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Drugs that Block Calmodulin Activity Inhibit Cell-to-Cell Coupling in the Epidermis of *Tenebrio molitor*

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Summary. In many cell systems, the permeability of membrane junctions is modulated by the cytoplasmic level of free Ca^{++}. To examine whether the calcium-dependent regulatory protein calmodulin is involved in this process, the ability of anticalmodulin drugs to influence the cell-to-cell passage of injected current and an organic tracer was tested using standard intracellular glass microelectrode techniques. Several antipsychotics and local anesthetics were found to block junctional communication in the epidermis of the beetle *Tenebrio molitor*. Treatment of the epidermis with chlorpromazine (0.25 mM) raised intercellular resistance two- to threefold within 20 to 25 min; cell-to-cell passage of electrical current was abolished within 41 ± 5 min. Loss of electrotonic coupling was accompanied by a block in the cell-to-cell movement of the organic tracer carboxyfluorescein. The reaction is fully reversible, with normal electrotonic coupling being restored within 2 to 4 hr. Other antipsychotics and local anesthetics had similar effects on cell coupling. The order of potency found was: trifluoperazine > thioridazine > D-butaclamol > chlorprothixine = chlorpromazine > L-butaclamol > dibucaine > tetracaine. The relative uncoupling potencies of these drugs correlate well with their known ability to inhibit calmodulin-dependent phosphodiesterase activity. Other anesthetic compounds, procaine and pentobarbital, did not block cell-to-cell communication. Altering the extracellular Ca^{++} concentration did not affect the rate of uncoupling by antipsychotics, while chelation of extracellular Ca^{++} with EGTA raised electrotonic coupling. The effect of three metabolic inhibitors on coupling was also examined. Iodoacetate uncoupled the epidermal cells while DNP and cyanide did not. These results are discussed in terms of possible mechanisms by which calmodulin may control junctional communication in this tissue.

Key Words cell-to-cell junctions · ionic coupling · calmodulin · anticalmodulin drugs · calcium.

Introduction

The epidermal cells of the beetle *Tenebrio molitor* are coupled by low-resistance channels that permit the cell-to-cell passage of electrical current and small organic dyes (Caveney, 1974; Caveney & Podgorski, 1975). Close membrane appositions, termed "gap junctions," have been detected between these cells by thin-section (Caveney & Podgorski, 1975) and freeze-fracture electron microscopy (Berdan & Caveney, 1981). These gap junctions are clusters of membrane particles with each particle believed to be a low-resistance channel (reviewed in Loewenstein, 1981).

Junctional conductance between epidermal cells is increased by the steroid hormone 20-hydroxyecdysone (Caveney & Blennerhassett, 1980), and this effect on coupling is not associated with an increase in the amount of gap junction between the cells (Caveney, Berdan & McLean, 1980), suggesting that the permeability of existing gap junctions is under physiological control.

Loewenstein (1966) proposed that the permeability of junctional membrane channels is regulated by the cytoplasmic concentration of free Ca^{++}. Raising the intracellular Ca^{++} concentration by injection of Ca^{++} causes junctional uncoupling in several systems, including *Chironomus* salivary gland (Loewenstein, Nakas & Socolar, 1967), sheep heart (De Mello, 1975), pancreatic acinar cells (Iwatsuki & Peterson, 1977), and *Tenebrio* epidermis (Popowich & Caveney, 1976). Rose and Loewenstein (1976) used aequorin (a protein which luminesces in the presence of Ca^{++}) to demonstrate that elevation of cytoplasmic free Ca^{++} only in the junctional locale leads to a decrease in junctional conductance.

To understand more clearly the control of junctional communication, it is important to understand how Ca^{++} influences the junctions and how its concentration is controlled within the cell. Recent studies have demonstrated the existence of a calcium-dependent regulatory protein, "calmodulin," which both mediates the effect of calcium in many cellular reactions and regulates intracellu-

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lar Ca^{++} concentration. (For reviews *see* Wang & Waisman, 1979; Means & Dedman, 1980.) Calmodulin was first isolated as an activator of mammalian brain phosphodiesterase (Cheung, 1970; Kakiuchi & Yamazaki, 1970). It is ubiquitous among the eucaryotes (Waisman, Stevens & Wang, 1975; Jamieson, Bronson, Shachat & Vanaman, 1980) with a highly conserved amino acid sequence (Dedman, Jackson, Schreiber & Means, 1977; Vanaman & Sharief, 1979; Watterson, Sharief & Vanaman, 1980). A calmodulin-like factor which activates bovine heart phosphodiesterase in a Ca^{++}-dependent manner has been isolated from *Tenebrio* (Waisman et al., 1975).

An opportunity to study the role of calmodulin in intact cell systems is facilitated by the recent discovery that a series of antipsychotic drugs binds to calmodulin in a calcium-dependent manner (Levin & Weiss, 1977) and prevents it from activating phosphodiesterase (Levin & Weiss, 1976) and several other calmodulin-dependent processes (reviewed by Weiss et al., 1980). A previous study has shown that chlorpromazine blocks electrical coupling between the cells of *Chironomus* salivary gland (Suzuki, Sangworasil & Higashino, 1978). The purpose of this study was to determine whether the anticalmodulin activity of various drugs could be correlated with their ability to inhibit junctional communication.

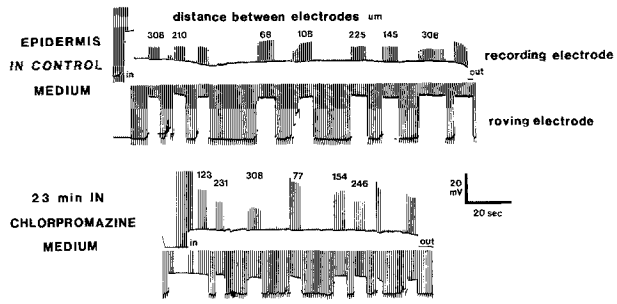


Fig. 1. Electrical recordings. Recordings of electrotonic potentials in the epidermis. This figure consists of two sets of recordings, one from a control and one from a chlorpromazine-treated preparation. Each set of recordings consists of an upper and lower trace. The vertical lines on the far left of the upper control trace indicate that the recording electrode is pulsing current into the medium. The upward deflection of the trace (*in*) results from insertion of the electrode into a cell. Its magnitude indicates the membrane potential of the cell. At this point the current pulsing is switched to the second, "roving" electrode, and a downward adjustment is made to the upper trace so that recordings of electrotonic potential will remain on scale. The roving electrode is then inserted into a second cell at a given distance from the first cell. This distance (μm) is indicated by the numbers in the upper trace. When the roving electrode is pulsing in the medium there is no response from the recording electrode. After the roving electrode is inserted into a cell there is an upward deflection of the upper trace with each current pulse delivered by the roving electrode. This is the electrotonic potential. At each interelectrode distance a different magnitude of electrotonic potential is recorded. The recordings taken in medium containing chlorpromazine are similar to the control recordings but the membrane potentials are smaller, electrotonic potentials are greater, and in this set of recordings the roving electrode was already in a cell when the recording electrode was inserted. (Injected current, 6×10^{-8} A)

Materials and Methods

Preparation

Tenebrio molitor, the yellow mealworm, was raised at 27 °C in a 12 hr light – 12 hr dark cycle. Newly-ecdysed larvae, weighing between 0.10 and 0.13 g, were collected and allowed to develop for a further 6 days. The larvae were then anesthetized and sterilized by immersion in methanol for 3 min. Squares of epidermis were excised from the ventral surface of abdominal segments II through VII and placed into *Tenebrio* culture medium (Caveney & Blennerhassett, 1980). This medium contains 1.26 mM Ca and 20.8 mM Mg.

Dissections were done under sterile conditions in a laminar flow hood. All fat body and most adhering muscle was removed. Preparations were then incubated for 16–30 hr at 27 °C in an atmosphere containing 2% CO_2. Each preparation consisted of a monolayer of at least 20,000 epidermal cells of which a small central region was utilized for electrical recordings.

Recording of Electrotonic Spread

The electrophysiological procedure is given in detail in Caveney and Blennerhassett (1980). Electrotonic spread of current within the epidermal sheet was measured by the use of two glass microelectrodes filled with 3 M KCl (tip resistance range, 15 to 40 MΩ). The roving electrode passed pulses of current ($I_o = 6 \times 10^{-8}$ A; 200 msec) into the cell sheet while the second stationary electrode recorded the steady-state membrane potential change (the electrotonic potential), at various distances from the current source (Fig. 1). Curves of electrotonic spread were plotted from between 4 and 10 electrotonic potentials recorded at distances (L) between 40 and 310 μm (5–30 cell diameters) from the current source. The microelectrodes also recorded membrane potential, which was used as an indication of the quality of electrode penetration and electrical stability of the cell sheet (Fig. 1).

The effective resistance of the intercellular route in the epidermis $r_i(\Omega)$, is directly proportional to the slope of the spatial decay in electrotonic potential (Caveney & Blennerhassett, 1980). Caveney and Blennerhassett determined r_i from the equation

$$V_L = \frac{I_o r_i}{2\pi} K_o\left(\frac{L}{\lambda}\right) \tag{1}$$

which describes the electrotonic spread of current in a model consisting of an infinite sheet of cytoplasm bounded on both sides by membrane, where K_o is a Bessel function and λ is the space constant (Eisenberg & Johnson, 1970). The space constant is defined as $\lambda = \sqrt{R_m/2r_i}$ where R_m is the specific resistance of the membrane bounding the cytoplasm. Since the extent of membrane folding in the epidermis is not known, calculated values of R_m are not particularly accurate and have

been excluded from this paper. Values for r_i and λ were then calculated by fitting a theoretical curve to each set of experimental points (Caveney & Blennerhassett, 1980).

In order to make the r_i value obtained from Eq. (1) applicable to a cell monolayer, the presence of intercellular junctions must be accounted for. In a discrete model of an hexagonally packed cell monolayer

$$r_i = \frac{R_c + R_j/d\sqrt{3}}{t}.$$

R_c is cytoplasmic resistance, R_j is junctional specific resistance, $d\sqrt{3}$ is a measure of cell density, and t is sheet thickness (Siegenbeek van Heukelom, Denier van der Gom & Prop, 1972. If R_c, $d\sqrt{3}$ and t are not changed by an experimental treatment then any change in r_i may be interpreted as a change in junctional specific resistance R_j.

The epidermal cells do not proliferate or die during short term culture (Caveney & Blennerhassett, 1980), and drug treatment did not cause detachment of the cells from the rigid cuticle, thus eliminating the possibility of cell density changes. In addition, we found no changes in sheet thickness of the magnitude required to explain experimentally-induced changes in r_i. Cytoplasmic resistance was not measured, as studies by Caveney and Blennerhassett (1980) demonstrated that cytoplasmic resistance made up only 15% of the total intercellular path resistance. Since unprecedented changes in cytoplasmic resistance would be required to explain the alterations in r_i reported in this paper (*see also* Discussion), we have used r_i as a direct index of the state of coupling.

Drug Application

Drugs were made up as aqueous stock solutions where possible. D- and L-Butaclamol were made up as 50 mM stock solutions in ethanol or DMSO, and chlorprothixene was made up in ethanol. Stock solutions were diluted at least 100× into medium. Control application of solvents alone had no effect on the electrical properties of the epidermal cells. Experiments were initiated by placing the epidermal preparation into 2.5 ml of drug-containing medium. When NaCN was used, it was added directly to medium immediately before the preparation was added.

Dye Injection

The fluorescent dye, 6-carboxyfluorescein (Eastman), was made up as a 50-mM aqueous solution and NaOH was added to give a pH of 7.5. It was then filtered through a Nalgene filter (0.2 μm pore size). Glass microelectrodes, pulled to the same specification as those for electrical recordings, were filled with carboxylfluorescein by capillary action, and then backfilled with 0.1 M KCl. Dye was injected into cells by iontophoresis, using hyperpolarizing pulses ($I_o = 6 \times 10^{-8}$ A/pulse/sec). The dye injection electrode also measured membrane potential. Carboxyfluorescein was excited by UV light from a mercury vapor lamp (HBO 50 W). Reichert epifluorescence optics were used. Blue wavelengths were selected with an FITC interference filter and a red absorbing filter. Dark field fluorescence was photographed on Kodak Tri-X 400 film through barrier filters OG1 and GG9.

Drug Sources

Several drugs were kindly provided free: trifluoperazine (Smith, Kline and French); chlorprothixene (Hoffman-La Roche); thioridazine (Sandoz); D- and L-butaclamol (Ayerst). Other drugs were pentobarbital (Abbott), NaCN (Fisher) and chlorpromazine, tetracaine, procaine, dibucaine, EGTA, iodoacetate, and DNP (Sigma).

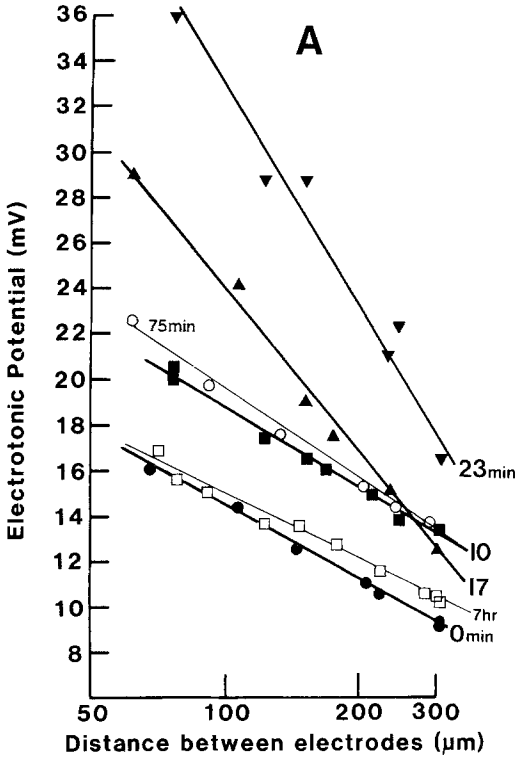
Abbreviations

DNP, 2–4, dinitrophenol; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N′-tetraacetic acid; NaCN, sodium cyanide; PIPES, piperazine-N,N′-bis (2-ethane sulphonic acid); DMSO, dimethyl sulfoxide.

Results

Effect of Chlorpromazine on Cell-to-Cell Coupling

Chlorpromazine is an antipsychotic drug known to inhibit calmodulin-dependent phosphodiesterase activity (Levin & Weiss, 1976). Since it is readily available and its effects on biological systems have been widely studied, it was our first choice in investigating the possible role of calmodulin in cell-to-cell communication. The control preparations of the chlorpromazine experiments had a mean intercellular resistance (r_i) of $5.1 \pm 1.1 \times 10^5\ \Omega$ ($n = 8$) and a membrane potential (E) of -40 ± 8 mV. These values compare favorably with the mean control values for the entire study ($r_i = 5.2 \pm 1.3 \times 10^5\ \Omega$ ($n = 116$), $E = -43 \pm 8$ mV). When epidermal preparations were exposed to 0.25 mM chlorpromazine there was a lag period of approximately 10 min during which little change in r_i occurred (Figs. 2 and 3). Within the next 10 to 15 min r_i rose rapidly to values 2 to 3 times that of the control (Figs. 2 and 3). This increase in r_i is seen in the recordings as an increase in the size of the electrotonic potentials and was associated with a two- to fourfold decrease in membrane potential (Fig. 1). Following the rise in intercellular resistance, there was a period when the cells were electrically unstable; a cell penetrated by an electrode lost its membrane potential and became uncoupled from the other cells in the sheet within 4 to 10 sec. A quantitative determination of electrotonic spread, which requires a stable membrane potential, was therefore not possible. However, a qualitative measurement of cell coupling could be obtained at this time by simultaneous insertion of both injecting and recording electrodes into the cell sheet. With the electrodes placed a distance of 50 μm (five cell diameters) apart, electrical coupling was detected up to 41 ± 5 min ($n = 18$) following addition of 0.25 mM chlorpromazine (Fig. 3), after which the epidermal cells became uncoupled. Uncoupled preparations had membrane potentials of -6 ± 2 mV. After



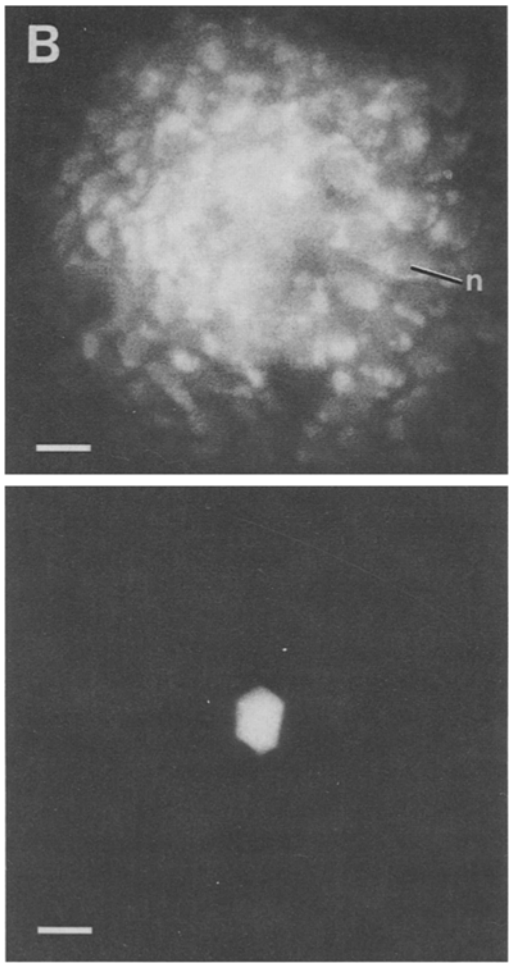


Fig. 2. (*left*) *A*: Effect of chlorpromazine on electrotonic spread. Readings were taken at 10, 17 and 23 min after exposure to 0.25 mM chlorpromazine began. Recovery readings were taken 75 min and 7 hr after the cells had been uncoupled and returned to normal medium. The fitted curves correspond to intercellular resistances of $5.2 \times 10^5\ \Omega$ (control, 0 min), $5.2 \times 10^5\ \Omega$ (10 min), $11.3 \times 10^5\ \Omega$ (17 min), $15.0 \times 10^5\ \Omega$ (23 min), $6.1 \times 10^5\ \Omega$ (75 min recovery) and $4.4 \times 10^5\ \Omega$ (7 hr recovery). (*B*): Fluorescence micrographs of the cell-to-cell movement of carboxyfluorescein in the absence (upper micrograph) and presence of 0.25 mM chlorpromazine (lower micrograph). In control cells the dye spreads rapidly through the cell sheet, imaging the cell nuclei (*n*). In cells uncoupled with chlorpromazine (>25 min exposure) the dye fails to pass out of the cell into which it is injected and delineates sharply the cell's boundaries. Scale bar, 10 μm

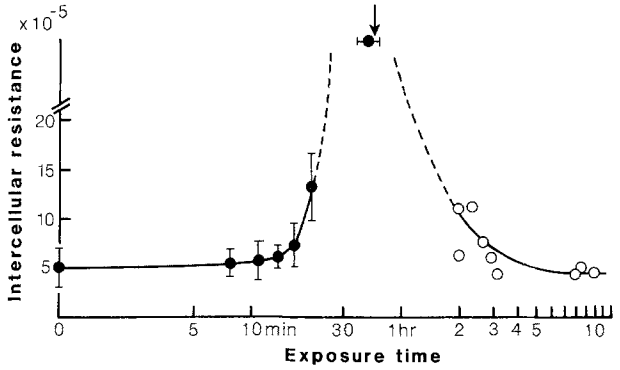


Fig. 3. Time-response curve of cellular uncoupling after exposure to 0.25 mM chlorpromazine. Each value plotted during the rise in intercellular resistance is the mean ±SD of at least five experiments. The upper point with horizontal error bar indicates the mean time to total uncoupling. No specific value for intercellular resistance could be determined at this time. ↓indicates return to normal medium. Recovery values (open circles) are from single experiments

coupling was no longer detectable, the preparations were returned to normal medium. Electrotonic spreads could subsequently be obtained within 1 hr of return, and normal coupling was restored within 2 to 4 hr (Figs. 2 and 3).

The uncoupling process was also visualized directly by injection of a fluorescent tracer, carboxyfluorescein (mol wt 376) into the cells. Nonjunctional membranes are highly impermeable to this tracer (Weinstein et al., 1977; Flagg-Newton, Simpson & Loewenstein, 1979), but it passes readily through cell junctions (Flagg-Newton et al., 1979). In control preparations carboxyfluorescein spread rapidly through the cell sheet to distances greater than five cell diameters from the source cell (Fig. 2). Dye can be detected in cells adjacent to the source cell within 2 sec of the application of iontophoretic current (i.e., one or two pulses of current). After 15 to 20 min in 0.2 mM chlorpromazine, the rate and extent of dye spread was

Table 1. Effect of anticalmodulin drugs on cell coupling

Class of drugs	Drug	Concentration to uncouple *Tenebrio* epidermis in 60 min. (μM)	Inhibition of calmodulin activated phosphodiesterase IC_{50} (μM)			Displacement of ^3H trifluoperazine from calmodulin IC_{50} (μM)
			(i)	(ii)	(iii)	(iv)
Phenothiazine	Trifluoperazine	100	6	10	–	1.5
	Thioridazine	135		18	–	–
	Chlorpromazine	225		42	6	8
Benzocycloheptapyridoisoquinoline	D-Butaclamol	140		–	15	75
	L-Butaclamol	310		–	15	350
Thioxanthene	Chlorprothixene	225		16	2.4–3.0	–
Local anesthetics	Dibucaine	900	180	–	–	–
Tertiary amines	Tetracaine	3,700	350	–	–	–
	Procaine	>50,000	–	–	–	–
Barbituate	Pentobarbital	>5,000	>10,000 μm			

(i) Volpi et al., 1981; (ii) Levin & Weiss, 1976; (iii) Norman et al., 1979; (iv) Levin & Weiss, 1979.

greatly reduced. Once chlorpromazine-treated cells reached the electrically-uncoupled state, no dye movement could be detected (Fig. 2). Chlorpromazine treatment caused no gross changes in cell ultrastructure during this period; in particular, the cell junctions remain intact.

Relative Potency of Several Antipsychotic Drugs that Disrupt Ionic Coupling

A large number of antipsychotic drugs have been tested for their ability to inhibit calmodulin-dependent processes in cell-free systems (Levin & Weiss, 1976). Some are powerful, others weak, inhibitors of calmodulin-dependent processes. The relative ability of several of these drugs to uncouple epidermal cells was tested.

All of the antipsychotic drugs tested had an effect on coupling similar to that of chlorpromazine. A rise in intercellular resistance was followed by uncoupling. Uncoupling was completely reversible in all cases with a time course similar to that of chlorpromazine-treated cells. From the dose-response curve for each drug (Fig. 4) the concentration required to uncouple the cell sheet within 60 min was derived. The relative potencies of the drugs were compared at 60 min (Table 1) because the drug concentration required to uncouple cells by this time approaches the lower limits at which the drugs have a detectable effect on intercellular resistance. The following order of potency of the antipsychotics was found: trifluoperazine > thioridazine > D-butaclamol > chlorprothixene = chlorpromazine > L-butaclamol. Table 2 compares

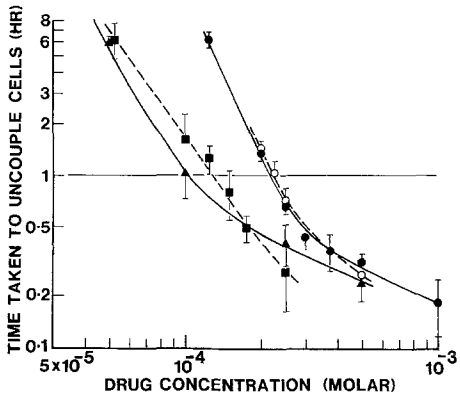


Fig. 4. Dose-response curves of the influence of several antipsychotics on cell coupling. The drugs tested were chlorpromazine (●, $n=87$), trifluoperazine (▲, $n=29$), thioridazine (■, $n=43$) and chlorprothixine (○, $n=17$), where n is the total number of preparations uncoupled to derive each dose-response curve. A horizontal line is drawn through the graph at 1 hr to indicate the time at which drug potencies were compared

the potency of the various drugs on cell coupling with their ability to block calmodulin-activated phosphodiesterase. Figure 5 demonstrates a close correlation between the concentration of the three phenothiazine compounds, thioridazine, trifluoperazine and chlorpromazine, required to uncouple epidermal cells and their ability to inhibit calmodulin activity ($R^2=99.7$). A higher concentration of chlorprothixene (225 μM) than expected (150 μM) was required to uncouple the cells, when compared with the phenothiazines. The butaclamol isomers showed a difference in uncoupling activity (L-butaclamol, 310 μM; D-butaclamol, 140 μM). This agrees with the data from calmodulin binding studies that suggest D-butaclamol

Table 2. Effect of external $[Ca^{++}]$ on uncoupling induced by phenothiazines

(a) Treatments that uncouple cells

Treatment	Time to uncoupling (min)	No. preparations
1.5×10^{-4} M thioridazine	47±20	9
*1.5×10^{-4} M thioridazine and 3×10^{-3} M EGTA	41± 6	9
1.5×10^{-4} M thioridazine and 1×10^{-2} M $CaCl_2$	48±11	9

(b) Treatments associated with the above experiments that do not uncouple the cells

Treatment	Time (min)	r_i (Ω, $\times10^{-5}$)	E (−mV)	No. preparations
1×10^{-2} M Ca^{++}	17–210	4.8±1.2	40±3	3
*3×10^{-3} M EGTA to give $[Ca^{++}]_e$ $<10^{-6}$ M	13–120	3.6+0.6	23+2	4

* In this experiment the Mg^{++} level in the medium was reduced from 20.8 mM to 0.8 mM.

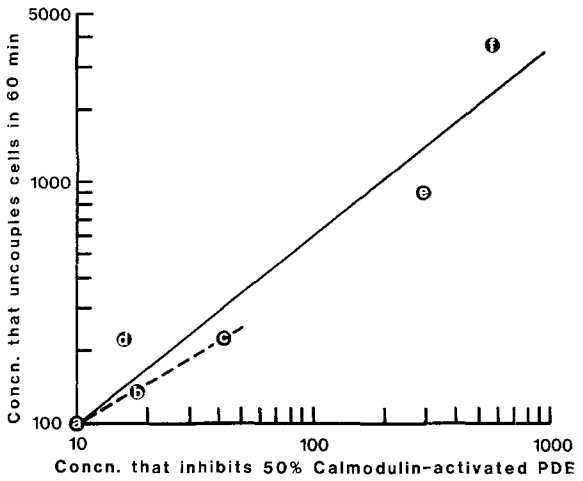


Fig. 5. Relationship between the drug concentration (μM) required to inhibit 50% of calmodulin-induced phosphodiesterase activity and the concentration (μM) required to uncouple the epidermal cells in 60 min. (*a*) trifluoperazine, (*b*) thioridazine, (*c*) chlorpromazine, (*d*) chlorprothixine, (*e*) dibucaine, and (*f*) tetracaine. R^2 (*a–f*) = 92%, R^2 (*a–c*) = 99.7%. Lines were plotted by linear regression. The results of Volpi et al. (1981) for dibucaine and tetracaine were standardized to the data of Levin and Weiss (1976) using trifluoperazine as the common drug. Unstandardized R^2 (*a–f*) = 92%

(IC_{50} = 75 μM)[1] is more effective than L-butaclamol (IC_{50} = 350 μM) in displacing trifluoperazine from calmodulin (Levin & Weiss, 1979). However, the finding that L- and D-isomers have different uncoupling activity disagrees with a separate study (Norman, Drummond & Moser, 1979) that indicates that they both inhibit calmodulin-activated phosphodiesterase to the same extent (IC_{50} = 15 μM) (Table 1). (We have not included the results of Norman et al. (1979) in Fig. 5 as they state that their results are not directly comparable to those of Levin and Weiss (1976) due to differences in experimental design.)

Effect of Anesthetics on Electrical Coupling

Anesthetics share with antipsychotics a hydrophobic nature and the ability to block nerve action potentials (Seeman, 1972). The calmodulin-inhibiting ability of several anesthetics has been tested (Levin & Weiss, 1976; Volpi et al., 1981). Therefore the effect of anesthetics on electrical coupling in the epidermis was tested. The positively-charged local anesthetics dibucaine and tetracaine had effects on coupling similar to those reported earlier for the antipsychotics. These anesthetics first raised intercellular resistance and then uncoupled the cells (Fig. 6). Once again, uncoupling was reversible (Fig. 6). A comparison of the relative concentrations of the two anesthetics and of several antipsychotics required to uncouple epidermal cells with that required to inhibit calmodulin-dependent PDE is shown in Fig. 5. A good correlation exists (R^2 = 92%).

Procaine, the third local anesthetic used, did not affect ionic coupling. Of all drugs used, however, it alone caused gross changes in cell appearance. At 20 mM, procaine caused the cells to vacuolate. These vacuoles are visible both in phase contrast images of the cells and in thin sections. Intracellular resistance did not rise at this concentration. Increasing the concentration to 50 mM caused rapid cell vacuolation but elevated r_i only slightly.

Pentobarbital, a negatively-charged anesthetic, was also tested for its ability to disrupt ionic coupling. Intercellular resistance was not affected in cells exposed to 1 mM pentobarbital. After exposure to 5 mM pentobarbital, a three- to fourfold increase in r_i was seen within 5 min (time 5–22 min, $r_i = 22\pm3\times10^5\ \Omega$, $n=3$). Surprisingly, this increased r_i was not followed by total uncoupling. This is exceptional since the rapid evation in r_i

[1] IC_{50}: concentration of inhibitor that inhibits calmodulin-dependent phosphodiesterase activity by 50%.

caused by other drugs was routinely followed by uncoupling. The three preparations tested remained coupled for at least 3 hr.

Effect of External [Ca^{++}] on Phenothiazine-Induced Uncoupling

Antipsychotics may uncouple epidermal cells by causing a net influx of Ca^{++} from the extracellular medium. To examine this possibility, the effects of thioridazine on cell coupling were studied in a series of experiments in which the level of external free [Ca^{++}] was altered. The concentration of Ca^{++} in normal medium is 1.25 mM. It was raised by adding 10 mM $CaCl_2$ to the medium; or the amount of free Ca^{++} in the medium was lowered by adding 3 mM EGTA (to medium containing a reduced level of 0.8 mM Mg^{++}) to give a free [Ca^{++}] of approximately 5×10^{-7} M at pH 6.8 (calculated from Portzehl, Caldwell & Ruegg, 1964).

Raising external [Ca^{++}] had little effect on the rate at which thioridazine induced uncoupling (Table 2). Raising external [Ca^{++}] alone had no effect on r_i or E (Table 2).

Lowering external free [Ca^{++}] with EGTA did not prevent 0.15 mM thioridazine from uncoupling the epidermal cells and actually caused a small (but statistically insignificant) decrease in the uncoupling time (Table 2). Lowering the external free [Ca^{++}] in the absence of the drug resulted in a drop in r_i and E (Fig. 7).

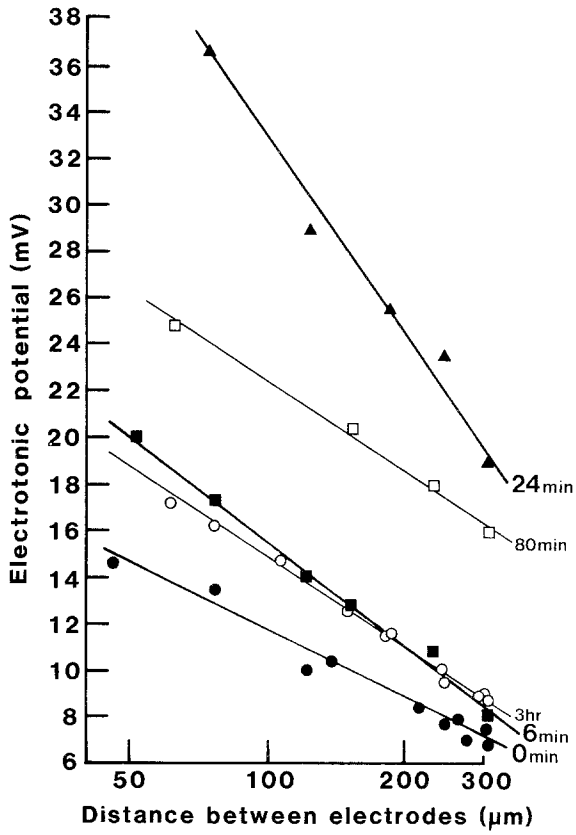


Fig. 6. Effect of dibucaine on electrotonic spread. Readings were taken in 1 mM dibucaine after 6 and 24 min exposure. Recovery readings were taken at 80 min and 3 hr after uncoupling and return to normal medium. The fitted curves correspond to intercellular resistances of 4.5×10^5 Ω (control, 0 min), 7.0×10^5 Ω (6 min), 13.2×10^5 Ω (24 min), 5.9×10^5 Ω (80 min recovery) and 6.1×10^5 Ω (3 hr recovery)

Influence of Inhibitors of Cell Metabolism on Ionic Coupling

Normal junctional conductance depends on a low $[Ca^{++}]_i$. In order to locate within the cell the source of energy required to maintain the normal Ca^{++} gradient across the plasma, mitochondrial, and ER membranes, the epidermis was exposed to several inhibitors of energy metabolism. Intercellular resistance and membrane potential were monitored in the presence of these metabolic inhibitors.

Cyanide. Cyanide (5 mM), an inhibitor of mitochondrial respiration, had no effect on intercellular resistance over a period of 60 min during which time the membrane potential dropped to approximately one half that of the control value (Table 3).

Dinitrophenol. An uncoupler of oxidative phosphorylation, DNP, at 0.1 mM did not affect either E or r_i (Table 3). Treatment of epidermal cells with

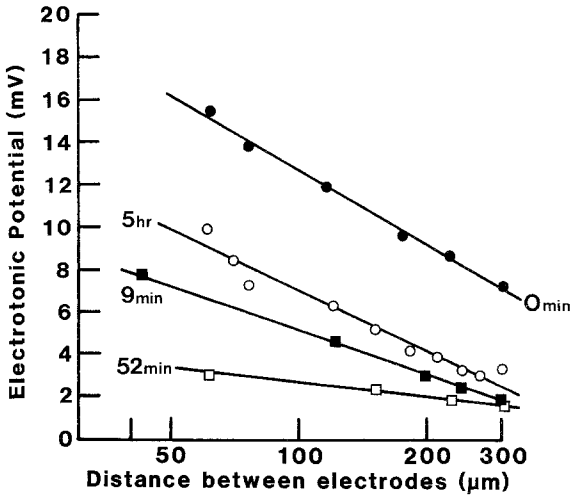


Fig. 7. Effect of EGTA on electrotonic spread. Readings in 10 mM EGTA were taken after 9 min, 52 min and 5 hr exposure to the chelator. The fitted curves correspond to intercellular resistances of 5.2×10^5 Ω (control, 0 min), 3.2×10^5 Ω (9 min), 1.1×10^5 Ω (52 min) and 4.5×10^5 Ω (5 hr)

Table 3. Effect of metabolic inhibitors on coupling

Drug	Conc^n (mM)	No. preparations	Time	Effect on coupling (r_i units: $\Omega \times 10^{-5}$)	Membrane potential ($-$mV)
Control		5		$r_i = 5.0 \pm 1.3$	42.4 ± 4.9
NaCN	5	7	60 min	$r_i = 4.7 \pm 1.3$	20.7 ± 4.5
DNP	0.1	5	60–120 min	$r_i = 5.3 \pm 1.2$	40.4 ± 5.9
	0.5	5	60 min	$r_i = 4.7$ to 18.2	13.4 ± 2.6
	1	4	up to 4 hr	Reduced, but cells not completely uncoupled, reversible	Drops rapidly to 5
Iodoacetate	1	4	125 ± 20 min	Uncoupled, irreversible	4–6
	2	6	23–145 min	Uncoupled, irreversible	3–5
	5	6	23–54 min	Uncoupled, irreversible	4–8

0.5 mM DNP caused a consistent drop in membrane potential to -13.4 ± 2.6 mV. Only in one case, however, was the drop in membrane potential accompanied by a large increase in r_i ($r_i = 18.2 \times 10^5$ Ω, 60 min). Exposure to 1 mM DNP caused a drop in membrane potential to less than half that the control value within 30 min. Cells treated with 1 mM DNP also became extremely thin, detached from the cuticle, and did not maintain a membrane potential following electrode penetration. The cells never became completely uncoupled even after 4 hr of exposure. Unfortunately, due to instability of membrane potentials after electrode penetration, no values of intercellular resistance could be determined for these preparations.

Iodoacetate. Iodoacetate is a SH-reacting reagent that inhibits the enzyme glyceraldehyde-3-phosphate dehydrogenase and, consequently, glycolysis (Racker, 1965). In contrast to the mitochondrial inhibitors that proved to be relatively ineffective in disrupting cell coupling, 1–2 mM iodoacetate consistently and completely uncoupled the epidermal cells (Table 3). Membrane potential dropped to -3 to -5 mV during uncoupling. Although these effects were irreversible, no obvious morphological changes were apparent.

Discussion

Phenothiazine antipsychotics interfere with many calmodulin-dependent reactions in cell-free systems, such as the activation of phosphodiesterase (Levin & Weiss, 1976), adenylate cyclase (Brostrom, Brostrom & Wolff, 1977), Ca^{++}-Mg^{++} ATPase (Raess & Vincenzi, 1980), and phosphorylase kinase (Shenolikar et al., 1979). Trifluoperazine binds to two high-affinity sites ($K_m = 1$ μM) on calmodulin in a calcium-dependent manner (Levin & Weiss, 1977). The affinity between phenothiazine and calmodulin is so high that a phenothiazine-sepharose affinity column may be used to partially purify calmodulin (Charbonneau & Cormier, 1979; Jamieson & Vanaman, 1979). Because of the high specificity of phenothiazine-calmodulin interactions in biochemical systems, we have used these drugs to investigate the possible role of calmodulin in regulating junctional conductance in an intact cell system.

The ability of antipsychotic drugs and anesthetics to block junctional communication in the epidermis correlates well with their calmodulin-inhibiting potency. To determine the validity of this correlation, several questions were asked, namely:

(a) Do these drugs really block junctional communication or do they change some other property of the cell sheet so that intracellular recording methods no longer measure junctional communication accurately? Since we were unable to measure the membrane resistance or cytoplasmic resistance when the cells were in the uncoupled state, apparent uncoupling could be caused by either a loss of resistance in the nonjunctional membrane or by a drastic rise in the cytoplasmic resistance, changing the core material from a conducting to a nonconducting medium by a mechanism not involving cell junctions. This is unlikely, since prior to total uncoupling there was a three- to fourfold increase in junctional resistance which was measured independently of changes in nonjunctional membrane resistance. Also, a normal spread of carboxyfluorescein through the sheet or leakage of the dye out of the source cell might be expected in epidermal cells that had lost their nonjunctional membrane resistance but maintained a normal junctional resistance. Figure 2*B* demonstrates that this did not happen. The dye banked up against the junctional membrane of the source cell, outlining its hexagonal shape. It is also evident that an increase in cytoplasmic resistivity cannot account

for the pattern of dye spread in uncoupled cells. Therefore, if the assumption is made that electrical current and fluorescent tracers follow the same cell-to-cell path in the epidermis, then uncoupling of electrical current spread between epidermal cells is a direct result of increased junctional resistance.

(b) Could a property of antipsychotics and anesthetics, other than binding to calmodulin, account for the relative uncoupling potencies of these drugs? Three properties of phenothiazines which could conceivably account for their uncoupling activity include (i) cytotoxicity at low concentrations (Naccache et al., 1980), (ii) extreme hydrophobicity – hydrophobic drugs partition into membranes to cause changes in fluidity, volume, deformability, susceptibility to osmotic lysis (reviewed by Seeman, 1972), and interfere with lipid-protein interactions (Mori et al., 1980), and (iii) their binding to proteins other than calmodulin (Jamieson & Vanaman, 1979; Weiss et al., 1980). The first criticism does not apply to *Tenebrio* epidermis, as the cells recover fully from phenothiazine treatment. Uncoupling is also not strictly dependent on drug hydrophobicity, as D and L-butaclamol, which have identical octanol/water partition coefficients (Norman et al., 1979) have considerably different uncoupling potencies. Pentobarbital, with a higher octanol/water partition coefficient than trifluoperazine (Leo, Hansch & Elkins, 1971) has less than 2% the uncoupling potency of trifluoperazine. Although tertiary amine anesthetics are also known for their membrane perturbing properties, 20 mM procaine does not uncouple epidermal cells, even after causing extensive changes in cell morphology (vacuolation). Two other local anesthetics, dibucaine and tetracaine, known to be calmodulin inhibitors (Volpi et al., 1981), are able to uncouple epidermal cells without causing vacuolation. These findings indicate that cellular uncoupling by phenothiazines is not strictly a hydrophobic interaction. Several recent studies using chlorpromazine derivatives (Roufogalis, 1981), antipsychotic isomers (Norman et al., 1979), non-phenothiazine antipsychotics (Raess & Vincenzi, 1980) and fluorescent dyes (Laporte, Wierman & Storm, 1980) have demonstrated, however, that the interaction of antipsychotics with calmodulin has an important hydrophobic component. Weiss et al. (1980) have refuted these studies by showing that 1 M ethanol does not affect the binding of trifluoperazine to calmodulin and that pentobarbital and diazapam, which have higher octanol/water partition coefficients than trifluoperazine bind calmodulin with a much lower affinity. It appears from these findings that inhibition of calmodulin by drugs depends on both a nonspecific hydrophobic component and also on a more specific component of drug structure. Therefore, phenothiazines can still be considered as useful probes of intracellular calmodulin activity.

Table 4. The relationship between intercellular resistance and membrane potentials during treatment with chlorpromazine and EGTA

Treatment	No. of trials	Selected membrane-potential range (mV)	r_i ($\times 10^{-5}\ \Omega$)	Difference from controls – levels of significance
Control	87	35–50	5.2 ± 1.3	
Chlorpromazine	27	15–20	10.3 ± 4.0	0.01
10 mM EGTA	22	6–15	4.1 ± 1.2	0.001

Although it is known that phenothiazines bind to proteins other than calmodulin, the identity of these proteins has yet to be determined. Weiss et al. (1980) have tested the ability of a wide range of purified proteins to bind trifluoperazine. They found that troponin C bound less than 10% of that bound by calmodulin, while other proteins did not show significant binding.

There are several possible mechanisms by which calmodulin may control junctional communication. Our finding that uncoupling by anticalmodulin agents is always accompanied by a drop in membrane potential may indicate that junctional communication is dependent on the maintenance of the membrane potential that is in turn dependent on calmodulin (Table 4). This possibility is unlikely, however, since varying medium K^+ to alter membrane potential in *Tenebrio* epidermis between -10 and -50 mV has no effect on junctional communication (Popowich & Caveney, 1977). Also, addition of EGTA to the medium causes a reduction in membrane potential to the same levels that are associated with anticalmodulin-induced uncoupling without increasing r_i in *Tenebrio* epidermis (Table 4).

It is more likely that junctional communication is regulated by calmodulin through one or more of the following three mechanisms:

1. Calmodulin May Act Indirectly on the Cell Junction by Controlling Intracellular Levels of Ca^{++}

Four potential sites for regulation of intracellular free Ca^{++} are extracellular Ca^{++}, bound cytoplas-

mic Ca^{++}, mitochondria, and endoplasmic reticulum. Calmodulin may act as a Ca^{++} storage site at the cell junction from which phenothiazines release Ca^{++} into the cytoplasm. This possibility does not seem likely as the binding of Ca^{++} to calmodulin is a prerequisite for phenothiazine-calmodulin interactions (Levin & Weiss, 1977). Calmodulin is known to regulate Ca^{++} transport by the plasma-membrane $(Ca^{++}+Mg^{++})$ATPase in red blood cells (Gopinpath & Vincenzi, 1977; Jarrett & Penniston, 1977), and adipocytes (Pershadsingh, Landt & McDonald, 1980). It has also been demonstrated to regulate Ca^{++} uptake by cardiac sarcoplasmic reticulum (Katz & Remtulla, 1978). Calmodulin binds strongly to mitochondria (Kaetzel, Pardue, Brinkley & Dedman, 1980).

We have conducted experiments to determine the source from which antipsychotics might induce net release of Ca^{++} into the cell cytoplasm. Since changing the external $[Ca^{++}]$ by either adding 3 mM EGTA or 10 mM $CaCl_2$ to the medium did not affect the rate at which phenothiazines uncoupled the cells, phenothiazines do not appear to act by inducing a net inward flux of Ca^{++} across the plasma membrane.

The possibility that phenothiazines cause a release of Ca^{++} from internal storage sites was investigated with metabolic inhibitors. Cyanide and DNP, both inhibitors of mitochondrial metabolism, cause a release of stored intracellular Ca^{++} and consequently are effective uncoupling agents of cell-to-cell communication in *Chironomus* salivary gland (Rose & Loewenstein, 1976). They are not effective uncoupling agents in *Tenebrio* epidermis. This difference in response may be explained by the fact that the epidermis is not well supplied by trachea, which carry oxygen to insect tissues, and mitochondria are relatively small and sparse when compared with ion-transporting epithelia such as salivary gland. Epidermal metabolism may be largely anaerobic. The epidermis does, however, contain large glycogen deposits and is uncoupled by iodoacetate, a nonspecific inhibitor of glycolysis. It appears likely that the endoplasmic reticulum (abundant in the epidermis, and, unlike the mitochondria, dependent on an external supply of ATP for Ca^{++} uptake) is a potential target for anticalmodulin drugs in this cell system. Iodoacetate (in combination with cyanide) has also been shown to uncouple mammalian cells *in vitro* (Flagg-Newton & Loewenstein, 1979) and another sulfhydryl reagent, N-ethylmaleimide, uncouples the salivary gland cells of *Chironomus* (Politoff, Socolar & Loewenstein, 1969).

These drugs could either interfere directly with calmodulin-dependent Ca^{++} fluxes across the endoplasmic reticulum membrane or deplete the ATP supply necessary for Ca^{++} uptake. ATP depletion may result from inhibition of phosphorylase kinase which is calmodulin-dependent (Shenolikar et al., 1979). Phosphorylase kinase is an important enzyme for maintaining ATP levels as it activates phosphorylase which converts glycogen to glucose-1-P. Depletion of intracellular ATP might also explain why phenothiazine-induced uncoupling is always accompanied by a drop in membrane potential. Measurement of ATP and free Ca^{++} concentrations in epidermis are necessary in order to substantiate these possibilities.

2. Calmodulin May Activate an Enzyme which Regulates the Permeability of Cell Junctions

Studies with mammalian cells in tissue culture (Larsen, 1975) and myometrium (Garfield, Merrett & Grover, 1980) have demonstrated the presence of calcium deposits adjacent to gap junctions. It was reasoned that these deposits might represent the activity of a phosphate releasing enzyme such as adenylate cyclase, ATPase, or phosphatase. The former two enzyme classes have members which are calmodulin-dependent (Wang & Waisman, 1979). We have been unable to find Ca^{++} deposits around the junction of *Tenebrio* epidermis despite employing a variety of possible stimulants, including 20-hydroxyecdysone.

3. Calmodulin May Control Junctional Communication Directly

This mechanism proposes that calmodulin may control both opening and closing of the junctional channel dependent on the local $[Ca^{++}]$.

Calmodulin is capable of binding 4 Ca^{++}, each with a different affinity (K_D ranging from 3×10^{-6} to 3×10^{-5} M in the presence of 3–5 mM Mg^{++}) (Klee & Kaiech, 1980; Huang et al., 1981). The binding of calmodulin to phosphodiesterase (PDE) requires the presence of Ca^{++}, and calmodulin must bind 4 Ca^{++} in order to activate PDE (Huang et al., 1981).

It has been proposed that calmodulin $-Ca^{++}_{1-2}$ may bind to, but not activate PDE (Klee & Kaiech, 1980). In this manner PDE activity can be regulated in the absence of relatively slow PDE-calmodulin dissociation. Another interesting example where calmodulin can have different activities at different Ca^{++} levels occurs with adenylate cyclase. Activation of adenylate cyclase by calmo-

dulin is dependent on the presence of Ca^{++} at 10^{-7} M. However, on increasing Ca^{++} levels to 10^{-6} M, calmodulin reduces adenylate cyclase activity to below that of controls from which calmodulin is absent (Potter et al., 1980).

Calmodulin may control junctional communication in a similar manner. The calmodulin-Ca^{++}_{1-3} complex may bind to and open the intercellular junction while calmodulin-Ca^{++}_4 would close the junctional channel. Calmodulin-Ca^{++}_{1-4}-phenothiazine would also result in a closed junction as would the absence of Ca^{++} or calmodulin. Recent support for this hypothesis comes from the work of Welsh et al. (1981) who have demonstrated that calmodulin binds to chick lens gap junctions and isolated gap junction protein. A cell could therefore regulate junctional communication by (i) controlling intracellular Ca^{++} levels, (ii) regulating the rate of calmodulin turnover or the turnover of a calmodulin-regulating protein, or (iii) by regulating gap junction turnover.

Exposing the epidermis to media containing EGTA raised junctional coupling (Fig. 7 and Table 3). If the preceding hypothesis is correct this increase could result from a conversion of calmodulin-Ca^{++}_4 (closed channel) to calmodulin-Ca^{++}_{1-3} (open channel) due to lowered intracellular Ca^{++} levels. The subsequent slow return of r_i to control values after prolonged EGTA exposure (Fig. 7) is also predicted since calmodulin would eventually be stripped of all Ca^{++} and dissociate from the channel.

The possibility that calmodulin controls junctional communication may have interesting implications in the development of *Tenebrio* epidermis. It was demonstrated previously that junctional communication in the epidermis of larval *Tenebrio* fluctuates during development (Caveney, 1976). A peak in communication occurs shortly before cell division. This peak can be induced *in vitro* by the application on an insect growth control hormone, 20-hydroxyecdysone (Caveney & Blennerhassett, 1980). It appears that the action of this hormone on junctional communication requires RNA synthesis; an increase in gap junctional area is not necessary (Caveney et al., 1980). It is possible that an RNA induced by 20-hydroxyecdysone codes for calmodulin. There is no direct evidence for increased calmodulin synthesis in the epidermis, but studies with chick embryo fibroblasts have demonstrated that calmodulin levels are higher in actively dividing cells *in vitro* and in cells transformed by Rous Sarcoma virus (RSV) when compared with confluent cultures of nontransformed cells (Watterson, Van Eldik, Smith & Vanaman, 1976). Thus an increase in calmodulin levels appears to be associated with cell growth. Cells transformed by RSV are also known to have reduced junctional communication (Atkinson et al., 1980), and Loewenstein's group has found many examples of transformed cell lines that are communication-defective (Loewenstein, 1979). The time is ripe for an examination of the effects of 20-hydroxyecdysone on calmodulin synthesis.

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