An Active Electrical Response in Fibroblasts

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ABSTRACT L cells have a resting potential of about −16 mv (internal negative) at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and a potassium concentration of 5.4 mM. Membrane resistivity is about 20,000 Ωcm² when the surface filopodia described by others are taken into account. Mechanical and electrical stimuli can evoke an active response from mouse L cells, cells of the 3T3 line, and normal fibroblasts which we have termed hyperpolarizing activation or the H.A. response. This consists of a prolonged (3–5 sec) increase in the membrane permeability by a factor of 2–10 with a parallel increase in membrane potential to about −50 mv. The reversal potential for the H.A. response is −80 mv. The resting cells are depolarized to about −12 mv when the external medium contains 27 mM potassium, and the potential reached at the peak of the H.A. response is about −30 mv. The reversal potential for the H.A. response is about −40 mv in 27 mM external potassium. This effect of potassium ions on the reversal potential of the H.A. response leads us to conclude that the response represents an increase in membrane permeability, predominantly to potassium, by at least a factor of five. This increase must be greater than 20-fold if previous measurements of the ratio of potassium permeability to chloride permeability in L cells are valid for the preparation used in the present study.

INTRODUCTION

L cells (Sanford et al., 1948) and 3T3 cells (Todaro and Green, 1963) are continuous lines of mouse fibroblasts, adapted to tissue culture, which have been widely used by mammalian cell biologists. Recently the surface membrane permeabilities of L cells to sodium, chloride, and potassium ions have been studied by Lamb and MacKinnon (1971 *a*, *b*). These workers found that Na^+ and K^+ permeability are roughly equal and that the chloride permeability is nearly an order of magnitude greater. We have investigated the electrical properties of the L-cell surface membranes using intracellular microelectrode techniques and have found an active hyperpolarizing elec-

trical response of these cells to electrical and mechanical stimulation. This active electrical response seems characteristic of a variety of fibroblasts for we have found it in 3T3 cells as well as in some normal fibroblastic cells from cultures of mouse central nervous system and muscle tissue. It is rare in neuroblastoma cells and we have not seen it in muscle cells, or normal neurons in cultures. We present here results bearing on the ionic basis for this active electrical response as well as estimates of passive membrane permeabilities and electrical resistivity.

MATERIALS AND METHODS

The L-929 strain of L cells was obtained from Microbiological Associates, Inc. (Cat. No. 71-131; Bethesda, Md.) and is the same strain as that used by Lamb and MacKinnon. Three mutant subclones of this line were also studied. These were the B82 and A9 lines, developed by Littlefield (1964, 1965), which lack the enzymes thymidine kinase (TK^-) and hypoxanthine guanine phosphoribosyl transferase ($HGPRT^-$), respectively, and the clone 1D (TK^-). Clone 1D and the 3T34E (TK^-) subclone of the 3T3 line of fibroblasts were given to us by H. Coon. Cell lines were grown in 250-ml Falcon plastic flasks (Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.) in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS) and antibiotics (10–50 units penicillin/ml and 10 μg streptomycin/ml). Ionic concentrations in this medium are given in Table II. For electrical study, cells were dissociated with Puck's D1 saline (adjusted to 340 mosmols with sucrose) containing trypsin (0.05%) and plated into 60-mm Falcon plastic Petri dishes under three culture conditions. In one condition, an 80–100% confluent culture was obtained by plating at a density of 1×10^5 cells/dish into DMEM + 10% FCS and waiting 3–5 days before study. If 24 hr after plating the medium was changed to DMEM without FCS, cell division slowed, and more large cells were evident. This large cell population could also be selected by plating 2×10^6 cells/dish into DMEM + 10% FCS + aminopterin (1×10^{-5}–4×10^{-7} M) in order to kill the small dividing cells (Peacock et al., 1972). Cultures in these conditions were maintained during the electrophysiologic experiments in a heated chamber (36°–38°C) on the stage of an inverted phase-contrast microscope with an atmosphere containing 10% CO_2 in air circulated over the culture dishes. Evaporation was prevented by a thin layer of mineral oil on the surface of the medium. The experimental arrangement, details of the intracellular recording and stimulating system, and methods of processing data have been described (Nelson et al., 1971). Intracellular recording was done with 3-M KCl–filled micropipette electrodes which were arranged in a bridge circuit so that currents could be passed through the impaling microelectrode and across the cell membrane to study the electrical responses of the cells. When the culture medium was changed during the electrophysiologic studies a flow system was used which heated the fluid so that a constant culture temperature was preserved. 35-mm photomicrographs were taken of each cell studied electrophysiologically and measurements of cell dimension were made on 4×5 inch enlargements of the 35 mm film.

RESULTS

An indication of the range of culture density and cell morphology under the various conditions is given in Fig. 1. Most of the studies were done with nearly confluent cultures in which a small percentage of the cells were multinucleated giant cells. These represented a more favorable technical situation for intracellular microelectrode studies than did the mononucleated cells, although the smaller cells were studied as well.

Passive Membrane Properties in Normal Extracellular K^+ (5.4 mmoles/liter)

The average resting membrane potential of 23 L cells was 16 ± 8.5 mv. These selected cells maintained steady potentials for at least 3 min and

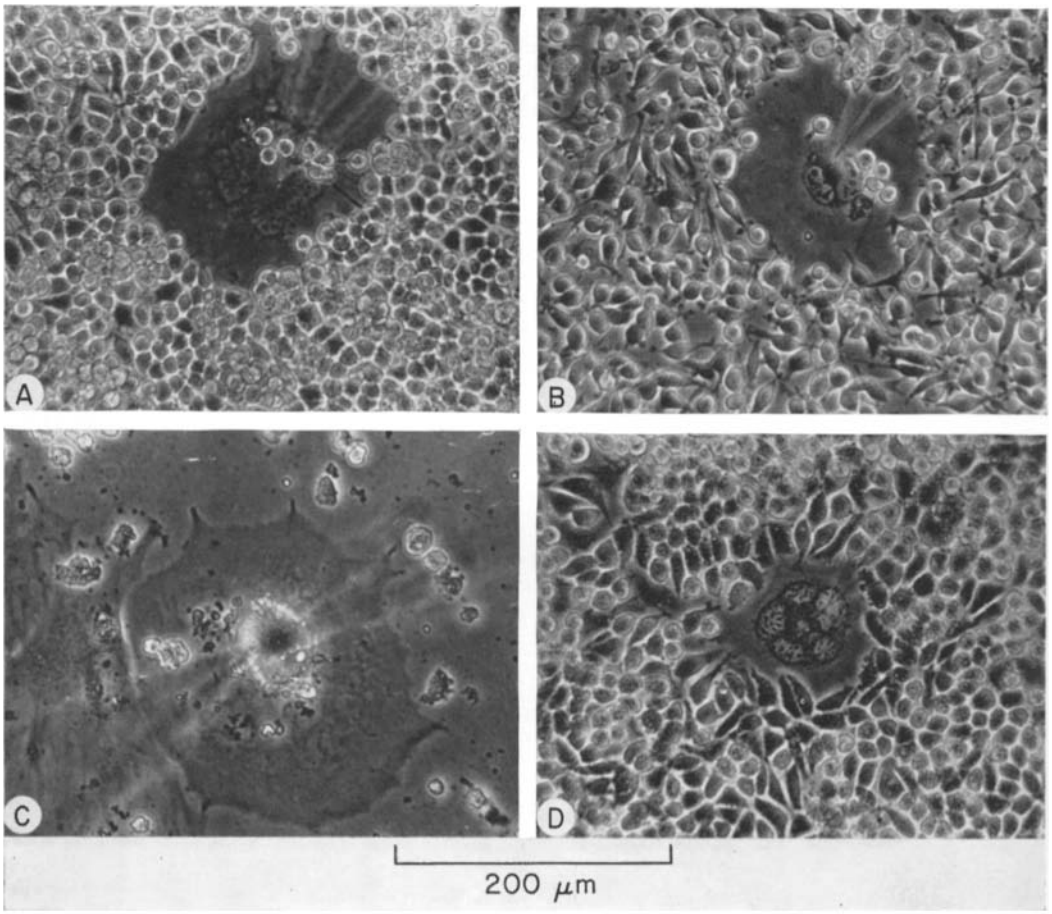

FIGURE 1. Examples of cells studied in (A)–(D): (A), large L cell (designated by microelectrode tip) in a confluent culture, clone A9. (B), similar type of cell in a confluent culture, clone 1D. (C), a surviving large L cell, clone B82, in culture medium containing aminopterin. (D), L-929 cell in a confluent culture.

usually for longer than 10 min. The membrane resistance and time constant were obtained by passing pulses of current across the cell membrane through the impaling microelectrode and by means of a bridge circuit measuring the change in transmembrane voltage produced by that current. The average resting input resistance of 15 cells studied in this way was 41 MΩ, and the membrane time constant (τ_m) was 9 msec. Assuming a relatively simple geometry for these cells corresponding to the smooth surface of a hemisphere, the specific surface membrane resistivity (R_m) would be 5000 Ωcm². Specific membrane capacity, C_m (equal to τ_m divided by R_m), is equal to 2.9 μF/cm². However, the surface of L cells is not smooth but is covered with densely packed filopodia (Price, 1970). The effect of these filopodia is to increase the surface area of the L cells by a factor of two to four over that of a smooth cell of a similar over-all dimension (Lamb and MacKinnon, 1971 *a*), so that the R_m of L cells should be between 10,000 and 20,000 Ωcm² and the capacitance is correspondingly reduced to about 1 μF/cm². Resistivity measurements as high as about 80,000 Ωcm² were obtained (assuming the actual surface area to be four times higher than the dimensions of the cells would indicate if the cell surface were smooth).

Active Membrane Hyperpolarization

L cells responded to mechanical and electrical stimuli with a marked increase in membrane potential. Features of the electrically evoked active response are shown in Fig. 2. A pulse of inward current lasting 100 msec produces the expected transient passive increase in membrane potential (upper traces, fast time base) coinciding with the pulse of current. After cessation of the current a prolonged phase of hyperpolarization occurred. This is an active response since it develops after termination of the stimulating current, and is shown in Fig. 2 B (slow time base). The threshold current for eliciting this active response ranged in different cells from 1 to 50 na. Some increase in stimulus threshold was seen following the occurrence of the active response, but no detailed study of the recovery process was done.

Membrane voltage and resistance are shown in Fig. 3 as a function of time during the active hyperpolarization and the decrease in cell membrane resistance which parallels the voltage change is clear. When large current pulses were used to elicit the H.A. response, an early depolarization, probably artefactual, was sometimes seen as a component of the response. Under these circumstances the fact that the membrane resistance and membrane potential change in parallel was sometimes obscured during the early phase of the response (Fig. 3 A). Particularly when the response was elicited by pressure from a second electrode the parallelism between resistance and potential changes was quite clear, as in Fig. 3 B. The decrease in membrane resistance is not a consequence of the membrane potential change, however,

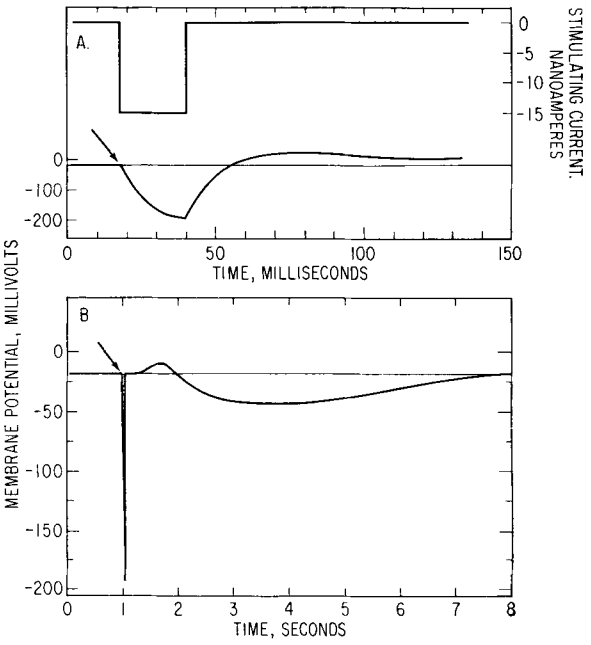

FIGURE 2. Line drawings from oscilloscope records of a hyperpolarizing response. (A) and (B) are simultaneous recordings at different time resolution (note abscissa). (A), record of the stimulus (upper trace) and passive response (lower trace). (B), record at slower time base and higher voltage gain. The arrow in (B) corresponds in time to the arrow in (A) and the large, short, downward deflection corresponds to the entire downward response in (A). Following this passive response is a brief depolarization (not a constant feature of the H. A. response) and a prolonged hyperpolarization. The overshoot following the passive response in (A) was obscured in the oscilloscope record in (B) and is not shown.

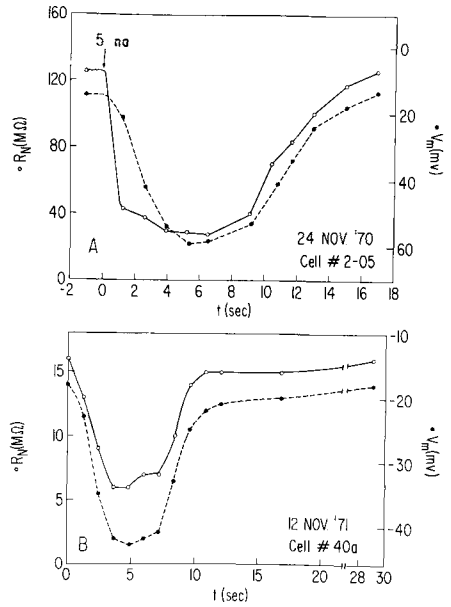

FIGURE 3. (A), graph of membrane potential (filled circles) and membrane resistance (open circles) during an H.A. elicited by a 100 msec, −5 na pulse of current delivered at the arrow. Resistance was measured by small brief test pulses of current during the course of the H.A. (B), similar graph for an H.A. response elicited by gentle pressure applied by a second pipette (filled with normal culture medium) to the cell which was being recorded from.

as is shown in Fig. 4. Membrane resistance as a function of membrane voltage is shown by the open circles; these values of membrane resistance were obtained by passing small test pulses of current across the cell membrane when the steady value of the membrane potential had been adjusted to various levels with steady currents passed through the recording microelectrode. The much larger change in membrane resistance occurring during the active

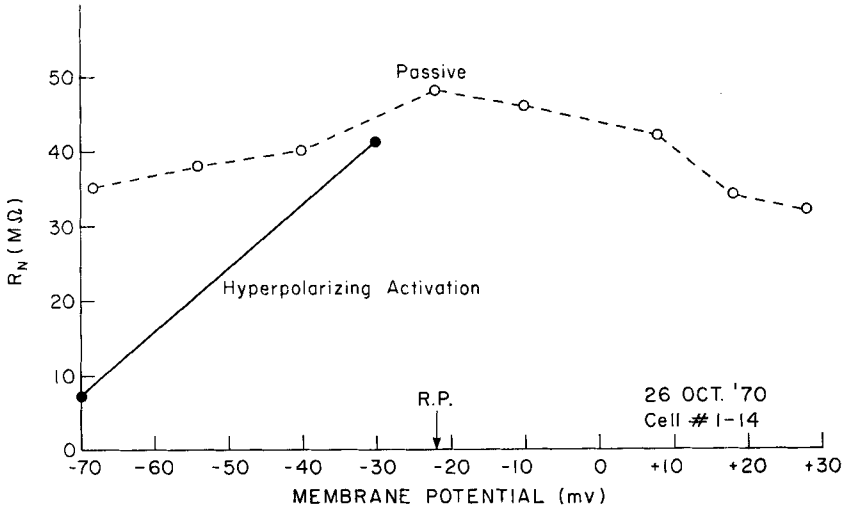

FIGURE 4. Comparison of membrane resistance changes occurring during the H.A. and during comparable passive changes in membrane potential. The filled circles indicate the membrane resistance and potential immediately before an H.A. is evoked (point corresponding to resting potential of −30 mv) and at the maximum change in membrane potential and resistance occurring during the H.A. (point corresponding to a membrane potential of −70 mv). The open circles show the variation in membrane resistance accompanying changes in membrane potential produced by steady currents delivered through the recording microelectrode. Resting membrane potential had shifted from −30 to −22 mv between the two sets of measurements.

hyperpolarizing response is shown by the filled circles. The resistance was measured during the hyperpolarizing response with small pulses of current as in the passive case above. The increase in membrane potential and large decrease in membrane resistance will be called the hyperpolarizing activation or H.A. response. The H.A. response could be elicited regularly by slight mechanical displacement of the recording electrode or pressure from a second electrode and in several cases appeared to occur spontaneously. The results of the present paper were obtained from cells in medium containing 10% fetal calf serum, but essentially similar H.A. responses were recorded from cells in serum-free media in an earlier study (Minna et al., 1971).

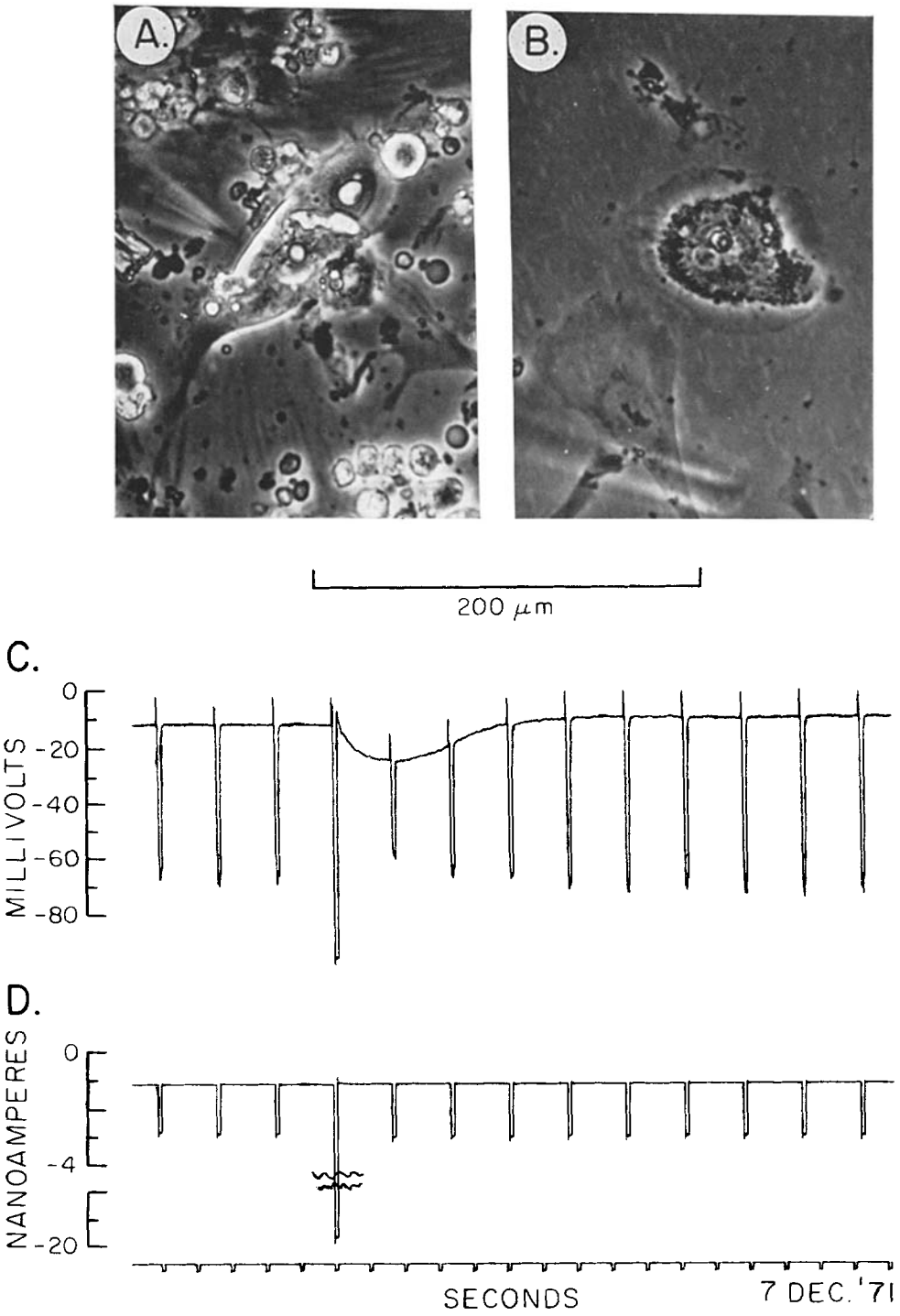

FIGURE 5. (A) and (B), examples of two different nonneuronal cells which exhibited H.A. responses. They were growing in cultures prepared by dissociation of normal fetal mouse muscle. (C) and (D), transmembrane voltage (C) and current (D) representing the H.A. response evoked in the cell shown in (A). The large stimulating pulse in (D) which evoked the response caused a voltage transient which saturated the pen recorder. Note the decreased voltage response to test pulses during the H.A. response, reflecting the decreased membrane resistance. Small upward deflections on voltage traces are 10-mv calibrating pulses.

The 3T34E cell line consisted of rather small cells under our culture conditions and extensive studies were not done due to technical limitations on the stability of the intracellular recordings. Nonetheless, clear evidence for the occurrence of the H.A. in this cell line was obtained. Similarly, the small cell population in the L cell cultures was not extensively studied, but the H.A. response was seen in the small cells. An example of the H.A. response recorded from a fibroblast growing in a culture from normal mouse muscle is shown in Fig. 5 (see Fischbach, 1972, and Peacock and Nelson, in

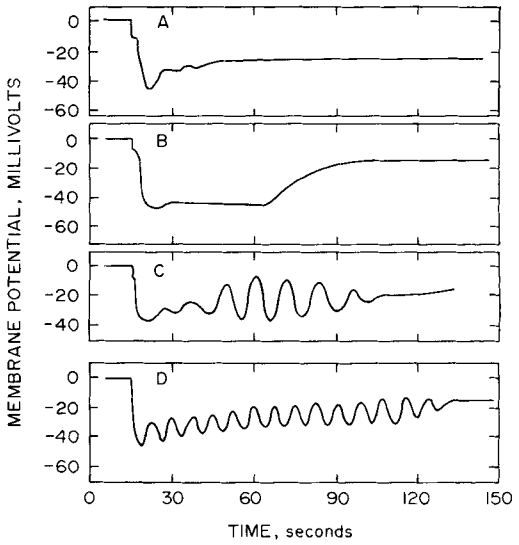

FIGURE 6. Various patterns of recorded transmembrane potential on penetration of L cells. Note the transient small negativities immediately after penetration (near the beginning of A, B and C). More prolonged, larger negativities were then seen followed by a variably timed return to a steady lesser negativity (A and B). Variable oscillatory potentials were seen in some cells, as shown in (C) and (D).

preparation, for methods of preparing such cultures). However, many fibroblastic cells in normal muscle and spinal cord cultures have large resting potentials and do not exhibit the H.A. response.

The changes in potential recorded during penetration of the L cells with a microelectrode appeared to result from an H.A. response elicited by penetration of the cells. Four different patterns of potential change during penetration of L cells are shown in Fig. 6. An initial, abrupt, and fairly small change in potential is followed by a slower increase in recorded potential lasting for several seconds (Fig. 6 A, B). Occasionally, L cells exhibited 10–20-mv oscillations in membrane potential (Fig. 6 C). As was the case with the electrically evoked H.A. responses, membrane resistivity was found to be de-

creased during the hyperpolarization associated with penetration of the cell and during the hyperpolarizing phase of the oscillations.

If the H.A. responses were due to an increase in membrane permeability to a specific ionic species, the polarity of the H.A. should be reversed when

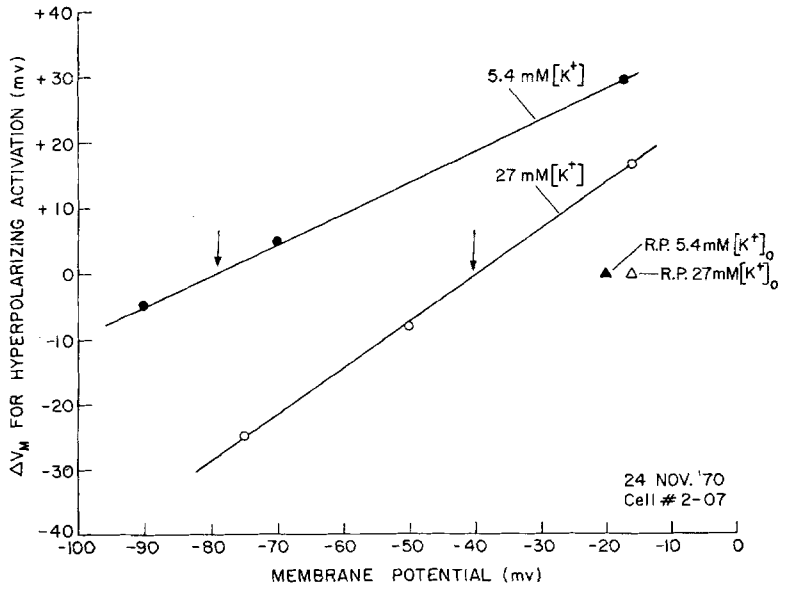

FIGURE 7. The membrane potential change occurring during an H.A. response as a function of steady membrane potential. ΔV_m = the steady membrane potential minus the membrane potential reached at the peak of the H.A. This steady membrane potential was adjusted by passing steady currents through the intracellular electrode. The open circles correspond to data obtained when the extracellular potassium concentration was 27 mM and chloride was 140 mM. The filled circles correspond to data obtained from the same cell within less than 10 min after the external medium was changed so that it contained 5.4 mM K^+, 118 mM Cl^-, and 44 mM mannitol. The arrows indicate the reversal potentials for the H.A. under the two conditions of extracellular potassium concentration. Resting potentials at the indicated external potassium concentrations are indicated by the triangles.

the steady membrane potential of the cell is made more negative than the equilibrium potential of that ionic species. The membrane potential at which the H.A. reverses would in fact be the equilibrium potential for the ions to which the L cell membrane becomes increasingly permeable during the H.A. The results of an experiment in normal medium with an external K^+ of 5.4 mM are shown in Fig. 7 (filled circles). The steady membrane potential was adjusted to various levels by steady current passed through the microelectrode and the H.A. response was then evoked by an additional large pulse of current. The amplitude of the H.A. potential measured from the

various steady baseline voltages established by the steady currents are plotted as a function of the steady membrane potential in Fig. 7. The ΔV of the H.A. varied linearly with changes in steady membrane potential and reversed at a membrane potential of −80 mv (filled circles, Fig. 7, arrow). This current clamping method used in the present study for obtaining the reversal potential of the H.A. is the method used for obtaining similar information about such membrane processes as the inhibitory postsynaptic potential (IPSP) in central neurons. The voltage-clamp technique could also give valuable additional information such as the instantaneous membrane conductance established during the H.A., but this method was not deemed necessary for the determination of the H.A. equilibrium potential.

Effects of Increased External K^+

The H.A. response was studied in solutions of high and normal potassium concentration in order to obtain some information as to the ionic basis of the response. Cells in a high K^+ solution (the usual culture medium with 3 M KCl added so as to raise the K^+ concentration to 27 mM and the Cl^- concentration to 162 mM) were compared to cells in a normal K^+ solution which was the usual culture medium containing 5.4 mM K^+ with 44 mM of mannitol added to provide an osmolarity comparable to the high K^+ solution.

The average resting potential of nine cells in 5.4 mM K^+ and 44 mM mannitol was −16 mv. This is the same as the resting potential found in 5.4 mM K^+ in the absence of mannitol. In 13 cells studied in the high K^+ solution the average steady resting potential was −12 mv. If this group of cells is compared with all the cells studied in normal K^+ (both with and without added mannitol) the decrease in resting potential seen in the cells studied in high K^+ is significant at the $<5\%$ level. As is shown in Fig. 7 (open circles, arrow), the reversal potential for the H.A. was shifted to about −40 mv, and peak voltage of the H.A. evoked at resting potential decreased from −50 mv in 5.4 mM K^+ to about −30 mv in 27 mM K^+. It should be noted that in the case of the data in Fig. 7, the cell was studied initially in the high K^+ solution and then restudied after replacement with the low K^+ mannitol solution. The lower H.A. amplitudes and reversal potential in the high K^+ solution thus cannot be explained on the basis of cell injury or deterioration following a second penetration in a different solution. Table I shows a comparison of resting potential, H.A. peak voltages, and H.A. reversal potential for three cells studied in 5.4 mM K^+ and three cells studied in 27 mM K^+. Only the cell the responses of which are given in Fig. 7 was studied in both solutions. Attempts to study changes in membrane properties during the changing of solutions were unsuccessful.

DISCUSSION

The present experiments have provided observations on L cell membrane potential and resistivity at two concentrations of external potassium chloride, at rest, and during the H.A. (hyperpolarizing activation) response. Qualitatively the results may be summarized as follows. (*a*) L cell resting membrane potential is low relative to many excitable cells and is decreased slightly by an increase in extracellular K^+. (*b*) The H.A. response consists of a decrease in membrane resistivity and an increase in potential which can be elicited by electrical and mechanical stimuli. (*c*) The amplitude of the H.A. is substantially decreased by raising extracellular K^+. (*d*) The equilibrium potential for the H.A. is decreased by nearly 40 mv for a fivefold increase in extracellular K^+ (Fig. 7 and Table I).

TABLE I

COMPARISONS OF RESTING POTENTIAL, H.A. PEAK VOLTAGES, AND H.A. REVERSAL POTENTIAL

External potassium concentration	Resting membrane potential	Membrane potential at peak of H.A. response	Reversal potential for H.A.	No. of cells
mmole/liter	*mv*	*mv*	*mv*	
5.4	−17	−50	−75	3
27	−10	−30	−38	3

These observations suggest the following interpretations. Since membrane potential during the H.A. is much more affected by changes in external K^+ than is the resting potential, the membrane permeability to K is substantially higher during the H.A. than it is at rest. In fact, the change in the equilibrium potential for the H.A. produced by high external potassium suggests that the H.A. represents an increase in membrane permeability largely or exclusively for potassium. Such a mechanism would result in a 40 mv shift in the equilibrium potential for a fivefold change in external K^+ and this is very nearly what is observed. The H.A. response can be clearly differentiated from the increase in potassium permeability that occurs in nerve and muscle cells as a component of the action potential on the basis of time-course and the means by which the response is elicited. Hyperpolarizing responses due to potassium activation have been described in other tissues and are discussed by Grundfest (1971). Our observations do not rule out the participation of other ions in the H.A. response. If chloride permeability were importantly involved, however, the increase in external chloride should have increased the H.A. in the experiment shown in Fig. 7 and the reverse was observed. A decreased Na^+ permeability, which could account for the membrane potential during the H.A., is clearly incompatible with

the observed large decrease in cell resistance. Combinations of decreased Na^+ permeability, increased chloride permeability, and increased potassium permeability cannot be ruled out on the basis of our data, however.

Utilizing the Hodgkin-Katz version of the constant field equations (Goldman, 1943; Hodgkin and Katz, 1949), one may compute the change in the cell membrane permeability to potassium, or P_K, required to produce a given change in membrane conductance and membrane potential for various potassium-to-chloride permeability, or P_K/P_{Cl}, ratios. Previous flux measurements had indicated that the L cell membrane is much more permeable to chloride than it is to potassium and the resting P_K/P_{Cl} ratio obtained by Lamb and MacKinnon was 0.12. If this ratio is characteristic of the L cell under the conditions used in the present experiments, then P_K would have to increase by a factor of 25 or so during the H.A. response, even in those cases where the cell membrane conductance increased by only a factor of two. If P_K and P_{Na} are comparable or even larger than P_{Cl}, then a 5- to 10-fold increase in P_K would be sufficient to generate the H.A. response. We have inferred that the reversal potential for the H.A. represents the equilibrium potential for K^+. This implies that, at rest, the permeability of some cation, such as Na^+, which has a concentration gradient across the membrane opposite to K^+ must have a permeability of the same order as K^+. This is required to explain the relatively low resting membrane potential and assumes that anions such as chloride are either impermeant or are at equilibrium.

Data from Lamb and MacKinnon are compared in Table II with representative data and calculated values of ionic permeabilities from the present work. Cases I and II represent a 10-fold range in the value for P_K/P_{Cl}. Values for both case I and case II are consistent with the measured membrane voltages and conductances and the resultant difference in the change of P_K which is required to account for the H.A. is shown in the last line of the table.

Our results are in general agreement with those of Lamb and MacKinnon, although some discrepancies are evident. Our measurements of membrane resistivity indicate that the resting conductance in our system is larger by a factor of two to three than the conductance calculated from Lamb and MacKinnon. The P_{Na}/P_K ratio is similar for both studies but sodium and potassium permeability as calculated in the present study are larger than in the Lamb and MacKinnon study. These permeability differences may be related to three differences between the culture conditions that were used in the two studies. Lamb and MacKinnon did their experiments in a Krebs's salt solution that included a tris(hydroxymethyl)amino methane–citrate buffer in which the free calcium ion concentration was 0.8 mM. Our medium was a complete growth medium and contained serum. The present experi-

ments were done at 37°C while the Lamb and MacKinnon experiments were done at about 20°C. Temperature dependence of the permeabilities would be expected and some differential effect on the different ionic permeabilities would not be unexpected. In addition, the data in the present study were obtained from a highly selected subgroup of the population (large multinucleated cells), while the flux measurements of Lamb and MacKinnon measured the permeability properties of the culture as a whole.

TABLE II

COMPARISON OF DATA FROM LAMB AND MacKINNON AND THIS PAPER

	Lamb and MacKinnon	Present work	
		Case I	Case II
$[K^+]_o$, *mmole/liter*	5.7	5.4	
$[Cl^-]_o$, *mmole/liter*	147	118	
$[Na^+]_o$, *mmole/liter*	137	145	
$[Ca^{++}]_o$, *mmole/liter*	0.8	1.8	
V_m (rest), *mv*	−15	−16	
G (rest), *mmho/cm²*	2×10^{-8}	5×10^{-8}*	
G (H.A.), *mmho/cm²*		10×10^{-8}*	
P_{Cl}, *cm/sec*	0.5×10^{-7}	0.22×10^{-7}	11×10^{-7}
P_{K_1}/P_{Cl}		5.0	0.55
$P_{Na}//P_{Cl}$		2.3	0.12
P_{K_2}/P_{K_1}		5.3	26

P_{K_1} = potassium permeability at rest.

P_{K_2} = potassium permeability during the H.A.

P_{Na} and P_{Cl} = sodium and chloride permeability which are assumed not to change during the H.A.

Case I assumes relatively low chloride permeability and an unchanging internal chloride concentration when external K^+ and Cl^- concentration are changed.

Case II assumes a relatively large chloride permeability and rapid equilibration of chloride when extracellular chloride concentration is changed.

* Area measurement includes a four-fold increase contributed by surface of filopodia (see Results).

It appears that the H.A. response can be elicited either by electrical stimuli or by mechanical stimuli. From an experimental standpoint, the H.A. response provides an electrical marker for four lines of cells suitable for use in cell fusion studies (Minna et al., 1971). Attributing any functional role to this electrical response can only be speculative at this point. However, the prolonged time-course of the H.A. response and the very large increase in potassium permeability that it represents means that large changes in transmembrane potassium fluxes should occur during this active response. The driving force on Na^+ ions is probably nearly doubled by the increase in membrane potential that occurs during the response. Thus, some altera-

tion in intracellular ionic concentrations would be expected if mechanical stimuli, for instance, produced a prolonged series of H.A. responses. The magnitude of the alteration in ionic concentrations would depend on the surface-to-volume ratios of the cells involved. The extremely flattened form of many fibroblastic cell types would tend to accentuate such alterations. Whether mechanical stimuli can induce such changes and whether they have any effect on cell metabolism or mitosis are experimentally approachable questions.

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