Transmission on an Active Electrical Response between Fibroblasts (L Cells) in Cell Culture

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ABSTRACT An active electrical response, the hyperpolarizing activation or H.A. response, is characteristic of L cells (a continuous line of fibroblasts) and is transmitted in a decremental manner between contiguous cells. Direct electrical coupling between pairs of L cells occurs occasionally, but transmission of the active electrical response is not dependent on such electrical connections. Some L cells are sensitive to acetylcholine but the transmitted response is not dependent on a cholinergic mechanism. 5-Iodosalicylate blocks the active electrical response. The response can be elicited readily by mechanical stimuli, and thus can serve both as a mechanical and chemical receptor mechanism and as a means of communication between cells.

INTRODUCTION

L cells, established cell lines of mouse fibroblastic origin, respond to mechanical, chemical, and electrical stimuli with a prolonged, 20–30 mV increase (hyperpolarization) in transmembrane potential (Minna et al., 1971; Nelson et al., 1972; Nelson and Peacock, 1972). This response is primarily due to a large increase in membrane permeability to potassium and is an active process in the sense that it outlasts the stimulus by several seconds. We have termed the response a hyperpolarizing activation or H.A. response (see Grundfest, 1971 for discussion of analogous responses in other tissues). A variety of L-cell clones exhibit the response as do some fibroblasts grown in culture from embryonic mouse spinal cord and muscle tissues. Neurons and muscle cells do not themselves generate the response, and mouse neuroblastoma cells do so in only a small minority of cells.

We present here observations relating to the transmission of the H.A. response from one L cell to another, as well as some pharmacologic properties of the response.

MATERIALS AND METHODS

The following cell types were used in the present study: the L929 clone of L cells obtained from Microbiological Associates, Inc., Bethesda, Md., the A9 and B82 mutant clone of L cells (courtesy of Dr. J. Littlefield), and the LM (TK^-) mutant clone of L cells (courtesy of Dr. F. A. McMorris). These cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Grand Island Biological Co., Grand Island, N.Y.) containing 10% fetal calf serum (Colorado Serum Co., Denver, Colo.) + 10 μg streptomycin and 10 U Na-penicillin G per ml. This basic medium will be called medium A. In some cultures selection of nondividing cells was accomplished by incubating the cells in medium A to which had been added 4×10^{-7} M aminopterin (Peacock et al., 1972). In others, cells were studied after growing to confluency in medium A. In still others, confluent cultures of large nondividing cells were obtained by exposing confluent cultures in flasks (Falcon Plastics, Oxnard, Calif., 250 ml) to 5,000 rad of X-irradiation. About 9 days after irradiation cells were dissociated using trypsin (Grand Island Biological Co.), 0.25% in Puck's saline D1, and plated at $1–2 \times 10^6$ cells per 60 mm plate. The cells were suitable for study within about 1 wk after plating.

Standard electrophysiologic techniques were used for intracellular recording and stimulation via 3 M KCl micropipette electrodes. Cultures were maintained at 36°–38°C and at a proper pH by circulating a heated mixture of 10% CO_2 in air over and around the culture dish. Evaporation from the culture dish was prevented by a thin layer of mineral oil placed on the surface of the culture medium at the time of the experiment. The recording and iontophoretic technique for applying drugs via an extracellular micropipette electrode has been described (Nelson et al., 1971). Solutions were changed while recording from single cells by a technique similar to that described by Steinhardt et al. (1971). Drugs were also occasionally added directly to the medium in the culture dish in concentrations such that, when diluted, they were at the selected effective concentration. This method results in less precise control of the actual concentrations reaching the cells, but is reproducible and provides very prompt delivery of the drugs. 5-Iodosalicylate (5-I-sal) was kindly supplied by Doctors Levitan and Barker.

RESULTS

A confluent population of large nondividing cells results from the X-irradiation and subsequent high density plating described in the Methods section. Intracellular recordings of transmembrane potential obtained from two cells in such confluent, postirradiation cultures are shown in Fig. 1. Patterns of potential change during penetration of these cells which are typical for L cells are shown near the middle of the traces in Fig. 1 A. These two cells were not electrically coupled, for current pulses injected into one cell of the pair did not produce any membrane voltage change in the other. When an H.A. response was elicited electrically in cell 1, no corresponding hyperpolarization was seen in cell 2. However, when electrical stimulation of cell 2 produced an H.A.

response, a large hyperpolarizing response was seen in cell 1 (near end of trace in Fig. 1 A). A subsequent H.A. in cell 2 was accompanied by a smaller hyperpolarization in cell 1 (Fig. 1 B; note that the time base is different from Fig. 1 A). A latency of about 15 s occurs between the H.A. in cell 2 and the hyperpolarization in cell 1. Note the very large decrease in the electrical

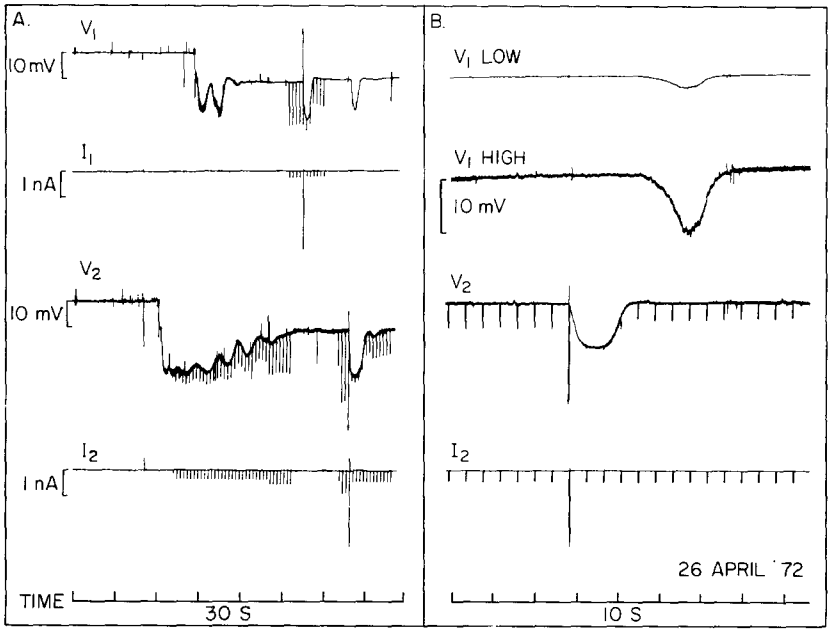

FIGURE 1. Intracellularly recorded transmembrane potentials obtained from a pair of LM (TK^-) cells selected by X-irradiation. A. V_1, potential recorded by microelectrode no. 1. I_1, current passed through electrode no. 1; V_2, potential recorded by microelectrode no. 2. I_2, current passed through electrode no. 2. Penetration of cells no. 1 and no. 2 by electrodes corresponding to V_1 and V_2 took place near middle of traces and typical penetration patterns of voltage change are shown. Large hyperpolarizing current pulses on I_1 and I_2 elicit hyperpolarizing responses on V_1 and V_2, respectively. H.A. response also occurs on V_1 after large pulse on I_2. B. Electrically elicited H.A. recorded on V_2 is followed after 10–20 s delay by a hyperpolarization on V_1. Response is lower amplitude and longer latency than was the case in A. (Note faster time scale in B than in A.) 10 mV calibration for V_1 in A holds for V_1 low in B.

membrane resistance of cell 2 (indicated by the voltage deflections produced by the test current pulse shown on I_2 of Fig. 1 B) and the absence of electrical coupling between cells throughout the recordings shown in both Figs. 1 A and B.

As shown in a previous study (Nelson and Peacock, 1972), iontophoretic application of acetylcholine (ACh) to L cells can elicit a hyperpolarizing response similar to the electrically elicited or propagated H.A. The reversal

potential for the ACh-induced H.A. response is about −80 mV as shown in Fig. 2. Extrapolated ACh reversal potentials in four other cells averaged −84 mV. This is essentially the same as the reversal potential for the electrically induced H.A. response (Nelson et al., 1972) and the electrically and chemically induced responses occlude with one another (Nelson and Peacock, 1972). Fig. 3 A shows a transmitted and an ACh-elicited hyperpolarization. A solution containing atropine sulfate (10^{-4} M) blocked the ACh response (Fig. 3 B,

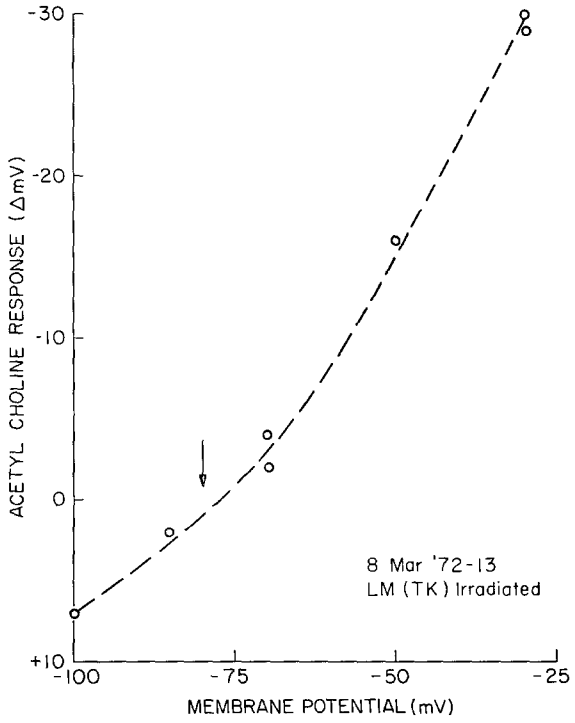

FIGURE 2. Plot of the amplitude of the ACh-elicited H.A. response as a function of the steady membrane potential occurring at the time the ACh was applied. The reversal potential for the ACh response is indicated by the arrow and is close to −80 mV.

beginning of trace) while the transmitted response (due to mechanical stimulation of an adjacent cell at time marked by arrow on Fig. 3 B) was not blocked. Thus, ACh would not appear to be involved in the conduction of the H.A. response from one cell to another. Curare (10^{-4} g/ml) failed to block either the transmitted H.A. or the ACh-elicited response (not shown). The ACh-elicited responses were not nonspecific responses due to current flows, because current from a choline chloride-filled pipette did not produce the H.A. response, even when tested on cells shown to be responsive to ACh.

We have tested the sensitivity of L cells to potassium ions iontophoretically applied to the cell surface from K-acetate-filled micropipette electrodes. We

calculate that, during an H.A. response, the total K^+ efflux in a single L cell is of the order of 1–10 nA, of which substantially less than 1 nA could be expected to impinge on any given neighboring cell. K^+ currents of this magnitude do not evoke an H.A. response (Fig. 3 C) and currents of over 20 nA have failed to elicit an H.A. response. This test, of course, does not rule out the

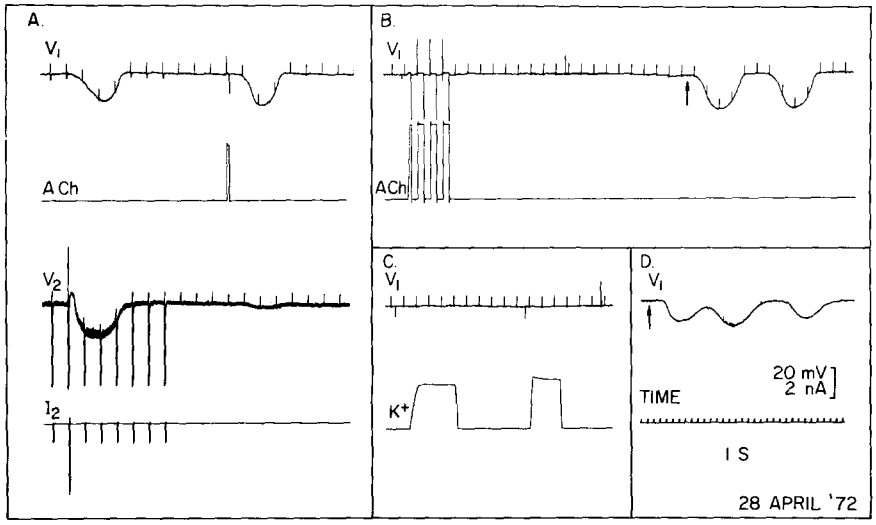

FIGURE 3. Transmembrane potential recordings from a pair of LM (TK^-) cells in an X-irradiated culture. A. V_1 and V_2 potential recordings from cell 1 and cell 2, respectively; ACh current passed out of an ACh-filled microelectrode in close apposition to the membrane of cell 1; I_2 current passed across the membrane of cell 2 through the microelectrode used to measure the membrane potential of that cell. Electrically evoked (large pulse on I_2) H.A. response in cell 2 is followed by H.A. in cell 1. ACh pulse elicits H.A. in cell 1 with no corresponding response in cell 2. B. Records obtained after perfusing the preparation with culture medium containing 10^{-4} M atropine sulfate. ACh pulses no longer elicit H.A., but mechanical stimulation of adjacent cell (arrow below V_1) does elicit repetitive H.A. response. C. Large prolonged currents from potassium acetate-filled micropipette electrode immediately adjacent to the recorded cell does not elicit an H.A. response. D. Mechanical stimulation of adjacent cell still elicits a prolonged H.A. response in the presence of 10 mM Mg^{++} in the culture medium. Calibrations in D apply to all records in the figure.

participation of K^+ ions in the conduction of the H.A. response, since K^+ ions released from a cell might be distributed very differently from those released from a micropipette. The test shown in Fig. 3 C demonstrates, however, that L cells are not generally responsive to localized application of K^+ ions. Perfusion with solution containing 27-mM K^+ ions also generally fails to elicit H.A. responses, although the mechanical disturbance associated with perfusion with any solution may elicit the response.

Transmission of the H.A. response was not blocked by 10-mM magnesium

ions (Fig. 3 D), and we have seen transmitted responses in cells incubated in 20 mM Mg^{++} + 1 mM EGTA. A very low ratio of calcium to magnesium ions thus does not block transmission of the H.A. response between L cells.

Although electrical coupling between L cells is not necessary for transmission of the H.A. response, electrical coupling was seen, and examples are shown in Fig. 4. Attenuation of the signal occurs between one cell and the other, and the coupling coefficient (defined as V_1/V_2 when current is injected into cell 2) is about 0.25 in the case of Fig. 4 A. The coupling coefficient ranged

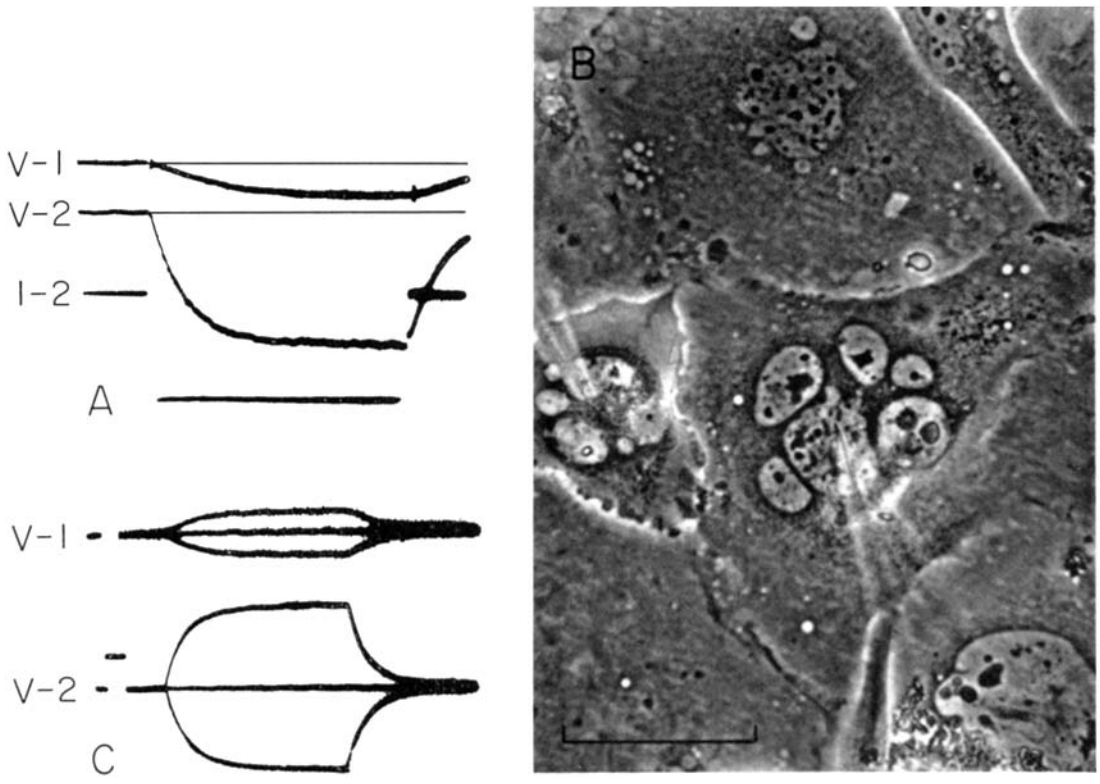

FIGURE 4. Recordings and microphotographs obtained from confluent irradiated cultures of LM (TK^-). A. Simultaneous recordings from two cells indicated by microelectrode shadows in B. V_1 is the transmembrane potential from cell near left edge of field; V_2 is the transmembrane potential recorded from next cell to the right. I_2 represents the current passed through the recording electrode V_2. Current pulse is about 3.5 nA. Baseline on V_1 and V_2 has been drawn in. B. Photomicrograph of culture. Calibration bar is 100 μm. × 250. C. Simultaneous recordings from another pair of cells. Three superimposed traces are shown: one with no current being passed, one with 1 nA of inward current being passed through the electrode recording V_2, and one with 1 nA of outward current through the same electrode. Note symmetry in hyperpolarizing and depolarizing potentials occurring in V_1 traces. Calibration pulse in V_2 represents 10 mV and 10 ms in all voltage traces in this figure.

from zero (most cell pairs examined) to nearly 1 in a few cases. No evidence of rectification in the electrical contacts was seen, as shown in Fig. 4 C.

Transmission of the H.A. response was not all-or-none, as was shown in Figs. 1 A and B, and spread of the response over distance was decremental. The greater the distance between two cells, the smaller the H.A. response occurring in one as a result of stimulation of the other. Some response was seen in cases in which several (up to eight) cells were interposed between the stimulated and recorded cells, and large transmitted responses were seen with two to three interposed cells (Fig. 5).

We have studied the effect on the H.A. response of two agents which may be related to K^+ movements across cell membranes. Diphenylhydantoin affects active K^+ transport in lobster axons (Fertziger et al., 1971) as well as stimulating Na^+, K^+-activated ATPase in brain (Festoff and Appel, 1968) and Na^+

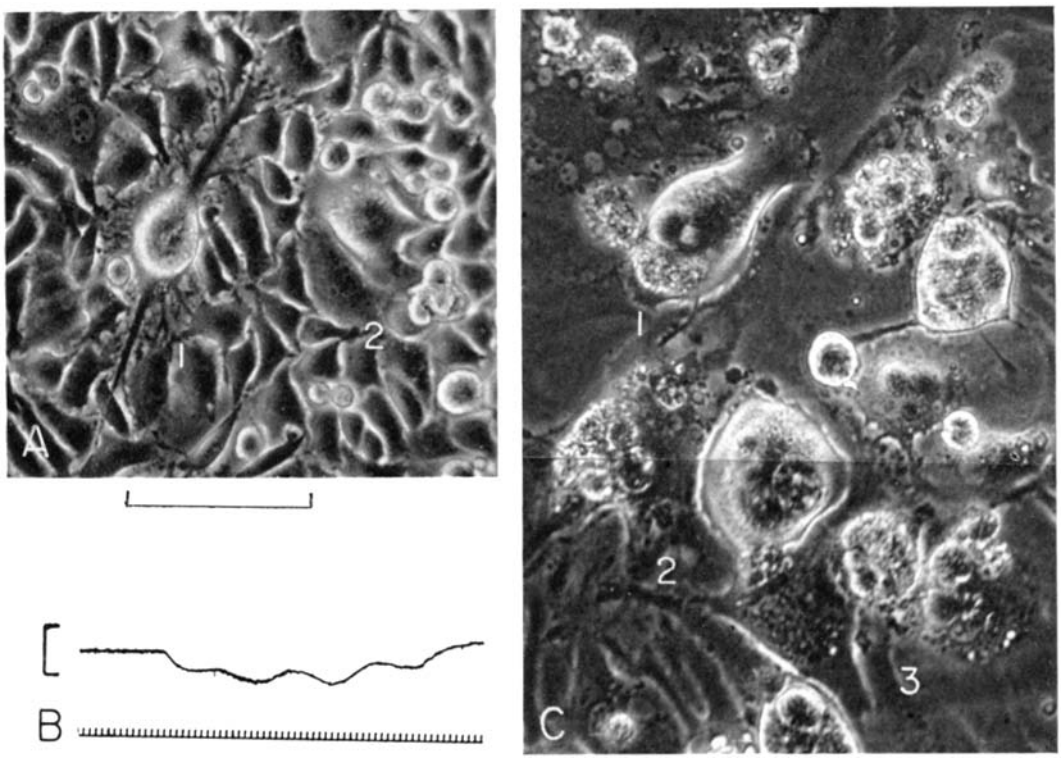

FIGURE 5. LM (TK^-) cultures at confluency (A) or X-irradiated (C). A, B. Cell labeled 1 in A gave the hyperpolarizing response shown in B when cell labeled 2 was mechanically stimulated. Time calibration in B, 1 s; voltage calibration, 20 mV. Resting potential level at beginning of trace, 15 mV. Bar under A represents 100 μm in A and C. × 250. C. Cell no. 1 gave large (>15 mV) hyperpolarizing responses when cell no. 2 or cell no. 3 were stimulated mechanically.

movements in rat brain (Woodbury, 1955) and frog skin (Watson and Woodbury, 1972). Salicylates have been shown to increase the resting K^+ permeability of molluscan neurons (Barker and Levitan, 1971; Levitan and Barker, 1972).

Diphenylhydantoin had no detectable effect on the membrane properties of L cells in doses of 50–100 μmol/liter, as shown in Table I. Resting membrane potential, membrane potential at the peak of the H.A. response, and resting cell membrane resistance are all similar for cells in control medium as compared to cells in medium containing diphenylhydantoin.

Salicylate (in the form of 5-I-sal) did significantly affect both the resting membrane electrical resistance and the occurrence of the H.A. response. Fig. 6 shows the changes in membrane potential and resistance occurring in two

TABLE I

MEMBRANE PROPERTIES OF L CELLS

Additions to medium A	Average resting potential (−mV)	Average H.A. potential (−mV)	Average cell resistance (MΩ)	no.
None	15	26	27	52
Diphenylhydantoin (50–100 μM)	15	24	26	20
5-I-sal (5 mM)	12	11	41	24

Differences between membrane properties of cells in medium A and medium A + diphenylhydantoin are not significant. Differences between the H.A. potential and cell resistances of cells in medium A and cells in medium A + 5-I-sal were significant at the $P < 0.001$ and $0.01 < P < 0.025$ level, respectively.

cells from which intracellular recordings were obtained during application of 2.5 mM and 5.0 mM 5-I-sal. A transient change in membrane potential occurred as membrane resistance increased three- to fourfold, and after treatment with 5 mM 5-I-sal, H.A. responses could not be elicited by electrical or mechanical stimulation. Table I shows the slight decrease in cell membrane potential, absence of H.A. response (the membrane potential after stimulation normally adequate to elicit an H.A. response is essentially the same as the resting membrane potential), and increase in cell membrane resistance produced in a population of L cells by application of 5 mM 5-I-sal. We have found that incubating L cells in a culture medium containing 1 mM or more 5-I-sal for 2–4 days markedly reduces cell mitotic activity and viability. No acute change in morphology was evident when 5-I-sal was added to the cultures during the electrophysiologic experiments, and since cell membrane electrical resistance was higher in the presence of 5-I-sal, the semipermeable nature of the membrane was enhanced. Acute toxicity or cell death would be

expected to increase the leakiness of the cell membranes and therefore reduce cell membrane resistance. The changes produced by the acute application of 5 mM 5-I-sal were promptly and completely reversed when the normal medium was reapplied to the cells.

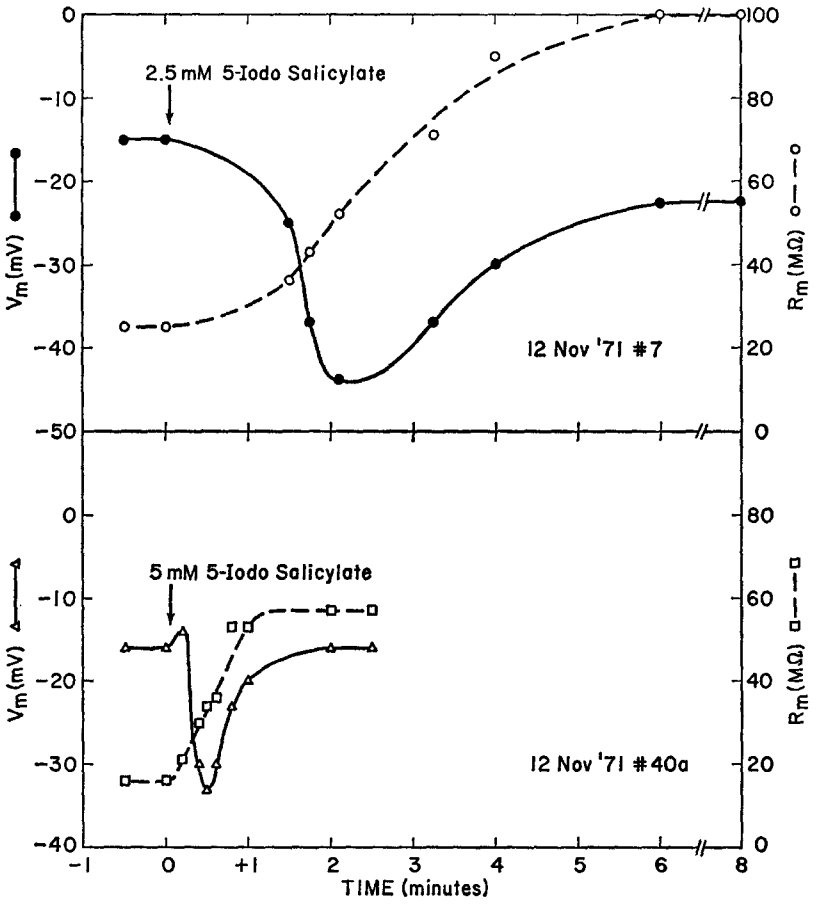

FIGURE 6. Plots of L-cell membrane resistance and potential during the course of changing the culture medium from normal (DMEM-10) to DMEM-10 plus 5-I-sal (2.5 mM in upper graph and 5 mM in lower graph). In each case 0.4 ml of × 10 concentrated 5-I-sal was added to 4 ml of culture medium in the culture dish.

DISCUSSION

The evidence presented here and in previous papers (Nelson et al., 1972; Nelson and Peacock, 1972) has dealt with the characteristics of an active electrical response in normal and continuous lines of fibroblasts. Electrical, mechanical, and chemical stimuli can elicit a prolonged (several seconds) increase in fibroblast membrane potential which is primarily due to an increase in membrane permeability to K^+ ions.

The primary result of the present work is the observation that this active response can be transmitted from one L cell to another. The occurrence of an H.A. in one L cell may elicit changes in the membrane potential of other adjacent cells, or cells one or more cell diameters away from the initiating cell. The basis for this transmission of the H.A. is not clear from the present work. ACh iontophoretically applied to the L-cell membrane can elicit a hyperpolarizing response, but ACh does not mediate the intercellular communication because atropine does not block transmission and it does block the ACh-elicited response. The ACh receptor mechanism is evidently not nicotinic in nature, for *d*-tubocurarine does not block the ACh-elicited response. Transmission of the H.A. response is not electrically mediated although definite electrical coupling between L cells does occur in a minority of cases. Transmission of the H.A. does occur in the absence of detectable electrical coupling (Fig. 1). It does not appear likely that the K^+ release accompanying an H.A. is responsible for the intercellular transmission of the response, although we have not ruled out this possibility. Iontophoretic application of K^+ ions to an L cell in amounts substantially greater than would be expected to impinge on a cell during adjacent H.A. activity does not produce the response. Alteration of the Ca^{++} and Mg^{++} ions did not appear to modify transmission of the response. We have used 10-mM Mg^{++} solutions and solutions containing 1 mM EGTA and 20 mM Mg^{++} without effect. While this would affect humoral release in many systems (Jenkinson, 1957; Katz and Miledi, 1965), it does not rule out a humoral mechanism of propagation in the present case of transmission between fibroblasts. The possibility exists of a mechanical basis for the transmission. Some contractile element could be activated in a cell undergoing an H.A. response, and a microscopic contraction could elicit a response in an adjacent cell. Preliminary experiments with cytochalasin B indicate that transmission is not entirely blocked by this compound even when striking morphologic changes have resulted from its application (Nelson and Godfrey, unpublished).

Our interpretation of the results of the present paper is that the hyperpolarizing response to electrical, chemical, and mechanical stimuli and that due to intercellular interaction all are due to a common mechanism. This mechanism, however, can be activated by these diverse sorts of stimulating agents. An analogy may be drawn to the action potential mechanism which can be activated by a variety of physical stimuli. In this case a common element involved in eliciting an action potential is membrane depolarization. In the case of the H.A. mechanism we have not identified the common, causal process.[1]

Salicylate (5 mM 5-I-sal) blocked the H.A. response, decreased cell mem-

[1] Work done subsequent to the submission of the present article indicates that intracellular injection of calcium ions can elicit the H. A. response. (Nelson, Shainberg, and Bullock, unpublished observations).

brane potential, and increased cell resistance. Our interpretation of this is that salicylates markedly decrease the large L-cell membrane permeability to chloride (Lamb and MacKinnon, 1971 *a*, *b*) as is the case with erythrocytes (Dalmark and Wieth, 1972). The K^+ and Na^+ permeability of molluscan neurons is increased by salicylates (Barker and Levitan, 1971) and if this is the case with L cells, it might explain the blockade of the response. If the K^+ permeability were increased by the salicylates, the potassium mechanism might be unavailable to participate in the H.A. The decrease in cell electrical conductance would be explained if the decrease in chloride conductance more than compensated for the increase in K^+ and Na^+ conductance. The transient hyperpolarization shown in Fig. 6 may well be artifactual and constitute essentially an H.A. response produced mechanically during addition of the salicylate. The slight degree of depolarization produced by the salicylates would be explainable by some Na^+ permeability increase which would counterbalance the hyperpolarization expected from a significant K^+ permeability increase. An alternative, simpler hypothesis would be that salicylates act to decrease L-cell membrane permeability to K^+. Our data do not contradict this explanation but, as mentioned, work on other preparations would argue against its validity. The concentration of salicylates which were required in order to block the H.A. response were high, about two- to fivefold greater than that giving a maximal effect on K^+ permeability in the molluscan system (Levitan and Barker, 1972). L-cell mitotic activity and viability is markedly reduced over a several day period by about 1 mM 5-I-sal (Nelson, Peacock and Godfrey, unpublished). The acute effect of salicylates is not a nonspecific toxic one, however, since cell membrane electrical resistance and permselectivity is increased and the salicylate effects are promptly and completely reversible. Whether the salicylate effects on fibroblast function demonstrated in the present work have any relevance for the in vivo mode of action of salicylates is not at all clear.

As a working hypothesis, we suggest that the hyperpolarizing response may subserve two functions: Firstly, it may represent a receptor potential which is generated in response to mechanical and chemical stimuli applied to the cells. Secondly, communication between L cells could be mediated by the hyperpolarizing response. The conduction of the response is decremental and only short range (ca. 0.5 mm) intercellular interactions could be mediated by it, but this might be important in ensuring a uniform response in a population of fibroblasts subjected to some nonhomogeneous stimulus.

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