—	
Rat diaphragm, denervated	ACh	20–22	−15	—	Eq. 1	—	Magazanik and Potapova, 1969
	Sub	20–22	−16	—	Eq. 2	—	
Rana pipiens, end plate	ACh	18–23	−15	Eq. 1	Eq. 1	—	Takeuchi and Takeuchi, 1960
Rana esculenta, end plate	ACh, CC, C10	15–18	−18	—	—	—	Feltz and Mallart, 1971
Rana esculenta, extrajunctional	C10	15–18	−42	Eq. 1	Eq. 1	—	—
Rana tempararia, end plate	ACh, CC, C4		−13 to −30	—	Eq. 1	—	Dunin-Barkovskii et al., 1969
	C10		−20	Eq. 1	Eq. 1	Impermeable	
	Sub		−20	—	Eq. 2	Permeable	
Rana tempararia, end plate	ACh	26	−18	—	Eq. 1	—	Bregestovski et al., 1972
	ACh	2	−18	—	Eq. 2	—	
Rana tempararia, end plate	ACh	22	−14	—	Eq. 1	—	Magazanik and Vyskocil, 1973
	ACh (partial α-BuTx block)	22	−16	—	Eq. 2	—	

*Abbreviations: ACh (acetylcholine), C4 (succinylcholine), Sub (suberyldicholine), C10 (Decamethonium), CC (carbachol), α-BuTx (alpha-bungarotoxin).

‡ E_r are reported for media containing approximately 5 mM K^+, 140 mM Na^+ (rats), and 2.5 mM K^+, 118 mM Na^+ (frogs).

sembles some of the properties reported for adult innervated and denervated muscle and also differs in many ways. Due to lack of in vivo data on fetal muscle before innervation, it is not clear whether these differences are peculiar to cultured myotubes or characteristic of muscle before innervation. It is also not known if these differences are due to changes in the molecular identity of the receptor or to differences in the microenvironment of the receptor.

Mechanisms of Ion Permeation

During the activation of a single ACh receptor there is a flow of approximately 2×10^7 ions, largely Na^+ and K^+, per second (Anderson and Stevens, 1973). The magnitude of the current indicates that ion permeation occurs through a channel rather than by a carrier mechanism. The selectivity of the receptor for cations further suggests that the channel is either lined with anionic sites, or contains a negatively charged selectivity barrier. The existence of separate channels for Na^+ and K^+ ions has also been proposed (Gage and Armstrong, 1968; Maeno, 1966), however, the data are not conclusive (Steinbach, 1968; Kordas, 1969; Magleby and Stevens, 1972). Recent studies on the time-course of end-plate potential decay, in fact, make a dual channel receptor very unlikely (Kordas, 1969; Magelby and Stevens, 1972).

One can further speculate on the nature of ion permeation based on the behavior of the reversal potential in media of different external Na^+ and K^+ ion concentrations. Eq. 2 can be employed to describe a charged, or uncharged, aqueous channel (or channels) with constant permeabilities for certain positively charged ions, namely Na^+ and K^+. Such channels exhibit conductances which vary in proportion to the concentration of the major permeant ions. Eq. 1 requires additional constraints since, in this case, conductances either remain constant, or vary but in such a manner that the conductance ratio to Na^+ and K^+ ions remains constant over a wide range of external Na^+ and K^+ ion concentrations.

In adult rat and frog muscle the receptors obey Eq. 1 but can reportedly be shifted to Eq. 2 behavior by special means. In cultured rat myotubes the response is mixed. The Na^+ dependence is described by Eq. 1 and the K^+ dependence by Eq. 2. At least three possible explanations for such behavior will be discussed and include (*a*) short channel open times, (*b*) ion-saturated channels, and (*c*) ion-sensitive gating mechanisms. Suggestion *a*, by Dunin-Barkovskii et al. (1969), proposes that the activated channel is open for such a short duration that channel conductances cannot change despite exposure to a wide concentration range of external Na^+ and K^+ ions (Eq. 1). Conditions which prolong the duration of the open channel, as shown by noise analysis for suberyldicholine and low temperature, (Katz and Miledi, 1973 *a*) could then allow the channel conductances to vary according to the external ion concentrations, hence conversion to Eq. 2 behavior. This proposal seems

unlikely for a number of reasons. Analysis of ACh noise (Katz and Miledi, 1972; Sachs and Lecar, 1973; Anderson and Stevens, 1973) indicates that channels are open for a relatively long period of time (between 1 and 10 ms at room temperature). This is sufficient time for the net flow of at least 2×10^4 ions (given a unit channel conductance estimated between 10^{-10} to 3×10^{-11} Ω^{-1}) which is likely to be several orders of magnitude greater than the number of ions present in the channel at any given moment. Furthermore, the receptor can be induced to go from Eq. 1 to Eq. 2 by a treatment, partial alpha-bungarotoxin blockade (Magazanik and Vyskocil, 1973), which does not prolong the mean duration of the channel open time (Katz and Miledi, 1973 *b*). Finally our attempts to manipulate the response of the receptor in cultured rat myotubes between Eq. 1 and Eq. 2 by methods which influence channel duration were unsuccessful. It is thus likely that some other time-independent constraint is responsible for the types of behavior observed.

Suggestion *b*, that the ion channels are saturated with ions at very low ion concentrations, quite readily explains Eq. 1 and could also explain conversion to Eq. 2, but is less likely to explain the mixed response present in rat myotubes. If affinity of the receptor for K^+ and Na^+ ions were very high, then the channels would saturate at low ion concentrations. Thus conductance would be maximal and independent of ion concentrations (Eq. 1). A proportional decrease in the affinity of the receptor for both Na^+ and K^+ could convert the channel to one described by Eq. 2. The mixed response (Eq. 1 for Na^+ and Eq. 2 for K^+) observed in cultured rat myotubes could result from a high affinity for Na^+ ions and a much lower affinity for K^+ ions. This idea seems unlikely in this case since the relative ion permeability to Na^+ and K^+ in normal medium is approximately 1.

The third possibility is the presence of ion-binding sites on the receptor for modulating ion permeabilities. Adult muscle would require sites for both Na^+ and K^+ ions. Such sites could be rendered ineffective or inaccessible under certain conditions (e.g., low temperature, partial alpha-bungarotoxin blockade, and suberyldicholine activation). In such a model, cultured myotubes require only an Na^+-sensitive site. The criticism of this model is that ion permeabilities must vary in response to Na^+ and K^+ ions in a very rigidly controlled manner in order to keep the Na^+ and K^+ conductance ratio constant.

None of the explanations offered are, at present, entirely satisfactory. The first is not likely in view of the recent data provided by ACh noise analysis. Certain features of models *b* and *c* can be tested by obtaining data on the magnitude and variability of the separate Na^+ and K^+ ion conductances. For example, if the Na^+ conductance, in particular, were found to vary in solutions of different Na^+ concentrations, this would rule out any mechanism such as *b*, which assumes that the conductance for this ion is already maximal.

Model *c* only requires that the ratio of Na^+ to K^+ conductance changes remain constant.

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