

Electrophysiological Properties of the Membrane and Acetylcholine Receptor in Developing Rat and Chick Myotubes

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ABSTRACT Membrane properties of rat and chick myotubes in various stages of development were studied. Resting membrane potentials (E_m) increased from -8 to -55 mV in both rat and chick as the myotubes developed from myoblasts to large multinucleated fibers. In the rat myotubes, this increase was not accompanied by significant changes in specific membrane resistivity or changes in Na^+ and K^+ ion distribution. Nor have we observed a significant electrogenic component to the resting E_m of mature rat myotubes under normal circumstances. A progressive increase in the passive permeability of the membrane to K^+ relative to Na^+ ions has been observed which can account for the changes in E_m with development. In contrast to the changes in the ionic selectivity of the membrane, we have found that the ionic selectivity of the ACh receptor of rat and chick myotubes remains constant during the same period of myotube development.

INTRODUCTION

Two important properties of mature skeletal muscle fibers are a large resting transmembrane potential and surface receptors which mediate depolarization of the end-plate region of muscle fibers when activated by the chemical transmitter, acetylcholine. The magnitude of both the resting membrane potential and potential changes in response to acetylcholine depend upon ion-selective channels in the membrane, and upon ion gradients (largely sodium and potassium) across the membrane. While these properties have been widely studied in the adult skeletal muscle, little is known regarding the development of these properties during cell differentiation. The ion channels responsible for the passive membrane permeability and the channel associated with the activated acetylcholine receptor might change, not only in number but in character, and the steady-state distribution of the ions across the membrane might also change as the membrane composition changes to accommodate the specialized function of the differentiated muscle fiber. Using electrophysio-

logical techniques, we have examined the development of these membrane properties during the maturation of myotubes maintained in culture. Tissue cultured cells are particularly well suited to such a study since differentiation and maturation of muscle in culture is easily observed, the extracellular ionic composition is easily controlled, and electrophysiological studies involving placement of more than one microelectrode are greatly facilitated.

Cultured myogenic cells first proliferate then fuse within 2–3 days to form multinucleated myotubes. The myotubes further differentiate into cross-striated muscle fibers capable of action potential generation and contraction (Konigsberg, 1963; a review). The development of acetylcholine sensitivity (Fambrough and Rash, 1971) and some electrophysiological properties of cultured myotubes (Fischbach et al., 1971; Powell and Fambrough, 1973; Kidokoro, 1973; Harris et al., 1973; Kano and Shimada, 1973; Land et al., 1973; Fukuda, 1974) have been described previously. In this paper we describe the ionic basis for the generation of the transmembrane potential and the acetylcholine potential during cell differentiation. A preliminary report was presented at the Fourth Annual Meeting of the Society for 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). The trypsin-dissociated cells were obtained from rat fore and hind limb of 17–18-day-old embryos (Sprague Dawley). The 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% CO₂ in air. The cells were plated at 5 × 10⁴ per culture dish.

Embryonic chick muscle was isolated from 11-day-old eggs (Truslow Farms, Chestertown, Md.). The cells were dissociated by vortex mixing for 1 min in 0.5 ml of medium per leg. The cell suspension was filtered through gauze and silk and plated at approximately 10⁵ cells per culture dish.

The L6 myoblast cell line was a gift from Dr. David Schubert of the Salk Institute, San Diego, Calif. These cells were grown in modified Eagle's medium containing 20% fetal calf serum (Vogt and Dulbecco, 1963).

Electrophysiology

Cultures were mounted on an inverted phase microscope with a heating stage to maintain cultures at 36°C. When rapid solution or temperature changes were desirable the medium was confined to a small rectangular area of the dish approximately 10 x 35 x 5 mm deep. A Holter roller pump (Extracorporeal Medical Specialties, Inc., King of Prussia, Pa.) was used to perfuse the dish with various solutions at a rate of 100 ml/h. The temperature of the solution was lowered by preequilibrating the

medium and circulating the heating stage with refrigerated water. Temperature was monitored with a thermocouple placed in the bathing medium.

Membrane potentials were measured with an intracellular recording electrode filled with 2 M KCl (Tasaki et al., 1968). The recording electrode was connected to a negative capacitance feedback ELSA-4 Bak amplifier (Electronics for Life Sciences, Inc., Hastings-on-Hudson, N. Y.) through a silver-silver chloride wire. Electrodes had resistances between 20 and 150 M Ω and time constants no greater than 300 μ s. The higher resistance electrodes were most often used with the younger myotubes. The bath electrode was a 150 mM NaCl agar bridge which was connected to ground via a silver-silver chloride 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.). An impalement was considered valid if the transmembrane potential (E_m) did not change by more than 5 mV over a period of at least 10 s or for the duration of the experiment. Stable membrane potentials have been recorded from individual myotubes for over 30 min.

Tip potentials were measured by breaking the tip of the electrode at the termination of the experiment and noting the change in ground potential. Data were discarded when tip potentials were found to be greater than 5 mV. However, these instances were rare.

Passive electrical properties of the membrane were determined by measuring the voltage response to hyperpolarizing current injected from a second intracellular microelectrode. This electrode was prepared like the recording electrode; however, the silver-silver chloride wire in this instance was connected to a pulse generator via a stimulus isolation unit (ISB 2.5, Bioelectric Instruments, Inc., Hastings-on-Hudson, N. Y.). The resistance to ground of this unit was greater than $10^9 \Omega$. When the membrane resistances of myotubes were measured in low chloride medium the stimulating electrode was filled with 2 M K citrate rather than KCl. The recording electrode was placed in the widest part of the myotube (usually near the middle of the myotube) and the current electrode was placed less than 20 μ m away. The input resistances (R_{in}) were calculated from linear voltage-current curves comprised of four to six points. Voltage-current curves which were nonlinear in the hyperpolarizing direction were discarded. The specific membrane resistance (R_m) was calculated by multiplying R_{in} by the surface area of the cell and assuming that the myotubes could be treated as spheres (see Results). A Polaroid photograph of each myotube was taken after the electrodes were withdrawn. The surface areas of these myotubes were estimated from the photographs by modeling each myotube as a series of right cones and cylinders. Membrane time constants (T_m) were obtained by determining the amount of time necessary to reach 63% of V_{max} (Stefani and Steinbach, 1969). V_{max} was approximately 20 mV in the hyperpolarizing direction. The specific membrane capacitance (C_m) was calculated from the relationship $T_m = R_m \times C_m$.

Acetylcholine reversal potentials (E_r) were measured with an intracellular recording electrode during iontophoretic application of acetylcholine (ACh). The steady-state membrane potential was altered by passing a 200–500-ms current through a

second intracellular electrode. Microelectrodes for iontophoresis were filled with 2 M AChCl and connected to a pulse generator via a stimulus isolation unit and a source of biasing current. The resistances of these electrodes were usually between 50 and 120 M Ω . All three electrodes were placed within 20 μm of each other. Relatively long-term application of ACh was achieved by removing the source of biasing current (up to 30 s) on the iontophoretic pipette or by adding AChCl to the bathing medium (up to 100 min).

Potassium-Specific Electrode

Potassium-specific ion electrodes were prepared with slight modifications of the method described by Brown et al. (1970). Blunt micropipettes were pulled from capillary tubing (Corning 7750, Corning Scientific Instruments, Medfield, Mass.) which had been washed in HCl and rinsed with distilled water. The tips were silicized with 2% Siliclad (Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N.J.) in alpha-chloronaphthalene (Fisher Scientific Co., Pittsburgh, Pa.) then dried for 2 h at 200°C. After filling a pipette with 500 mM KCl, liquid K⁺ ion exchanger (Corning 477317) was drawn into the pipette tip by gentle suction, making a column 100–1,000 μm in height. The pipette was connected to a high impedance electrometer amplifier (Analog Devices 311J, Analog Devices, Inc., Norwood, Mass.) via a silver-silver chloride wire. The response of the electrode was monitored on a Tektronix oscilloscope. The ground electrode was a silver-silver chloride wire connected to the bath via a 500 mM KCl agar bridge. The response of the electrode in KCl standards between 2 and 500 mM was 58 mV per decade at 36°C. This was less than the theoretical slope of 61 mV per decade expected at that temperature. Each electrode was calibrated in perfusion media at 36°C containing different amounts of Na⁺ and K⁺; the selectivity varied between 40:1 and 100:1 in favor of K⁺, depending on the individual electrode. The time constant of the electrode was measured by introducing a 20-mV potential change between the bath and ground. The time constants varied between 5 and 100 ms, depending on the fineness of the tip and the height of the ion exchange column. The potassium electrode was used to measure the K⁺ concentration immediately surrounding a myotube while the potassium ion concentration of the medium was being changed by replacement of regular medium (Table I, *b*) with a solution of high potassium and low sodium concentration (Table I, *c*).

Sodium and Potassium Determinations

The Na⁺ and K⁺ content of the cultured cells was analyzed by flame photometry, using a modification of a procedure described by McDonald and DeHaan (1973). Myotubes were grown on four to six culture plates (9-cm diameter) containing 6–8 $\times 10^5$ cells per plate. After 1 day in culture cells were treated for 2 days with 1 $\mu\text{g}/\text{ml}$ of cytosine arabinoside (Sigma Chemical Co., St. Louis, Mo.) to eliminate almost all of the fibroblast population of the cultures (Fischbach, 1972). Myotubes were removed from the plates at the appropriate time by treatment with collagenase (Worthington Biochemical Corp., Freehold, N.J.) for 30 min at a final concentration of 40 U/ml. The cells were collected by centrifugation at 1,800 rpm (IEC PR2, International Equipment Co., Boston, Mass.) for 2 min at 4°C. The supernatant was aspirated and

TABLE I
IONIC COMPOSITION OF MEDIA

	K ⁺	Na ⁺	Li ⁺	Cl ⁻	SO ₄ ⁻	Mg ⁺⁺	Ca ⁺⁺	PO ₄ ⁻	CH ₃ SO ₄ ⁻	Pro- pionate	Sucrose
(a)	5.1	153	—	147	0.33	0.8	1.0	0.7	—	—	—
(b)	5.3	142	—	150	0.41	0.9	1.3	0.8	—	—	—
(c)	148	0.4	—	150	0.41	0.9	1.3	0.8	—	—	—
(d)	10	138	—	80	32.5	0.9	1.3	0.8	—	—	32
(e)	25	123	—	32	56.5	0.9	1.3	0.8	—	—	56
(f)	50	98	—	16	64.5	0.9	1.3	0.8	—	—	64
(g)	100	48	—	8	68.5	0.9	1.3	0.8	—	—	68
(h)	148	0.4	—	5.4	69.8	0.9	1.3	0.8	—	—	69
(i)	5.3	142	—	—	71.2	0.9	1.3	0.8	—	—	71
(j)	148	0.4	—	—	74.2	0.9	1.3	0.8	—	—	71
(k)	5.3	142	—	5.3	0.41	0.9	1.3	0.8	144	—	—
(l)	5.3	0.4	141	150	0.41	0.9	1.3	0.8	—	—	—
(m)	148	0.4	—	3.5	0.41	0.9	1.3	0.8	—	147	—

All concentrations are listed as millimoles per liter. These solutions also contain 18 mM HEPES buffer, pH 7.2, 0.5% BSA, 0.2% glucose, and penicillin, streptomycin, and fungizone. Varying amounts of sucrose are present in the medium where necessary to maintain isotonicity. Solution *a* is a HEPES buffered modified Ham's F12 medium which contains various amino acids and vitamins in addition to the items already indicated. Solutions of varying Na⁺ and K⁺ content were prepared by mixing *b* and *c*. Solutions *d* through *h* contain varying amounts of K⁺ and Na⁺ while maintaining a constant [K⁺] × [Cl⁻] product equivalent to the product which is present in *b*, the standard solution. Solutions of varying K⁺ and Na⁺ concentration in Cl-free medium were prepared by mixing *i* and *j*.

the cells were resuspended in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonate) buffered Ham's medium for 60 min at 37°C to allow the cells to recover from the collagenase treatment. The cells were then collected by centrifugation and resuspended in 1.0 ml of medium containing 0.1 μCi/ml of [¹⁴C]inulin (New England Nuclear, 5 mCi/mmol). Aliquots of 0.3 ml were added to four small-bore polyethylene centrifuge tubes and centrifuged for 2 min at 1,800 rpm. Aliquots of the supernatant were counted by scintillation spectrometry in triton X-100:toluene scintillation cocktail (1:2). The four pellets were extruded onto two small tared coverslips and into two tared low-alkali test tubes (Thermal American Fused Quartz Co., Montville, N.J.) and weighed after 45, 60, 90, and 120 s. The wet weight of the pellet was determined by linear extrapolation to time zero. The coverslips were dried overnight at 110°C, cooled in a dessicator, then weighed. The water content of the pellet was taken as the difference between the wet and dry weight. The coverslips were then placed in a scintillation vial overnight with 0.15 ml distilled water and 1.0 ml of NCS solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.). After the addition of 10 ml of toluene scintillation cocktail (16 g PPO (2,5-diphenyloxazole), 0.2 g POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene) in 1 gallon toluene), radioactivity was counted on a Packard Tri Carb Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The counting efficiency, determined by addition of standard [¹⁴C]toluene to each sample, was 85% for samples counted in

triton-toluene cocktail. The efficiency of NCS solubilized pellets counted in toluene cocktail was a few percent lower depending on the size of the pellet. The extracellular fluid was calculated from these measurements.

Pellets which had been extruded into low-alkali test tubes were digested with nitric acid in a heating block after determination of the wet weight. The dried residue was dissolved in a lithium chloride diluent and the Na^+ and K^+ contents were analyzed by flame photometry (National Instrument Laboratories, Fredricksburg, Va.). Values were rejected when a reasonable agreement between duplicate samples was not obtained.

Solutions

The composition of the various types of media used in this study are shown in Table I. The Na^+ and K^+ concentrations were determined by flame photometry. Electrophysiological experiments were generally performed in medium *a*, a HEPES buffered modified Ham's F12 medium. Perfusion experiments requiring large volumes of medium were performed in the simpler solutions indicated by *b* through *m* of Table I. In older cultures, 1 $\mu\text{g}/\text{ml}$ of tetrodotoxin (Calbiochem, San Diego, Calif.) was added to the recording medium to suppress action potential generation. Other compounds which have been added to the medium where indicated are ouabain (Nutritional Biochemical Corp., Cleveland, Ohio.), 2,4-dinitrophenol (Eastman Organic Chemicals, Div. Eastman Kodak Co., Rochester, N.Y.), atropine sulfate (Merck & Co., Inc., Rahway, N.J.), and iodoacetate (Eastman Organic Chemicals).

RESULTS

Passive Electrical Properties

The passive electrical properties were measured in developing rat myotubes at different ages in culture. Since immature myotubes are very short and spindle shaped, they do not meet the criteria necessary for either finite or infinite cable analysis. For this reason relatively short (less than 330 μm in length) cells of simple geometry were chosen for all ages studied and analyzed by assuming that such cells can be approximated by spheres of the same surface area with uniform distribution of current over the entire surface. This assumption seems justified by our observation that little, if any, change with distance was found in the voltage response to constant current injected into seven myotubes between 220 and 310 μm in length. Even the longest myotubes used in this study are also well within the space constant of 600 μm which has been reported for cultured mouse myotubes as determined by finite cable analysis (Powell and Fambrough, 1973).

R_{in} was generally determined from the slope of linear V vs. I curves, as shown in Fig. 1. Membrane time constants were determined only in cells which exhibited simple on-transient curves. The off-transient response to hyperpolarizing current was often complicated, especially in older myotubes, by the generation of an action potential (Fig. 1). Other complicated on- and

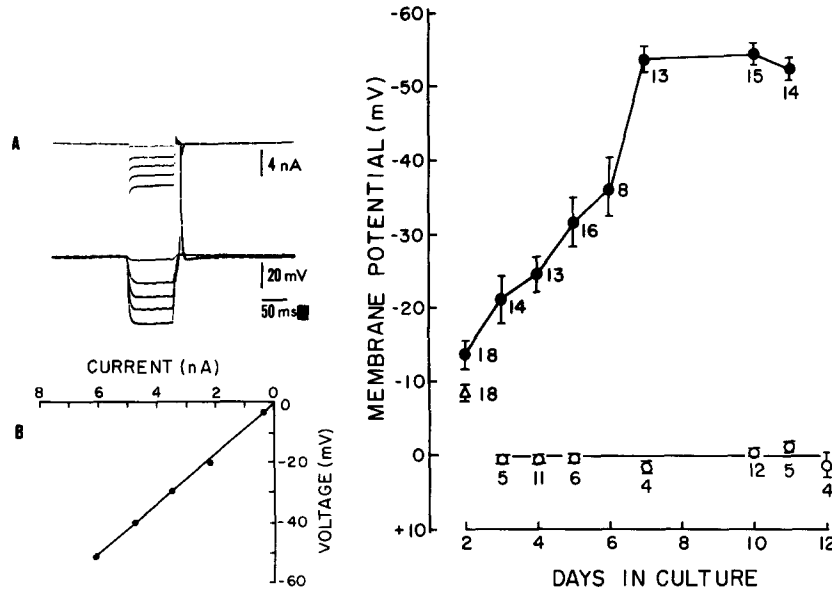


FIGURE 1

FIGURE 2

FIGURE 1. Voltage-current curve for a 7-day-old rat myotube. (A) Record showing the hyperpolarizing electrotonic potentials (lower lines) produced by 100-ms current pulses (upper lines) injected through a second intracellular electrode. The off-transient responses are complicated by the generation of an anode break action potential with a positive overshoot of 20 mV. (B) Voltage-current plot of the myotube recorded in A. The R_{in} calculated from the slope of this line was 8.9 M Ω . The surface area of this myotube was 10.9×10^{-5} cm 2 .

FIGURE 2. Membrane potentials and ACh reversal potentials of rat myotubes as a function of the age of the culture. The membrane potentials are represented by closed circles for myotubes and by the triangle for myoblasts. The open circles represent the ACh reversal potentials. Each point is represented as the mean \pm SEM with the number of myotubes indicated by the adjacent number. These measurements were made at 37°C in HEPES buffered Ham's medium (Table I, a).

off-transient responses were also observed (See Powell and Fambrough, 1973) but are not shown in this paper.

Membrane constants and cell dimensions are summarized in Table II for cultured rat myotubes. As the myotubes matured there was a trend towards increasing E_m which became maximal after 7 days in culture. During this time the specific membrane resistance also increased from 958 Ω cm 2 to 1,567 Ω cm 2 . The membrane time constant ranged between 2.4 and 5.1 ms with an overall mean of 3.8 ms. The specific membrane capacitance varied between 3.0 and 5.3 μ F/cm 2 with a mean of 3.7 μ F/cm 2 .

Membrane Potential vs. Age

The increase in E_m of cultured rat myotubes as a function of cell development has been studied in greater detail. The mean E_m of rat myoblasts in 2-day-old

cultures was -8 mV. There was a progressive increase in the absolute value of E_m as the myotubes matured (Fig. 2). A plateau was reached after approximately 7 days in culture with a mean E_m of -55 mV. Changes in E_m of similar magnitude and time-course were also observed in cultured chick myotubes (Table III).

It is conceivable that the smaller membrane potentials observed in younger myotubes were the result of a leakage shunt produced during impalement. Such leakage would cause a proportionately larger diminution in the measured transmembrane potentials of the smaller and, therefore, younger myo-

TABLE II
ELECTRICAL CONSTANTS FOR RAT MYOTUBES IN CULTURES OF DIFFERENT AGE

Age	E_m	Length	Width	Area	R_m	N	T_m	C_m	N
days	mV	μm	μm	$cm^2 \times 10^5$	Ωcm^2		ms	$\mu F/cm^2$	
3	-23.5 ± 2.5	135 ± 15	20 ± 1	4.8 ± 0.7	958 ± 127	15	3.4 ± 0.4	5.3 ± 1.3	7*
4	-31.1 ± 2.2	197 ± 17	22 ± 1	7.4 ± 0.7	974 ± 113	12	2.4 ± 0.3	3.2 ± 0.7	8*
5	-46.1 ± 1.4	237 ± 18	27 ± 2	9.6 ± 1.0	$1,000 \pm 94$	12	3.1 ± 0.1	3.0 ± 0.3	5
7	-51.2 ± 1.5	246 ± 28	30 ± 2	12.1 ± 1.4	$1,046 \pm 158$	11	4.9 ± 0.5	4.0 ± 0.3	6
7†	-53.8 ± 2.5	260 ± 18	32 ± 2	13.5 ± 0.4	$1,119 \pm 177$	5			
9	-51.2 ± 1.5	227 ± 23	33 ± 2	13.0 ± 1.6	$1,567 \pm 258$	6	5.1 ± 0.4	3.1 ± 0.4	4
9-14§	-45.7 ± 1.6	151 ± 25	22 ± 2	5.1 ± 0.4	654 ± 107	6			

These measurements were performed in standard medium (Table I, b) at 37°C. Each value represents the mean \pm SEM. With the exception of seven myotubes in the 3-day cultures and two myotubes in the 4-day cultures, all resistances were determined from the slope of four to six hyperpolarizations which were linear on a $V-I$ plot. Membrane time constants were only measured on a portion of the total number of cells for which R_m was determined and are indicated by the smaller number of N .

* C_m and R_m were determined in these myotubes from a single hyperpolarizing current. The R_m of these myotubes were very similar to the mean R_m of myotubes determined from four to six hyperpolarizations.

† These measurements were performed in low Cl^- , methylsulfate-substituted medium (k of Table I) with 2 M K citrate in the current-passing electrode.

§ These cells were plated at low density ($1 \times 10^4/35$ -mm dish) and subsequently grown for 2 days in $1 \mu M$ cytosine arabinoside.

TABLE III
MEMBRANE POTENTIALS AND ACETYLCHOLINE REVERSAL POTENTIALS IN DEVELOPING CHICK MYOTUBES

Age	E_m	N	E_r	N
days	mV		mV	
1-2	-11.8 ± 0.9	25	-4.1 ± 0.44	3
3	-19.6 ± 2.6	20	—	
4	-37.4 ± 2.9	21	-1.2 ± 1.2	5
5	-53.5 ± 1.2	18	—	
6	—		-0.5 ± 1.0	4
7	-52.3 ± 1.9	22	-3.0 ± 1.2	4
8	-54.5 ± 1.3	22	—	

These experiments were performed in HEPES buffered modified Ham's medium at 37°C. These values are reported as the mean \pm SEM for the number of observations indicated by N .

tubes. Although the small myotubes are more readily damaged, we feel that the contribution of leakage to the small E_m measured in young myotubes is minimal and that small membrane potentials are a developmental property of our primary muscle cultures for the following reasons. (a) If young myotubes did have high resting potentials then this would probably be evident from occasional successful impalements. In fact we have never recorded large resting potentials in young myotubes. (b) Unlike myoblasts of our primary rat cultures the myoblasts of the rat L6 clonal cell line have mean resting potentials of -60 mV (Kidokoro, 1973). To ensure that the difference observed between our cultures and the L6 cell line was a property of the cultures and not a difference in technique, we measured the E_m of L6 myoblasts 3 days after plating and also found a mean resting membrane potential of -55.9 ± 1.6 mV (mean \pm SEM; $N = 31$). (c) Further indication that size alone cannot be a sufficient cause for the measurement of small membrane potentials in young cells is that equally small but differentiated cells of our primary rat cultures had relatively large membrane potentials. Nine- to fourteen-day-old cells which had been plated at low density and had been treated subsequently with cytosine arabinoside were usually small due to the low index of fusion. The E_m of these cells was -47 mV (Table II) compared with the small E_m (-23 mV) of similar size cells which had been in culture for only 3 days.¹ While a small leakage component may contribute somewhat to smaller membrane potentials in small cells the magnitude of this effect is clearly not great enough to account for the very small resting potentials of young myotubes. (d) As will be shown later, the response of the E_m to media of varying external K^+ concentrations was nearly identical to the theoretical response predicted by the Goldman equation and yielded extrapolated values for internal K^+ ion concentrations which were similar for both newly formed and mature myotubes. Such behavior would not be expected of a very small myotube with a considerable leakage shunt. (e) The resting potentials which are calculated after correction for a shunt resistance produced by microelectrode impalement are still substantially lower in younger myotubes. Shunt resistance (R_s) was measured by making the assumption that R_s is the same for each of two electrodes inserted into the same myotube. If R_{in} is the "true" input resistance of the cell, R_1 the resistance measured with one electrode in place (via a bridge circuit) and R_2 the resistance measured with both electrodes in place, then $1/R_1 = 1/R_{in} + 1/R_s$ and $1/R_2 = 1/R_{in} + 2/R_s$. The mean R_1 and R_2 for five such myotubes were 30 and 23 M Ω , respectively. R_s ranged between 70 and 420 M Ω with a mean of 170 M Ω and the mean true input resistance was found to equal 42 M Ω . The true E_m (E) would then be -30 mV according to

¹ Cells treated with cytosine arabinoside seem to be more fragile than nontreated cells as judged by a higher frequency of unsuccessful impalements and this may account for the somewhat smaller R_m measured in these cells (Table II) compared with untreated myotubes of similar age.

the following relationship: $E = E_{obs} (R_{in}/R_1)$ where E_{obs} is equal to the mean observed E_m of -22 mV. By using similar assumptions we have also determined R_s by supposing a maximum decrease of 5 mV in E_m (see Methods) caused by insertion of the second microelectrode for those experiments reported in Table II. From the mean R_{in} ($R_2 = 26$ M Ω) and E_m ($E_{obs} = -23$ mV) found in 3-day myotubes an R_s of 95 M Ω was calculated. Such cells could have a true E_m of -32 mV and R_{in} of 36 M Ω . For 7-day myotubes ($R_2 = 8.6$ M Ω and $E_{obs} = -51$ mV) an R_s of 88 M Ω was calculated so that the true E_m and R_{in} in these cells could be -57 mV and 11 M Ω , respectively. Therefore, while some leakage current may contribute to the measurement of lower membrane potentials in all age cells, the extent of this leakage is of insufficient magnitude to account for the observed increase in E_m with age. The tendency for the microelectrode to create a shunt resistance, however, can lead to fairly large errors (twofold) in the measured R_m reported in Table II, especially in younger myotubes. One can still criticize the validity of this argument: for example, damage caused by insertion of the first electrode may be on average much larger than damage caused by insertion of the second electrode (i.e., the R_s for the second microelectrode could be much greater than, rather than equal to, R_s for the first electrode). However, further objections can only be resolved by techniques which are not dependent on the use of intracellular microelectrodes.

Effect of Temperature and Ouabain on Membrane Potentials

The resting membrane potential which results from the distribution of ions across a passively permeable membrane is relatively insensitive to large decreases in temperature over a short period of time. Although a decrease in temperature will inhibit the electrically neutral Na^+ - K^+ exchange pump the resulting change in Na^+ and K^+ ion distribution requires hours before a noticeable effect is observed on the resting E_m . A sodium electrogenic pump, however, directly contributes to the membrane potential by transporting more Na^+ ions out of the cell than the number of K^+ ions transported into the cells for each pump cycle. This unequal transport of Na^+ and K^+ ions results in a current across the membrane which hyperpolarizes the cell beyond the passively determined membrane potential. Such a component to the resting E_m would be immediately sensitive to decreases in temperature. Sodium electrogenic pumps are also sensitive to inhibition by ouabain (Thomas, 1972, a review). The influence of temperature and ouabain on E_m was therefore studied in order to determine if an electrogenic pump was contributing to the resting E_m . The E_m of rat myotubes was not affected either by a short-term (up to 20 min) decrease in temperature to 15°C or exposure to ouabain concentrations as high as 10^{-3} M (Table IV). The lack of any immediate effect on E_m by either treatment suggests that the resting E_m is determined by the ion distribution and the passive permeability properties of the membrane.

Both ouabain (5×10^{-4} M) and incubation at low temperatures were effective in decreasing the E_m by approximately 50% after $3\frac{1}{2}$ – $4\frac{1}{2}$ h of incubation. This long-term effect is attributable to a gradual accumulation of Na^+ and loss of K^+ ions which occurs when the Na^+ - K^+ exchange pump is inhibited. Myotubes which have been incubated for $4\frac{1}{2}$ h at 15°C exhibit a markedly rapid recovery of their original resting potentials upon rewarming to 37°C . This recovery (to -53 mV) occurred within 2 min after warming the cells. Fifteen minutes after warming the dish to 37°C , when the temperature was again lowered to 15° , the mean E_m decreased to -44 mV as compared to the -21 mV recorded before warming. Preincubation with either ouabain or metabolic inhibitors prevented the recovery of cells maintained for long periods at 15°C . The recovery at 37°C in the presence of Li^+ -substituted medium, however, was identical to the recovery of cells in control medium. These results are summarized in Table IV. It thus appears likely that a Na^+ electrogenic pump, presumably activated by rising internal concentrations of Na^+ , aids in the rapid recovery of the membrane potential of rat myotubes

TABLE IV
EFFECT OF TEMPERATURE AND OUABAIN ON THE RESTING POTENTIAL OF RAT MYOTUBES

Conditions	Temper- ature	E_m	<i>N</i>
	$^\circ\text{C}$	<i>mV</i>	
Immediate effect (2–20 min)			
Control	37	-46.8 ± 0.9	41
Low temperature	15	-47.3 ± 1.5	30
Ouabain, 5×10^{-4} M	37	-46.2 ± 2.0	29
Ouabain, 1×10^{-3} M	37	-44.5 ± 2.5	31
Ouabain for 3.5 h			
Control, 3.5 h at 37°C	37	-49.7 ± 0.9	30
Ouabain, 5×10^{-4} M	37	-28.3 ± 1.5	32
Ouabain, 1×10^{-4} M	37	-20.6 ± 1.4	28
Low temperature for 4.5 h and subsequent recovery			
Control, 4.5 h at 37°C	37	-50.9 ± 1.6	20
Low temperature (15°C) for 4.5 h	15	-20.6 ± 1.9	24
Recovery at 37°C (2–12 min)	37	-52.7 ± 0.9	24
Return to 15°C after 15-min recovery period at 37°C	15	-44.5 ± 2.8	28
Recovery in the presence of inhibitors after 4.5 h at low temperature (15°C)			
No additions	37	-45.1 ± 1.0	19
Dinitrophenol, 2×10^{-4} M + iodoacetate, 2×10^{-3} M	37	-20.5 ± 2.7	19
Ouabain, 5×10^{-4} M	37	-17.0 ± 2.5	31
LiCl (Table I, <i>l</i>)	37	-43.5 ± 1.2	27

These experiments were performed in medium *b* of Table I. These values are reported as the mean \pm SEM with the number of observations indicated by *N*.

which have been maintained for long periods at 15°C. This is indicated by the temperature-sensitive portion of the E_m upon subsequent recooling. A portion of the early recovery can also be attributed to partial reestablishment of ion gradients. This is indicated by the temperature-insensitive portion of the recovery recorded 15 min after warming to 37°C.

The presence of a Na^+ electrogenic pump was also revealed in cultured chick myotubes by a less conventional method. After prolonged (20–50-s) depolarization by acetylcholine applied iontophoretically, the transmembrane potential of young chick myotubes became much larger than the pre-ACh level (Fig. 3). This hyperpolarization has been observed only in young chick myotubes and is notably absent in older chick myotubes or rat myotubes at all stages of development. The increase in E_m was accompanied by a slight decrease in R_{in} (Fig. 3) indicating a small increase in membrane permeability.

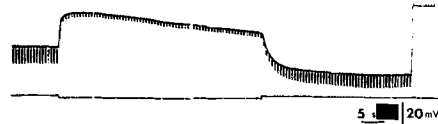


FIGURE 3. Hyperpolarization of chick myotube after ACh-induced depolarization. The transmembrane potential was recorded with one intracellular microelectrode (-38 mV) and then another, current-passing microelectrode was introduced into the myotube and current pulses of 200-ms duration, given once every 800 ms, were adjusted to hyperpolarize the myotube by about 15 mV. Then ACh was applied iontophoretically to the myotube for about 45 s. This initially depolarized the myotube to -7 mV and decreased the input resistance to 0.25 of the original value. After the ACh-induced depolarization, the transmembrane potential dropped to -62 mV and the input resistance rose to about 0.75 of the initial value. Finally the recording electrode was withdrawn from the myotube. The lower trace represents the current for the iontophoretic pipette containing ACh. This record shows a larger than average change in resting potential and is slightly atypical in that the input resistance did not increase substantially during application of ACh. Record is from 5-day-old chick myotube in medium *a* (Table I) at 35°C.

The ACh-induced hyperpolarization occurred when the extracellular medium contained only NaCl but did not occur in LiCl, sucrose, or other low Na^+ medium (Table V). The hyperpolarization did not require external K^+ , Cl^- , or Ca^{++} ions. The effect was blocked by 10^{-3} M 2,4-dinitrophenol and treatment with 10^{-4} M ouabain (although the blockade was slow to develop). The effect was also less frequently and reversibly blocked at 25°C. The response was not blocked by 5×10^{-6} M atropine. A possible explanation for the phenomena is that the increased influx of Na^+ ions during the ACh depolarization activated an electrogenic metabolic mechanism for Na^+ ion extrusion.

Ionic Determinants of the Membrane Potential

If the resting membrane potential is determined only by the passive membrane permeabilities and the ion distribution across the membrane, then the E_m can

TABLE V
HYPERPOLARIZATION OF CHICK MYOTUBES AFTER PROLONGED
DEPOLARIZATION WITH IONTOPHORETICALLY APPLIED
ACETYLCHOLINE

Medium	Resting transmembrane potential (mean \pm SEM)		N*
	Before	After	
	mV	mV	
<i>a</i> (Table I)	-33.2 \pm 2.1	-43.6 \pm 1.9	45
<i>b</i> with propionate substituted for chloride	-26.8 \pm 5.3	-33.1 \pm 4.9	12
150 mM NaCl	-30.9 \pm 3.4	-50.0 \pm 5.3	7
350 mM Sucrose	-27.7 \pm 4.2	-24.0 \pm 4.0	3
150 mM LiCl	-18.0 \pm 2.5	-18.6 \pm 3.3	6
<i>a</i> + Ouabain 10 ⁻⁴ M 20 min or more	-29.9 \pm 1.5	-28.4 \pm 1.5	17
<i>a</i> + 2,4-DNP 10 ⁻³ M 10 min or more	-29.9 \pm 4.7	-31.5 \pm 2.5	6
<i>a</i> + 5 \times 10 ⁻⁶ M Atropine	-26.7 \pm 3.1	38.2 \pm 3.9	6

* N = number of observations.

Young chick myotubes were maintained at 37°C in the indicated media. Resting potentials were recorded with intracellular microelectrodes. Only myotubes with very stable resting potentials were examined. Myotubes were depolarized by iontophoretic application of ACh from a low resistance pipette close to the myotubes. Twenty- to thirty-second pulses were used. Transmembrane potentials were recorded until a new stable resting membrane potential was achieved. Then the recording electrode was withdrawn and the final transmembrane potential was determined by the potential change at that moment (see Fig. 3).

be described by the Goldman constant field equation (Goldman, 1943; Hodgkin, 1951) for Na⁺ and K⁺ ions:

$$E_m = \frac{RT}{F} \ln \frac{[K^+]_o + (pNa/pK)[Na^+]_o}{[K^+]_i + (pNa/pK)[Na^+]_i}, \quad (1)$$

where pK and pNa refer to the membrane permeabilities of Na⁺ and K⁺, *T* is the temperature, *R* the universal gas constant, and *F* the faraday constant. The notations []_o and []_i represent the external and internal concentrations, respectively, of the ions indicated. Since we have shown that the resting *E_m* is largely determined by the passive properties of the membrane, the Goldman equation should be useful in determining the origin of the observed changes in *E_m* with age.

ION CONTENT OF MYOTUBES. In adult frog skeletal muscle the large resting *E_m* is mainly due to a high internal concentration of K⁺ relative to the outside of the cell and to a very small pNa/pK ratio (Hodgkin and Horowicz, 1959). It is thus possible that the low membrane potential of young myotubes is due to a smaller chemical gradient for K⁺ ions. Such could be the case if, for example, the Na⁺-K⁺ exchange pump was poorly developed in myoblasts and newly formed myotubes. The internal Na⁺ and K⁺ concentrations as

determined by flame photometry, are given in Table VI for rat myotubes after 3–9 days in culture. The mean $[K^+]_i$ was 153 ± 5 mV (\pm SEM for $N = 6$) and the mean $[Na^+]_i$ was 13 ± 1 mM. These values were relatively constant throughout the course of cell development. Not shown in Table VI are the results of a 6-day culture which was found to have a mean $[K^+]_i$ of 105 mM and a $[Na^+]_i$ of 23.4 mM. We presume that the higher $[Na^+]_i$ and lower $[K^+]_i$ obtained during this particular assay was due to cell damage and subsequent leakage of these ions.

E_m vs. $\log [K^+]_o$. The relative permeability of the membrane to Na^+ and K^+ was determined by observing the relationship between E_m and $[K^+]_o$, described by Eq. 1. The E_m of a single myotube was monitored through an intracellular microelectrode while perfusing the culture dish with a continuous ion gradient in which the Na^+ content in the medium was completely replaced by K^+ within 3 min. The extracellular K^+ concentration was measured within

TABLE VI
SODIUM AND POTASSIUM CONTENT OF CULTURED RAT MYOTUBES

Age	Wet wt	H ₂ O content	Extracellular space	$[K^+]_i$	$[Na^+]_i$
days	mg	% wet wt	% H ₂ O content	mM	mM
3	5.1	84.7	37.2	144	13.6
	4.1			159	15.5
4	5.76	87.7	38.7	137	14.9
	5.05			146	7.4
9	2.99	86.3	43.0	156	16.3
	2.75			175	12.7

15 μ m of the myotube by simultaneously recording with an extracellular K^+ -selective ion electrode. All solutions were changed in the direction of increasing $[K^+]_o$. A plot of E_m vs. $\log [K^+]_o$ is shown in Fig. 4 A for four myotubes of different ages. Examination of the Goldman equation reveals that when the term $(pNa/pK) [Na^+]_i$ is small relative to $[K^+]_i$, then the internal K^+ concentration can be estimated by extrapolation to zero potential where $[K^+]_i$ will be approximately equal to $[K^+]_o$. However, a further linear transformation of Eq. 1 to Eq. 2 (Williams, 1970) greatly facilitates the estimation of both $[K^+]_i$ and the pNa/pK ratio.

$$e^{E_m F / RT} = \frac{[K^+]_o (1 - pNa/pK) + (pNa/pK) (M)}{[K^+]_i} \quad (2)$$

In Eq. 2, $M = [K^+]_o + [Na^+]_o$ or 148 mM, and $(pNa/pK) [Na^+]_i$ is assumed to be negligible relative to $[K^+]_i$. According to Eq. 2 a plot of $e^{E_m F / RT}$ vs. $[K^+]_o$ should produce a straight line. Such a plot is shown in Fig. 4 B for the

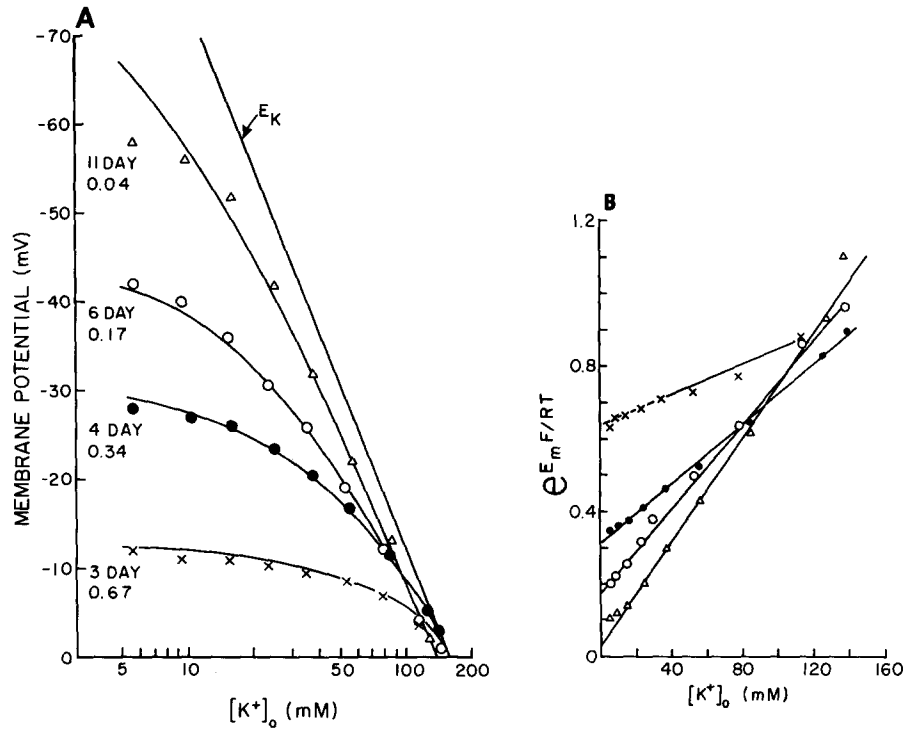


FIGURE 4. The relationship between E_m and external K^+ ion concentration for four myotubes of different age. The E_m of each myotube was recorded during perfusion of the myotube with a continuous K^+ ion gradient between 5.3 and 148 mM. The external K^+ concentration was simultaneously monitored with a K^+ -selective ion electrode. The entire gradient was completed within 3 min. The external Cl^- concentration was maintained constant. (A) E_m is plotted against $\log [K^+]_o$ for each myotube. The Nernst equilibrium potential for K^+ is indicated by the straight line labeled E_K . The solid line for each myotube is the theoretical curve predicted by the Goldman equation using the $[K^+]_i$ and the pNa/pK ratio estimated from the graph in Fig. 4 B and using an $[Na^+]_i$ of 13 mM. (B) $e^{E_m F/RT}$ is plotted against $[K^+]_o$ for each myotube shown in 4 A. Identical symbols were used to represent each myotube in both 4 A and B. These experiments were performed at 37°C.

same four myotubes represented in Fig. 4 A. A value for $[K^+]_i$ for each myotube was estimated by determining the $[K^+]_o$ where the straight line intercepted the value of 1.0 on the ordinate. At this point the E_m is equal to zero and $[K^+]_i$ should be approximately equal to $[K^+]_o$. The pNa/pK ratio was determined from the y intercept which is equal to $(pNa/pK) (148)/[K^+]_i$. Values for $[K^+]_i$ and pNa/pK obtained by this method are reported in Table VII for those myotubes which produced straight lines, or lines which were only slightly concave at low $[K^+]_o$. (See Fig. 4 B). The entire line was markedly concave for approximately 30% of all myotubes studied, most likely due to

TABLE VII
PERMEABILITY RATIOS AND $[K^+]_i$ ESTIMATED FROM CURVES OF MEMBRANE
POTENTIAL vs. LOG $[K^+]_o$ FOR MYOTUBES OF DIFFERENT AGES

Age	E_m	$[K^+]_i$	pNa/pK	<i>N</i>
<i>days</i>	<i>mV</i>	<i>mM</i>		
3	-24±4	153±3	0.40±0.08	5*
4	-35±5	151±5	0.25±0.05	3*
6	-40±2	143±5	0.19±0.03	4*
6	-48	139	0.17	1‡
7	-55±3	143±3	0.05±0.01	3§
8	-60	141	0.05	1*
8	-54±1	137±1	0.073±0.003	3§
8	-61±2	139±2	0.04±0.01	2
9	-59±2	149±5	0.06±0.01	3
10	-55±2	140±3	0.085±0.005	2§
10	-50±8	131±8	0.12±0.05	2‡
11	-51±6	131±6	0.07±0.03	2*

* E_m was recorded from single cells during perfusion with a continuous K^+ gradient in which $[NaCl] + [KCl]$ remained constant.

‡ E_m was recorded from single cells during perfusion with a chloride-free K^+ gradient in which $[K^+]_o + [Na^+]_o$ remained constant. Cl^- was replaced by sulfate ions with the addition of sucrose to maintain isotonicity.

§ The average E_m was determined from 10 or more myotubes which had equilibrated for 10 min in media at six different $[K^+]_o$. $[NaCl] + [KCl]$ remained constant. A separate culture dish was used for each different concentration.

|| Continuous recording of E_m from single cells during perfusion with six different concentrations of $[K^+]_o$ in which the $[K^+]_o \times [Cl^-]_o$ product remained constant. $[Na^+]_o + [K^+]_o$ also remained constant. The chloride concentration was varied by replacement with sulfate ions with the addition of sucrose to maintain isotonicity. On one occasion the average E_m was determined from 10 or more myotubes which had equilibrated for 10 min at each K^+ concentration.

These values are reported as the mean \pm SEM with the number of observations indicated by *N*.

changing the external K^+ too rapidly. Markedly concave lines were never observed when myotubes were allowed to equilibrate for 2–20 min in media of different $[K^+]_o$. The estimated $[K^+]_i$ was 153 mM for 3-day myotubes and decreased slightly to a mean of 139 mM for myotubes which were 7 days or older. The values for $[K^+]_i$ obtained by this method are very similar to the internal K^+ concentrations which were determined by flame photometry. The mean permeability ratio for 3-day cells was 0.4 and decreased to a mean of 0.07 after 7 days in culture. The largest ratio observed was 0.66 for a 3-day myotube in which the E_m was -12 mV. The lowest ratio was 0.04 for three

myotubes which were 7 days or older with membrane potentials near -60 mV.

The measurements of E_m vs. $[K^+]_o$ were made under a variety of conditions (Table VII) in addition to the conditions already described, i.e., rapid perfusion with a continuous K^+ gradient in which $[Na^+]_o$ and $[K^+]_o$ are varied with a constant external Cl^- ion concentration. Similar results were obtained when $[K^+]_o$ was changed while myotubes were perfused in Cl^- -free medium, or when myotubes were allowed to equilibrate for 1–10 min in media of different $[K^+]_o$ with either constant $[Cl^-]_o$ or when maintaining a constant external $[K^+]_o \times [Cl^-]_o$ product. The latter comparison is also shown in Fig. 5. In all instances $[K^+]_o + [Na^+]_o$ was 148 mM.

CHLORIDE PERMEABILITY. In frog twitch skeletal muscle where the distribution of Cl^- is found to be in equilibrium with the E_m , the contribution

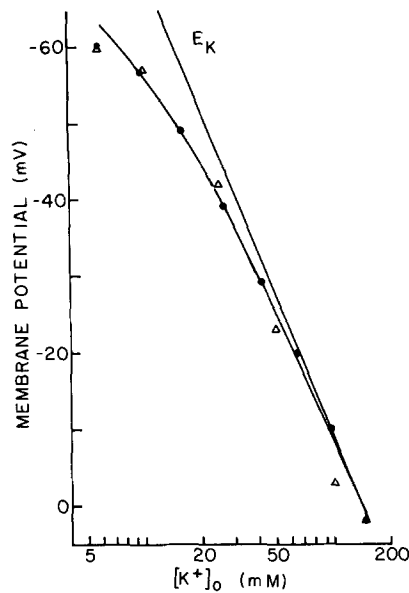


FIGURE 5. The effect of external Cl^- ions on the relationship between E_m and $\log [K^+]_o$ for two different rat myotubes. $[K^+]_o$ was varied under conditions in which $[Cl^-]_o$ was maintained constant (closed circles) or where the $[K^+]_o \times [Cl^-]_o$ product was maintained constant (triangles). The points indicated by the closed circles were measured during rapid perfusion with a continuous $[K^+]_o$ ion gradient. The triangles were measured during perfusion with a step gradient for six different K^+ concentrations with approximately 2 min allowed for equilibration at each step. The external K^+ concentration in the immediate environment of the myotube being examined was monitored in both instances with a K^+ -selective ion electrode. The curve is the theoretical line predicted by the Goldman equation using a pNa/pK ratio of 0.048 and a $[K^+]_i$ of 141 mM estimated with the aid of Eq. 2 (see text for further details). Both myotubes were 8 days old. The temperature was $37^\circ C$.

of Cl^- to the resting potential is negligible and, therefore, is not represented in the Goldman equation (Hodgkin and Horowicz, 1959). In the frog twitch muscle very rapid changes in the external Cl^- concentration produce immediate changes in E_m . The changes, however, are transient since the E_m returns to the potential established by Na^+ and K^+ as the Cl^- ions redistribute across the membrane.

The continuously recorded E_m , monitored in single rat myotubes, remained invariant when the external Cl^- concentration was changed from medium containing 150 mM Cl^- (Table I, *b*) to one containing 5.3 mM Cl^- (Table I, *k*). In this medium Cl^- was replaced by the impermeant anion, methylsulfate. The membrane potentials of these mature myotubes were generally between -50 and -60 mV.

When myotubes were allowed to equilibrate for 10 min in high K^+ , high Cl^- medium (Table I, *c*), subsequent replacement of this medium with low external Cl^- medium (Table I, *m*) also failed to produce a transient positive polarization of the cell. The same cells, however, could be repolarized in the negative direction by replacement with standard medium containing 5.3 mM K^+ , consistent with its established permeability to K^+ ions (Table VIII).

The failure to observe transient changes in E_m under the conditions described could be indicative of very low Cl^- permeability or to a very large $p\text{Cl}^-$ resulting in extremely rapid equilibration of $[\text{Cl}^-]_i/[\text{Cl}^-]_o$ with E_m . If Cl^- permeability were indeed substantial, then the membrane resistance ought

TABLE VIII
EFFECT OF CHLORIDE AND POTASSIUM CONCENTRATIONS ON THE MEMBRANE POTENTIAL OF RAT MYOTUBES

Medium	Myotube no.			Expected response if permeable to K^+ and Cl^-	Expected response if permeable to K^+ but not Cl^-
	1	2	3		
5.3 mM K^+ , 150 mM Cl^- (Table I, <i>b</i>)	-50	-60	-60	-50 to -60	-50 to -60
148 mM K^+ , 150 mM Cl^- (Table I, <i>c</i>)	-3	+1	0	0	0
148 mM K^+ , 3 mM Cl^- (Table I, <i>m</i>)	-3	+1	-9	Transient positive potential	0
5.3 mM K^+ , 150 mM Cl^- (Table I, <i>b</i>)	-50	-49	-56	-50 to -60	-50 to -60

The E_m of an individual myotube was recorded intracellularly while changing the external Cl^- and K^+ ion concentration of the perfusion medium. A 3-6-min equilibration was allowed before each medium change. The recording electrode remained inside the myotube during the entire experiment which involved three changes in the composition of the external medium. In each of the myotubes reported above, the E_m (mV) shown in each medium also represents the E_m measured at equilibrium since no transient changes were observed. The expected responses for a cell permeable to both K^+ and Cl^- or to K^+ only are indicated in the two right-hand columns. These experiments were performed at 37°C.

to increase in low Cl^- medium (Hutter and Nobel, 1960; Stefani and Steinbach, 1969). When R_{in} was monitored in individual rat myotubes by passing hyperpolarizing currents through a second intracellular electrode, replacement of Cl^- with methylsulfate produced no change in either E_m or R_{in} (Fig. 6 A). This is in contrast to the results obtained from changes in external K^+ . In this case an increase in $[\text{K}^+]_o$ depolarized the myotube and also decreased the R_{in} (Fig. 6 B) measured by hyperpolarizing responses to injected current. In this example the E_m returned to the original resting potential of -50 mV

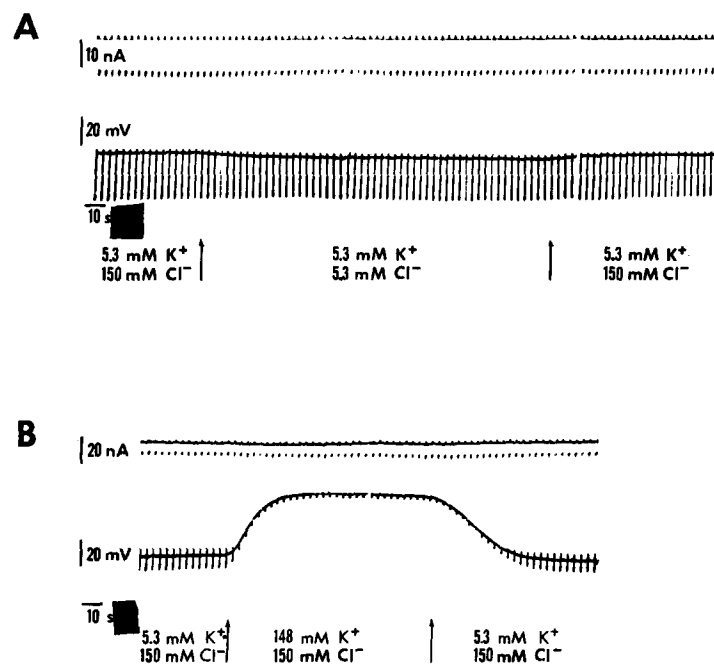


FIGURE 6. The effect of external K^+ and Cl^- on the E_m and R_{in} of rat myotubes. The upper line in each trace represents 100-ms current pulses passed through an intracellular microelectrode filled with 2 M K citrate. The middle line is a record of the membrane potential with hyperpolarizations in the downward direction. The arrows indicate changes in the composition of the medium perfusing the myotubes. These figures were produced by placing consecutive oscilloscope traces adjacent to each other. (A) The resting E_m of this myotube in control medium (Table I, *b*) was -50 mV. A slight hyperpolarization (<4 mV) of the membrane potential occurred as the medium was changed from 150 mM Cl^- to 5.3 mM Cl^- (Table I, *k*). This slight hyperpolarization is the result of a 3–4-mV difference in the ground potential recorded in these two solutions. The hyperpolarization disappeared upon return to the original control medium. (B) The resting E_m of this myotube was -48 mV in control medium. The medium was changed from one containing 5.3 mM K^+ to medium containing 148 mM K^+ (Table I, *c*). After several minutes the medium was again replaced with control medium. These myotubes were 10 days old. The temperature was 37°C . All media contained $1 \mu\text{g}/\text{ml}$ of tetrodotoxin.

when $[K^+]_o$ was again decreased to normal levels, however, there was a very slight decrease in R_{in} possibly due to some cell damage.

Finally, the specific membrane resistance, determined in 5.3 mM Cl^- (Table I, k) for 7-day-old myotubes (Table II), gave values similar to those obtained in standard medium containing 150 mM Cl^- . From these data, it is concluded that the resting pCl^- of the rat myotube membrane is very low.

Acetylcholine Reversal Potentials

Activation of ACh receptors in skeletal muscle results in a depolarization due to the opening of channels permeable to both Na^+ and K^+ ions. A measure of the ionic selectivity of such channels, that is, the relative conductance change to Na^+ and K^+ ions ($\Delta g_{Na}/\Delta g_K$), can be estimated from the ACh reversal potential (E_r). This relationship is given by Eq. 3:

$$E_r = [E_K + (\Delta g_{Na}/\Delta g_K) E_{Na}][1 + \Delta g_{Na}/\Delta g_K]^{-1}, \quad (3)$$

where E_K and E_{Na} refer to the K^+ and Na^+ Nernst equilibrium potentials (Takeuchi and Takeuchi, 1960). Since our measurements of the internal Na^+ and K^+ content of rat myotubes indicate that E_K and E_{Na} remain constant during development, any changes in E_r can be interpreted as changes in ion selectivity.

The potential change in response to iontophoretically applied ACh was measured while simultaneously changing the membrane potential with an intracellular polarizing electrode (Fig. 7). In most cases a reversal of the ACh response was obtained and a plot of E_m vs. ACh response was linear (Fig. 7). The membrane potential where the straight line intercepted the abscissa was taken as the reversal potential. At this potential the net inward movement of Na^+ is exactly balanced by the net outward movement of K^+ , thus, no potential change is observed. In the example shown in Fig. 7 the ACh E_r was 0 mV. Occasional instances when reversal of the ACh response could not be obtained or when the response was not linear, were discarded.

The ACh reversal potentials measured in cultured rat myotubes as a function of the age of the culture are shown by the open circles in Fig. 2. The ACh reversal potential for rat myotubes remained at approximately 0 mV for myotubes between 3 and 12 days in culture. Similar, although slightly more negative, values were found for E_r in cultured chick myotubes. These results are indicated in Table III. The constancy of E_r with age indicates that the ionic selectivity of the ACh receptor, $\Delta g_{Na}/\Delta g_K$, does not change during cell development.

Desensitization of ACh Receptors

Adult frog, rat, and chick muscle become desensitized in the continued presence of ACh, as indicated by the gradual decrease in the extent of ACh-

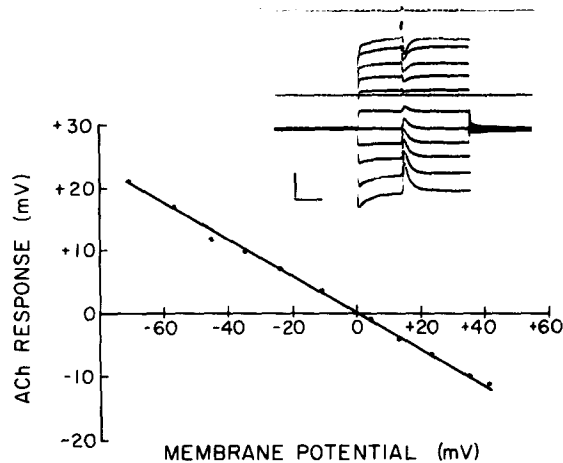


FIGURE 7. Determination of the ACh reversal potential of a 4-day-old cultured rat myotube. The inset is a record of the oscilloscope traces. The upper line represents the current trace for the iontophoretic pipette containing ACh. The small downward deflection from this line is equivalent to 8 nA of current passed for 3 ms. The middle line is ground potential determined at the end of the experiment. The lower line represents the membrane potential which has been displaced to various levels with a second intracellular current-passing electrode. The vertical calibration represents 20 mV and the horizontal calibration 100 ms. The ACh response at each membrane potential is plotted in the graph below. The ACh reversal potential determined from this plot was 0 mV. The ACh sensitivity of this myotube was 310 mV/nC at the resting membrane potential which was -30 mV.

induced depolarizations (Katz and Thesleff, 1957; Rang and Ritter, 1970; Freeman and Turner, 1972). This is also true of cultured chick myotubes (Fig. 8). Mature chick myotubes (8 days), with a mean resting E_m of -57 mV, depolarized upon the addition of AChCl to the bath (10^{-5} M). The depolarization was short-lived despite the continued presence of ACh in the bathing medium, due to the rapid desensitization of the receptors. Within 1 min after bath application the membrane potentials had returned to control values and remained there when tested 90 min later. One hundred minutes after the initial addition of ACh the dish was washed repeatedly with fresh ACh-free medium. The mean E_m of these myotubes was identical to the pretreated control value.

The behavior of cultured rat myotubes under similar conditions was very different (Fig. 9). Bath application of ACh (10^{-5} M) depolarized the myotubes to their reversal potential, which was approximately -3 mV in this medium. This depolarization could be sustained for periods as long as 100 min with only a slight decrease in the extent of depolarization. Upon subsequent removal of ACh from the bath, the E_m immediately returned to the control level of -49 mV. Thus, there is a species difference in the ability of chick and rat myotubes to sustain ACh-induced depolarizations.

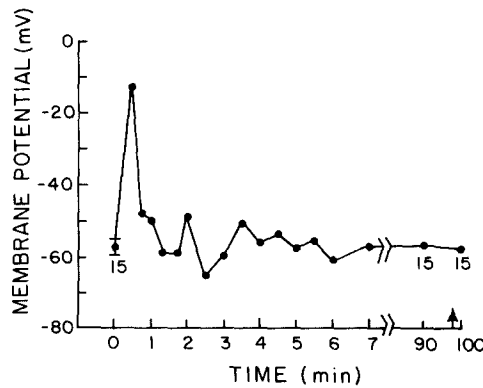


FIGURE 8

FIGURE 8. Effect of prolonged application of ACh on the membrane potential of a mature cultured chick myotube. The mean resting membrane potential in standard medium (Table I, *b*) at 37°C is shown at time zero. AChCl at a final concentration of 10^{-5} M was then added to the culture dish. The first three time points were measured continuously in a single myotube. Other time points represent sampling of E_m in different myotubes up to 10 min after the addition of ACh. The mean E_m was again determined after 90 min in ACh. The culture dish was then rinsed several times with fresh, ACh-free medium (indicated by the arrow) and the mean E_m of these myotubes is shown. Each point containing vertical bars represents the mean \pm SEM for the number of myotubes indicated adjacent to the point.

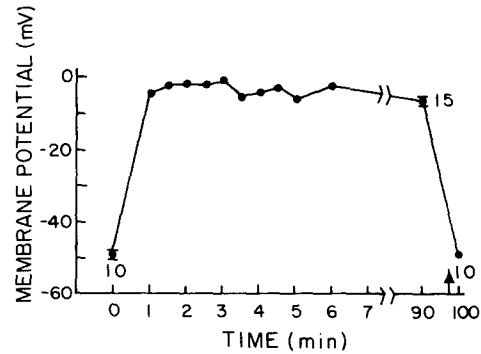


FIGURE 9

FIGURE 9. Effect of prolonged application of ACh on the membrane potential of a mature cultured rat myotube. All conditions are identical to those indicated by the legend to Fig. 8 except that every time point after the initial addition of ACh was recorded from a separate myotube.

DISCUSSION

Development of the Resting Transmembrane Potential

An increase in E_m from -8 mV to approximately -55 mV has been found in cultured rat and chick myotubes as a function of cell maturation. A similar increase in E_m with development has been reported by others for primary rat (Fambrough and Rash, 1971), mouse (Powell and Fambrough, 1973), and chick (Fischbach et al., 1971) cultures, in newborn rats (Boethius, 1969; Fudel-Osipova and Martynenko, 1962; Hazelwood and Nichols, 1969) and mice (Harris and Luff, 1970), and chick embryos (Boethius and Knutsson, 1970), *in vivo*. The rat L6 clonal cell line is different in this respect in that myoblasts already have fairly high resting potentials, near -60 mV (Kidokoro, 1973; this study), while the mature myotubes have a mean E_m which is slightly lower (Kidokoro, 1973).

During the period of change in the E_m of postnatal rats, the internal K^+ concentration is near 150–160 mM and remains fairly constant with age (Vernadakis and Woodbury, 1964; Fudel-Osipova and Martynenko, 1965).

The internal Na^+ reportedly decreases as much as 10-fold to values less than 11 mM during the same period. Since the membrane potential is largely determined by the K^+ ion distribution it seemed unlikely that differences in ion distribution alone could account for the large changes in membrane potential. Our results on development of cultured myotubes substantiate this conclusion.

Our measurements of $[\text{K}^+]_i$ and $[\text{Na}]_i$ by flame photometry revealed very little change in the Na^+ and K^+ content of rat myotubes with cell development, $[\text{K}^+]_i$ being 153 mM and $[\text{Na}]_i$ being 13 mM. Estimations of $[\text{K}^+]_i$ independently determined by extrapolation or interpolation to zero membrane potential when changing external K^+ concentrations ranged from a mean of 153 mM in 3-day cultures to 139 mM after 7 days in culture. The differences between the two methods for the determination of $[\text{K}^+]_i$, however, are slight and probably due to the variability inherent in both methods. The results reported here for cultured rat myotubes are in general agreement with the results obtained by others *in vivo* for $[\text{K}^+]_i$ (cited above). We did not find high internal Na^+ concentrations in young myotubes, which were reported by Vernadakis and Woodbury (1964) for postnatal rat muscle. The reasons for this discrepancy are not clear.

In view of the relatively constant ion distribution, the gradual development of E_m with age could be due to changes in specific ion permeabilities or to the establishment of an electrogenic pump. We have found that the development of the E_m is most readily explained by changes we have observed in specific ion permeabilities. Very young rat myotubes, after 3 days in culture, had a mean pNa/pK ratio of 0.4 for five myotubes tested with a mean E_m of -24 mV. This decreased progressively to a mean ratio of 0.07 for 18 myotubes examined between 7 and 11 days of age with a mean E_m of -55 mV. Thus, the membrane becomes relatively more permeable to K^+ as the myotubes develop into mature fibers. In adult frog skeletal muscle the pNa/pK ratio is 0.01 and the E_m is -90 mV (Hodgkin and Horowicz, 1959).

The passive electrical constants were also determined for cultured rat myotubes at different stages of development. There was a tendency for the R_m of cultured rat myotubes to increase between 3 and 9 days in culture from a mean value of 958 to 1,567 Ωcm^2 . The most dramatic changes in E_m , and therefore, in specific ion permeabilities, occurred between 3 and 7 days in culture when the measured R_m showed the least change. The most compatible interpretation of these results is that the early change in relative specific ion permeabilities is largely due to a decrease in P_{Na} while the later increase in R_m (day 9) could be associated with a decrease in both pNa and pK . However, because of the variability and possible systematic error in the measurement of R_m (see discussion of leakage artifacts in Results) such an interpretation is tentative.

Similar changes in E_m and permeability ratios have been observed in developing heart cells (Sperelakis and Shigenobu, 1972; McDonald and DeHaan, 1973) and soon after fertilization of echinoderm eggs (Steinhardt et al., 1971; Tupper, 1972). In the developing heart cells (Sperelakis and Shigenobu, 1972) and the *Asterias* embryo (Steinhardt et al., 1972; Tupper and Powers, 1973) the decline in the pNa/pK ratio was attributed to a selective increase in pK.

The relative Cl^- permeability of rat myotubes appears to be negligible since both the E_m and the membrane resistance of mature fibers were insensitive to changes in external Cl^- concentration. The R_m for cultured rat myotube (overall mean of $1,100 \Omega\text{cm}^2$) is, nevertheless, relatively low for a cell with low Cl^- permeability. In frog twitch muscle where chloride permeability accounts for approximately 68% of the total membrane conductance, R_m is $2,500\text{--}5,000 \Omega\text{cm}^2$ in the presence of Cl^- and approximately three times greater when Cl^- is replaced by an impermeant anion (Hutter and Noble, 1960; Stefani and Steinbach, 1969). In frog twitch muscle transient decreases in E_m could also be measured as the external Cl^- concentration was decreased (Hutter and Noble, 1960; Stefani and Steinbach, 1969). Large Cl^- conductance, however, is not a general property of skeletal muscle. Low chloride permeability has been found in frog slow muscle (Stefani and Steinbach, 1969) and appears likely in rat diaphragm muscle (Kernan, 1968). In our studies of E_m vs. $[\text{K}^+]_o$ we also found very little difference in the determination of $[\text{K}^+]_i$ or pNa/pK ratios when $[\text{K}^+]_o$ was changed in medium of constant external Cl^- , Cl^- -free medium, or medium in which the $[\text{K}^+]_o \times [\text{Cl}^-]_o$ product was maintained constant. The contribution of Cl^- to the membrane potential of cultured myotubes can, therefore, be considered as negligible.

We found no significant electrogenic component to the resting E_m of mature rat myotubes as short-term exposure to low temperature or ouabain generally had no effect on E_m . A sodium electrogenic pump, however, can be activated in rat myotubes which have been exposed for long periods of time to low temperature. The mean E_m of myotubes maintained for 3.5–4.5 h at 15°C was very low due to the loss of Na^+ and K^+ ion gradients. The recovery of the E_m of these myotubes after warming to 36°C was very rapid and also sensitive to subsequent relowering of temperature. The recovery from cold treatment could be prevented by pretreatment with ouabain or inhibitors of metabolism. Lithium chloride medium failed to inhibit the recovery. Since Li^+ presumably inhibits the Na^+ pump from the inside of the cell it is possible that an insufficient amount of Li^+ entered the cell to effect the blockade. The activation of the Na^+ electrogenic pump was probably due to the increase in internal Na^+ content which occurred during the prolonged incubation at 15°C .

A contribution of a sodium electrogenic pump to the membrane potential has been described in sodium-enriched frog sartorius muscle (Kernan, 1962;

Frumento, 1965; Mullins and Awad, 1965; Adrian and Slayman, 1966) and rat diaphragm muscle (Kernan, 1968). There is also a report of a 15-mV sodium electrogenic component to the normal resting E_m of skeletal muscle (Luderitz and Bolte, 1968) in rats maintained on K^+ -deficient diets.

A sodium electrogenic pump was also found to exist in the membrane of cultured chick myotubes. A mean hyperpolarization of approximately 10 mV beyond the original resting E_m has been observed in young chick myotubes after a 30-s ACh-induced depolarization. The hyperpolarization appears to be due to an electrogenic pump since it can be blocked by ouabain, 2,4-dinitrophenol, and LiCl. The activation of the Na^+ pump is most likely due to the increased influx of Na^+ ions which occurs during the ACh depolarization. The ability of Li^+ to inactivate the electrogenic pump in this case, as compared to its ineffective blockade of the recovery of cold incubated rat myotubes, could result from the increased influx of Li^+ into the cell during the activation of the ACh receptor of the chick myotubes and not to a difference in the passive permeability of rat and chick myotubes to Li^+ . This phenomena was not blocked by atropine and is unrelated in all aspects to the slow hyperpolarizing response observed in rat myotubes (Fambrough and Rash, 1971), L6 myoblasts (Patrick et al., 1972) and mouse L cells (Nelson et al., 1972). The latter phenomena has been attributed to an ACh-activated fourfold increase in K^+ permeability (Nelson et al., 1972). Although a slight increase in membrane conductance is associated with the hyperpolarization, its contribution to the response is not substantial. This phenomenon cannot be induced in rat myotubes or in mature chick myotubes. It is possible that the internal Na^+ content of young chick myotubes is more readily increased due possibly to a less active membrane Na^+K^+ -exchange pump.

Properties of the ACh Receptor in Developing Myotubes

The ACh receptor appears very early in the development of cultured myotubes. ACh sensitivity can be measured within 2 days after plating and coincident with the onset of cell fusion (Fambrough and Rash, 1971). As the myotubes mature the density of ACh receptors in the membrane increases (Hartzell and Fambrough, 1973). When the ACh receptors are activated, ion channels in the membrane are opened which are selectively permeable to Na^+ and K^+ ions (Ritchie and Fambrough, 1975). Possible changes in the relative ion selectivity of the receptor to Na^+ and K^+ ions during cell development were studied by measuring the ACh reversal potential. This potential was near 0 mV for rat myotubes and -2 mV for chick myotubes examined between 1 and 12 days in culture. Values between $+10$ and -10 mV have been reported for the reversal potential in cultured chick myotubes before (Harris et al., 1973) and after innervation by chick spinal cord neurons (Fischbach, 1972) and in the rat L6 myoblast cell line before and after inter-

actions with neuroblastoma cells (Steinbach, 1975). These values are not greatly different from the value of -7 to -20 mV reported for the innervated and denervated rat muscle in vivo (Magazanik and Potapova, 1969; Thesleff and Albuquerque, 1967). Although the membrane composition is presumably changing as the myoblast differentiates into a mature muscle fiber, these changes do not influence the ionic selectivity of the ACh receptor, as indicated by the constancy of the reversal potential in rat and chick myotubes during development.

The ACh receptors of adult frog (Katz and Thesleff, 1957; Nastuk et al., 1966), rat (Freeman and Turner, 1972), and chick muscle (Rang and Ritter, 1970) desensitize in the continued presence of ACh. Cultured chick myotubes (Harvey and Dryden, 1974) and myotubes from the rat L6 myoblast cell line (Steinbach, 1975) are also known to desensitize in the presence of ACh. We have also found that chick myotubes undergo rapid desensitization by observing the transient nature of the ACh-induced depolarization in the continued presence of ACh. Within 1 min after the bath application of ACh (10^{-5} M) the membrane potential of chick myotubes had returned to control levels. A marked difference was found in rat myotubes where ACh-induced depolarizations remained nearly constant for a period as long as 100 min in the presence of 10^{-5} M ACh. Stability of the ACh-induced depolarization, however, does not necessarily indicate the absence of desensitization. We have occasionally observed some desensitization in rat myotubes by a decline in the ACh-induced current during prolonged iontophoretic application of ACh without a concomitant change in the ACh-induced depolarization. The difference which we have found between rat and chick myotubes could, therefore, be explained by differences in the electrical properties of the membrane, differences in receptor density, or to other effects which are related to the microenvironment of the receptor. A wide spectrum of compounds regarded as "membrane stabilizers" reportedly enhance the rate of receptor desensitization in frog muscle (Vyskocil and Magazanik, 1972; Magazanik and Vyskocil, 1972). We have found that pretreatment of rat myotubes with partial alpha-bungarotoxin blockade of ACh receptors is effective in causing a measurable decline in the ACh-induced depolarization with prolonged application of ACh (unpublished observations). While this effect in our rat myotubes could be due to the effective decrease in receptor density produced by blockade, Vyskocil and Magazanik (1972) have shown that alpha-bungarotoxin is capable of enhancing the rate of ACh receptor desensitization in frog muscle by a possible "membrane stabilizing" action which is independent of the ability of this compound to inhibit ACh receptor activation.

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REFERENCES

- ADRIAN, R. H., and C. L. SLAYMAN. 1966. Membrane potential and conductance during transport of sodium, potassium and rubidium in frog muscle. *J. Physiol. (Lond.)*. **184**:970.
- BOETHIUS, J. 1969. Resting membrane potential in neck and leg muscles of young rats. *Acta Physiol. Scand.* **75**:253.
- BOETHIUS, J., and E. KNUTSSON. 1970. Resting membrane potential in chick muscle cells during ontogeny. *J. Exp. Zool.* **174**:281.
- BROWN, A. M., J. L. WALKER, JR., and R. B. SUTTON. 1970. Increased chloride conductance as the proximate cause of hydrogen ion concentration effects in *Aplysia* neurons. *J. Gen. Physiol.* **56**:559.
- FAMBROUGH, D. M., and J. RASH. 1971. Development of acetylcholine sensitivity during myogenesis. *Dev. Biol.* **26**:55.
- FISCHBACH, G. D. 1972. Synapse formation between dissociated nerve and muscle cells in low density cell cultures. *Dev. Biol.* **28**:407.
- FISCHBACH, G. D., M. NAMEROFF, and P. G. NELSON. 1971. Electrical properties of chick skeletal muscle fibers developing in cell culture. *J. Cell Physiol.* **78**:289.
- FREEMAN, S. E., and R. J. TURNER. 1972. Agonist-antagonist interaction at the cholinergic receptor of denervated diaphragm. *Aust. J. Exp. Biol. Med. Sci.* **50**:21.
- FRUMENTO, A. S. 1965. Sodium pump; its electrical effect in skeletal muscle. *Science (Wash. D. C.)*. **147**:1442.
- FUDEL-OSIPOVA, S. I., and O. A. MARTYNYENKO. 1962. Early ontogenic development of membrane potential of muscle fibers in rat. *Fiziol. Zh. Akad. Nauk Ukr. RSR.* **8**:442.
- FUDEL-OSIPOVA, S. I., and O. A. MARTYNYENKO. 1965. Change in the water-salt composition of rat muscles during ontogenesis. *Biofizika.* **10**:796.
- FUKUDA, J. 1974. Chloride spike: A third type of action potential in tissue-cultured skeletal muscle cells from chick. *Science (Wash. D. C.)*. **185**:76.
- GOLDMAN, D. E. 1943. Potential, impedance, and rectification in membranes. *J. Gen. Physiol.* **27**:37.
- HARRIS, J. B., and A. R. LUFF. 1970. The resting membrane potential of fast and slow muscle fibers in developing mouse. *Comp. Biochem. Physiol.* **33**:923.
- HARRIS, J. B., M. W. MARSHALL, and P. WILSON. 1973. A physiological study of chick myotubes grown in tissue culture. *J. Physiol. (Lond.)*. **229**:751.
- HARTZELL, H. C., and D. M. FAMBROUGH. 1973. Acetylcholine receptor production and incorporation into membranes of developing muscle. *Dev. Biol.* **30**:153.
- HARVEY, A. L., and W. F. DRYDEN. 1974. Depolarization, desensitization and the effects of tubocurarine and neostigmine in cultured skeletal muscle. *Eur. J. Pharmacol.* **27**:5.
- HAZLEWOOD, C. F., and B. L. NICHOLS. 1969. Changes in muscle sodium, potassium chloride, water and voltage during maturation in the rat: An experimental and theoretical study. *Johns Hopkins Med. J.* **125**:119.
- HODGKIN, A. L. 1951. The ionic basis of electrical activity in nerve and muscle. *Biol. Rev.* **26**:339.
- HODGKIN, A. L., and P. HOROWICZ. 1959. The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol. (Lond.)*. **148**:127.
- HUTTER, O. F., and D. NOBLE. 1960. The chloride conductance of frog skeletal muscle. *J. Physiol. (Lond.)*. **151**:89.
- KANO, M., and Y. SHIMADA. 1973. Tetrodotoxin-resistant electric activity in chick skeletal muscle cells differentiated *in vitro*. *J. Cell Physiol.* **81**:85.
- KATZ, B., and S. THESLEFF. 1957. A study of the desensitization produced by acetylcholine at the motor end-plate. *J. Physiol. (Lond.)*. **138**:63.
- KERNAN, R. P. 1962. Membrane potential changes during sodium transport in frog sartorius muscle. *Nature. (Lond.)*. **193**:986.
- KERNAN, R. P. 1968. Membrane potential and chemical transmitter in active transport of ions by rat skeletal muscle. *J. Gen. Physiol.* **51**:204s.

- KIDOKORO, Y. 1973. Development of action potentials in a clonal rat skeletal muscle cell line. *Nat. New Biol.* **241**:158.
- KONIGSBERG, I. R. 1963. Clonal analysis of myogenesis. *Science (Wash. D. C.)*. **140**:1273.
- LAND, B. R., A. SASTRE, and T. R. PODLESKI. 1973. Testrodoxin-sensitive and -insensitive action potentials in myotubes. *J. Cell Physiol.* **82**:497.
- LUDERITZ, B., and H. D. BOLTE. 1968. Ouabaininflus auf das membranpotential bei alimentärem Kaliummangel. *Pflüegers Archiv. Gesamte Physiol. Menschen Tiere.* **304**:69.
- MAGAZANIK, L. G., and T. V. POTAPOVA. 1969. Effect of changes in extracellular ionic medium on equilibrium potentials of the extrasynaptic membrane of denervated muscle. *Biofizika.* **14**:658.
- MAGAZANIK, L. G., and F. VYSKOCIL. 1972. The loci of α -bungarotoxin action on the muscle postjunctional membrane. *Brain Res.* **48**:420.
- MCDONALD, T. F., and R. L. DEHAAN. 1973. Ion levels and membrane potential in chick heart tissue and cultured cells. *J. Gen. Physiol.* **61**:89.
- MULLINS, M. J., and M. Z. AWAD. 1965. The control of the membrane potential of muscle fibers by the sodium pump. *J. Gen. Physiol.* **48**:761.
- NASTUK, W. L., A. A. MANTHEY, and A. J. GISSEN. 1966. Activation and inactivation of post-junctional membrane receptors. *Ann. N. Y. Acad. Sci.* **137**:999.
- NELSON, P. G., J. PEACOCK, and J. MINNA. 1972. An active electrical response in fibroblasts. *J. Gen. Physiol.* **60**:58.
- PATRICK, J., S. F. HEINEMANN, J. LINDSTROM, D. SCHUBERT, and J. H. STEINBACH. 1972. Appearance of acetylcholine receptors during differentiation of a myogenic cell line. *Proc. Natl. Acad. Sci. U. S. A.* **69**:2762.
- POWELL, J. A., and D. M. FAMBROUGH. 1973. Electrical properties of normal and dysgenic mouse skeletal muscle in culture. *J. Cell Physiol.* **82**:21.
- RANG, H. P., and J. M. RITTER. 1970. On the mechanism of desensitization at cholinergic receptors. *Mol. Pharmacol.* **6**:357.
- RITCHIE, A. K., and D. M. FAMBROUGH. 1974. Electrophysiological properties of the membrane and cholinergic receptor in developing rat myotubes. Fourth Annual Meeting of the Society for Neuroscience. St. Louis, Missouri, October, 1974.
- RITCHIE, A. K., and D. M. FAMBROUGH. 1975. Ionic properties of the acetylcholine receptor in cultured rat myotubes. *J. Gen. Physiol.* **65**:751.
- SPERELAKIS, N., and K. SHIGENOBU. 1972. Changes in membrane properties of chick embryonic hearts during development. *J. Gen. Physiol.* **60**:430.
- STEFANI, E., and A. B. STEINBACH. 1969. Resting potential and electrical properties of frog slow muscle fibres. Effect of different external solutions. *J. Physiol. (Lond.)*. **203**:383.
- STEINBACH, J. H. 1975. Acetylcholine responses on clonal myogenic cells *in vitro*. *J. Physiol. (Lond.)*. In press.
- STEINHARDT, R. A., L. LUNDIN, and D. MAZIA. 1971. Bioelectric responses of the echinoderm egg to fertilization. *Proc. Natl. Acad. Sci. U. S. A.* **68**:2426.
- STEINHARDT, R. A., S. SHEN, and D. MAZIA. 1972. Membrane potential, membrane resistance and an energy requirement for the development of potassium conductance in the fertilization reaction of echinoderm eggs. *Exp. Cell Res.* **72**:195.
- TAKEUCHI, A., and N. TAKEUCHI. 1960. On the permeability of end-plate membrane during the action of transmitter. *J. Physiol. (Lond.)*. **154**:52.
- TASAKI, K., Y. TSUKAHARA, S. ITO, M. J. WAYNER, and W. Y. YU. 1968. A simple, direct and rapid method for filling microelectrodes. *Physiol. Behav.* **3**:1009.
- THESLEFF, S., and E. X. ALBUQUERQUE. 1967. Influence of phospholipase C on some electrical properties of the skeletal muscle membrane. *J. Physiol. (Lond.)*. **190**:123.
- THOMAS, R. C. 1972. Electrogenic sodium pump in nerve and muscle cells. *Physiol. Rev.* **52**:563.
- TUPPER, J. T. 1972. The ionic basis of the membrane potential in the early *Asterias* embryo. *Dev. Biol.* **29**:273.
- TUPPER, J. T., and R. D. POWERS. 1973. Na and K permeability during early echinoderm development: electrophysiological and tracer-flux determinations. *J. Exp. Zool.* **184**:356.

- VERNADAKIS, A., and D. M. WOODBURY. 1964. Electrolyte and nitrogen changes in skeletal muscle of developing rats. *Am. J. Physiol.* **206**:1365.
- VOGT, M., and R. DULBECCO. 1963. Steps in the neoplastic transformation of hamster embryo cells by polyoma virus. *Proc. Natl. Acad. Sci. U. S. A.* **49**:171.
- VYSKOCIL, F., and L. G. MAGAZANIK. 1972. The desensitization of postjunctional muscle membrane after intracellular application of membrane stabilizers and snake venom polypeptides. *Brain Res.* **48**:417.
- WILLIAMS, J. A. 1970. Origin of transmembrane potentials in non-excitabile cells. *J. Theor. Biol.* **28**:287.