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Permeability of a Cell Junction and the Local Cytoplasmic Free Ionized Calcium Concentration: A Study with Aequorin

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Summary. A technique is devised to determine the spatial distribution of the free ionized cytoplasmic calcium concentration ($[Ca^{2+}]_i$) inside a cell: *Chironomus* salivary gland cells are loaded with aequorin, and the Ca^{2+}-dependent light emission of the aequorin is scanned with an image-intensifier/television system. With this technique, the $[Ca^{2+}]_i$ is determined simultaneously with junctional electrical coupling when Ca^{2+} is microinjected into the cells, or when the cells are exposed to metabolic inhibitors, Ca-transporting ionophores, or Ca-free medium. Ca microinjections elevating the $[Ca^{2+}]_i$ in the junctional locale produce depression of junctional membrane conductance. When the $[Ca^{2+}]_i$ elevation is confined to the vicinity of one cell junction, the conductance of that junction alone is depressed; other junctions of the same cell are not affected. The depression sets in as the $[Ca^{2+}]_i$ rises in the junctional locale, and reverses after the $[Ca^{2+}]_i$ falls to baseline. When the $[Ca^{2+}]_i$ elevation is diffuse throughout the cell, the conductances of all junctions of the cell are depressed. The Ca injections produce no detectable $[Ca^{2+}]_i$ elevations in cells adjacent to the injected one; the Ca-induced change in junctional membrane permeability seems fast enough to block appreciable transjunctional flow of Ca^{2+}. Control injections of Cl^- or K^+ do not affect junctional conductance. The Ca injections that elevate $[Ca^{2+}]_i$ sufficiently to depress junctional conductance also produce under the usual conditions an increase in nonjunctional membrane conductance and, hence, depolarization. But injections that elevate $[Ca^{2+}]_i$ at the junction while largely avoiding nonjunctional membrane cause depression of junctional conductance with little or no depolarization. Moreover, elevations of $[Ca^{2+}]_i$ in cells clamped near resting potential produce the depression, too. On the other hand, complete depolarization in K medium does not produce the depression, unless accompanied by $[Ca^{2+}]_i$ elevation. Thus, the depolarization is neither necessary nor sufficient for depression of junctional conductance. Treatment with cyanide, dinitrophenol and ionophores X537A or A23187 produces diffuse elevation of $[Ca^{2+}]_i$ associated with depression of junctional conductance. Prolonged exposure to Ca-free medium leads to fluctuation in $[Ca^{2+}]_i$ where rise and fall of $[Ca^{2+}]_i$ correlate respectively with fall and rise in junctional conductance.

It has previously been shown that the permeability of the membrane junction between *Chironomus* salivary gland cells is decreased when the cells are poisoned with cyanide or dinitrophenol or when they are exposed for some time to Li medium or to medium lacking Ca and Mg (Politoff,

Socolar & Loewenstein, 1969; Rose & Loewenstein, 1971). Under each of these conditions, a rise in the concentration of cytoplasmic free Ca^{2+} may be expected in the junctional region on the basis of known properties of cellular Ca^{2+} metabolism (*cf.* Baker, 1972). It has furthermore been shown that the decrease in junctional permeability can be brought about by direct injection of Ca^{2+} into the cell (Loewenstein, Nakas & Socolar, 1967; Délèze & Loewenstein, 1976). Here again a rise in cytoplasmic Ca^{2+} concentration may be expected at the junction, if the injection overcomes the Ca-buffering capacity of the cell. In the present work, we aim to find out whether in these various conditions the junctional uncoupling indeed correlates with an elevation of the Ca^{2+} concentration in the junctional locale. We use aequorin (Shimomura, Johnson & Saiga, 1962; Hastings, Mitchell, Mattingly, Blinks & Van Leeuwen, 1969) as an indicator of cytoplasmic Ca^{2+} and view the light emission of this protein inside the cell with the aid of an image-intensifier/television system. The present aequorin method is sensitive to Ca^{2+} concentrations between $10^{-6}-10^{-4}$ M, a range over which the cytoplasmic Ca^{2+} may be expected to change during junctional uncoupling (Oliveira-Castro & Loewenstein, 1971); and it gives the spatial distribution of the light emission inside the giant salivary gland cells (100–200 μ in diameter) with a resolution of 1–2 μ. By simultaneous determinations of aequorin light emission and electrical coupling and other electrical parameters, we obtain information on junctional membrane permeability and local cytoplasmic Ca^{2+} concentration. Preliminary reports of the results have already appeared (Rose & Loewenstein, 1974; 1975*a*).

Materials and Methods

Preparation and Media

Salivary glands of *Chironomus thummi*, mid-fourth instar, were isolated (Rose, 1971) in Ca-free medium and set up in a perfusion chamber. The glands were kept in this medium until the aequorin had been injected and all intracellular microelectrodes were in place. The perfusion system allowed vibration-free exchange of the medium; about 95% of the medium was exchanged within 3 min (Rose & Loewenstein, 1971). The composition of the media used in the various experiments is given in Table 1. Sodium cyanide (CN) was added to media immediately before use. 2,4-dinitrophenol (DNP) was prepared as a stock solution (10^{-2} M) and diluted in Ca, Mg-medium before use. Ionophore X 537 A (Hoffman La Roche), adsorbed to albumin, was added to Ca-medium containing 1 mg/ml bovine serum albumin. Ionophore A 23187 (Lilly) was dissolved in 1:1 dimethyl sulfoxide/ethanol and then diluted to 2×10^{-6} M in Ca-free medium, 0.1% final solvent concentration. Control application of the solvent had no detectable effects on junctional coupling.

All experiments were carried out at room temperature ranging 22–25 °C.

Table 1. Composition of Media (mM)

Medium	KCl	NaCl	Na_2-Fumarate	$CaCl_2$	Glutamine	TES[a]	Na_2-Succinate	Mg-Succinate	Ca-Succinate	LiCl	Li_2-Succinate	Succinic Acid	KH-Fumarate	Na-Propionate	Titrated to pH 7.4 with
Ca-medium (control)	2	28	28	5	80	5			7						NaOH
Ca-free medium	2	38	28		80	5	7								NaOH
K-medium	30			5	80	5		7					28		KOH
Ca-free K-medium	50				80	5						7	28		KOH
Ca-free Li-medium	2				80	5				38	38				LiOH
Ca-free propionate medium	2		28		80	5								45	NaOH
Ca, Mg-medium[b]	2	28	28	5	80	5		7							NaOH

[a] N-Tris-(hydroxymethyl) methyl-2-amino-ethane sulfonic acid.
[b] Used only in experiments with dinitrophenol.

Aequorin Technique

Aequorin. Aequorin, highly purified and precipitated with ammonium sulfate in the presence of 0.1 M ethylenediaminetetraacetic acid (EDTA) (Shimomura, Johnson & Saiga, 1962; Shimomura & Johnson, 1969), was kindly given to us by Dr. Osamu Shimomura, Princeton University. The ammonium sulfate and EDTA were eliminated in a miniature Sephadex G-25-fine column, and the aequorin, 1 mg in 0.1 ml of 10 mM potassium phosphate buffer (pH 7.0), was subdivided into small droplets which were stored in separate dishes under filtered mineral oil at −25 °C. Just before use, one aequorin droplet was thawed under the oil on a cold plate. With the exception of the injection micropipettes, only plastic pipettes and containers were used for handling of the aequorin in order to minimize Ca contamination by glass.

Injection. Aequorin was microinjected with short-tapered micropipettes of 1–2 μ tip diameter. The micropipettes were filled by capillarity and negative pressure: the tip was placed into the aequorin droplet and suction was applied to the other end of the micropipette with a syringe, while the rise of the meniscus in the micropipette was observed in a dissecting microscope. In a fair number of cases, the tips became clogged at some point during the filling, as indicated by the standstill of the meniscus. Such pipettes were used for microinjection; unclogged ones were not useful because their aequorin became diluted by the bathing medium before cell impalement.

Aequorin was injected into the cells by pneumatic pressure pulses. The pressure pulse system consisted essentially of a tank of compressed gas (CO_2, air, or N_2) and a three-way valve controlled by a solenoid (ASCO GS 8320 A21) which was switched by a square pulse generator-relay system (Fig. 1). The system delivered pressure pulses variable in strength, duration and frequency. In the "pressure-off" position of the valve, the micropipette was shunted to room pressure, providing rapid pressure release (Fig. 1). To begin the cell injections, brief, repetitive high-pressure pulses (up to 100 ppsi) were applied until movement of the micropipette meniscus indicated that the tip had unclogged. Further injection pulses were of lower pressure. The difference in height of the pipette meniscus provided an index of the injected volume. The amounts of aequorin injected and the aequorin concentrations in the cells varied in different experiments.

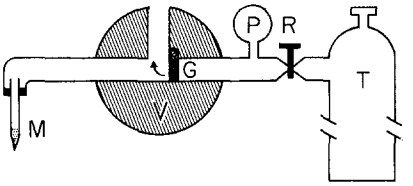


Fig. 1. Diagram of pneumatic pressure injection system. *T*, tank of compressed gas. *R*, reduction valve. *P*, pressure gauge. *V*, three-way valve. *G*, valve gate controlled by a solenoid, connecting micropipette (*M*) to room pressure or to gas tank. The entire system is gas-filled up to the solution in the micropipette

After aequorin injection, the pipette was retracted. The experiments were begun not sooner than 1/2 hr thereafter. By then the aequorin had spread throughout the cell, as demonstrated by Ca injections into various cell regions. The injected aequorin (33,000 Dalton) did not seem to pass to adjacent cells; there was no glow in these cells when they were injected with Ca or treated with cyanide or with Ca ionophores.

The aequorin injections had no obvious ill effects: the cells retained their transparency, membrane potentials and electrical input resistance. This was so even with relatively massive injections in which the cell radius increased transiently by about 10 % during the injections.

Optics. The insertions of the aequorin micropipettes and microelectrodes into the cells, and the aequorin injections were done under a metallurgical microscope (Leitz) with a 20 × objective of 15 mm working distance. For work with the television system, a saltwater immersion objective (25 × ; working distance, 1.67 mm; numerical aperture, 0.60) was rotated into place. A prism diverted part of the light to the oculars for direct observation. In the television mode, the prism was removed for maximum light transmission. The microscope optics were free to move in the vertical and horizontal planes.

Image-intensifier/television system. For viewing of the aequorin luminescence inside the cells, a television camera (Dynavision, Model 2001) with a three-stage image intensifier was used in combination with the microscope (Fig. 2). The image intensifier was coupled through fiber optics to a 1-inch Vidicon. The system was focussed with a lens (Schneider, Tele-Xenar 1:2.8/100) through the phototube of the microscope onto the image plane of the immersion objective. The entire setup was enclosed in a dark chamber; the cells were not illuminated during the experiments. The cells could also be viewed through the TV system by simply illuminating them from below (e.g. Fig. 5 *Ab*); the image intensifier gain was automatically reduced. Such "brightfield" viewing was done routinely before the experiments to permit tracing of the outlines of the cells on the screen of the TV monitor. The output of the TV camera was fed into a video tape recorder and a TV monitor.

Photomultiplier. The spatially integrated aequorin luminescence in the cells was measured with the aid of a photomultiplier (EMI 9502SA operated at 1310 V). This way of measuring the lumped light output of the cells has been the general technique in earlier work utilizing aequorin as an indicator of cytoplasmic Ca^{2+} concentration during muscle contraction, nerve and receptor cell activity (Ashley & Ridgway, 1970; Baker, Hodgkin & Ridgway, 1971; Llinás, Blinks & Nicholson, 1972; Brown & Blinks, 1974). The light was collected by a wave guide of high transmission efficiency (Fig. 2). The wave guide consisted of a lucite rod bent to right angle, without residual stress, and sheathed in aluminum foil. A drop of silicone oil (Dow Corning 710; refractive index equal to that of lucite) on each end of the rod provided optical continuity with the photomultiplier window and the preparation dish. The photomultiplier output was fed through a DC Ammeter (time constant 250 msec) into a storage oscilloscope, a potentiometric chart recorder and/or an oscillographic chart recorder.

Sensitivity and spatial resolution. The method was sensitive over a range of cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) of $10^{-6} - 10^{-4}$ M. The lower limit of sensitivity for diffuse

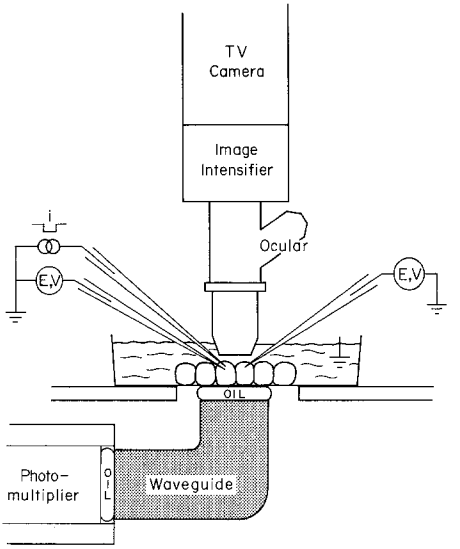


Fig. 2. Setup. Television camera coupled by fiber optics to image intensifier views the aequorin luminescence through microscope. Luminescence is also measured by photomultiplier. Electrical coupling is measured with the aid of three or four microelectrodes by pulsing current (i) between the inside of one cell and the grounded bath, and measuring the resulting steady-state displacements (V) of membrane potential (E) in this cell and an adjacent one

aequorin glows inside the cells was determined by large injections of EGTA-Ca buffers of various EGTA/Ca ratios. The limit, as determined with the image intensifier-TV system (which viewed an optical section of the cell) was a $[Ca^{2+}]_i$ of 5×10^{-7} M. This was also the limit as determined with the photomultiplier system in the case of cells of average size (cell volume $\approx 2 \times 10^{-7}$ cm^3). For smaller cells, the sensitivity of the photomultiplier system (which integrates light emission over a whole cell) was lower.

The spatial resolution of the image-intensifier/TV system was 1–2 μ. Nearly as good was the definition with which the limits of the spherical aequorin glows produced by the Ca injection could be seen. Because of the presence of fast energized cytoplasmic Ca sinks, the boundary of the glow was rather sharp (Rose & Loewenstein, 1975*b*); the spatial resolution for discriminating a $[Ca^{2+}]_i$ above the sensitivity limit at the boundary was 1–2 μ. The precision with which the junctional cell boundaries could be located was lower. The junctional cell surfaces are somewhat corrugated; the image boundary location in an optical cell section may vary by as much as 4 μ. Therefore, in dealing with the position of the junctional borders in relation to the aequorin glow, we will generally use the term *junctional locale* in the *Results*, implying a blurring of up to 4 μ. In some cases, there was evidently less variation in the junctional boundaries in the optical cell sections; the cell boundary appeared then as a sharply defined border in the glow sphere, resolvable with an accuracy of 1–2 μ (Fig. 11*a*). These cases were useful in the experiments dealing with the question of transjunctional flow of Ca^{2+}.

Test for aequorin depletion. The amount of aequorin injected into a cell was usually sufficient to serve as Ca^{2+} indicator without appreciable depletion for several hundred Ca^{2+} pulses of the kind shown in Fig. 4*A*, or for some twenty pulses of the kind of Fig. 4*B*, or for several minutes of diffuse elevation of $[Ca^{2+}]_i$, such as shown in Fig. 12*c–e*. Even though Ca was injected from a point source, the rate of Ca introduction, averaged over an entire experimental run, was apparently small enough so that the still active aequorin molecules kept themselves nearly uniformly distributed. In experiments in which there was a decline in

aequorin glow, we tested routinely whether this was due to a fall in $[Ca^{2+}]_i$ or to a depletion of active aequorin, by recording the light output in response to a standard Ca injection. For testing aequorin exhaustion in terminal experiments, the cell was perforated in Ca medium.

Ca-microinjection

Injection. Ca was injected either by pressure, with the system used for injection of aequorin (Fig. 1), or by iontophoresis. For pressure injection, the micropipettes were filled with Ca-EGTA buffers (Ethyleneglycol-bis-β(aminoethylether)-N, N'-tetraacetic acid) prepared with $CaCl_2$ and EGTA, pH 7.3 adjusted by KOH. The $EGTA_{total}$ was in all cases 0.1 M. Aside from stabilizing the free Ca^{2+} concentration, the EGTA was also helpful in preventing the micropipettes from becoming clogged inside the cells. Such clogging occurred much less frequently than in the experiments described in the preceding paper (Délèze & Loewenstein, 1976) in which the Ca in the micropipettes was unbuffered. For this reason, we used EGTA also in the microelectrodes for iontophoretic injection of Ca. The microelectrodes were filled with 0.2 M $CaCl_2$ and 0.1 M EGTA, pH 7 with KOH. The current for iontophoresis was supplied by an optically coupled current source *(see below)* and flowed between the microelectrode inside the cell and the grounded exterior. Unless otherwise noted, the iontophoresis was by continuous current.

The cells were in Ca-free medium during the Ca injections in order to minimize background cytoplasmic Ca^{2+} activity and Ca^{2+} leakage at the micropipette insertion sites. The micropipette tip was always in the focal plane of the image-intensifier/TV system, and the local spherical aequorin glows were centered in that plane.

Estimation of $[Ca^{2+}]_i$. The free $[Ca^{2+}]_i$ in the injected Ca buffers were calculated from the known concentrations of Ca_{total} and $EGTA_{total}$ in the micropipettes and assuming a concentration of free cytoplasmic Mg^{2+} of 5 mM (*see* Baker & Crawford, 1972) and a cytoplasmic pH of 7.0, by solution of the simultaneous equations expressing the binding equilibria of the three cations (Ca, Mg and H) with EGTA (Chabereck & Martell, 1959; Donaldson & Kerrick, 1975). The binding constants used were: $[Ca\,A]/[Ca]\,[A]=10^{11}$; $[Mg\,A]/[Mg]\,[A]=10^{5.2}$; $[H\,A]/[H]\,[A]=10^{9.43}$; $[H_2\,A]/[H]\,[H\,A]=10^{8.85}$; where A is the concentration of the ligand EGTA. The computer solutions were kindly provided to us by Dr. John Barrett and Mr. Ming Ferng. Curve *1*, Fig. 3, gives the computed free $[Ca^{2+}]_i$ for the whole buffered range of Ca_{total} used in the various experiments (EGTA, 0.1 M in all cases).

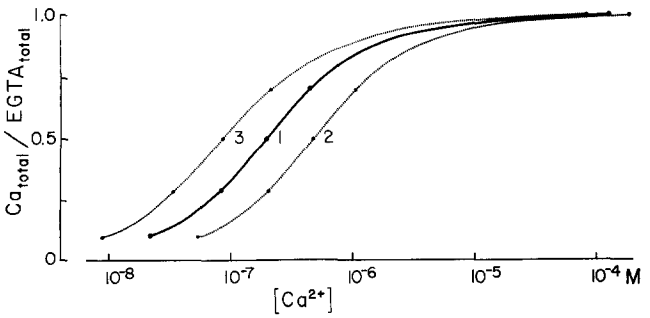


Fig. 3. Free Ca^{2+} concentration *vs.* $Ca_{total}/EGTA_{total}$ of the buffer, computed for a free Mg^{2+} concentration of 5 mM and pH 7.0 (curve *1*), pH 6.8 (curve *2*) and pH 7.2 (curve *3*). Curve *1* was used for estimating the cytoplasmic free Ca^{2+} concentrations in the experiments. Variation of free Mg^{2+} concentration from 0 to 10 mM deviates the computed values of curve *1* by less than the diameter of the plotted points. The curves were visually fitted to the computed points

The errors in using Curve *1* were assessed by considering limits for free $[Mg^{2+}]_i$ and pH_i. The lower limit for free $[Mg^{2+}]_i$ is 0 and a plausible upper limit is 10 mM, where, respectively, none or all of the cytoplasmic Mg is in free ionized form (for instance, in squid axoplasm the total Mg is about 6 mM; Baker & Crawford, 1972). The width of the points on Curve *1* gives the corresponding maximal deviations in the free $[Ca^{2+}]_i$ over the entire experimental range. It is evident that $[Mg^{2+}]_i$ has little effect on $[Ca^{2+}]_i$. The pH_i has a substantial effect, particularly in the lower range of Ca_{total}. Curves *2* and *3* give, for example, the deviations in $[Ca^{2+}]_i$ corresponding to actual pH_i of 6.8 and 7.2, respectively.

The free Ca^{2+} concentrations inside the micropipettes (Mg=0; pH 7) for the various $Ca_{total}/EGTA_{total}$ used are equal to the $[Ca^{2+}]$ given by Curve *1*.

Electrical Measurement, Recording and TV Display

The electrical coupling was determined by injecting rectangular current pulses (*i*) (150 or 200 msec duration) into one cell and measuring the resulting "steady-state" displacements (*V*) of membrane potential (*E*) in this cell and one or two adjacent cells, as diagrammed in Fig. 2 (Loewenstein & Kanno, 1964; Loewenstein *et al.*, 1967). The current-passing electrodes were filled with 3 M KCl or, when used also for Ca injection, with Ca-EGTA. The current was delivered by a voltage-controlled device driven by a square pulse generator and coupled electrooptically to the microelectrodes (Baird, 1967). When used in conjunction with the KCl electrodes, the device provided reliably constant current output. The potential-recording electrodes were filled with 3 M KCl and had resistances of 10–30 MΩ; the tip potentials, generally <5 mV, were offset by dc voltage. The specific electrode arrangements employed in the various experiments are diagrammed in the illustrations of the *Results*. In these diagrams, the current-passing electrodes containing KCl are designated *i*, and those containing Ca-EGTA are designated as appropriate, *Ca* or *Ca-EGTA*.

Currents, membrane potentials, and photomultiplier currents were recorded on a potentiometric chart recorder and/or an oscillographic chart recorder. They were besides displayed on a storage oscilloscope onto which a TV camera was focussed. The output of this camera and that of the image-intensifier camera were video-taped together and displayed on a monitor. Thus, we had simultaneous records of the electrical parameters and of the aequorin luminescence, including its spatial distribution.

The samples of aequorin luminescence illustrated in the figures (e.g. Fig. 5 *Aa*) are photographs taken of the screen of the television monitor during the replay of continuous video tapes. The procedure was to display the cells in brightfield at the start of the experiment (e.g. Fig. 5 *Ab*) and to trace their outlines on the screen; and then to superpose the aequorin luminescence of the cells in darkfield, and to photograph the particular video-taped sequence.

Results

Ca^{2+} Injection

$[Ca^{2+}]_i$ elevation and uncoupling. The aequorin-loaded cells had no detectable resting glow; the concentration of free ionized calcium in the cytoplasm, $[Ca^{2+}]_i$, was below 5×10^{-7} M, the limit of resolution of the method. On this zero luminescence background, a small pulse of Ca^{2+} produced a transient glow which did not spread much beyond the site of the

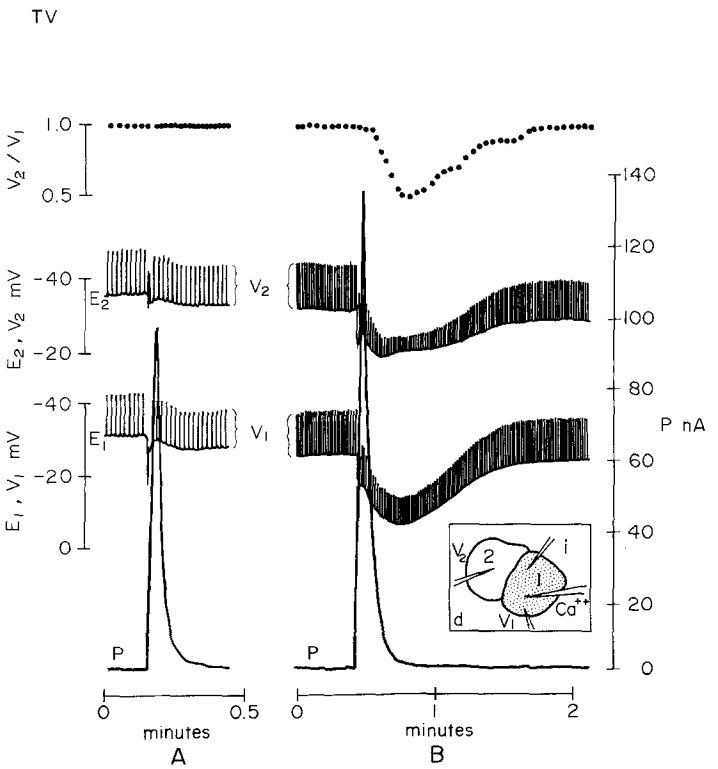


Fig. 4. Uncoupling by Ca injection. A pulse of Ca^{2+} (0.15 M Ca and 0.1 M EGTA) is injected (pressure) into cell *1* while electrical coupling between cells *1* and *2* is monitored. *A* and *B* show the effects respectively of a small and large Ca pulse delivered to the same intracellular site. *Top:* TV pictures of the resulting aequorin glows (*A*, *B*) at time of their maximum spatial spread (corresponding with peak of *P* curve); and brightfield TV picture (*c*) of the cells and micropipettes (diagrammed in *d*; dotted cell contains aequorin). Calibration, 100 μ. *Below:* Chart record tracings of the photomultiplier current (*P*), membrane potentials (E_1, E_2) of the two cells and their displacements (V_1, V_2) produced by the test current pulses ($i = 4 \times 10^{-8}$ A; 1/sec), and plot of V_2/V_1 (coupling ratio). In *A* the aequorin glow, i.e., the detectable $[Ca^{2+}]_i$ elevation, is restricted to a radius of *ca.* 15 μ from the injection pipette and does not reach the region of cell junction 1:2. The cells stay coupled. In *B* the $[Ca^{2+}]_i$ elevation extends to the junction. The cells uncouple. Note different time scales in *A* and *B*

injection. We showed elsewhere that the brevity of the $[Ca^{2+}]_i$ elevation and its local confinement are mainly due to rapid, energy-dependent Ca sequestering (Rose & Loewenstein, 1975*b*). In the example given in Fig. 4, a Ca^{2+} pulse thus produces a sphere of aequorin luminescence with a rather sharply defined boundary. At the time of maximum spread, the sphere, in the case of a short pulse, had a radius of about 15 μ and its boundary was about 50 μ away from the cell junction; that is, over this distance the $[Ca^{2+}]_i$ was below 5×10^{-7} M. Such Ca^{2+} pulses had no noticeable effect on the electrical coupling across the junction (Fig. 4*A*).

With larger or longer Ca^{2+} pulses the glow spread farther, and if it reached the junctional region, the coupling fell (Fig. 4*B*). Rise of $[Ca^{2+}]_i$ at junction, as indicated by the aequorin glow in this locale, and uncoupling went hand in hand in all 18 experiments. The cells started to recouple when the $[Ca^{2+}]_i$ had fallen again below detectable level (Fig. 4*B*), full recoupling lagging sometimes several minutes behind (Fig. 7).

Single-junction and multiple junction uncoupling. When the rise in $[Ca^{2+}]_i$ was sufficiently local, it was possible to show that the uncoupling was confined to the junction where the rise had taken place. In these experiments, the electrical coupling was measured at two opposite junctions of a given cell while injecting Ca^{2+} close to one of them. In the example of Fig. 5*A*, the $[Ca^{2+}]_i$ was thus elevated at junction *1:2* but not at junction *1:3*, about 115 μ away. Only junction *1:2* of this cell showed decreased coupling.

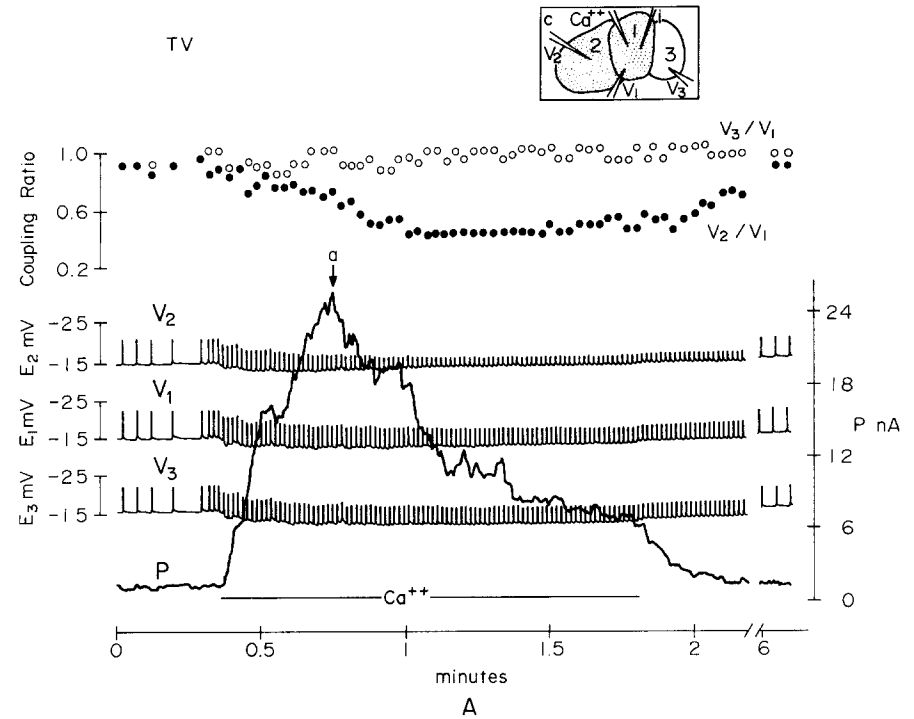
With a Ca pulse of a greater rate of rise delivered to the same site, the $[Ca^{2+}]_i$ elevation extended also to junction 1:3. The coupling across this junction was then affected too (Fig. 5*B*). In general, whenever the $[Ca^{2+}]_i$ elevation was diffuse throughout the cell, all junctions of the cell seemed to uncouple.

Under these conditions, it was also most evident that the junctional conductance had been reduced: the fall in coupling ratio V_2/V_1 was associated with a rise in V_1 (e.g. Figs. 5*B* and 10). While a fall in coupling ratio alone could in principle be due to nonjunctional factors, the combination with a rise in V_1, as analyzed elsewhere (Loewenstein *et al.*, 1967), is compatible only with a marked fall in junctional conductance.

In other cases of uncoupling there was an initial rise in V_1 (see first V_1 pulse after Ca injection in Fig. 7) followed by a fall, as the effect of Ca^{2+} on nonjunctional membrane conductance became dominant *(see below)*; but even here V_1 was found to increase again while the coupling ratio was still depressed, after the cell had rid itself of the excess Ca^{2+} and had begun to recouple.

A rise of V_1 was invariably present when its masking by nonjunctional Ca effects was avoided. Thus, in the experiment of Fig. 6, Ca^{2+} was injected into cell *2* (instead of cell *1* as in the preceding experiments) while test current was injected into cell *1*. V_1 rose as $[Ca^{2+}]_i$ was elevated on side *2* of junction 1:2.

The decrease in junctional conductance was further evidenced by a divergence in the resting potential of adjacent cells. Well-coupled adjacent cells had about the same resting potential. During uncoupling by Ca^{2+} injection, the resting potentials generally diverged. For example, in the


TV
c
Ca++
1
2
3
V2
V1
V3
V3 / V1
V2 / V1
Coupling Ratio
1.0
0.6
0.2
a
V2
E2 mV
-25
-15
V1
E1 mV
-25
-15
V3
E3 mV
-25
-15
P
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24
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A

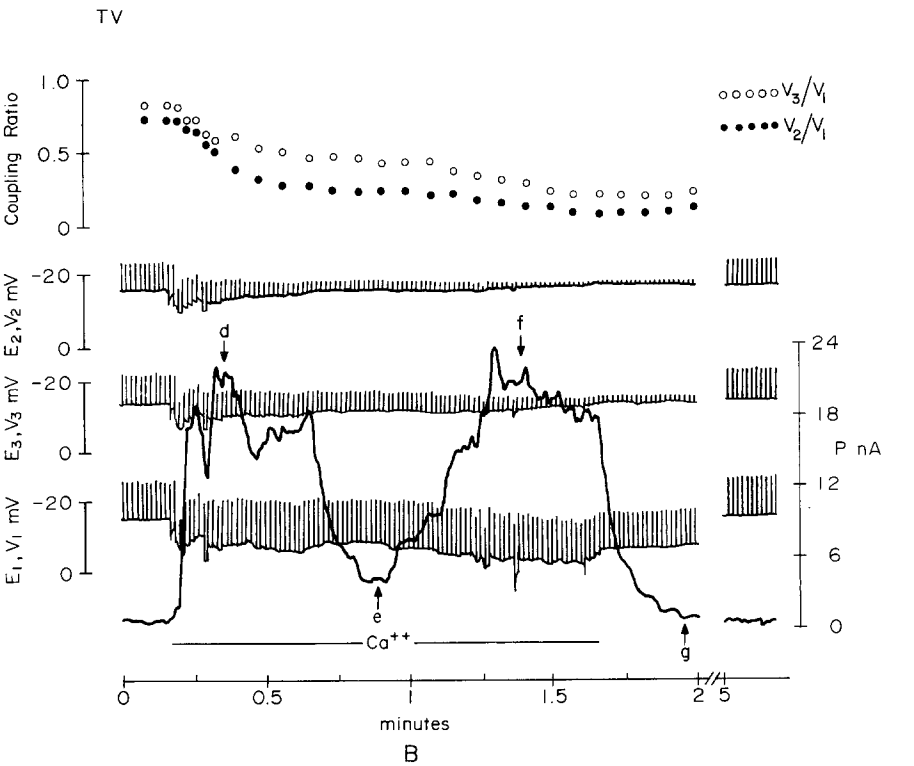
TV
Coupling Ratio
1.0
0.5
0
V3/V1
V2/V1
E2, V2 mV
-20
0
E3, V3 mV
-20
0
E1, V1 mV
-20
0
d
e
f
g
Ca++
24
18
12
6
0
P nA
0
0.5
1
1.5
2
5
minutes
B

Fig. 5 (left). *A.* Single-junction uncoupling by Ca injection. Ca is injected (iontophoresis) close to one cell junction (1:2) elevating $[Ca^{2+}]_i$ locally in the region of this junction. Coupling is measured across junctions 1:2 and 1:3. TV picture (*a*) shows the aequorin glow at the time of maximal spatial spread. Below are the chart record tracings of the photomultiplier current (*P*), the membrane potentials (*E*) and their displacements (*V*) in the three cells (test current pulses $i = 4 \times 10^{-8}$ A; 1/sec), and the coupling ratios V_2/V_1 and V_3/V_1.
B. Multiple junction uncoupling by Ca injection. The rate of Ca injection is increased causing the $[Ca^{2+}]_i$ elevation to spread farther reaching both junctions 1:2 and 1:3 (TV picture *d*). The coupling is now depressed at both junctions. During a second peak of the Ca^{2+} injection, the Ca^{2+} swamps the cell (TV picture *f*) depressing the coupling further. *In this and subsequent figures, the bar marks the duration of the Ca current, the arrows on the P-curve indicate the time correspondence of TV pictures, and the calibration bar on the TV pictures equals* 100 μ

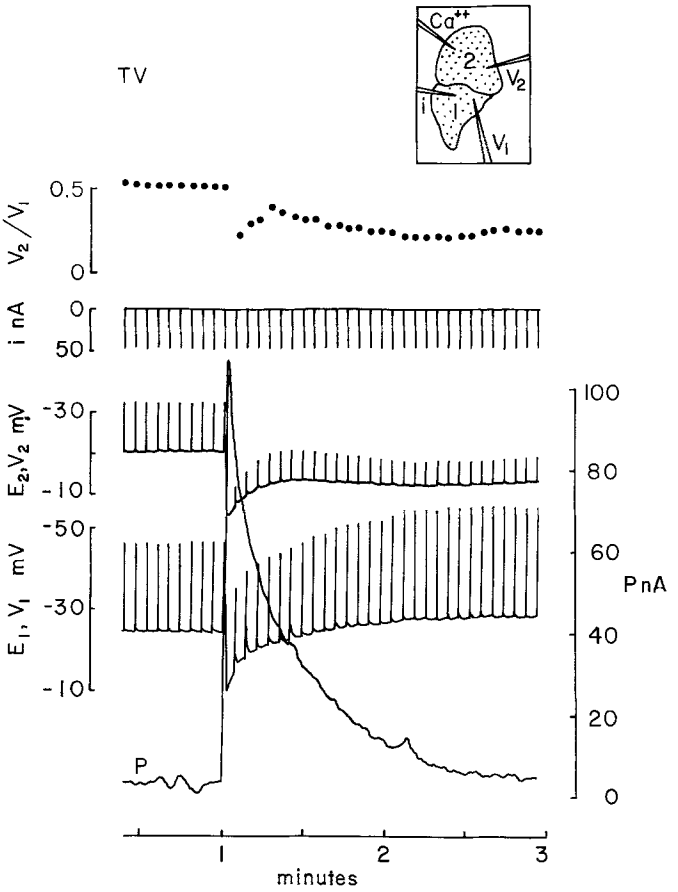

Fig. 6. Uncoupling by Ca injection with unmasked rise in input resistance. Injection of Ca^{2+} into cell *2*; test current into cell *1*. V_1 rises as the coupling ratio falls during the elevation of $[Ca^{2+}]_i$ in cell *2*

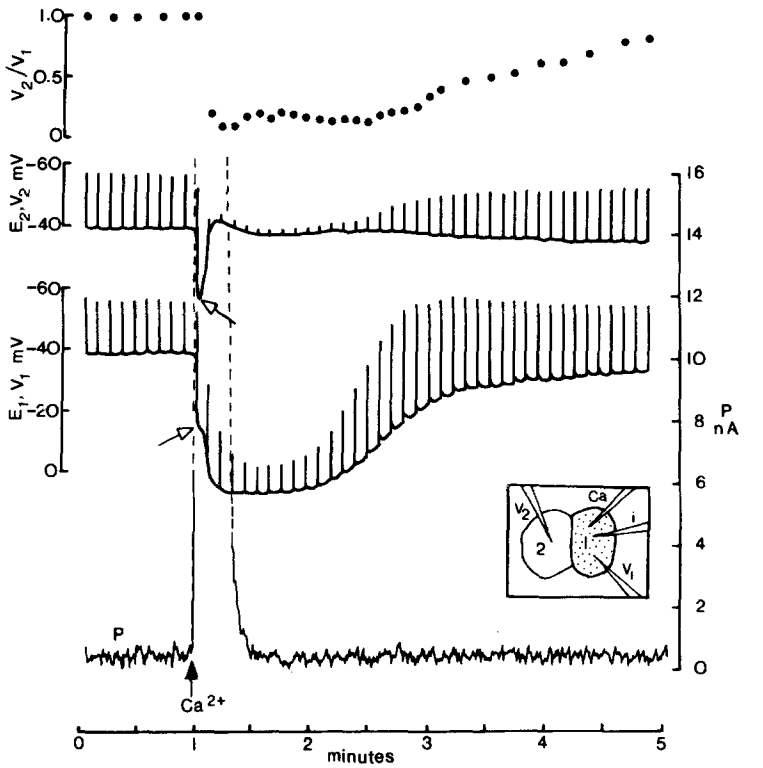

Fig. 7. Diverging membrane potentials during uncoupling by Ca injection. Injection (pressure) of buffered Ca^{2+} into cell *1* causes depolarization of this cell and of the coupled cell *2*. Then (at open arrow) cell *1* continues to depolarize while cell *2* repolarizes as the coupling ratio falls. $i = 4 \times 10^{-8}$ A

experiment of Fig. 7, cells *1* and *2* (initial resting potential −40 mV) depolarized together and nearly to the same extent during the first 2 sec of the Ca^{2+} injection while the coupling between the cells was still high. But then, cell *2* abruptly repolarized while cell *1* depolarized further, as the coupling fell. In the case of single-junction uncoupling, the resting potentials did not diverge as much; but there was then often at least some degree of divergence signalling the decrease in junctional conductance even when V_1 had not risen.

When $[Ca^{2+}]_i$ was raised locally at one junction, the uncoupling cells retained some degree of coupling, often a substantial one (e.g. Fig. 5 *A*). A substantial residual coupling is to be expected under these conditions, because a pair of giant cells – the cells used in our experiments – is coupled directly via their common junction and indirectly via junctions with the flat cells spanning the lumen (Rose, 1971). The junctions mediating direct and indirect couplings are sufficiently far apart in a given giant cell so that

the indirect couplings may not be affected by a local rise in $[Ca^{2+}]_i$ at one giant cell junction. However, when the Ca^{2+} injection swamped the cell and a $[Ca^{2+}]_i$ elevation was seen throughout the cell, all or nearly all coupling was abolished (e.g. Figs. 5*B* and 11).

Effects on nonjunctional membrane. The Ca^{2+} injections in the present experiments as in those reported in the first paper of this series (Délèze & Loewenstein, 1976) were generally made just below nonjunctional membrane in order to minimize membrane damage by the insertion of the micropipettes. Under these conditions, the Ca^{2+} spread over a substantial area of nonjunctional membrane on the inside. The general effect was a decrease in input resistance (V_1/i) accompanied by depolarization. The fall in input resistance was due to a decrease in resistance of nonjunctional membrane, for it was present also when the $[Ca^{2+}]_i$ elevation was restricted to a nonjunctional locale (and not accompanied by uncoupling) (Fig. 4*A*). Thus, as in red blood cells (Romero & Whittam, 1971), and certain nerve cells (Meech & Standen, 1975; Barrett & Barrett, 1976), Ca^{2+} on the inside causes the nonjunctional membrane permeability of the gland cells to rise. It was this fall in nonjunctional membrane resistance that masked the rise in V_1 (due to fall in junctional conductance) during uncoupling.

We do not know what specific ion permeabilities are involved in the change in nonjunctional membrane resistance. The experimental situation in the gland cells is complicated by the presence of a lumen which is not easily accessible; the ion composition is readily controlled in the medium in contact with the cells' base, but not in the lumen. Probably Na permeability is increased since the cells depolarize, but other ion permeabilities may be affected too.

Uncoupling in voltage-clamped cells. Since the uncouplings by Ca^{2+} injection were generally accompanied by depolarization, the question arose whether Ca^{2+} or depolarization is the primary cause of uncoupling. To resolve this point, Ca^{2+} was injected while the cells were (loosely) voltage-clamped with the aid of a feedback system.

The voltage-sensing electrode (V_1) and the feedback-current electrode (i_{clamp}) were in the cell (*1*) into which Ca^{2+} was injected from a Ca-EGTA-filled electrode by continuous iontophoretic outward current (Fig. 8). The coupling was monitored with periodic inward pulses passed through the latter electrode. The inward pulses, carried in part by $EGTA^-$, were small and infrequent enough to permit a sufficient buildup of $[Ca^{2+}]_i$. The feedback gain was adjusted to counteract the depolarizations produced by the effect of Ca^{2+} on nonjunctional membrane; and the current

pulses that tested the coupling were made large enough to produce measurable (hyperpolarizing) V_1 and V_2 pulses.[1]

The general result of the voltage-clamp experiments was that uncoupling occurred at or near resting membrane potential as well as when the cells depolarized, whenever $[Ca^{2+}]_i$ was elevated at a junction. In the example illustrated in Fig. 8, while the coupling ratio diminished from 0.95 to 0.64, the membrane potential did not diminish by more than 10 mV (which by itself is never sufficient for uncoupling; Socolar & Politoff, 1971). Depolarization is thus not necessary for uncoupling.

A similar conclusion follows even more simply and directly from the results of two experiments (obtained after the voltage-clamp experiments had been completed) in which small Ca injections close to a junction caused minimal depolarization (<5 mV). Here the junctions uncoupled nonetheless whenever the $[Ca^{2+}]_i$ was seen to rise at the junctional regions (Fig. 5*A*).

Maintenance of coupling in depolarized cells. In a complementing series of experiments, the cells were exposed to medium with high K concentration (K-medium, Table 1). Within 2–5 min, the membrane potential fell to near zero or overshot zero by 5–10 mV. The depolarized cells nevertheless stayed well-coupled much longer; in the example of Fig. 9, for nearly 4 hr. Eventually and rather abruptly, they uncoupled, and this was invariably associated with an abrupt rise in $[Ca^{2+}]_i$ (Fig. 9).[2] Hence, depolarization, except as it may on its own produce elevation of $[Ca^{2+}]_i$, is also not sufficient for uncoupling.

This conclusion is also consistent with the results of an experiment in which $[Ca^{2+}]_i$ was elevated locally at one giant cell junction by Ca injection. In this experiment the other junction of the injected cell stayed fully coupled in spite of depolarization. Moreover, in those cases in which

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1 In the example illustrated in Fig. 8, Ca^{2+} was injected locally at one giant cell junction (1:2), keeping the other junctional pathways between cells *1* and *2* open so that both cells stayed effectively voltage-clamped during uncoupling of junction 1:2. However, the presence of residual junctional pathways was not an essential condition for holding the membrane potentials near their resting levels. Even when the uncoupling was more pronounced than in the experiment of Fig. 8 and it involved more than one junction, the membrane potential of cell *2* stayed fairly close to resting level during uncoupling; the nonjunctional membrane of cell *2* was not affected by injection of Ca^{2+} into cell *1* because Ca^{2+} does not pass through junction *(see next section)*. Thus, the membrane potential of cell *2*, which followed the depolarization of cell *1* while the two cells were coupled, returned by itself to the resting level as the cells uncoupled more completely (*see* Fig. 7).

2 In the experiment of Fig. 9, the uncoupling in K medium was preceded by an exposure to Ca-free Li medium. This step was only incidental; the rise in $[Ca^{2+}]_i$ and uncoupling occurred also in other experiments in which this step was lacking.

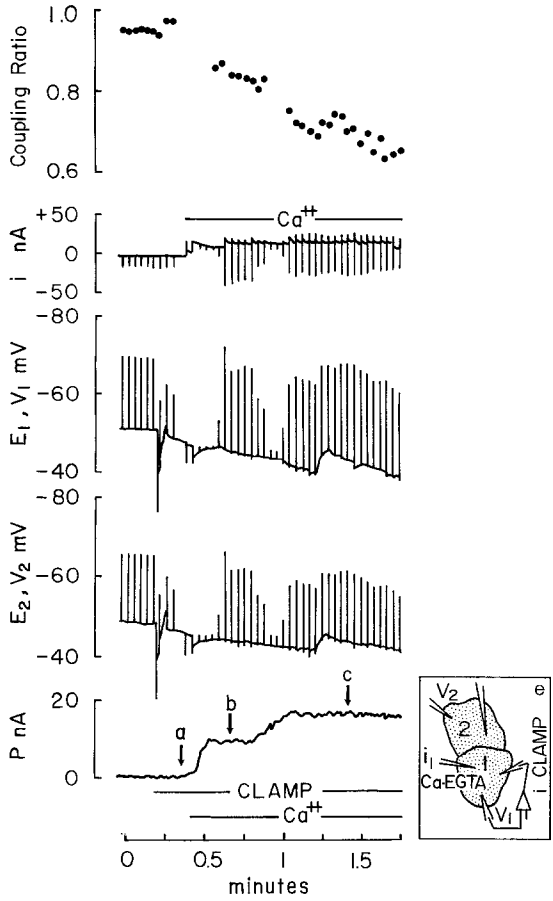

Fig. 8. Uncoupling by Ca injection in voltage-clamped cells. Ca^{2+} is injected (iontophoresis) into cell *1* close to junction *1:2* causing a local elevation of $[Ca^{2+}]_i$, while the membrane potential is held near resting value by a feed-back controlled inward current (i_{clamp}) for the period marked *clamp*. Coupling is measured with inward test current pulses into cell *1*. Electrode i_1 filled with Ca-EGTA, serves to inject Ca^{2+} (outward current) and to pass the (inward) test current pulses into cell *1* (200 msec duration, 0.4/sec). All other electrodes are filled with KCl. With the feed-back gain used, the test current is associated with attenuated *V* pulses (compare first three *V* values after onset of clamp with those preceding clamp). The trace *i* displays the test current pulses and the dc Ca-current (outward current, upward). The test currents are adjusted during the experiment to compensate for changes in feed-back gain. Both cells contain aequorin

$[Ca^{2+}]_i$ elevations in *nonjunctional* cell regions caused substantial depolarization, there was no uncoupling.

Control injections of K^+ and Cl^-. Injections of K^+ and Cl^- had no noticeable junctional effects. In the experiment of Fig. 10, K^+ and Cl^- were iontophoresed on either side of a junction of a pair of cells. The outward and inward dc currents carrying these ions were passed simul-

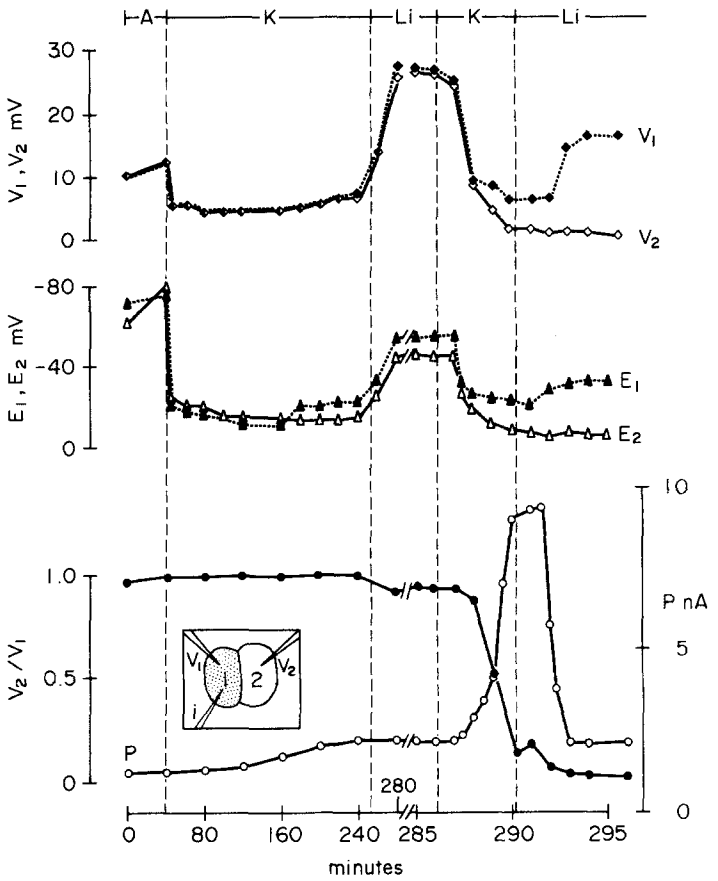

Fig. 9. Maintenance of coupling during depolarization. Cells were exposed to Ca medium (*A*)→ →K medium→Ca-free Li medium→K medium→Ca-free Li medium (see Footnote 2, page 100). *Top to Bottom:* Schedule of media; V_1 and V_2; membrane potential E_1, E_2; coupling ratio V_2/V_1; photomultiplier current *P*. (TV system not used.) During the first exposure to K medium the cells depolarize (from 75 to 15 mV), and maintain their coupling for 4 hr. They are repolarized in the Ca-free Li medium. The subsequent second exposure to K medium produces uncoupling associated with a rise in $[Ca^{2+}]_i$. $i = 4 \times 10^{-8}$ A. Note change in time scale

taneously into the two cells and were adjusted so as to null the deviations in membrane potential produced by the dc currents. In this way, the effects of K^+ and Cl^- on junctional coupling could be tested at constant membrane potential. Unlike Ca^{2+}, neither K^+ nor Cl^- affected coupling.[3]

Similarly ineffective were pressure injections of KCl (0.15 M) so long as they were not accompanied by rise in $[Ca^{2+}]_i$. However, massive KCl

3 The iontophoresis of Cl^- produced an aequorin glow. About a minute after the start of Cl^- iontophoresis, a faint glow became visible over a radius of 2–4 μ around the electrode, well away from the junction (Fig. 10*a*). Presumably the Cl^- caused some Ca release from intracellular stores.

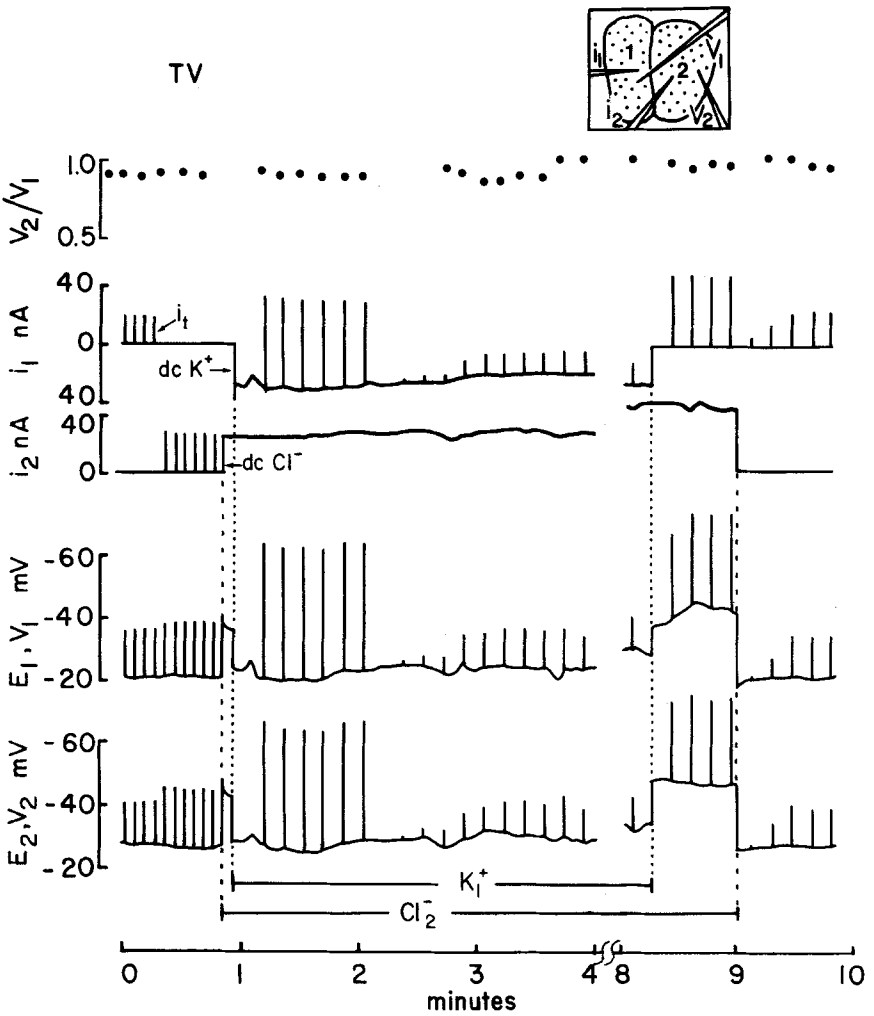

Fig. 10. Control K and Cl injections do not produce uncoupling. K^+ and Cl^- are iontophoresed into cells *1* and *2*, respectively, with dc currents passed between inside and outside (grounded) with KCl-filled electrodes. The dc currents in the two coupled cells are adjusted to approximately null the membrane potential changes produced by the dc currents. Inward test current pulses (i_t) into cell *1* of varying amplitude are superposed on the dc current. Shown are the chart record tracings of the currents (i), membrane potentials (E) and their displacements (V) produced by i_t, plot of coupling ratio V_2/V_1, and TV pictures. Note (*a*) the faint, local aequorin glow at the electrode i_1 during dc iontophoresis of Cl^-

injection led in three trials to rise of $[Ca^{2+}]_i$ and uncoupling. In these trials, the $[Ca^{2+}]_i$ began to rise at a site away from the junction and spread slowly throughout the cell; the uncoupling ensued when the rise in $[Ca^{2+}]_i$ had visibly reached the junction. Presumably the elevation of $[Ca^{2+}]_i$ was caused by release of Ca^{2+} from intracellular stores. An alternative, that the elevation was due to divalent cation contamination

of the KCl solution, is unlikely. The analysis by the manufacturer (Fisher Scientific) reports the total Ca, Mg, Sr, Ba contamination as 0.5%; thus Ca contamination could have been at most 1×10^{-6} M which, even before dilution in the cell, should have been well below the uncoupling threshold (Oliveira-Castro & Loewenstein, 1971). Besides, in one of the cases, the $[Ca^{2+}]_i$ elevation was seen to originate at an intracellular site away from the tip of the injection pipette.

To summarize the experiments of Ca injection, *the junctions uncoupled whenever* $[Ca^{2+}]_i$ *rose in the junctional locale, and only then.*

Lack of Transjunctional Ca^{2+} Flux

Does Ca^{2+} pass through the junction? The series of experiments addressed to this question was designed to determine the $[Ca^{2+}]_i$ on both sides of a cell junction when Ca^{2+} was injected on one side. The basic experimental sequence was (*i*) to preinject two adjacent cells with aequorin; (*ii*) to inject Ca^{2+} into cell *1* close to the cell junction 1:2 while continuously monitoring the aequorin glow on both sides of the junction with the image-intensifier/TV system; and, after completion of the tests, (*iii*) to inject Ca^{2+} into cell *2*, or to perforate the cell in Ca-containing medium to ascertain that the cell, in fact, contained sufficient aequorin.

In these experiments (step *ii*), the Ca^{2+} reached visibly the junctional region on the injected side within fractions of a second. In no instance (10 cases) was Ca^{2+} detectable on the other side of the junction (cell *2*), although on the injected side of the junction Ca^{2+} had built up to concentrations of 10^{-4} or 10^{-3} M, more than two or three orders of magnitude above detection threshold (Figs. 5*Bd, f*, and 11*a*). By contrast, fluorescein, a larger fluorescent molecule (330 MW), was found with the same method, to traverse the junction in fractions of a second. The best demonstration of the lack of transjunctional Ca flow was afforded by preparations in which the junctional boundaries were definable with an accuracy of 1 μ in the optical cross sections of the image-intensifier/TV system *(see Methods, sensitivity and spatial resolution)*. Thus, in the example of Fig. 11*a* the boundary is a sharp border in the aequorin glow.

In a variant of these experiments, Ca^{2+} was injected while the cells were in Ca-free medium containing 2 mM cyanide. At this concentration the cyanide by itself caused no detectable rise in $[Ca^{2+}]_i$ or junctional uncoupling during a 5-min exposure, but the injected Ca^{2+} diffused more freely through the cytoplasm (*see* Fig. 1 of Rose & Loewenstein, 1975*b*). Here again no sign of transjunctional passage of Ca^{2+} was found.

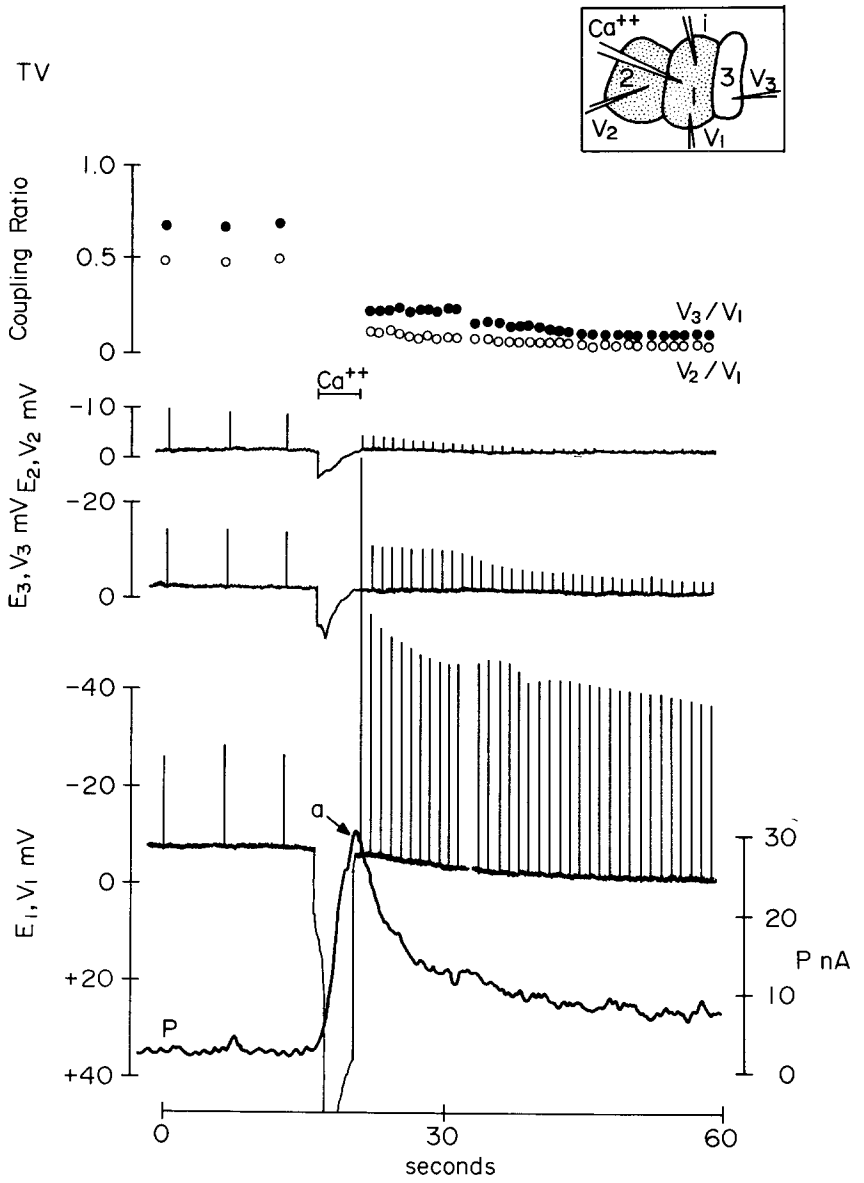

Fig. 11. Lack of transjunctional Ca^{2+} flow. Cells *1* and *2* are preinjected with aequorin. Ca^{2+} injection (iontophoresis) into cell *1* produces elevation of $[Ca^{2+}]_i$ along the entire length of junction 1:2 in the injected cell side but not on the other side (TV picture *a*). The junctional boundary here is seen as a sharply defined border of the glow sphere. The $[Ca^{2+}]_i$ elevation reaches also junction 1:3. Both junctions uncouple, with marked rise in V_1. $i=4\times10^{-8}$ A

Thus, Ca^{2+} does not seem to pass through junctional membrane in appreciable amounts. Apparently the Ca^{2+}-induced change in junctional membrane permeability is fast enough to block appreciable transjunctional flux of this ion.

Metabolic Inhibitors

Cyanide. Exposure to Ca-free medium containing cyanide (2–5 mM) led to diffuse rise in $[Ca^{2+}]_i$ and uncoupling. Fig. 12 illustrates a case in which, 10 min after application of the medium, the $[Ca^{2+}]_i$ was seen to rise, first locally (at the region where the aequorin pipette had formerly been inserted) and then diffusely throughout the cell. During the local phase, which lasted 6 min, the elevation of $[Ca^{2+}]_i$ was fairly well circumscribed and away from junction. During the diffuse phase, the elevation reached all junctions of the cell within a minute. This phase was associated with electrical uncoupling.

Uncoupling went hand in hand with rise in $[Ca^{2+}]_i$ in all experiments with cyanide (10 trials). Typically the coupling ratio fell to near zero, while V_1 rose initially, unambiguously showing a marked decrease in junctional conductance. Upon washout of the cyanide, the $[Ca^{2+}]_i$ fell rapidly again and this was followed by recoupling (Fig. 12).

In one experiment, in which the washout of cyanide (5 mM) was delayed, $[Ca^{2+}]_i$ fell spontaneously to baseline level 3 min after cyanide application. (Aequorin had not been exhausted.) The cells stayed nonetheless uncoupled. Recoupling ensued only after washout of the cyanide. However, it is quite possible that the $[Ca^{2+}]_i$ here may still have been above normal before the washout. The baseline of our method is 5×10^{-7} M, and the normal $[Ca^{2+}]_i$ may well be lower (Baker, 1972). That the $[Ca^{2+}]_i$ in this experiment might not have yet returned to normal level is also suggested by the fact that the fall in input resistance (and depolarization) was reversed only after washout of the cyanide.

As in the case of Ca injection, the rise in $[Ca^{2+}]_i$ produced by cyanide was accompanied by depolarization and eventually by a decrease in input resistance. The latter reflected an increase in nonjunctional membrane conductance which, by analogy with the Ca injection results, presumably ran parallel with a decrease in junctional conductance. However, as in Fig. 12, a rise in V_1 was generally detectable before the increase in nonjunctional membrane conductance took dominance.

It is not certain whether the sources that nurtured the rise in $[Ca^{2+}]_i$ in the Ca-free cyanide medium were entirely intracellular, because we do not know to what extent the medium in the lumen was exchanged with the external medium. We are inclined to believe that the source here was mainly intracellular because the diffuse $[Ca^{2+}]_i$ elevations occurred in relatively brief shots (of 2–5 min duration). Had luminal Ca been a major source, one would expect the $[Ca^{2+}]_i$ elevation to have been more sus-

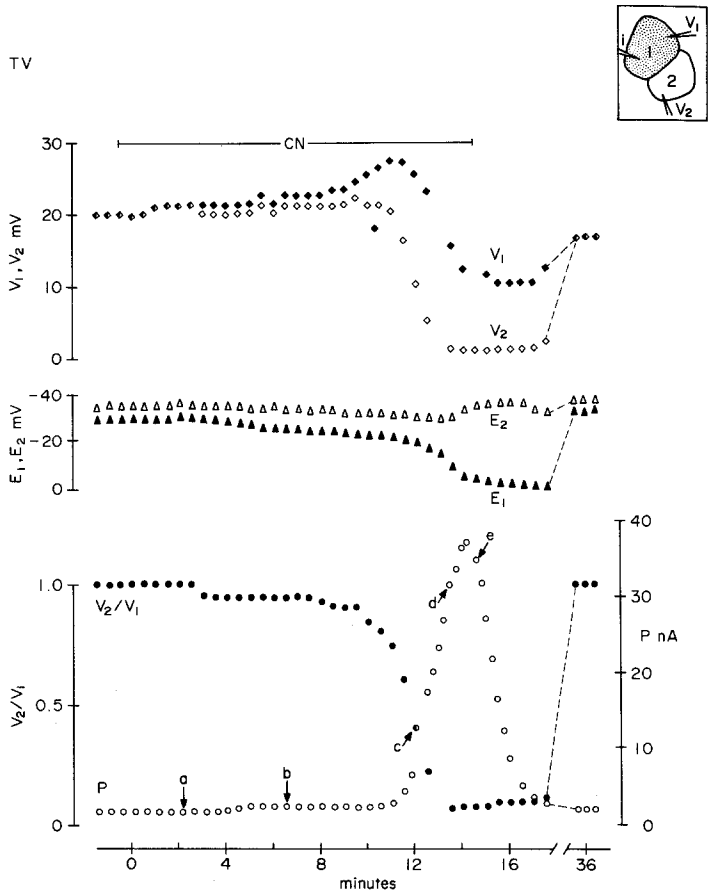

Fig. 12. Uncoupling by cyanide. Cells are exposed to 5 mM Na cyanide for the period indicated by the bar. They are in Ca-free medium throughout the experiment. $[Ca^{2+}]_i$ rises at first locally (TV picture *b*), and then diffusely associated with uncoupling (*c*–*e*). $[Ca^{2+}]_i$ falls upon washout of cyanide and recoupling ensues. $i = 4 \times 10^{-8}$ A

tained, as indeed it was when the external medium contained Ca. (The volume of the lumen is roughly of the order of 10^{-4} cm^3.)

In Ca-containing cyanide medium, the diffuse $[Ca^{2+}]_i$ elevation set in earlier and was detectable for as long as there was reactive aequorin inside the cells (2 experiments). The effects on coupling were the same as in Ca-free cyanide medium.

Dinitrophenol. In another set of experiments, the cells were treated with dinitrophenol (0.1 mM) in Ca-containing medium (4 experiments). In these experiments, the image intensifier system was not used; the aequorin glow was monitored only with the aid of the photomultiplier. Results similar to those obtained with cyanide and an equally good correlation between rising $[Ca^{2+}]_i$ and uncoupling were obtained (Fig. 13).

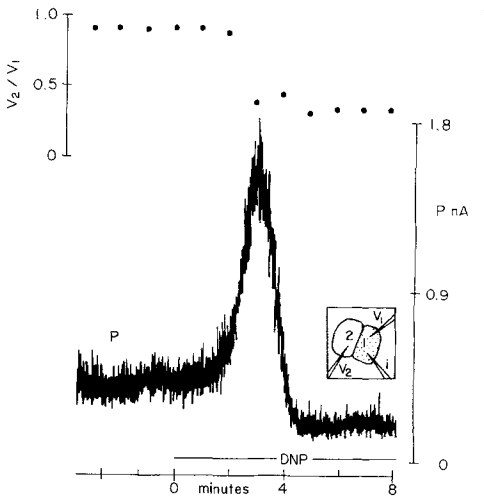

Fig. 13. Uncoupling by dinitrophenol. Cells are exposed to 0.1 mM dinitrophenol for the period indicated by the bar. They are in Ca, Mg medium throughout the experiment. Shown are the chart record of the photomultiplier current (P) and the plot of V_2/V_1. In this case there is a resting aequorin luminescence presumably due to Ca leakage at the micropipette impalement sites. TV system not used. The fall in luminescence was due to depletion of aequorin (*see* Methods for tests of depletion) $i = 4 \times 10^{-8}$ M

Ionophores

Ionophore X537A. Application of ionophore X537A (Caswell & Pressman, 1972; Scarpa & Inesi, 1972) produced diffuse rise in $[Ca^{2+}]_i$ and uncoupling (4 experiments). Here again the $[Ca^{2+}]_i$ elevation showed two phases: an early phase in which the elevation was local and a second one in which the elevation became rapidly diffuse and reached the junctions. The second phase was invariably associated with uncoupling (Fig. 14). In one experiment, the ionophore was washed out decreasing $[Ca^{2+}]_i$ to below 5×10^{-7} M; this was associated with partial junctional recoupling.

Ionophore A23187. A single experiment was done with ionophore A23187 (Reed & Lardy, 1972) in Ca-free medium. In this case, $[Ca^{2+}]_i$ rose rapidly and diffusely upon application of the ionophore (there was no distinct local phase) together with junctional uncoupling (Fig. 15).

The Local Phase of $[Ca^{2+}]_i$ Elevation during Treatment with Metabolic Inhibitors or Ionophores

The early $[Ca^{2+}]_i$ elevations in Ca-free cyanide medium or in Ca-containing X537A medium were generally localized at the regions where the micropipettes had been inserted. The region of the relatively large

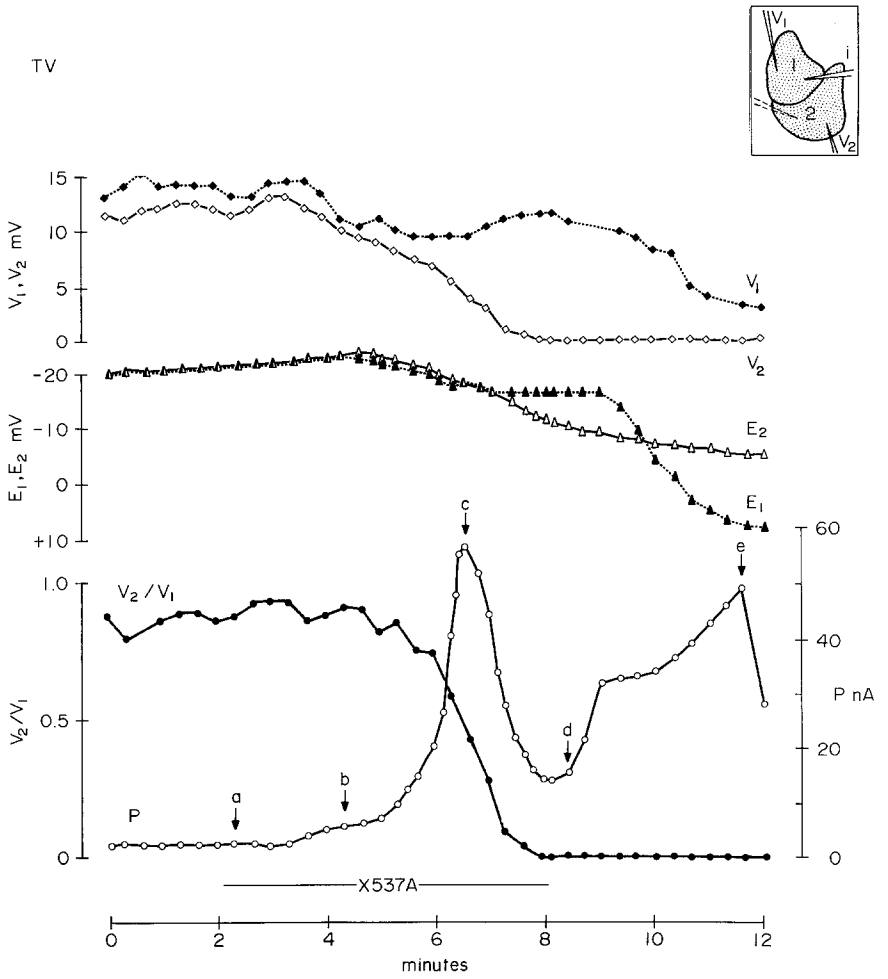

Fig. 14. Uncoupling by ionophore X537A. Bar signals exposure to 0.01 mM X537A. Cells in Ca-medium throughout. Local elevation of $[Ca^{2+}]_i$ is seen in cell *2* around the V_2 electrode. The first diffuse elevation occurs in cell *1* and correlates with uncoupling. Diffuse elevation in cell *2* begins 3 min later. TV pictures *c* and *e* show the peak luminescence in each cell. The decline in luminescence in each cell is due to aequorin depletion. TV picture *a* has some background light from the TV screen. $i = 4 \times 10^{-8}$ A (Cell *2* contained a second electrode not used here)

aequorin pipette (1–2 μ tip diameter) was the most common site, even though this pipette was no longer in the cell at the time of the $[Ca^{2+}]_i$ elevation. (The pipette was retracted after the cells were loaded with aequorin.) The $[Ca^{2+}]_i$ was below detectable level so long as the cyanide or the ionophore were absent from the medium (Figs. 12*a* and 14*a*). The local $[Ca^{2+}]_i$ elevations were, therefore, clearly caused by the cyanide or the ionophore, but the sites of micropipette impalement seemed to have

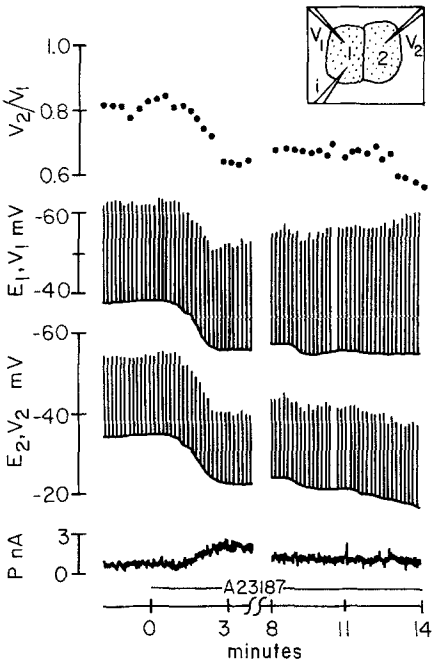

Fig. 15. Uncoupling by ionophore A23187. Bar indicates exposure to 2 μM A23187. Cells in in Ca-free medium throughout. $i = 4 \times 10^{-8}$ A

been more susceptible to these agents. A plausible explanation is that the micropipettes disturbed intracellular Ca sequestering elements at the region of impalement, depressing their Ca storing capacity and hence rendering the energized sequestering elements more sensitive to cyanide or to X537A.[4] The $[Ca^{2+}]_i$ elevation may thus have been kept circumscribed by the neighboring undisturbed sequestering elements until the cytoplasmic concentration of cyanide or X537A rose sufficiently to affect all of the energized sequestering elements in the cell. Another possibility is that the cyanide or the ionophore penetrated first into the cell at the sites of micropipette insertion. However, it would then be hard to see why the $[Ca^{2+}]_i$ elevation stayed localized several minutes in the experiment of Fig. 12; for a diffusible molecule, such as cyanide, one would expect a more rapid equilibration in the cytosol.

In some experiments with ionophore X537A, local $[Ca^{2+}]_i$ elevations were observed in cell regions in which no micropipettes had been placed. This suggests that different cell regions may differ in their Ca-buffering

4 Besides the best-known energized Ca-sequestering element, the mitochondrion (*cf.* Lehninger, 1970), the endoplasmic reticulum, which is very abundant in the salivary gland cells, may be an element of this kind (Moore, Knapp & Landa, 1975). Ca sequestering independent of metabolic energy may here be effected by cytoplasmic proteins (Baker & Schlaepfer, 1976).

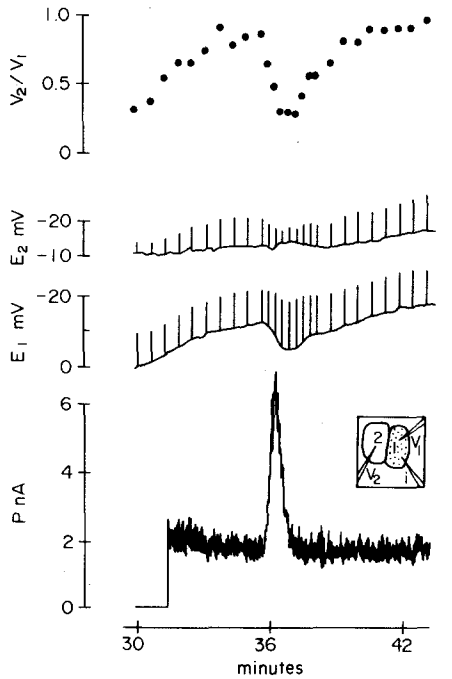

Fig. 16. Uncoupling in Ca-free Na medium. Cells in Ca-free Na medium undergo fluctuation in $[Ca^{2+}]_i$ and coupling. The start of the photomultiplier recording (*P*) caught the first $[Ca^{2+}]_i$ fluctuation on its return to baseline. The second fluctuation is recorded in full. $i = 4 \times 10^{-8}$ A

ability perhaps normally. Other kinds of evidence showing differences in the extent and configuration of the spread of Ca^{2+} in various regions inside the cell (not treated with cyanide or ionophore) point in the same direction (Rose & Loewenstein, 1975*b*).

We should also like to point out that both the local and diffuse $[Ca^{2+}]_i$ elevations can occur at widely different times in adjacent cells exposed simultaneously to a solution of cyanide or X537A. For example, in the experiment of Fig. 14, diffuse elevation of $[Ca^{2+}]_i$ in cell *2* occurred about 3 min after that in cell *1*. This, of course, does not necessarily reflect an intrinsic difference between the cells. The cells had been impaled on the aequorin pipettes and microelectrodes, and variations in these factors and their possible associated local disturbances could well account alone for the asynchronies in the $[Ca^{2+}]_i$ elevations.

Ca-free Medium

In a set of 4 experiments in which the aequorin glow was determined by means of the photomultiplier only, the cells were placed in Ca-free Na medium. This led to uncoupling. Two of these cases showed a transient

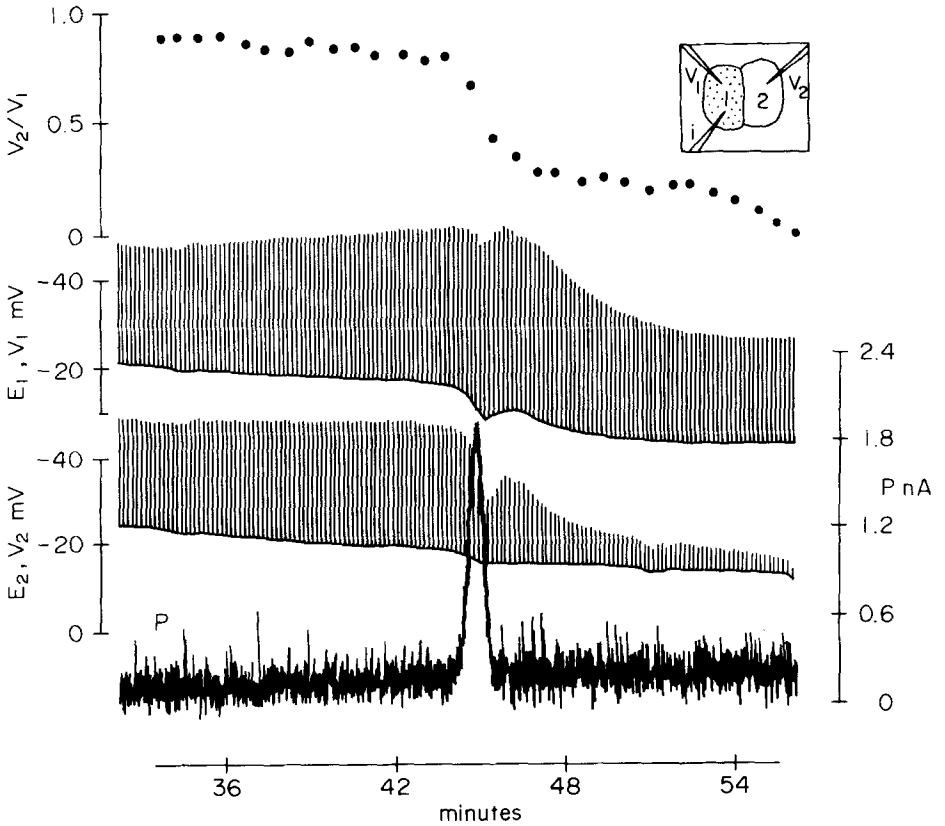

Fig. 17. Uncoupling in Ca-free Li medium. Onset of uncoupling correlates with rise in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ falls again, but remains above baseline for the remainder of the exposure to the medium. Time zero corresponds to start of exposure to the medium. $i = 4 \times 10^{-8}$ A

rise in $[Ca^{2+}]_i$ where the rising and falling phases were associated, respectively, with uncoupling and recoupling (Fig. 16). As in the case of Ca injection and treatment with metabolic poisons and ionophore, the rising phase of $[Ca^{2+}]_i$ went hand in hand with the fall in coupling; recoupling began after the aequorin luminescence returned to baseline. However, in the other two cases, no change in $[Ca^{2+}]_i$ was detected although the cells clearly uncoupled (and had reactive aequorin).

In two other experiments, the cells were uncoupled in Ca-free K medium. This led in both cases to a rise in $[Ca^{2+}]_i$ associated with uncoupling.

Ca-free Li Medium

In Ca-free Li medium, the cells uncoupled (9 experiments). The onset of uncoupling correlated with $[Ca^{2+}]_i$ elevation in one experiment

(Fig. 17). The $[Ca^{2+}]_i$ fell again rapidly, but remained above baseline level for the remainder of the exposure to the Li medium; and the cells stayed uncoupled. In all other experiments, no $[Ca^{2+}]_i$ elevation was detectable during the entire period of exposure to this medium, which extended 10 to 50 min beyond the onset of uncoupling.

Discussion

On the Site and Mode of Ca^{2+} Action

The present results demonstrate a close correlation between depression of junctional conductance and elevation of cytoplasmic Ca^{2+} concentration in the junctional locale. The correlation was clearest in the experiments of Ca injection, metabolic inhibition and ionophore treatment: depression of junctional conductance ensued whenever the $[Ca^{2+}]_i$ was seen to rise in the junctional locale, and only then. Here Ca^{2+} fulfilled the requirements of the sufficient cause of the depression of junctional permeability. Depolarization, the other frequent companion of uncoupling, was shown to be neither sufficient nor necessary by the experiments in which the cells were depolarized in K medium, and by the experiments of Ca injection in which nonjunctional membrane changes were avoided.

A plausible mechanism of the Ca^{2+} action is that the ion binds to junctional membrane (Loewenstein, 1967) and that, as a result of this binding, the junctional membrane, at $[Ca^{2+}]_i \gtrsim 5 \times 10^{-5}$ M, undergoes a change in conformation effectively occluding the junctional channels to all molecules. In this light, the finding that Ca^{2+} itself does not pass through the junction in detectable amounts (Fig. 11) suggests a rapid Ca-binding/membrane conformation reaction; and the apparent lag of junctional conductance recovery behind the decline of $[Ca^{2+}]_i$ (e.g. Fig. 7) may reflect a relatively slow release of the junction-bound Ca^{2+}. The former phenomenon would constitute an interesting case in which an ion limits its own membrane flux; and the latter is, of course, to be expected if the binding occurs at high free $[Ca^{2+}]_i$, and particularly so if the binding sites are in a narrow cleft or the release mechanism involves cooperativity (*cf.* divalent cation binding and release by actin or phosphatidic acid; Loscalzo, Reed & Weber, 1975; Haynes, 1976).

As to the mode of channel occlusion by Ca^{2+}, two general mechanisms are readily envisaged: (*a*) the bore of the membrane channel is reduced; or (*b*) the channels in the two membranes (the hemichannels) are misaligned (without loss of junctional insulation). Structural alterations of this sort

could conceivably be brought about by Ca binding to the channels themselves or, somewhat less directly, by Ca^{2+} binding to other constituents of the junctional membrane whose change in conformation is transmitted to the channels. In this connection, it is important to note the electronmicroscopical findings of Peracchia and Dulhunty (1974 and *personal communication*) in uncoupled crayfish electrical synapse: the "gap" junctional membrane particles, generally assumed to contain the channels (*cf.* McNutt & Weinstein, 1973; Gilula, 1975) become smaller and more closely packed after uncoupling by dinitrophenol or other agents. There are also electronmicroscopical and spectroscopical findings that may reflect a Ca-binding capacity of junctional membrane: Oschman and Wall (1972; 1973) and Oschman, Hall, Peters and Wall (1974) showed Ca deposits along the "septate" junction of the present cells when these were exposed to Ca^{2+}; and Larsen (1975) using Oschman and Wall's technique, has given evidence for such deposits in these conditions at the "gap" junction of certain cells in tissue culture.

Because the cytoplasmic Ca^{2+} concentration is sensitive to a broad variety of factors (e.g. cellular energy metabolism, Ca permeability of nonjunctional cell membrane, Ca-holding capacity of mitochondria and cell membrane) the calcium ion is probably a frequent determinant of uncoupling; but of course not the only possible one. In principle, junctional uncoupling may come about by (1) junctional channel occlusion (2) by leakage in the junctional insulation or (3) by actual separation of the junctional membranes. Among the various experimental conditions known to bring about uncoupling, treatment with anisotonic medium (Barr, Dewey & Berger, 1965; Dreifuss, Girardier & Forssmann, 1966; Loewenstein *et al.*, 1967) may produce the uncoupling directly through separation of the junctional membranes; there is electronmicroscopic evidence that the membranes of "gap junction" come apart after treatment with hypertonic sucrose (Barr, Berger & Dewey, 1968; Goodenough & Gilula, 1974). There is also evidence for membrane separation (Pappas, Asada & Bennett, 1971) for the uncoupling produced by medium in which propionate$^-$ substitutes for Cl^- (Dreifuss *et al.*, 1966; Asada & Bennett, 1971). However, a question difficult to answer is whether the membrane separation is the actual causal event of these uncouplings or whether it is merely a secondary one. We have examined the uncoupling by propionate medium with the present aequorin method to see whether the uncoupling is accompanied by elevation of $[Ca^{2+}]_i$. In the 2 trials made, the *Chironomus* salivary gland cell junction uncoupled without sign of $[Ca^{2+}]_i$ elevation. This negative result, unfortunately, does not advance

us any further. However, a related result of these trials may be more revealing: the uncoupling by propionate medium could be largely reversed by passing a hyperpolarizing current through nonjunctional cell membrane; and, as in the case of uncoupling mediated by elevation of $[Ca^{2+}]_i$ (Rose and Loewenstein, 1971), the reversal occurs within 1–10 sec of the current passage. Thus, by switching the current off and on while the cells were in the propionate medium, uncoupling and recoupling could be produced in rapid succession. Thus, if membrane separation were the primary event in the uncoupling here, the junctional membranes would need to be capable of unzipping and zipping up (with alignment of hemichannels) within 1 sec.

On the Physiological Role of Ca-mediated Uncoupling

Since the Ca concentration in the normal extracellular fluids is high ($\geqq 10^{-3}$ M), Ca^{2+} is likely to play a part in the closing of the junctional channels even in those modalities of junctional uncoupling (modes *2* or *3* above) which are not primarily caused by a rise in $[Ca^{2+}]_i$. In fact, when the junctional insulation is rendered leaky by chemical treatment or when coupled cells are mechanically separated, the hemichannels or whatever is left over of the former membrane junction after separation, seal in the presence of Ca^{2+} in the external medium; the input resistance rises (Loewenstein *et al.*, 1967; Ito, Sato & Loewenstein, 1974*a*, *b*).

It is thus easy to see two possible functional roles of Ca^{2+} in the regulation of intercellular communication: (i) that of Ca^{2+} uncoupling a cell community from a cell member with defective Ca pumping or sequestering capacities, and (*ii*) of uncoupling it from a cell member with a damaged membrane. The first is likely to apply to cells reaching the end of their life span, and the second, in fact, is known to apply to tissue injury (Loewenstein & Penn, 1967; Oliveira-Castro & Loewenstein, 1971). A further possibility, that of Ca^{2+} acting in a more subtle form of regulation of intercellular communication, is discussed in the companion paper (Délèze & Loewenstein, 1976).

In many types of coupled cells, such as heart muscle, smooth muscle, nerve, visual, and gland cells, Ca^{2+} serves as an intracellular messenger and the $[Ca^{2+}]_i$ rises during physiological activity. During heart- and smooth-muscle contraction, for instance, the $[Ca^{2+}]_i$ elevation is probably quite high. This, however, does not necessarily mean that the cells become transiently uncoupled. An important facet of cellular Ca handling enters here. The Ca ion is unique in that it is rapidly sequestered by

energized cellular organelles of high capacity and hence it spreads only over very short distances in the cell (cytosol) (*cf.* Baker & Crawford, 1972; Rose & Loewenstein, 1975*b*). As illustrated in the example of Fig. 4*A* by Ca^{2+} spreading from a point source, the $[Ca^{2+}]_i$, which is $\geqq 10^{-4}$ M within a radius of 15 μ, falls precipitously over the next 1–2 μ to $< 5 \times 10^{-7}$ M. Hence, it is entirely possible in cells with sufficient density of sequestering elements – particularly if these are close to junction – that the permeability of the junction not be affected by $[Ca^{2+}]_i$ elevation occurring somewhere else inside the cell. For the present cell type this is strikingly demonstrated in the experiment of Fig. 5*A* in which a $[Ca^{2+}]_i$ elevation in the region of the junction causes uncoupling of this junction but not of other junctions of the same cell. This question is discussed further elsewhere in relation to the broader problem of intracellular communication by Ca^{2+} signals (Loewenstein, 1975).

Uncoupling in Ca-free Medium and Li Medium

In the experiments with Ca-free medium or Li medium, uncoupling occurred in all cases in which a rise in Ca^{2+} concentration was seen in the junctional locale. In fact, the experiments with Ca-free medium in which $[Ca^{2+}]_i$ fluctuated (Fig. 16) showed the close dependence of junctional conductance on $[Ca^{2+}]_i$ most strikingly.

But not in all cases of uncoupling was a rise in Ca^{2+} seen; in fact, in the experiments with Li medium the rise was seen only in one case out of nine. A rise in $[Ca^{2+}]_i$ here may be expected on the following grounds: In Ca-free medium, Na extrusion is depressed and this ion accumulates inside the cells (Baker, Blaustein, Hodgkin & Steinhardt, 1969). In the presence of Na or Li, the mitochondria release their Ca^{2+} (Carafoli, Tiozzo, Lugli, Crovelli & Kratzing, 1974). This also accounts satisfactorily for the fact that $[Ca^{2+}]_i$ elevation, when it occurred, was a single-shot affair. The absence of a detectable Ca^{2+} response may be explained in several ways. A simple possibility is that the $[Ca^{2+}]_i$ elevation here may not have been high enough for detection by our method. In the uncouplings produced by the metabolic inhibitors or ionophores, the $[Ca^{2+}]_i$ conveniently rose to many times the threshold concentration for electrical uncoupling, far above the normal sensitivity limit of the method. But in the experiments with Ca-free medium or Li medium, the elevation may have been smaller and/or the Ca sensitivity may have been reduced by elevation of $[Mg^{2+}]_i$. Such a reduction in sensitivity is plausible because in the presence of Na or Li, the mitochondria release also Mg (Carafoli

et al., 1974), and this may have been sufficient to compete with Ca for the aequorin. Aequorin has nearly as high an affinity for Mg^{2+} as for Ca^{2+}, but the Mg reaction produces no luminescence (Shimomura *et al.*, 1962). Thus, variations in Ca^{2+} release and/or Mg^{2+} release may account for the variations in Ca^{2+} response in the experiments with Ca-free medium or Li medium.

With the thought of a Mg interference in mind we did 4 experiments in which we tried to deplete the mitochondrial stores of Mg by the following preliminary steps: (1) application of Ca, Mg-free medium plus 0.05 mM warfarin (15 min); (2) application of Ca, Mg-free medium plus warfarin plus 5 mM cyanide ($[Ca^{2+}]_i$ rose and the cells uncoupled; the step was terminated when $[Ca^{2+}]_i$ returned to below the detectable level). (3) Mg-free medium plus warfarin (10–20 min) (the cells recoupled). (4) Mg-free medium. Thereupon followed the application of the Ca, Mg-free Li test medium. Step 2 was intended to deplete the mitochondria of Ca and Mg and step 4 to reload them with Ca alone. In two experiments, warfarin was replaced by 1 mg/50 ml antimycin-A. These agents block Ca and Mg uptake in isolated liver mitochondria (*cf.* Lehninger, Carafoli & Rossi, 1967; Lehninger, 1970) and their presence in steps 1 through 4 were intended to prevent mitochondrial Mg uptake. In spite of these efforts, the treatment with Ca, Mg-free Li medium produced no detectable aequorin glow. The negative outcome of these experiments may, of course, have been due to various reasons in this complex situation. For instance, the intracellular stores may not have been depleted of Mg in step 2, or warfarin and antimycin A may not have entered the cell.

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