Evidence for an Intracellular Calcium Store Releasable by Surface Stimuli in Fibroblasts (L Cells)

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ABSTRACT   A spontaneously occurring or electrically elicited hyperpolarizing activation (HA) in L cells was previously shown to be due to a specific increase in the membrane K^+ permeability (Nelson et al. 1972. *J. Gen. Physiol.* **60**:58-71). Intracellular injection of Ca^{++} elicits an identical hyperpolarizing response which suggests that the increased K^+ permeability associated with the HA is mediated by an increase in cytoplasmic Ca^{++}. In zero-Ca, EGTA-containing saline the proportion of cells in which HA's can be evoked decreases, but the amplitude of those HA's that are produced is comparable to that of HA's in normal Ca saline. Co^{++} does block the HA but only after a period of 2 h or longer; D-600 does not affect the HA. These observations, with others, suggest that the primary source of the Ca mediating the HA response is intracellular. In L cells the endoplasmic reticulum forms morphologically specialized appositions with the surface membrane which resemble structures at the triads of muscle that are thought to mediate coupling between surface membrane electrical activity and contraction via Ca release from the sarcoplasmic reticulum. The similar structures in L cells may mediate coupling between surface membrane electrical, mechanical, or chemical stimuli and the HA response via release of Ca from the endoplasmic reticulum. Surface-coupled release of Ca from intracellular stores might also regulate a number of other intracellular functions in nonmuscle cells.

L cells are a continuous cell line of fibroblast origin whose surface membrane properties can be investigated with intracellular microelectrode techniques. Previous studies have shown that these cells have a low membrane potential (20 mV inside negative), a high membrane resistivity, and are capable of generating an active electrical membrane response to mechanical, electrical, or chemical (acetylcholine) stimuli (Nelson et al., 1972; Nelson and Peacock, 1972; Okada, et al., 1977 a). The response consists of a hyperpolarization lasting several seconds, accompanied by a decrease in membrane resistance which appears to be due to a specific increase in the surface membrane permeability to K^+ ions (Nelson et al., 1972; Okada et al., 1977 b). We have termed the response the hyperpolarizing activation or HA response and have shown that it can be transmitted (by an unknown mechanism) from one L cell to another (Nelson and Peacock, 1973).

The concentration of free intracellular Ca^{++} ion has been shown in a number of cell types to regulate surface membrane permeability to K^+ (Whittam, 1968; Romero and Whittam, 1971; Meech and Strumwasser, 1970; Meech, 1974; Brown and Brown, 1973; Krnjevic and Lisiewicz, 1972), and it seemed plausible that the HA response might be caused by an increase in free intracellular Ca^{++}. As we shall show, this appears to be the case. Furthermore, several observations suggest that the increase in free intracellular Ca may be due not exclusively or even primarily to entry of Ca from the extracellular fluid, but may be due to release of Ca from intracellular stores.

The concept that surface stimulation might be coupled to release of calcium from intracellular stores was made more attractive by the observation of a possible fine structural basis for coupling of surface stimuli to release of internal calcium. The electron microscope shows that in these cells the endoplasmic reticulum approaches the surface membrane, forming morphologically specialized appositions similar to appositions between the surface or transverse tubule membrane and the sarcoplasmic reticulum of muscle. In muscle such junctions are thought to be sites at which surface membrane excitation is in some way coupled to Ca release from the sarcoplasmic reticulum (Franzini-Armstrong, 1964; Peachey, 1964). We suggest that these structures in L cells may be sites which mediate coupling of surface stimulation with release of Ca from the endoplasmic reticulum.

Preliminary reports of some of these findings have been presented (Henkart et al., 1974; Nelson et al., 1974)

METHODS

Culture Techniques

L cells were grown at 35-37°C in 75 cm^2 Falcon flasks in Dulbecco's Modified Eagles Medium (DMEM, Grand Island Biological Co., Grand Island, N.Y.) containing 10% fetal calf serum (DMEM-10) at 35° in an atmosphere containing 8-10% CO_2. Cells were routinely passaged at confluence using 0.25% trypsin and Puck's D1 saline, adjusted to 340 mosmol with sucrose. Morphologic and electrophysiologic experiments were done on L cells which had been caused to stop dividing and increase in size by exposure to 5,000 rad of X-irradiation (Whitmore et al., 1958). This method has been described earlier (Nelson and Peacock, 1973).

Standard electrophysiologic techniques of intracellular microelectrode recording and stimulation were used in measuring surface membrane properties. Electrodes were filled with either 3 M KCl or 4 M K acetate. Culture plates were maintained at 37°C and were equilibrated with 10% CO_2 and 90% air on the stage of a Zeiss inverted phase-contrast microscope (Carl Zeiss, Inc., N.Y.). The experimental arrangement has been described (Nelson et al., 1971). A single microelectrode in a bridge circuit was used to measure membrane resistance and time constant. Bridge balance was usually excellent with the recording microelectrode extracellular (see Fig. 3 C) and, in cells such as the L cells which have relatively long time constants and high membrane resistances, the bridge technique gives quite reliable results. Intracellular Ca injection was by iontophoresis from microelectrodes containing 0.5-2 M $CaCl_2$. Experiments with altered Ca concentrations were done in modifications of a physiological salt solution (BSS) having the following composition (millimolar): NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 11, Hepes 5, pH 7.4. Altered Ca concentrations were compensated osmotically by changing the Na

concentration appropriately. Drugs were usually added to complete growth medium, but other solutions were used as noted. The ionphore A-23187 was obtained from Eli Lilly and Co. (Indianapolis, Ind.) and dissolved in DMSO. Physiologic determinations consisted of the following: (a) resting membrane potential, V_m; (b) the peak membrane potential reached during an HA elicited by hyperpolarizing current pulses, V_{HA}; (c) cell membrane input resistance, R_{in} (defined as the ratio of the membrane potential change produced by a pulse of current to the magnitude of the current pulse); and (d) a convenient index of membrane time constant, the $T_{2/3}$, which is defined as the time after the onset of a current pulse that the voltage change reaches two-thirds of its maximum value. This was useful, in conjunction with the R_{in} measurement, in providing an indicator of specific membrane resistivity.

The X-irradiated L cells survive 2-3 wk after irradiation. It was important to do the morphologic and electrophysiologic experiments 5-10 d post-irradiation. Reproducibility was good under these conditions as data from three separate experiments (shown in Table I) indicate. Cells selected for physiological studies were mostly large, rather rounded, phase-bright cells, but similar recordings were obtained from flatter, more phase-dark cells. In some experiments in which the cells were studied at relatively early times after irradiation, the cells were small, and the input resistance was high. It was

TABLE I
MEMBRANE PROPERTIES AND HA RESPONSES OF L CELLS IN DMEM-10

Experiment		V_m	V_{HA}	V_m-V_{HA}	R_{in}	$T_{2/3}$	Cells tested
No.		mV	mV	mV	MΩ	ms	n
1	Mean	14.9	37.6	22.8	27.0	6.8	16
	SE	1.2	2.9	2.4	5.7	0.3	
2	Mean	16.4	37.9	21.4	21.6	6.	13
	SE	1.8	2.9	1.8	3.8	0.6	
3	Mean	21.5	44.6	23.2	21.4	9.2	11
	SE	1.3	3.0	3.2	2.1	1.2	

necessary, therefore, to obtain control data from each batch of irradiated cells to compare with similar cells exposed to the different experimental conditions. All conclusions are based on such paired comparisons.

For electron microscopy cells were fixed *in situ* in 2.5% glutaraldehyde in 0.10 or 0.15 M cacodylate buffer, pH 7.4. After a buffer rinse they were postfixed in 1% OsO_4 in 0.1 M cacodylate buffer. The cells were block-stained in 1% uranyl acetate in 0.05 M acetate buffer, pH 5, dehydrated in ethanol and embedded in Epon (Shell Chemical Co., Div. of Shell Oil Co., New York). Sections were cut parallel or perpendicular to the plane of the culture dish. Cells comparable to those used for physiologic studies were selected, marked and sectioned.

RESULTS

Injection of calcium into L cells regularly elicited HA-like responses. An example is shown in Fig. 1. The amplitude of hyperpolarizations produced was approximately proportional to the outward (+) current passed through the $CaCl_2$-filled electrode. Passage of inward current through the $CaCl_2$ electrode or pulses of comparable magnitude of either polarity delivered through the KAc-filled microelectrode failed to produce HA-like responses. Larger negative

pulses represent an adequate stimulus for HA generation (Nelson et al., 1972). These observations suggested that increased intracellular calcium might mediate the increase in K^+ conductance which determines the membrane potential during HA's. To study the mechanism of HA generation further, the effects of altered extracellular calcium concentrations were studied.

Replacement of the normal growth medium with medium made up without added calcium and containing 1.5 mM Na ethylene glycol-bis-(β-aminoethyl
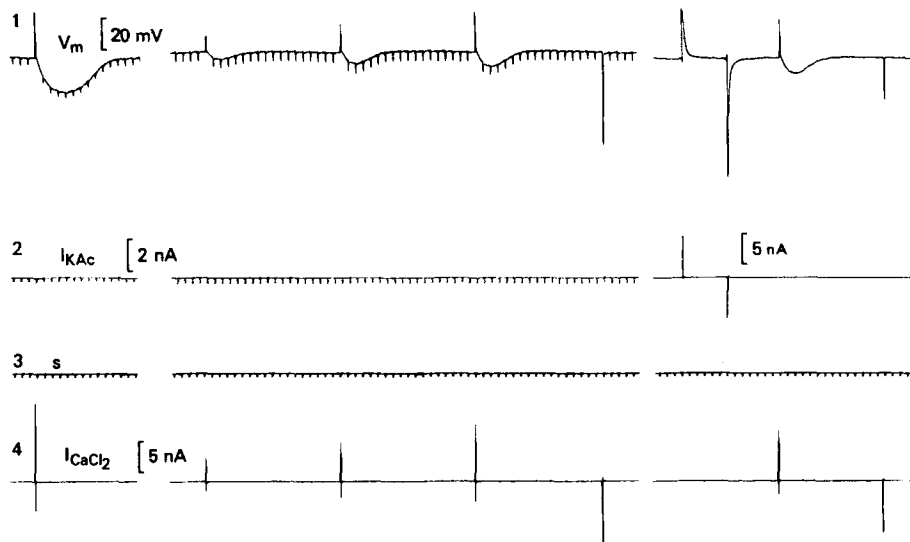

FIGURE 1.   Effect of calcium injection into L cells. Two electrodes, one filled with 2 M K acetate (KAc) and one filled with 92 mM $CaCl_2$, were inserted into a single L cell. The four traces are simultaneous records. Line 1 (V_m) is membrane potential recorded by the KAc pipette. Line 2 (I_{KAc}) is current passed through the KAc-filled pipette. In this and subsequent figures, small constant current pulses are passed throughout most of the record from the KAc pipette to measure membrane resistance. Line 3 is a time marker, and line 4 (I_{Ca}) is current passed through the $CaCl_2$ pipette. Outward current pulses (injecting Ca^{++}) through the $CaCl_2$ pipette produce hyperpolarizations seen in trace 1. The amplitude of the hyperpolarizations is proportional to the size of the current pulses. Inward current pulses through the $CaCl_2$ electrode did not produce any active membrane response. Current pulses of comparable magnitudes of either polarity through the KAc pipette (end of trace 2) failed to elicit hyperpolarizing responses.

ether) N, N'-tetraacetate (EGTA) did not immediately affect the HA response, but more prolonged incubation in zero Ca^{++} medium with EGTA reversibly abolished the HA in most cells. Inasmuch as the definition of free Ca^{++} concentration in serum-containing medium is uncertain, further experiments with altered Ca^{++} were done in artificial saline solutions. Table II summarizes the results of a series of measurements on a single plate in which the resting membrane properties and HA parameters were measured in growth medium, in the standard saline (containing 2 mM Ca and 1 mM Mg), briefly in nominally

Ca-free saline, in Ca-free saline with 0.3 mM Na EGTA added, and after return to growth medium. The potential changes associated with HA's produced just after penetration, those elicited electrically, and those in response to gentle mechanical stimulation of an adjacent cell were comparable in amplitude in growth medium and in 2 mM Ca-containing saline. In zero Ca, EGTA-containing saline, the resting membrane potential and membrane input resistance were low. Surprisingly, however, the amplitudes of potential changes associated with the HA's that were produced in response to all types of stimulation were not significantly reduced. This is particularly clear when the decrease in membrane input resistance is taken into account. Results with five other plates were similar: the amplitudes of HA's produced in Ca-free saline for as long as 2 h (the duration of the experiments in saline solutions) were not significantly reduced. Fig. 2 A and B are recordings from a cell after 4 h in zero Ca^{++} medium. The resting membrane potential was low while the membrane

TABLE II

EFFECTS OF REDUCED EXTRACELLULAR CALCIUM ON MEMBRANE PROPERTIES AND HA's OF L CELLS

Solution	Cells tested	V_m	V_{HA} at penetration*	ΔV_{HA} at penetration	V_{HA} electrically evoked*	V_{HA} to mechanical stimulation*	ΔV_{HA} largest evoked	R_{in}	$T_{2/3}$
	n	mV	mV	mV	mV	mV	mV	MΩ	ms
DMEM-10 2 Ca, 1 Mg	11	15.5 ± 1.6	35.2 ± 2.7	19.7 ± 2.5	36.2 ± 4.3	41.6 ± 8.5	22.2 ± 3.0	28.6 ± 3.3	8.7 ± 1.1
BSS 0 Ca, 3 Mg	7	15.6 ± 1.5	38.7 ± 3.2	22.8 ± 3.9	36.0 ± 3.6	32.3 ± 5.8	20.7 ± 4.2	34.7 ± 5.6	7.8 ± 1.0
BSS 0 Ca, 3 Mg	3	13.7 ± 0.9	34.7 ± 1.8	21.0 ± 2.1	26.3 ± 1.2	not done	12.7 ± 0.3	23.7 ± 0.9	8.0
0.3 mM NaEGTA	15	9.9 ± 0.5‡	29.9 ± 1.6‡	19.9 ± 1.7	25.0 ± 4.7	27.0 ± 3.0	15.0 ± 3.4	21.5 ± 1.8‡	8.6 ± 0.8
DMEM-10	4	17.5 ± 1.3	43.0 ± 6.6	25.5 ± 5.5	31.0 ± 4.4	45.3 ± 0.7	22.0 ± 4.7	15.0 ± 2.5‡	7.3 ± 1.5

Values in the body of the table are mean ± standard error. * The mean amplitudes of HA's produced at penetration and evoked by electrical or mechanical stimulation were calculated on the basis of those that were produced. See Table III for data on incidence of HA's. $\Delta V_{HA} = V_m - V_{HA}$. ‡ Values are significantly different from the appropriate control at P <0.05 (t test). The apparent significance of the V_{HA} at penetration is due to the reduction in resting potential, V_m. No significant decrease in ΔV_{HA} occurred.

resistance and time constant were within the normal range, indicating that the cells do not simply deteriorate in low Ca^{++} solutions. 12 h after return to normal medium (Fig. 2 C) the membrane properties and HA had returned to normal. Fig. 2 D illustrates an HA in response to stimulation in a cell that had been in Ca-free saline with 0.3 mM EGTA for 90 min. In Ca-free, EGTA-containing medium spontaneous HA's were observed in 2 of 56 cells as long as 1 h after transfer into that solution. Membrane potential oscillations often follow HA's produced in normal medium. Oscillations were also seen occasionally in Ca-free, EGTA-containing solutions.

Although the HA's that did occur in Ca-free solutions were comparable in amplitude to those in normal Ca medium, the proportion of cells that produced HA's decreased in Ca-free, EGTA-containing solutions as shown in Table III. It further appeared that after incubation in Ca-free solution for a few minutes the number of HA's that a single cell could produce was limited to one or two,
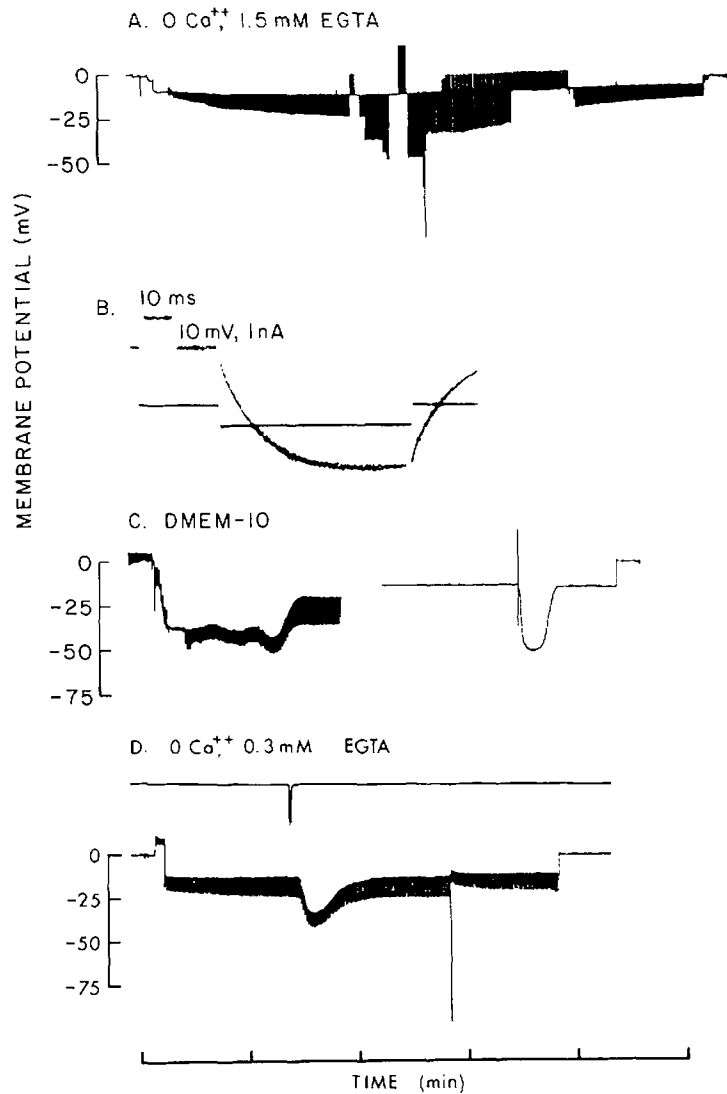
FIGURE 2. Effects of low Ca^{++} solutions. (A) After several hours in 0 Ca^{++}, 1.5 mM EGTA growth medium, a recording shows a low resting potential with no HA response. (B) Oscilloscope recording shows that membrane resistance and time constant are normal. This indicates that even though this cell does not produce HA's it has not simply deteriorated. (C) After recovery for ~12 h in normal medium a cell from the same plate produced an HA on penetration. The current pulses show that, as usual, the HA was associated with a decrease in membrane resistance. After several minutes when the cell was stimulated electrically (the small current pulses were not being applied in this case), the HA produced in response to the stimulus was normal. (D) A cell after 90 min in a saline solution containing 0 Ca^{++} and 0.3 mM Na EGTA. The lower trace is an intracellular recording showing a typical HA produced in response to mechanical stimulation of an adjacent cell. The top trace indicates the mechanical stimulus to the adjacent cell.

whereas in normal Ca solutions HA's could be elicited repeatedly. Return to Ca-containing saline resulted within a few minutes in recovery of the cells' ability to produce HA's in response to various stimuli.

Increasing the extracellular calcium concentration from 2 to 10 mM resulted in a significant, reversible increase in the membrane input resistance, but no increase in the amplitude of HA's, as shown in Table IV. This lack of increase in HA amplitude might have been expected if the peak HA potential had been approaching the K^+ equilibrium potential. This is not the case, however, because in L cells the reversal potential for the HA response is in the vicinity of -80 mV (Nelson et al., 1972), while the HA peak potentials in both 2 and 10 mM Ca are in the range of -30 to -60 mV.

Cobalt ions have been shown to block Ca entry through voltage-sensitive Ca

TABLE III

EFFECT OF REDUCED EXTRACELLULAR CALCIUM ON THE PROPORTION OF CELLS PRODUCING HA's

Condition	Cells tested	Cells producing HA's at penetration	Cells producing HA's to electrical or mechanical stimulation	Time in solution at which HA last evoked
	n	n	n	*min*
2 Ca, 1 Mg BSS (five plates)	40	33 (83%)	31 (78%)	
Nominally Ca free BSS (one plate)	3	3 (100%)	3 (100%)	
0 Ca, 3 Mg 0.3 EGTA BSS (five plates)	71	38 (54%)	27 (38%)	115
0 Ca, 3 Mg 1.5 mM EGTA BSS (one plate)	16	6 (38%)	10 (63%)	90

Data above are from a total of six plates in which low Ca salines were tested. (Data on HA amplitudes from one of the above plates is shown in Table II.)

channels in certain muscles (Hagiwara and Takahashi, 1967; Hagiwara and Kidokoro, 1971; Miyazaki et al., 1972) and in nerve cells (Geduldig and Junge, 1968). In L cells, 2 mM Co^{++} decreased the amplitude of the HA progressively with time, but even after 6 h had not blocked completely. 5 mM Co^{++} blocked completely within 4 h. The membrane properties of a cell in 5 mM Co^{++} are illustrated in Fig. 3 A-C. When cells were tested immediately after addition of 15 mM Co^{++} two of four cells produced HA's with amplitudes between 20 and 30 mV (Table V). Cells in medium containing Sr^{++}, in contrast, produced HA responses of larger than normal amplitude and exhibited increased resting membrane resistance and time constant (Fig. 3 C and D and Table VI).

D-600, a blocker of voltage-sensitive Ca channels in some preparations (Kohlhardt et al., 1972) failed to block the HA (not shown) when used at a concentration of 4 μg/ml for a period in excess of 1 h.

The ionophore, A-23187 (Reed and Lardy, 1972), was used in an attempt to alter the intracellular Ca^{++} concentration. When 10-40 μmol/liter ionophore was added to the normal medium (which contained ~1.2 mM Ca^{++}) after incubations of 4 h, a slight increase in membrane potential but no significant change in membrane resting resistance or $T_{2/3}$ was evident (Table VI). The fact that neither input resistance nor membrane time constant changes with ionophore treatment argues strongly that the specific resistivity has not been affected by the treatment. The HA response, however, was nearly eliminated. This somewhat surprising result will be discussed further below.

As can be seen from the electron micrographs Figs. 4 a, 5 a and b, and 6, there is no apparent diffusion barrier between the surface of the cells and the culture medium. The long times over which the amplitude of HA is maintained in the absence of extracellular Ca, and the time required for the effects of Co^{++} to develop suggest that Ca in some less accessible compartment is involved in the HA response.

Taken together, the physiologic observations suggest that the HA response is mediated by an increase in intracellular calcium, but all the results are not

TABLE IV

EFFECTS OF INCREASED EXTRACELLULAR CALCIUM ON MEMBRANE PROPERTIES AND HA's OF L CELLS

Condition	V_m	V_{HA}	ΔV_{HA}	R_{in}	$T_{2/3}$	Cells
	mV			$M\Omega$	ms	n
2 Ca, 1 Mg	20.6 ± 1.6	45.7 ± 4.1	25.1 ± 3.2	21.2 ± 4.1	10.6 ± 0.4	7
10 Ca	22.3 ± 1.2	47.0 ± 3.9	24.8 ± 3.9	60.3 ± 15.7*	25.3 ± 7.1	8
2 Ca, 1 Mg	18.7 ± 1.4	37.7 ± 2.7	19.0 ± 2.9	21.2 ± 2.5	12.0 ± 1.9	6

V_{HA} was the membrane potential achieved by the largest HA evoked in each cell.

* Significantly different from value in 2 Ca, 1 Mg BSS. $P < 0.05$ (t test).

simply consistent with the interpretation that the increase in G_K responsible for HA generation results from influx of Ca from the extracellular fluid. An alternative source of Ca would be intracellular stores.

Fig. 4 illustrates the structure that we believe is a likely candidate for the site of coupling between stimulation across the surface membrane and Ca release from intracellular stores. In L cells the endoplasmic reticulum (ER) often approached the surface membrane and formed morphologically specialized appositions. These appositions were characterized by paralleling of the surface and outer ER membranes within 16–20 nm over an area of a few tenths to ~1 μm^2. There was a fuzzy material in the gap, and the gap material sometimes appeared as strands extending between the two membranes. Because the structure of these appositions between ER and surface membranes is very similar to the arrangement of ER and surface membrane frequently seen in neurons and called "subsurface cisterns" of the ER (Rosenbluth, 1962), we feel that it is appropriate to apply the same descriptive term to these structures in L cells. As in neurons, the subsurface cisterns (SSC) in L cells are continuous with rough ER and may, themselves, bear ribosomes on the cytoplasmic side of the cistern membrane not apposed to the surface. Subsurface cisterns occur on both

the upper surface of the cell (Fig. 4 a) and on the surface of the cell facing the culture dish (Fig. 4 b).

Fig. 5 compares the morphology of a control cell and a cell treated with 10 μmol/liter A-23187 in normal medium (~1.8 mM Ca) for 4 h. Cells treated with the ionophore became filled with large "vacuoles." Higher magnification shows that some of the "vacuoles" are derived by distension of elements of the Golgi apparatus (Fig. 5 C), and some are pinocytotic or phagocytic vesicles (Fig. 6).
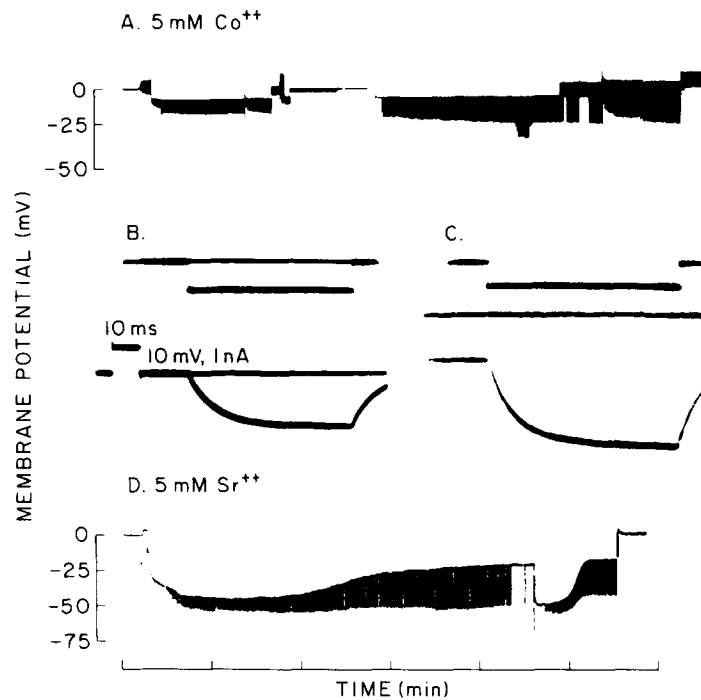

FIGURE 3. Effects of Co^{++} and Sr^{++} on L cells. (A) Penwriter recordings from two successive cells after 4 h in medium containing 5 mM Co^{++} showing low membrane potential and lack of HA response. (B and C) Oscilloscope recordings of two cells under Co^{++} to show normal resistance and normal time constant of the cells. Middle trace in C is voltage record with microelectrode located extracellularly, to show excellent bridge balance. Calibration is indicated in B. (D) Penwriter recordings from L cells in medium containing 5 mM Sr^{++}. A prominent HA response occurred during penetration (beginning of trace) and another HA was elicited electrically near the end of the recording.

The endoplasmic reticulum in ionophore-treated cells is generally more distended than in untreated cells. The distended ER contains granular material having the same staining properties as the control ER. Subsurface cisterns are found less frequently adjacent to the surface of ionophore-treated cells, but those that are found are similar to the SSC in untreated cells. A striking finding was that these very specific structures are quite systematically associated with pinocytotic and phagocytic vesicles. Each pinocytotic vesicle in the vicinity of

those shown in Fig. 6, when studied in serial sections, was found to have one or more SSC applied to it. (inset Fig. 6). The mitochondria of ionophore-treated cells generally appeared very condensed, and microtubules were common in both ionophore-treated and untreated cells.

DISCUSSION

The control of membrane K^+ permeability by intracellular Ca^{++} has now been demonstrated in a number of cell types including erythrocytes (Whittam, 1968; Romero and Whittam, 1971), neurons (Meech and Strumwasser, 1970; Meech, 1974; Feltz et al., 1972; Krnjevic and Lisiewicz, 1972), and macrophages (Gallin et al., 1975). In a previous report (Nelson et al., 1972) it has been shown that the

TABLE V

EFFECTS OF COBALT ON HA AMPLITUDE AND
MEMBRANE INPUT RESISTANCE OF L CELLS

Cobalt concentration	Time after treatment	ΔV_{HA}	R_{in}	Cells tested
mM	h	mV	MΩ	n
2	0-2	18	17	8
2	3.5-5.5	14	12	6
2	5.5-6.5	8	19	8
5	>4	0	20	10
15	<1	12*		4

* Two cells exhibited ΔV_{HA} of ≥20 mV.

TABLE VI

EFFECTS OF STRONTIUM AND A-23187
ON MEMBRANE PROPERTIES AND HA's OF L CELLS

	V_m	V_{HA}	ΔV_{HA}	R_{in}	$T_{2/3}$	Cells tested
	mV	mV	mV	MΩ	ms	n
Control	16.4	37.9	21.4	21.6	6	14
Strontium, 5 mM	19.9	48.2*	28.3*	33.0*	9.6	10
Control 1-2:100 DMSO	19.1	39.9	20.4	37.9	9.1	20
A-23187, 10-40 μM	24.2*	29.9*	5.9*	42.7	10.3	30

* Value is significantly different from the appropriate control value at P <0.05 level (t test).

hyperpolarizing activation in L cells is due to an increase in membrane conductance to K^+ ions. Experiments reported here show that Ca^{++} ions injected into the cytoplasm of L cells can give rise to a long-lasting hyperpolarization indistinguishable from the HA elicited by other means. This suggests that the HA response is produced in these cells by a transient increase in cytoplasmic Ca^{++} concentration. The most obvious hypothesis as to the source of this Ca is that it enters from the external solution through stimulus-sensitive Ca channels. Several pieces of evidence, however, suggest that the Ca responsible for turning on the HA does not enter from the external medium but is released from intracellular stores in response to a stimulus. (a) Frequently and especially when the HA response was elicited by propagation from an adjacent

cell or by acetylcholine application, the hyperpolarization clearly was not preceded by any depolarizing phase (Nelson and Peacock, 1973). This would argue against ingress of Ca^{++} ions as the major source of Ca^{++} involved in initiating the HA response. (b) Occasionally spontaneous HA's and oscillations following evoked HA's were observed in Ca-free, EGTA-containing external solutions. (c) In Ca-free, EGTA-containing solutions the proportion of cells in which HA's could be evoked was reduced, but the HA's that were produced were regularly comparable in amplitude to HA's in normal Ca medium. (d) High Ca^{++} concentrations did not increase the HA amplitude significantly even though the membrane potential at the peak of the HA was still far from the
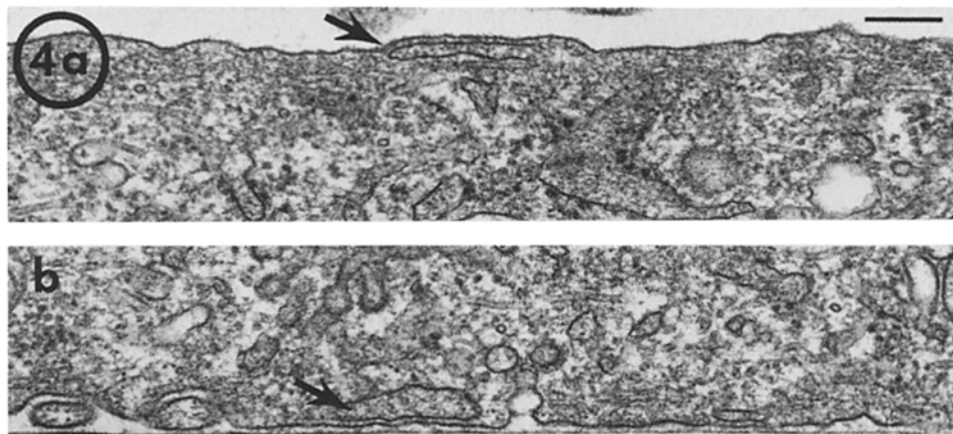

FIGURE 4. Electron micrographs of regions of the (a) upper surface and (b) lower surface of L cells cut perpendicular to the plane of the culture dish. The arrows indicate areas in which cisterns of the endoplasmic reticulum form morphologically specialized appositions with the surface membrane (subsurface cisterns). At these appositions the membranes are parallel over some distance and there is a fuzzy material in the gap. The distance between the ER and surface membranes is 16–20 nm. In the peripheral cytoplasm are filaments (~5 nm in diameter) and microtubules. The dark line below the cell in part b represents the interface between the culture fluid and the plastic dish and probably is produced by protein adsorbed to the plastic surface. The bar indicates 0.2 μm.

determined reversal potential for the HA response. (e) Solutions containing D-600, a blocker of plasma membrane voltage-sensitive Ca channels in some systems (Kohlhardt et al., 1972), failed to block the response. We consider it likely, therefore, that the response is due primarily to intracellular release of Ca^{++} rather than ingress of Ca^{++} across the surface membrane. The observations that in Ca-free, EGTA-containing solutions there is a rapid drop (within a few minutes) in the proportion of cells in which HA's can be evoked, whereas HA amplitudes are unaffected for much longer times (at least 2 h) suggests that Ca also plays a permissive role in allowing surface stimuli to result in HA generation. Another way of expressing this is: a step in the coupling of surface stimulation to intracellular Ca release is Ca dependent. Although this is
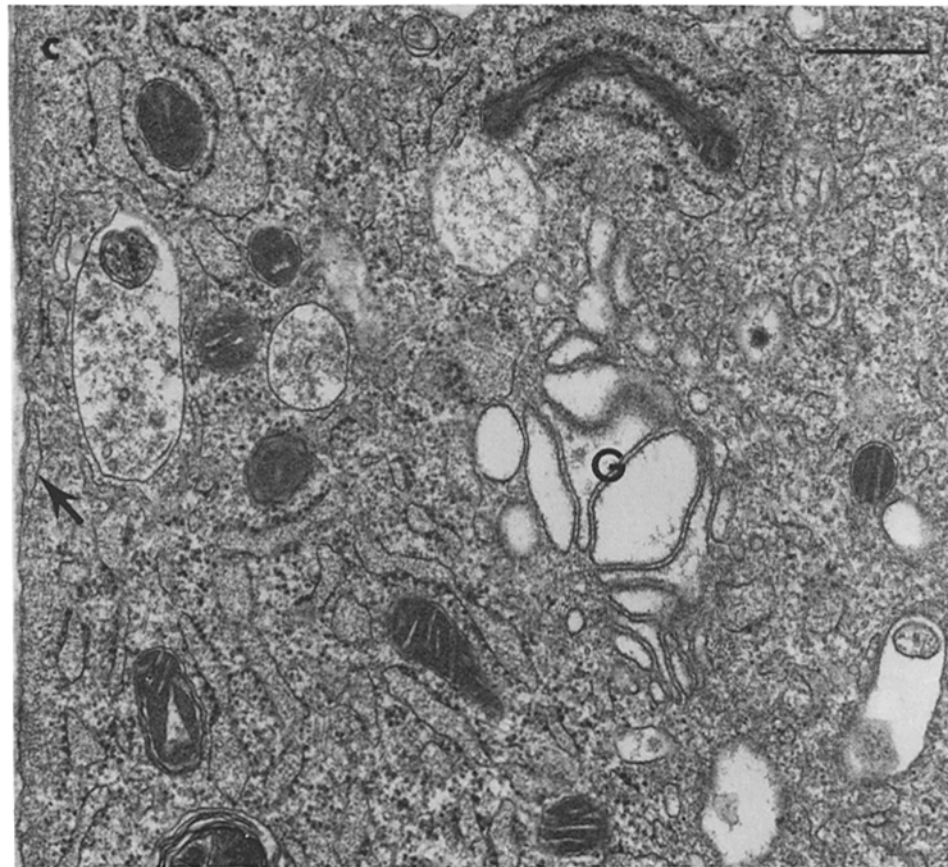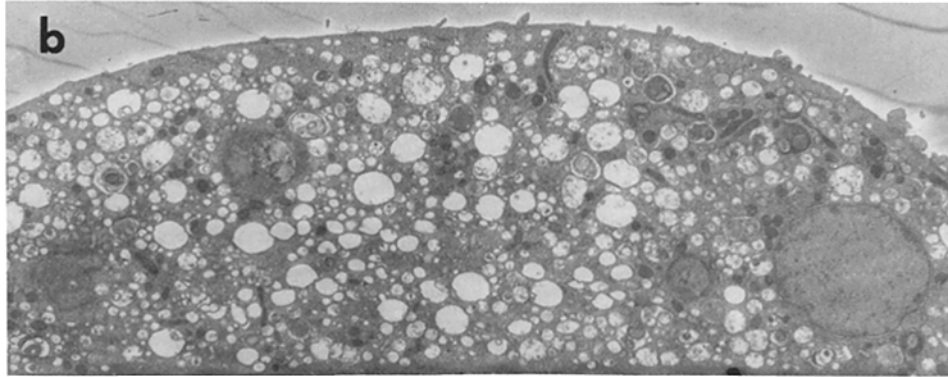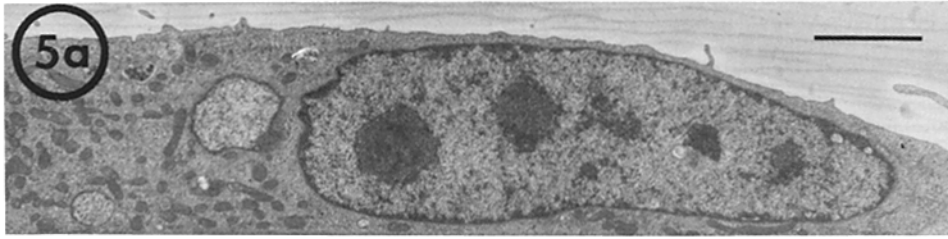
somewhat reminiscent of a "calcium release by calcium" mechanism such as has been suggested as a mechanism of excitation-contraction coupling in muscle (see Endo, 1977 and Stephenson and Podolsky, 1978 for review and discussion), we prefer to use the term "permissive" calcium because it does not imply any particular mechanism. We can only speculate as to the location of this "permissive" Ca. It must be located in a more superficial site than the Ca store that produces the HA, but it is clearly more inaccessible or more tightly bound than Ca free in the external solution. A reasonable guess might be that the permissive Ca is somewhere in association with the surface membrane: either at the inner surface or associated with intrinsic membrane components where it exchanges with external Ca relatively slowly.

It should be noted that external Ca, as expected, also affects the resting membrane input resistance, presumably by nonspecific divalent ion effects on the surface potential.

Co^{++} blocks Ca^{++} fluxes across surface membranes (Hagiwara and Takahashi, 1967; Geduldig and Junge, 1968), and the fact that it blocked the HA could be taken as evidence that Ca influx is the trigger for the HA. The length of time required for development of Co^{++} block even in fairly high concentrations, however, might suggest a more internal site of action. Co^{++} could block the HA by affecting the coupling mechanism or by penetrating to intracellular sites of Ca storage and blocking its release. When the squid giant axon is treated with Co^{++}, then fixed, prepared for electron microscopy, and observed without futher heavy metal staining, membranes of the endoplasmic reticulum appear unusually electron opaque. This suggests that in this preparation Co^{++} may penetrate into the ER (Henkart, 1975 and footnote 1). Under Co^{++} the membrane resistance was little changed, but the resting membrane potential was substantially reduced. This could be due to a decrease in membrane conductance to potassium (G_K). Inasmuch as G_K is relatively low in L cells normally, and the membrane potential is not near the potassium equilibrium potential, the change in G_K required to produce the observed changes in potential would not necessarily produce a large change in overall membrane resistance. Strontium, on the other hand, augments the HA response while increasing resting membrane resistance, presumably by a nonspecific divalent ion extracellular effect. These results are compatible with data from a variety of systems in which cobalt blocks Ca^{++}-mediated effects while strontium can substitute for calcium in regulating membrane function (reviewed by Hagiwara, 1973 and Reuter, 1973).

FIGURE 5.   Electron micrographs of L cells cut perpendicular to the plane of the culture dish. (a and b) Low power micrographs illustrating the general appearance of (a) untreated and (b) A-23187-treated cells. The ionophore-treated cell is filled with membrane-bound vacuoles. The bar indicates 2 μm. (c) Higher magnification view of an area of the cytoplasm of an ionophore-treated cell. The cell's contact with the plate forms the left-hand border of the picture. A subsurface cistern (arrow) is adjacent to the surface membrane. An area of the Golgi apparatus (G) appears distended and contributes to the vacuolated appearance of the ionophore-treated cell. The mitochondria appear very condensed. The bar indicates 0.5 μm.

When L cells were incubated with the ionophore, A-23187 for 4 h, the surface membrane resistance and time constant were not changed and the resting membrane potential was altered only slightly despite the formation of large
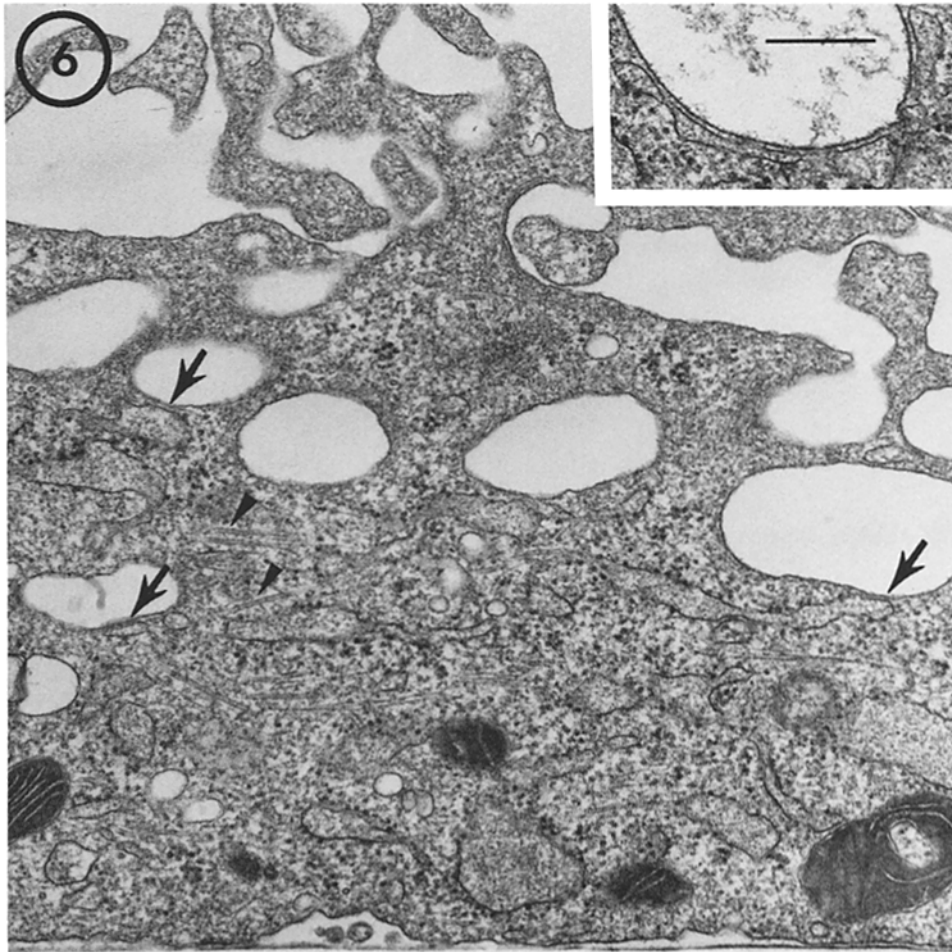

FIGURE 6.   Electron micrograph of a cell treated with A-23187. In this region the cell surface appears to be active in pinocytosis. The pinocytotic vesicles probably contribute to the vacuolated appearance of the cell. The arrows point to areas where the ER is apposed to the membrane of pinocytotic vesicles. Arrowheads indicate microtubules which are still plentiful in ionophore-treated cells. The *inset* shows at higher magnification a portion of an entirely internal vesicle with a cistern of the endoplasmic reticulum applied to its surface. The bar indicates 0.5 μm in the main figure and 0.33 μm in the inset.

vacuoles in the cytoplasm. We conclude from the electrophysiologic result that the free intracellular Ca^{++} concentration was altered very little despite a presumably substantial increase in the Ca^{++} influx across the surface membrane as indicated by vacuole formation. The presence of normal numbers of

microtubules in these ionophore-treated cells also suggests that the cytoplasmic free Ca concentration may not be increasing substantially (Schliwa, 1976). Thus, intracellular systems for sequestration of Ca must compensate for the increased influx of Ca across the surface membrane induced by the ionophore. These effects of A-23187 suggest that these cells have a large capacity for sequestration of calcium. The HA amplitude was markedly reduced by ionophore treatment. If the subsurface cisterns (SSC) are responsible for coupling surface stimulation to the release of Ca from intracellular stores, then the reduction in number of SSC observed adjacent to the surface membrane in ionophore-treated cells could lead to the observed decrease in HA amplitude by reducing the effectiveness of coupling. The ionophore seems to stimulate pinocytosis, and the association of SSC with pinocytotic vesicles suggests that the SSC may be internalized. It is also possible, however, that SSC become disconnected from some sites and new SSC form in association with pinocytotic vesicles. More extensive quantitation of these structures and their distribution in cells subjected to more closely timed drug treatments would be required to establish this point.
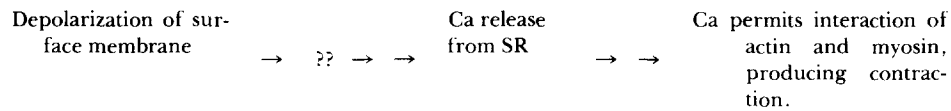
^{45}Ca flux studies suggest the existence of multiple Ca compartments in L cells (Lamb and Lindsay, 1971). Furthermore, there is evidence that a nonmitochondrial subcellular (microsomal) fraction from fibroblasts is capable of accumulating Ca by an ATP-dependent mechanism (Hoffman-Berling, 1964; Moore and Pastan, 1977). Our findings are compatible with the concept of a nonmitochondrial, intracellular, calcium-accumulating system and extend it by presenting some evidence that calcium may be released from intracellular stores in response to surface membrane stimulation. We have also demonstrated morphologic sites of apposition between the endoplasmic reticulum (presumably one source of the microsomal Ca uptake system) and the surface membrane which could mediate coupling of surface stimulation to Ca release.

The primary reason for suggesting that the SSC are sites of coupling between cell surface stimulation and Ca release is their striking resemblance to the sites (triads, diads, surface couplings) in muscle where the sarcoplasmic reticulum is apposed to the surface or transverse tubule membrane. Such sites in muscle are thought to mediate excitation-contraction coupling. As was noted above, similar SSC structures are found in neurons where the endoplasmic reticulum has also been shown to accumulate calcium (Henkart et al., 1978 a and b; McGraw et al., 1978) and where SSC have also been proposed as mediators of coupling between surface membrane electrical activity and intracellular processes (Rosenbluth, 1962; Henkart et al., 1976). Subsurface cisterns are found not only in X-irradiated L cells, but in non-irradiated L cells, and normal mouse fibroblasts[1] which also produce HA responses (Nelson et al., 1972). Mouse and human macrophages which produce HA responses (Gallin et al., 1975) also have subsurface cisterns (an example is shown but not mentioned in Reaven and Axline, 1973, Fig. 1 B; and footnote 1). SSC are not conspicuous in all cell types and HA have, so far, not been looked for extensively. Thus, we do not yet know how general these phenomena or their correlation may be.
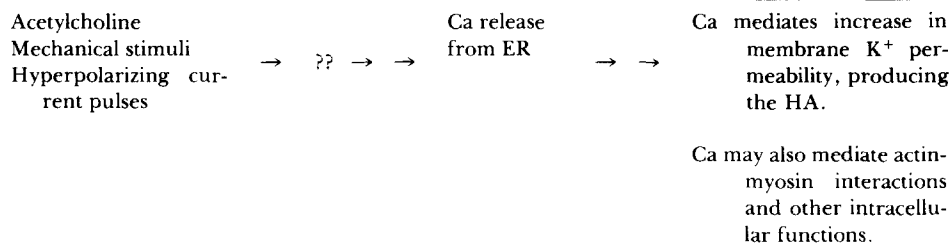
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[1] Henkart, M. Unpublished observations.

The sequence of events we propose can be summarized and compared with excitation-contraction coupling of muscle as follows:

Excitation-contraction coupling:

Depolarization of surface membrane	→ ?? → →	Ca release from SR	→ →	Ca permits interaction of actin and myosin, producing contraction.

Stimulus-HA coupling in L cells:

Acetylcholine Mechanical stimuli Hyperpolarizing current pulses	→ ?? → →	Ca release from ER	→ →	Ca mediates increase in membrane K⁺ permeability, producing the HA.
				Ca may also mediate actin-myosin interactions and other intracellular functions.

"??" indicate in both cases unknown steps in the coupling mechanisms which may require Ca located at "permissive" sites (see discussion above for the case in L cells; Stephenson and Podolsky, 1978, for the case in muscle.)

The primary function of the release of calcium from intracellular stores in addition to the control of membrane permeability to other ions may be to control cellular motility by regulation of the interactions of actin and myosin-like proteins (reviewed by Hitchcock, 1977). The importance of this Ca-regulating system probably extends beyond the control of motility, however, because Ca has been implicated as a second messenger or coupling factor in the regulation of a variety of other cellular processes (reviewed by Berridge, 1975 b; Rasmussen and Goodman, 1977) such as secretion (Douglas, 1968; Rubin, 1970), control of microtubule polymerization and function possibly including chromosome movement in mitosis (Gallin and Rosenthal, 1974; Schliwa, 1976; Harris, 1978), and control of cell proliferation (MacManus et al., 1975; Berridge, 1975 a; Rebhun, 1977).

The continuous relation between the amount of Ca injected and the HA amplitude, in combination with the occurrence of full amplitude HA's in Ca-free, EGTA-containing solutions and the lack of an increase in HA amplitude in high external Ca are incompatible with simple Ca ingress from the extracellular fluid as the major source of Ca producing the HA response. On the other hand, the evidence implies a Ca-sensitive step between surface stimulation and release of intracellular Ca. This makes it somewhat difficult to design experiments that distinguish functions related to intracellular release of Ca from those actually requiring Ca entry. In fact, the methods most commonly used to study the effects of Ca on cellular functions probably tend to obscure possible contributions from intracellular sources. Thus, although at present the evidence for surface-coupled Ca release from intracellular stores in cells other than muscle is indirect, we feel that the physiological implications of such a mechanism are of sufficient importance that the possibility should be examined further.

REFERENCES

BERRIDGE, M. J. 1975 a. Control of cell division: a unifying hypothesis. *J. Cyclic Nucleotide Res.* 1:305-320.

BERRIDGE, M. J. 1975 b. The interaction of cyclic nucleotides and calcium in the control of cellular activity. *Adv. Cyclic Nucleotide Res.* 6:1-98.

BROWN, A. M., and H. M. BROWN. 1973. Light response of a giant *Aplysia* neuron. *J. Gen. Physiol.* 62:239-254.

DOUGLAS, W. W. 1968. Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. *Br. J. Pharmacol.* 34:451-474.

ENDO, M. 1977. Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* 57:71-108.

FELTZ, A., K. KRNJEVIC, and A. LISIEWICZ. 1972. Intracellular free Ca^{++} and membrane properties of motoneurons. *Nat. New Biol.* 237:179-181.

FRANZINI-ARMSTRONG, C. 1964. Fine structure of sarcoplasmic reticulum and transverse tubular system in muscle fibers. *Fed. Proc.* 23:887-895.

GALLIN, J. F., and A. S. ROSENTHAL. 1974. The regulatory role of divalent cations in human granulocyte chemotaxis: evidence for an association between calcium exchanges and microtubule assembly. *J. Cell Biol.* 62:594-609.

GALLIN, E. K., M. L. WIEDERHOLD, P. E. LIPSKY, and A. S. ROSENTHAL. 1975. Spontaneous and induced membrane hyperpolarizations in macrophages. *J. Cell Physiol.* 86:653-661.

GEDULDIG, D., and D. JUNGE. 1968. Sodium and calcium components of action potentials in the *Aplysia* giant neurone. *J. Physiol. (Lond.).* 19:347-365.

HAGIWARA, S. 1973. Ca spike. *Adv. Biophys.* 4:71-102.

HAGIWARA, S., and KIDOKORO, Y. 1971. Na and Ca components of action potential in amphioxus muscle cells. *J. Physiol. (Lond.).* 219:217-232.

HAGIWARA, S., and K. TAKAHASHI. 1967. Surface density of calcium ions and calcium spikes in the barnacle muscle fiber membrane. *J. Gen. Physiol.* 50:583-601.

HARRIS, P. 1978. Triggers, trigger waves, and mitosis: a new model. *In* Cell Cycle Regulation. Monographs on Cell Biology. J. R. Jeter, I. L. Cameron, G. M. Padilla, and A. M. Zimmerman, editors. Academic Press, Inc., New York. 75-104.

HENKART, M. 1975. The endoplasmic reticulum of neurons as a calcium sequestering and releasing system: morphological evidence. *Biophys. J.* 15:267a.

HENKART, M., A. BREUER, and P. NELSON. 1974. Time lapse and electron microscope studies of process formation in L-cells: a model for morphogenesis of process-bearing cells. *Abstr. Soc. Neurosci.* 253.

HENKART, M., D. M. D. LANDIS, and T. S. REESE. 1976. Similarity of junctions between plasma membranes and endoplasmic reticulum in muscle and neurons. *J. Cell Biol.* 70:338-347.

HENKART, M., T. S. REESE, and F. J. BRINLEY, JR. 1978 a. Oxalate produces precipitates in endoplasmic reticulum of Ca-loaded squid axons. *Biophys. J.* 21:187a.

HENKART, M., T. S. REESE, and F. J. BRINLEY, JR. 1978 b. Endoplasmic reticulum sequesters calcium in the squid giant axon. *Science (Wash. D.C.).* 202:1300-1303.

HITCHCOCK, S. E. 1977. Regulation of motility in nonmuscle cells. *J. Cell Biol.* 74:1-15.

HOFFMAN-BERLING, H. 1964. Relaxation of fibroblast cells. In: Primitive Motile Systems

in Cell Biology. R. D. Allen and N. Kamiya, editors. Academic Press, Inc., New York. 365–375.

KOLHARDT, M., B. BAUER, H. KRAUSE, and A. FLECKENSTEIN. 1972. New selective inhibitors of the transmembrane Ca conductivity in mammalian myocardial fibers. Studies with the voltage clamp technique. *Experientia (Basel)*. **28**:288–289.

KRNJEVIC, K., and A. LISIEWICZ. 1972. Injections of calcium ions into spinal motoneurons. *J. Physiol. (Lond.).* **225**:363–390.

LAMB, J. F., and R. LINDSAY. 1971. Effect of Na, metabolic inhibitors and ATP on Ca movements in L cells. *J. Physiol. (Lond.)* **218**:691–708.

MACMANUS, J. P., J. F. WHITFIELD, A. L. BOYNTON, and R. H. RIXON. 1975. Role of cyclic nucleotides and calcium in the positive control of cell proliferation. *Adv. Cyclic Nucleotide Res.* **5**:719–734.

McGRAW, C. F., A. V. SOMLYO, and M. P. BLAUSTEIN. 1978. Ultrastructural localization of calcium by electron probe analysis in presynaptic nerve terminals. *Abstr. Soc. Neurosci.* **4**:332.

MEECH, R. W. 1974. The sensitivity of *Helix aspersa* neurons to injected calcium ions. *J. Physiol. (Lond.)* **237**:259–277.

MEECH, R. W., and F. STRUMWASSER. 1970. Intracellular calcium injection activates potassium conductance in *Aplysia* nerve cells. *Fed. Proc.* **29**:834.

MIYAZAKI, S. I., K. TAKAHASHI, and K. TSUDA. 1972. Calcium and sodium contributions to regenerative responses in the embryonic excitable cell membrane. *Science (Wash. D.C.).* **176**:1441–1443.

MOORE, L., and I. PASTAN. 1977. Regulation of intracellular calcium in chick embryo fibroblast: calcium uptake by the microsomal fraction. *J. Cell Physiol.* **91**:289–296.

NELSON, P. G., M. HENKART, and B. RANSOM. 1974. Membrane electrical properties associated with morphogenesis in L cells: Relationship between divalent cations cyclic nucleotides, and prostaglandin E₁. *Abstr. Soc. Neurosci.* 353.

NELSON, P. G., and J.H. PEACOCK. 1972. Acetylcholine responses in L cells. *Science (Wash. D.C.).* **177**:1005–1007.

NELSON, P. G., and J. H. PEACOCK. 1973. Transmission of an active electrical response between fibroblasts (L cells) in cell culture. *J. Gen. Physiol.* **62**:25–36.

NELSON, P. G., J. H. PEACOCK, T. AMANO, and J. MINNA. 1971. Electrogenesis in mouse neuroblastoma cells *in vitro. J. Cell. Physiol.* **77**:337–352.

NELSON, P. G., J. PEACOCK, and J. MINNA. 1972. An active electrical response in fibroblasts. *J. Gen. Physiol.* **60**:58–71.

OKADA, Y., Y. DOIDA, G. ROY, W. TSUCHIYA, K. INOUYE, and A. INOUYE. 1977 a. Oscillations of membrane potential in L cells I. Basic characteristics. *J. Membr. Biol.* **35**:319–335.

OKADA, Y., G. ROY, W. TSUCHIYA, Y. DOIDA, and A. INOUYE. 1977 b. Oscillations of membrane potential in L cells. II. Effect of monovalent ion concentrations and conductance changes associated with oscillations. *J. Membr. Biol.* **35**:337–350.

PEACHEY, L. D. 1964. Transverse tubules in excitation-contraction coupling. *Fed Proc.* **24**:1124–1134.

RASMUSSEN, H., and D. B. P. GOODMAN. 1977. Relationships between calcium and cyclic nucleotides in cell activation. *Physiol. Rev.* **57**:421–509.

REAVEN, E. P., and S. G. AXLINE. 1973. Subplasmalemmal microfilaments and microtubules in resting and phagocytizing cultivated macrophages. *J. Cell Biol.* **59**:12–27.

REBHUN, L. I. 1977. Cyclic nucleotides, calcium, and cell division. *Int. Rev. Cytol.* **49**:1–54.

REED, P. W., and H. A. LARDY. 1972. A-23187: a divalent cation ionophore. *J. Biol. Chem.* **247:**6970–6977.

REUTER, H. 1973. Divalent cations as charge carriers in excitable membranes. *Prog. Biophys. Mol. Biol.* **26:**3–43.

ROMERO, P. J., and R. WHITTAM. 1971. The control by internal calcium of membrane permeability to sodium and potassium. *J. Physiol. (Lond.).* **214:**481–507.

ROSENBLUTH, J. 1962. Subsurface cisterns and their relationship to the neuronal plasma membrane. *J. Cell Biol.* **13:**405–422.

RUBIN, R. P. 1970. The role of calcium in the release of neurotransmitter substances and hormones. *Pharmacol. Rev.* **22:**389–428.

SCHLIWA, M. 1976. The role of divalent cations in the regulation of microtubule assembly: *In vivo* studies on microtubules of the heliozoan axopodium using the ionophore A-23187. *J. Cell Biol.* **70:**527–540.

STEPHENSON, E., and R. J. PODOLSKY. 1978. The regulation of Ca^{++} in skeletal muscle. *Ann. N.Y. Acad. Sci.* **307:**462–476.

WHITMORE, G. F., J. E. TILL, R. B. L. GWATKINS, L. SIMINOVITCH, and A. F. GRAHAM. 1958. Increase of cellular constituents in X-irradiated mammalian cells. *Biochim. Biophys. Acta.* **30:**583–590.

WHITTAM, R. 1968. Control of membrane permeability to potassium in red blood cells. *Nature (Lond.).* **219:**610.