Acetylcholine Responses in L Cells

Abstract. *L cells, a family of continuous cell lines of mouse fibroblastic origin, generate a prolonged active membrane hyperpolarization (the hyperpolarizing activation response) when stimulated mechanically or electrically. Iontophoretically applied acetylcholine elicits a similar response; atropine blocks the acetylcholine but not the electrically or mechanically elicited responses. The hyperpolarizing activation response can also be elicited by electrical, mechanical, or acetylcholine stimulation of cells adjacent to the recorded cell. Propagation of the response from one cell to another is not dependent on direct electrical coupling between cells and is not blocked by application of a bath containing atropine or curare. These results show that L cells are capable of generating an active electrical response, that they are sensitive to at least one neurotransmitter (acetylcholine), and that humorally mediated interaction (probably noncholinergic) between L cells occurs.*

A monolayer of large, nondividing L cells can be obtained by x-irradiation of confluent cultures. These large cells are favorable for electrophysiologic study, and stable intracellular recordings of the membrane potential can be obtained readily. We have shown (*1*) that, whereas these cells have low resting potentials (10 to 30 mv, inside negative) and exhibit no evidence whatever of action potential generation, they do produce an active electrical response when appropriately stimulated (Fig. 1A). This response consists of an increase in membrane conductance and potential that lasts 5 to 10 seconds. We now show that this response can be elicited by iontophoretically applied acetylcholine (ACh) and that the ACh response is blocked by atropine.

Conventional intracellular recording and stimulating techniques were used with either 3*M* KCl or 4*M* potassium acetate electrodes (resistance, 30 to 100 megohms). Acetylcholine chloride (0.2 g/ml) electrodes with resistances of greater than 70 megohms and atropine sulfate (0.1 g/ml) electrodes with resistances usually greater than 100 megohms were connected to pulse and d-c generators through 1000 megohms limiting resistors so that these drugs could be delivered by iontophoresis. Cultures of the LM line of L cells were grown to confluency in Dulbecco's modified Eagle's medium (Gibco) containing 10 percent fetal calf serum (Colorado Serum) with 10 units of sodium penicillin G and 10 μg of streptomycin sulfate per milliliter. The cultures, in 250-ml Falcon tissue culture flasks, were subjected to 5000 rads of x-irradiation and plated 9 days later into 60-mm Falcon tissue culture plates at a density of 1.2×10^6 cells per plate (5.7×10^4 cell/cm^2). The cells were studied electrophysiologically about 2 weeks later. During the electrophysiologic experiments the cultures were maintained at 37 to 39°C on the stage of an inverted phase-contrast microscope with 10 percent CO_2 in air being

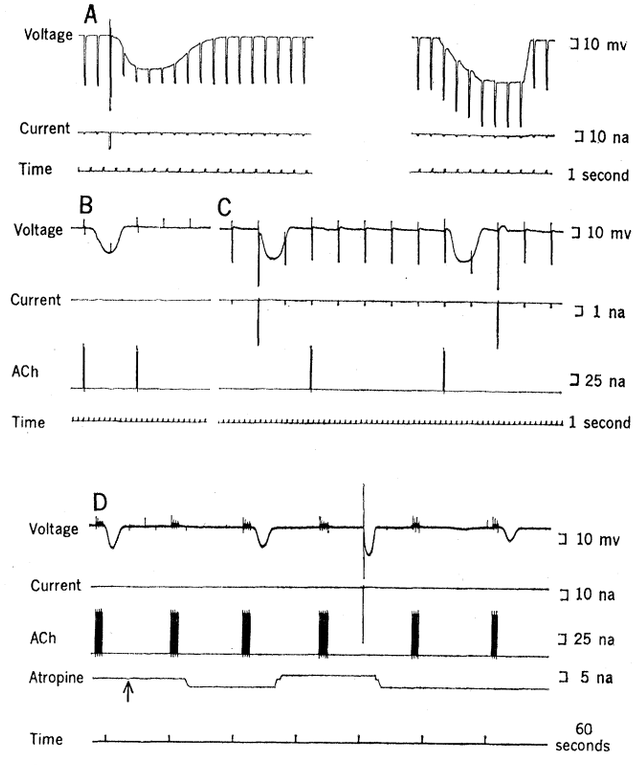

Fig. 1. Records from three different cells shown in (A), in (B) and (C), and in (D). Traces labeled *Voltage* represent transmembrane voltage (negative downward) measured with an intracellular pipette. Traces labeled *Current* represent current passed across the membrane (inward current down) by the intracellular pipette arranged in a bridge circuit. Traces labeled *Acetylcholine* represent total current passed through an ACh-containing micropipette (outward current up). Steady levels of negative holding current of about 5 na were passed from the ACh pipette between pulses. Trace labeled *Atropine* represents total current passed through an atropine sulfate electrode (outward current up). All calibrations as marked. (A) Left, HA response. Small pulses of current are used to test membrane resistance and the larger pulse (third pulse on current trace) elicits the HA response. Note decrease in voltage response to small test current pulse during the HA response. Right, membrane potential is increased by small steady current while testing with small pulses to show that voltage responses to these test pulses are essentially unchanged as a result of the increase in membrane potential as contrasted to the result during the HA response. Resting potential in this cell, −25 mv. (B) Response to ACh. Second pulse fails to elicit a change in membrane potential. Upward deflection on voltage traces are calibration pulses. (C) Interaction between directly elicited HA and ACh responses. The ACh pulse that follows an HA response by some 10 seconds fails to elicit a response even though membrane potential and resistance have returned to normal. Similarly a previously adequate direct stimulus does not elicit an HA response when preceded by an ACh elicited response. Resting membrane potential in (B) and (C), −30 mv. (D) The ACh but not the HA responses are blocked by atropine in consecutive trials of continuous recording. First series of ACh pulses elicited a response. At the arrow below the atropine trace the atropine electrode was positioned with its tip close to the tip of the ACh electrode with no negative steady current flowing so that some leakage of atropine would occur. The subsequent ACh pulses (second application of ACh) did not elicit a response (the refractory period of the ACh response was shown in other trials to be much shorter than the 70-second period between these two ACh applications). Atropine flow was turned off when a negative current was passed through the atropine pipette and the ACh response was recovered (third application of ACh). Positive atropine current released more atropine than before and again resulted in abolition of the ACh response (fourth application of ACh) but the HA response could still be elicited (note large stimulus pulse on current trace). After negative current is passed through the atropine electrode for about 1.5 minutes, the ACh response recovered (sixth application of ACh). Resting membrane potential, −20 mv.

circulated over the cultures. The experimental technique has been described (*2*).

About 20 percent of 64 L cells tested responded to pulses of ACh with a transient increase in membrane potential, the time course of which was similar to that of the hyperpolarizing activation response (HA response) (Fig. 1B). A second pulse of ACh delivered within about 10 seconds of an initial pulse produced no response (Fig. 1B), and a reciprocal occlusion of responsiveness between the HA response and the ACh response was demonstrated (Fig. 1C). A directly elicited HA response blocked an ACh response (left portion of Fig. 1C), and an ACh response blocks the response to a subsequent direct stimulation (right portion of Fig. 1C). The reversal potential of the ACh potential was quite similar to that of the HA response, that is, about -80 mv (*1*). The mechanism for the HA and ACh responses is, by these tests, identical. When an ACh pulse was preceded by the application of atropine from an atropine-filled electrode, the response to ACh was blocked, although an HA response could still be elicited by direct stimulation (Fig. 1D). Responsiveness to ACh recovered several seconds after atropine application was terminated (Fig. 1D, end of tracing).

The latency, amplitude, and time course of the ACh-elicited hyperpolarization was related to the size of the ACh pulse that was applied (C1 and C2 in Fig. 2). The hyperpolarizing potential could be elicited in some cases by applying ACh to an adjacent cell (A, B, C3, and C4 in Fig. 2). This was due to the ACh effect on the adjacent cell rather than diffusion of ACh to the recorded cell because pulling the ACh electrode back into the culture medium over the recorded cell resulted in loss of the response, even when this distance was substantially less than the distance to the adjacent cell from which which the response could be elicited. A difference in the latency of the response was seen, depending on whether the ACh was applied to the cell body or cell process of the adjacent cell in the case illustrated in Fig. 2 (A, C3, and C4). When several cells in the immediate vicinity of a recorded cell were tested with ACh pulses as many as three out of five gave responses propagated into the recorded cell (B in Fig. 2).

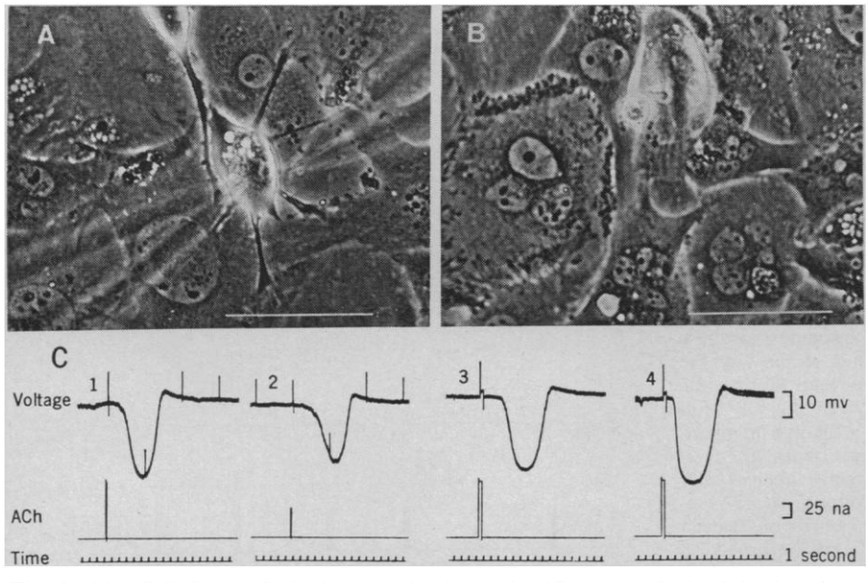

Fig. 2. (A) Cell from which the records shown in (C) were obtained is the phase bright cell in the center of the field. Electrode shadow coming from lower left is from the intracellular microelectrode; that from the upper right from ACh electrode. (B) Phase bright cell in middle top portion of the field exhibited hyperpolarizing responses when three out of five of the cells adjacent to it were stimulated with acetylcholine. Scale is 100 micrometers. (C1) Membrane potential response of cell when ACh was applied to the cell body as shown in (A). (C2) Slower similar response obtained with smaller ACh pulse. (C3) Response from same cell when ACh was applied to the cell body of the phase bright cell at upper edge of field. (C4) Faster, larger response when ACh was applied to the process of the upper cell. Response was abolished by withdrawing the electrode several micrometers into the culture medium. Resting membrane potential of this cell, -30 mv.

It has been shown that the electrically or mechanically elicited HA response may be propagated over a distance of several cell diameters. We have found that this propagation of the HA response is not blocked when a bath containing atropine ($2 \times 10^{-4} M$) is applied. High external K^+ (27 mM) applied by bath perfusion does not evoke the response (*1*).

The L cells are thus capable of generating an active hyperpolarizing response (HA response) when stimulated by mechanical, electrical, or chemical agents. Whereas the response can be elicited by ACh, the propagation of the response that we see between cells does not appear to be mediated by ACh, or at least it is not blocked by atropine in high doses. A large efflux of K^+ probably accompanies the response, but this does not seem to be the basis for the response propagation between cells, because high K^+ solutions do not elicit the response. Electrical coupling does occur between some pairs of L cells under our conditions of culture, but we feel that this is not the basis of the propagation of the HA and ACh responses because (i) we have seen propagation of the hyperpolarizing response in the case of two pairs of cells in which we could detect no direct electrical coupling; (ii) even in cells with some degree of direct electrical coupling, the hyperpolarizing response in the receiving cell could be larger than the response in the initiating cell; (iii) direct stimulating currents much larger than can be accounted for by the hyperpolarizing response are required to initiate an active response; and (iv) the latency between a response in one cell and the initiation of a response in an adjacent cell is too long to be explained on the basis of direct coupling. Since ACh release, direct flow of electrical current, or K^+ accumulation do not seem to account for the propagation of the hyperpolarizing response between L cells, we suggest that some other, presumably humoral, agent must be involved.

The HA response has been seen both in other continuous lines of fibroblasts and in fibroblasts in primary platings of dissociated normal mouse muscle and neuronal tissue (*1*). We hypothesize that this response may be characteristic of at least certain types or stages of fibroblasts. If so, it represents a mechanism by which these cells

can respond to a variety of chemical, mechanical, and electrical stimuli. The hyperpolarizing response in L cells differs from the responses to ACh found in a number of neuronal and other cell types (*3*). We have no evidence as to whether this hyperpolarizing response serves as a regulatory mechanism with regard to fibroblast metabolism or mitotic activity, but the more than fourfold increase in cell K^+ permeability and the probable doubling of Na^+ influx as a result of the membrane potential change (*1*) could result in appreciable changes in the ionic milieu of the cell. The recent observations with regard to changes in cell membrane permeability and potential during the onset of cell division in sea urchin eggs are indicative of a close relation between cell membrane properties and mitosis (*4*). We suggest that the HA response, capable of being elicited by different modalities of stimulation and being propagated by electrical and humoral means, could reflect some significant control mechanism for the regulation of connective tissue disposition and, hence, of organ growth.

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Social Control of Sex Reversal in a Coral-Reef Fish

Abstract. *Males of* Labroides dimidiatus *control the process of sex reversal within social groups. Each group consists of a male with a harem of females, among which larger individuals dominate smaller ones. The male in each harem suppresses the tendency of the females to change sex by actively dominating them. Death of the male releases this suppression and the dominant female of the harem changes sex immediately. Possible genetic advantages of the system are considered.*

Sex reversal is widespread in a number of tropical fishes included in the families Labridae, Scaridae, and Serranidae (*1*, *2*). In this report I describe the pattern of protogynous sex reversal in the labrid fish *Labroides dimidiatus*. The species is a member of a small but widespread genus, the species of which are termed "cleaner fish" because they remove ectoparasites from the skin of other fishes (*3*). Choat (*2*) established that the species is protogynous, with far more females than males, and that probably all the males were secondarily derived from females.

The basic social unit is a male with a harem of usually three to six mature females and several immature individuals living within the male's territory. At Heron Island, Great Barrier Reef, detailed field records were kept on 11 groups for up to 25 months; 48 sex reversals were recorded in these and another eight groups. Individual adults were recognized by unchanging variations in their color patterns.

All individuals exhibit territoriality, but its expression varies with age and sex. The largest, oldest individual is the male, which dominates all the females in the group. Larger, older females of the group dominate smaller ones, which usually results in a linear dominance hierarchy. Thus territoriality is only fully expressed in males and is directed mainly toward other males. Usually there is one dominant female in each group, but sometimes two equal-sized females are codominant and can successfully defend their territories against each other. The dominant female lives in the center of the male's territory, with the other females scattered around. The male is socially very active. It makes frequent excursions throughout its territory both to the feeding areas of the females and to points on the territory border where the male is likely to meet neighboring males. During these excursions the male feeds in the females' areas and actively initiates aggressive encounters with them and other individuals. Females, on the other hand, are more sedentary and passive. When a male meets a female of the same group, the male frequently performs a distinctive aggressive display toward the female. This display has not been seen in encounters between males and only very rarely in encounters between females, when it was given by dominant females.

Some males and large females have maintained nearly the same territories and feeding sites for almost 2 years. Small adults and large juveniles are more mobile. Deaths of individuals high in the hierarchy result in more marked changes in the distribution of other high-status individuals than do deaths of low-status individuals. With the death of a high-status female, the vacated area may be incorporated into the territory of an individual of equal status or taken over by an individual immediately below the deceased in status, the lower status female deserts its own territory in the process. This shift can result in the immediate redistribution of three or four high-status females.

Sex reversal frequently occurs as a part of the reorganization of the group following the death of the male. The success of an initiated reversal depends upon both inter- and intragroup social pressures. Intergroup social pressures take the form of territory invasion and takeover attempts by neighboring males, and if these pressures are successfully resisted by the dominant female it changes sex. Groups with codominant females sometimes divide when both dominants change into males. In all, 26 cases of single dominant females reversing sex were observed (five naturally occurring and 21 experimentally induced by removing the male), and four cases of reversal of pairs of codominants were also seen (all induced by removing the male).

Observations of five dominant females after the removal of their males have shown that the first behavioral signs of sex reversal appear rapidly and that the behavioral changeover can be completed within a few days. For approximately half an hour after the death of the male the dominant female continues to behave aggressively as a