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# INTERCELLULAR COMMUNICATION IN AN INSECT ENDOCRINE GLAND

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## SUMMARY

1. The parenchymal cells of the corpora allata (CA) of the cockroach *Diploptera punctata* are coupled through low-resistance intercellular pathways.

2. Extensive dye coupling of cells of CA from fourth instar and adult female cockroaches was revealed following iontophoretic injection of Lucifer Yellow.

3. Freeze-etch electron microscopy revealed numerous gap-junction-like particles in CA cell plasma membranes.

4. The spread of ionic current from cell to cell was demonstrated by injecting current pulses into one cell and recording electrotonic potentials from other cells. The amplitude of electrotonic potentials elicited by negative current injection varied inversely with the rate of juvenile hormone biosynthesis by the CA.

5. The 'length constant' (distance at which an electrotonic potential in CA cells decays to 37% of its magnitude at the site of current injection) could not be measured accurately, but was found to be much greater than the length of the CA.

6. Forskolin- and IBMX-induced elevation of intracellular cAMP increased the electrotonic potential but reduced juvenile hormone release of day 8 CA.

## INTRODUCTION

Juvenile hormone (JH) biosynthesis by the corpora allata (CA) in insects appears to be regulated by a complex of nervous and humoral signals (see Tobe & Feyereisen, 1983 and Tobe & Stay, 1985 for reviews). In response to such signals, hormone biosynthesis may be regulated by either a modulation of the JH biosynthetic activity of all cells in the CA or by a change in the number of biosynthetically active cells (Tobe & Pratt, 1976; Tobe & Saleuddin, 1977). A precise cycle of JH biosynthesis is exhibited in mated females of the cockroach *Diploptera punctata* (Tobe & Stay, 1977). However, in virgin females, hormone biosynthesis remains low, and no cycle is apparent (Stay & Tobe, 1977; Tobe & Stay, 1980). Thus CA activity may be regulated through the intercellular coordination of JH biosynthesis. The presence of gap junctions connecting CA cells during the reproductive cycle (Johnson, Stay & Rankin, 1985) represents a possible mechanism for intercellular communication.

Ionic and dye coupling between insect epithelial cells through low resistance pathways is a well-documented phenomenon (Caveney, 1976, 1978; Safranyos &

Key words: cell coupling, corpus allatum, *Diploptera punctata*, forskolin, IBMX, Lucifer Yellow.

Caveney, 1985). Although coupling has been reported to result in coordinated electrical activity in islets of Langerhans in vertebrate pancreas (Meda *et al.* 1983*a*,*b*; Eddlestone, Goncalves, Bangham & Rojas, 1984), there have been no reports of coupling between cells in endocrine organs comprising non-excitable cells.

In this investigation, we demonstrate that the cells of the CA are electrically coupled at all times during the cycle of JH biosynthesis associated with the gonadotrophic cycle of *D. punctata*. Injected current and the fluorescent dye Lucifer Yellow pass from cell to cell through low-resistance pathways. Electrical coupling varies in inverse relationship to the rates of JH biosynthesis by the CA, and elevated levels of intracellular cAMP cause an increase in the intercellular electrotonic conductance.

## MATERIALS AND METHODS

The CA and part of the corpora cardiaca (CC) were dissected from *D. punctata* of known age, ensuring that all connective tissue and muscle was removed. The sheath of the CA was removed by treatment for 15 min with 0·1 % collagenase II (Sigma) in cockroach saline (Krauthamer, 1980), containing (in mmol $l^{-1}$) NaCl, 150; KCl, 12; $CaCl_2$, 15; $MgCl_2$, 3; glucose, 40; and HEPES 10 at pH 7·4. In some experiments TC 199 (Gibco) containing 5 mmol $l^{-1}$ $Ca^{2+}$ was substituted for cockroach saline.

### *Freeze-etching*

Fifteen pairs of CA from day 5 mated females and day 13 fourth instar females were dissected, and pre-fixed for electron microscopy as described by Lococo & Tobe (1984). The tissue was rinsed in 20 % glycerol in 0·1 mol $l^{-1}$ sodium cacodylate (pH 7·2), frozen and fractured as described by Shivers (1976). Replicas were observed with a Philips 201 transmission electron microscope.

### *Dye coupling*

Lucifer Yellow CH (Sigma) (3 % in 0·05 % LiCl) was injected *via* a bridge circuit (WP Instruments Model M707 Microprobe System) into a surface cell of the CA by iontophoresis (−5 nA, 500-ms pulses at 1 Hz) for 5 min. The preparations were fixed in 4 % formaldehyde in 0·1 mol $l^{-1}$ sodium cacodylate (pH 7·2) overnight, washed in cacodylate buffer for 1 h, dehydrated in an ethanol series, cleared in methyl salicylate and photographed with epifluorescence optics on a Zeiss Standard compound microscope. The video recording system utilized to determine the dynamics of dye diffusion was that described by Safranyos & Caveney (1985).

### *Electrical coupling*

Single CA cells were impaled with glass capillary microelectrodes (resistance 50–100 MΩ) filled with 3 mol $l^{-1}$ KCl. Electrical coupling of CA cells was demonstrated by passing current with one electrode and recording membrane responses from other cells with a second microelectrode. Current was measured with an I/V

converter in the ground circuit (WP Instruments Model IVA). Current/voltage relationships were determined by injecting 1·5-s square-wave current pulses of varying amplitude and polarity into one cell and recording the responses from another cell. The decay of electrotonic potential with distance from the current-passing electrode was determined by injecting hyperpolarizing current pulses of uniform amplitude into one cell and recording responses from other cells at varying inter-electrode distances (IED). IED was measured with an ocular micrometer.

The spread of hyperpolarizing electrical current in different glands was compared by injecting uniform current pulses (5·0 nA, 1·5 s) and recording responses with a uniform IED (200 $\mu$m). CA from females during the first 11 days of the first gonadotrophic cycle were compared.

### *Elevation of intracellular cyclic adenosine monophosphate (cAMP)*

To examine the effects of elevated levels of cAMP on membrane potential, electrical coupling and JH release, glands were exposed to 50 $\mu$mol $l^{-1}$ forskolin (Seamon, Padgett & Daly, 1981) and 100 $\mu$mol $l^{-1}$ isobutyl 1-methylxanthine (IBMX) (Beavo *et al.* 1979) dissolved in TC 199 containing 0·2% dimethyl-sulphoxide (DMSO). JH release was measured by the method of Tobe & Stay (1977) using the modification of Feyereisen & Tobe (1981).

## RESULTS

### *The CA: basic features*

The CA (Fig. 1) of *D. punctata* were enveloped with a fibrous non-cellular sheath, which was removed easily with collagenase to facilitate penetration by intracellular microelectrodes. Collagenase treatment removed the sheath except for some fibrous material on the cell surface (Fig. 2). The three-dimensional arrangement of the cells in the CA was maintained after collagenase treatment. The cells were irregularly shaped and were between 5 and 9 $\mu$m long. Some cells were spindle-shaped and as long as 15 $\mu$m, with widths tapering from 5 to 1 $\mu$m. CA ranged in length from 200 to 350 $\mu$m.

### *Lucifer Yellow injections*

Lucifer Yellow was injected into cells of the CA to determine the extent of dye coupling. Dye passed rapidly from the impaled cell to adjacent cells (Figs 5, 6). Video recordings (not shown) of the process of dye injection demonstrated that within 5 min, the advancing front of dye spread appeared to stop. Owing to the high background fluorescence and the exponential dilution of the dye, it was impossible to resolve the interface between the advancing front of dye and unfilled cells. Injection for 30 min showed no detectable advance of the dye. This was not due to blocked electrodes because current continued to pass throughout the injection period as observed by a current monitor.

In all preparations, the dye spread readily at an injection current of 5 nA.

*Freeze-etching of the CA*

Morphological evidence for gap junctions between CA cells in *D. punctata* was reported by Johnson *et al.* (1985) using conventional transmission electron microscopy. We confirm this finding using freeze-fracture techniques. Freeze-etched replicas were prepared from CA of adult and fourth instar females. Gap junctions were observed in CA from both fourth instars and from adult females (0–5 days) (Figs 3, 4). E-type gap junctions were observed in groups of 8–10 at the basal end of the outer parenchymal cells of the CA at day 5. Smaller numbers (1–3) were located on the lateral membranes of these roughly columnar cells. Fragments of P-face membrane, sometimes associated with mitochondria, often adhered to gap junctions on the E-face (Fig. 4). All the cells observed had large areas of membrane with sparse intra-membrane particles and small regional groups of gap junctions. No other junctional specializations were seen in these samples.

*Electrical coupling*

Pairs of CA cells were impaled with intracellular microelectrodes at inter-electrode distances of 50–250 $\mu$m. Membrane potentials ranged from $-45$ to $-65$ mV. The lower values were probably the result of damage to the CA cell membranes by the electrodes. For six glands from day 4 mated females, the mean resting potential of the cells was $-55{\cdot}1 \pm 4{\cdot}7$ mV (S.D., $N = 51$). Typical current/voltage plots are shown in Fig. 7. Voltage responses were linear in the hyperpolarizing direction, but membrane resistance (or gap junctional conductance) began to decrease when the cells were depolarized by more than 10–15 mV. The input resistance of the entire network of electrically coupled cells (the slope resistance of I/V plots in the hyperpolarizing direction) ranged from 1 to $3 \times 10^6\,\Omega$. No membrane responses were observed when the current-passing electrode was withdrawn from a cell and current injected into the extracellular space. The time constant of decay of electrotonic potentials ranged from 100 to 200 ms. Electrical coupling of CA cells was observed in every case examined, in animals from the fourth stadium up to (and including) day 13 of adulthood.

Fig. 1. Scanning electron micrograph of a corpus allatum de-sheathed in 0·1 % solution of collagenase II in saline. Scale bar, 30 $\mu$m.

Fig. 2. Higher magnification scanning micrograph illustrating variability in cell shape. Incompletely digested sheath has strand-like appearance and adheres to cells. The cells of the corpus allatum retain their cell-to-cell association after collagenase treatment. Scale bar, 3 $\mu$m.

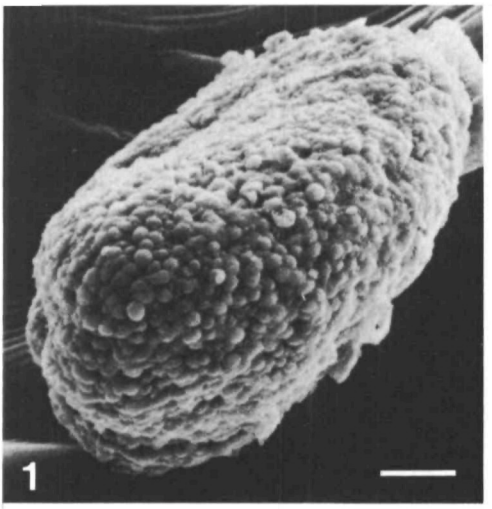
Figs 3, 4. Freeze-etch micrograph of plasma membrane of surface corpora allata cells. E-type gap junctions appear in groups of 8–10 in discrete regions whereas the majority of the membrane is nearly devoid of intramembrane particles. Membrane fragments associated with cell organelles adhere to gap junctions suggesting a strong adhesiveness.

Fig. 3. Day 13 fourth stadium CA membrane showing gap junctions. Scale bar, 50 nm.

Fig. 4. Day 4 mated adult female CA showing gap junctions. Scale bar, 50 nm.

Fig. 5. Whole mount phase-contrast micrograph of day 4 mated adult female CA. Scale bar, 100 $\mu$m.

Fig. 6. Whole mount fluorescence micrograph of the same day 4 mated adult female CA as in Fig. 5, injected with Lucifer Yellow. Scale bar, 100 $\mu$m.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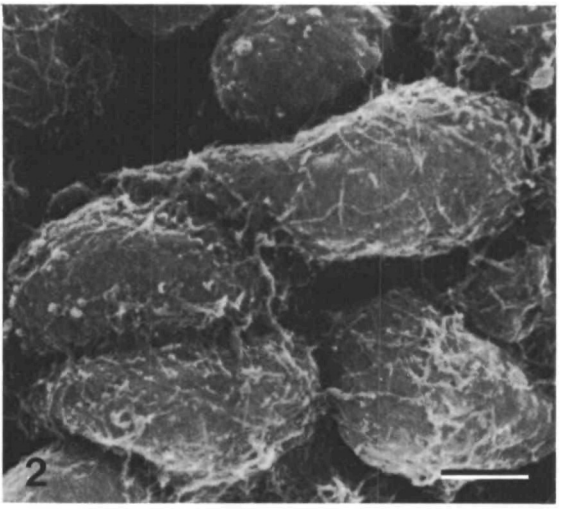
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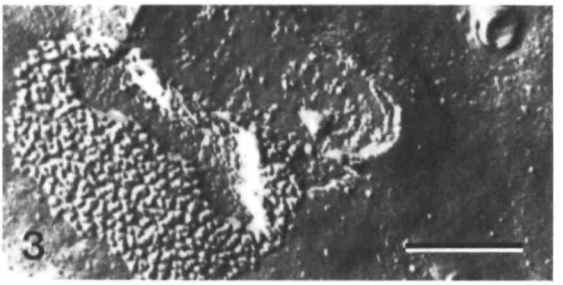
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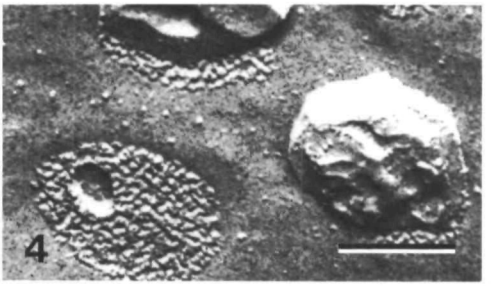
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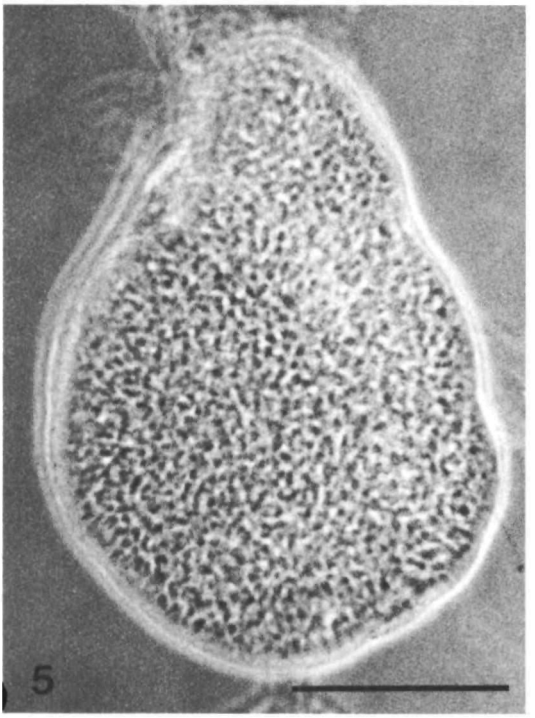
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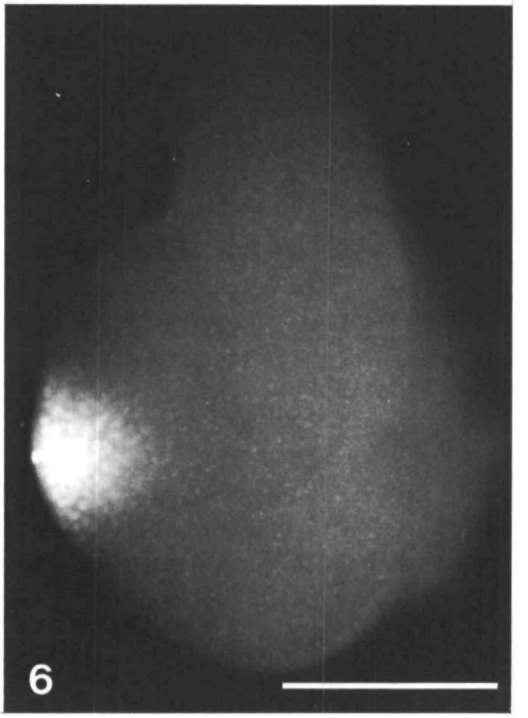
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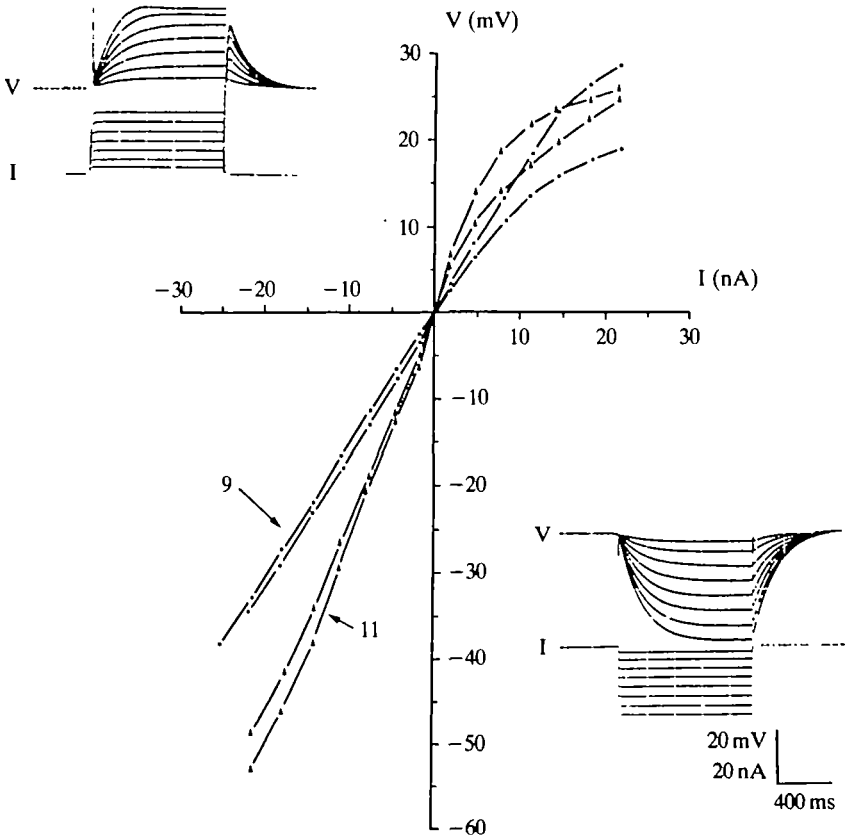


Fig. 7. Typical current/voltage relationships of CA. Two of the four curves shown are from day 11 mated females and two are from day 9 mated females. I/V relationships are linear in the hyperpolarizing direction but show a pronounced decrease in slope in the depolarizing direction. Examples of data from which these curves were obtained are shown in the upper left (depolarizing) and lower right (hyperpolarizing) quadrants.

The decay of electrotonic potential with distance is illustrated in Fig. 8. Uniform negative current pulses were injected into one cell near one end of a gland and the response recorded from other cells at various inter-electrode distances. The variability in amplitude of the responses recorded from various cells was too great to permit an accurate determination of the length constant, but it is apparent that it is much greater than the length of the glands themselves (200–350 $\mu$m). Because the electrotonic potential does not decay rapidly with distance, the input resistance of a single cell must be very high relative to the resistance of the alternative pathway for current flow to ground (i.e. through the gap junctions with contiguous cells into the network of electrically coupled cells). The correlation between the size of the electrotonic potential and the resting potential of the cells (inset, Fig. 8) suggests that electrode-induced damage to the cell membranes is responsible for most of the variability observed.

To compare the spread of electrical current in glands producing different amounts of JH, CA were examined from mated females of different ages (Fig. 9). Seven to ten

different recordings were taken from each of five pairs of CA for each age group (day 0, 1, 3, 4, 5, 6, 8 and 11). The magnitude of the response to constant current pulses was found to decrease with increasing JH biosynthesis.

### *Elevation of cAMP*

The effects of elevated levels of intracellular cAMP on membrane potential, electrotonic potential and JH release were investigated by exposing glands to a mixture of forskolin (50 $\mu$mol l$^{-1}$) and IBMX (100 $\mu$mol l$^{-1}$). In one series of experiments, the membrane potential and electrotonic potential (−5·0 nA, IED 100 $\mu$m) were measured from several cell pairs in each of 10 glands from day 8 mated female cockroaches, both before and 40–60 min after exposure to forskolin and IBMX. Both membrane potential and electrotonic potential were significantly increased by this procedure (Table 1). To measure the time course of this effect,

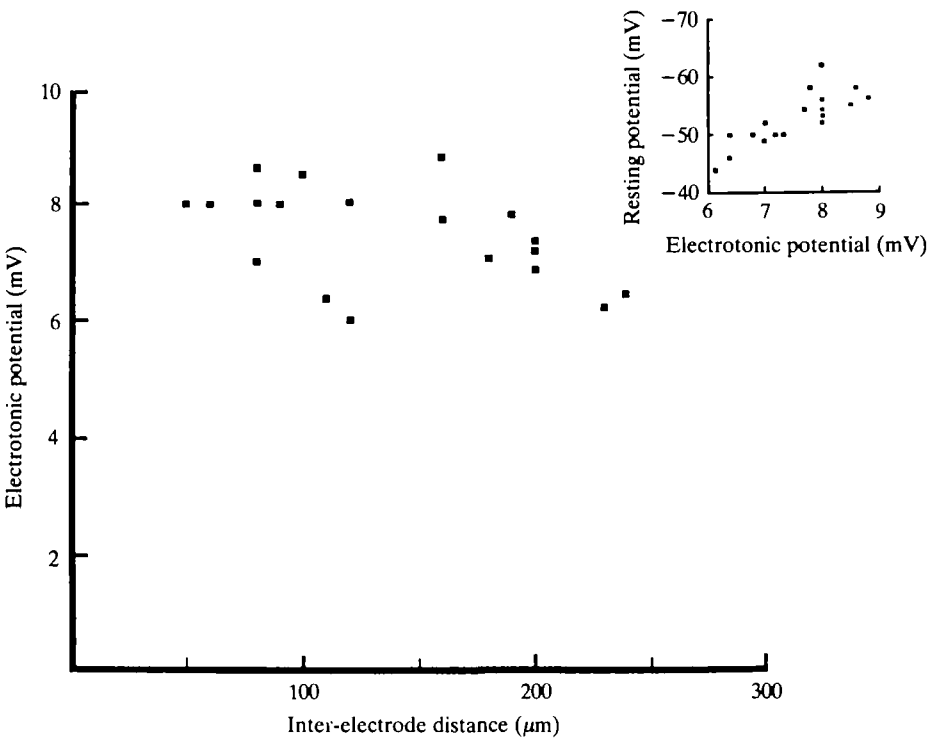


Fig. 8. Electrotonic potential as a function of inter-electrode distance as typically recorded from a day 4 CA in which the stimulating electrode remained stationary while the recording electrode was moved laterally along the length of the CA. Electrotonic potential does not appear to vary with increasing inter-electrode distance. Each point represents the potential recorded intracellularly from a single cell injected with 5 nA for 500 ms. The magnitude of the voltage response depended more upon the resting membrane potential than the inter-electrode distance (inset). The length of this gland was 340 $\mu$m.

electrodes were kept in a single pair of cells and a current pulse (−5·0 nA) injected every 15 s during exposure to forskolin and IBMX (Fig. 10). In four cases, electrodes were maintained in place for more than 20 min after exposure to the drugs and all four trials showed the same complex effect. Both membrane potential and electrotonic potential initially increased but after a few minutes decreased to control values or lower. Within 5–7 min, however, both began to increase again and, after 15 min, exceeded control values (Fig. 10A). Control trials in which glands were exposed to 0·2% DMSO in saline showed no effect on membrane potential or electrotonic potential (Fig. 10B).

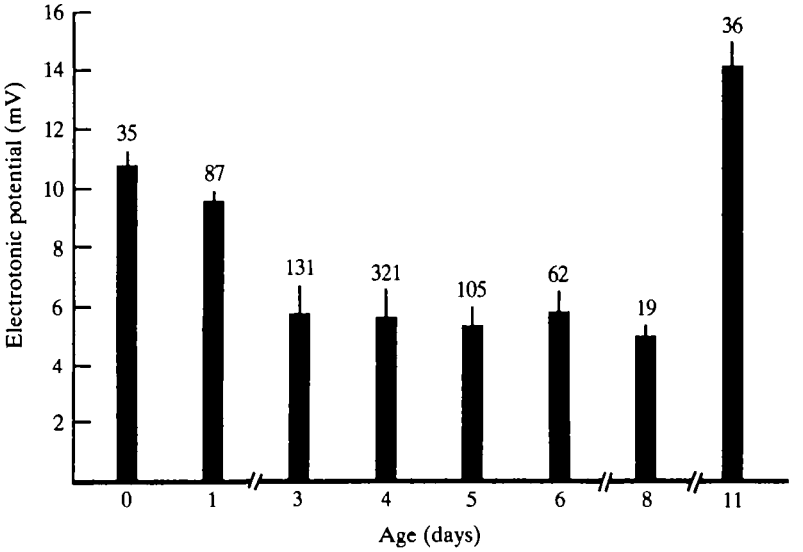


Fig. 9. Electrotonic potential at various days after the imaginal moult. Coupling is lower during days 3–5, when rates of JH biosynthesis are high, but is high at days 0, 1 and 11, when rates of biosynthesis are low. Maintenance of a low electrotonic potential in CA from day 6 and day 8 animals (low rates of JH biosynthesis) suggests that a decrease in JH biosynthesis does not result in an increase in coupling. Numbers above bar plots represent sample size. The vertical lines above the bars represent 1 S.D.

Table 1. *Effects of IBMX/forskolin on membrane potential, electrotonic potential and JH release*

| | Control | IBMX/forskolin treatment | % Change |
|---|---|---|---|
| Resting membrane potential (mV)* | 49·24 ± 5·97 ($N$ = 95) | 59·95 ± 8·95 ($N$ = 99) | +21·7 |
| Electrotonic potential (mV)* | 5·88 ± 1·6 ($N$ = 61) | 10·22 ± 3·51 ($N$ = 60) | +73·8 |
| JH release (pmol $h^{-1}$ $pair^{-1}$)† | 10·0 ± 1·9 ($N$ = 5) | 1·4 ± 1·0 ($N$ = 5) | −86·0 |

* $\bar{x}$ S.D.; $P < 0{\cdot}0005$, Student's $t$-test.
† $\bar{x}$ S.E.M.; $P < 0{\cdot}001$, Student's $t$-test.

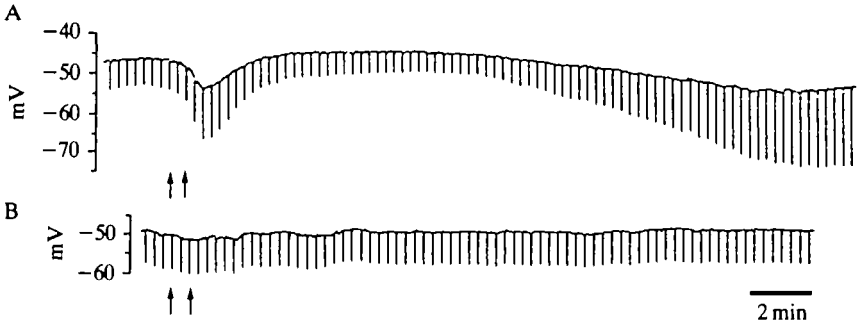


Fig. 10. Time course of the effect of elevating intracellular cAMP. (A) 250 μl of a stock solution of forskolin and IBMX dissolved in DMSO in saline was slowly added to the preparation dish (arrows). Both membrane potential and electrotonic potential were significantly increased by this procedure. (B) Control trial in which the addition of DMSO alone (arrows) produced no effect.

In another experiment, using five pairs of CA from day 8 mated females, elevated cAMP was found to cause a significant inhibition of JH release ($P < 0{\cdot}001$, Table 1) (see also Meller, Aucoin, Tobe & Feyereisen, 1985).

## DISCUSSION

Experimental evidence presented here demonstrates that the cells of the CA are coupled by low-resistance pathways which are permeable to ions and the dye Lucifer Yellow. The small size (6–8 μm) and irregular stellate shape of CA cells (Johnson *et al.* 1985) make it difficult to impale individual cells with two intracellular microelectrodes. Thus the coupling coefficient between contiguous cells (Socolar, 1977) cannot be calculated, nor can the input resistance of single cells be determined. The input resistance of the entire system of electrically coupled cells ranged from 1 to 3 MΩ. The apparent decrease in input resistance which occurs with depolarizing current pulses is probably not due to rectification of cell-to-cell junctions. Rectifying electrical junctions are known to exist but rectification is associated with a potential difference across the junction (Giaume & Korn, 1983). Slight differences in resting potential from cell to cell were observed in the present study but these are probably due to electrode-induced damage to the cell membranes. A more likely explanation for the decrease in input resistance with depolarization is the presence of voltage-sensitive potassium channels which are characteristic of many types of excitable cells (Hille, 1984).

Lucifer Yellow injections indicate that at all times during the fourth stadium and the first gonadotrophic cycle of JH biosynthesis coupling is sufficient to allow extensive dye diffusion in three dimensions. The rate of dye diffusion is not quantifiable due to the inherent high background fluorescence of the CA. However, in day 4–5 adults, in which JH biosynthesis is maximal, dye diffusion appears less extensive. This could be caused by a decrease in gap junction channel size or a decrease in the number of open junction channels. During development of grasshopper embryo

neuroblasts, dye coupling decreases to the point of uncoupling, whereas ionic or electrical coupling remains (Goodman & Spitzer, 1979). In *Oncopeltus fasciatus*, Blennerhassett & Caveney (1984) showed physiologically that Lucifer Yellow does not cross body segments following injection into epidermal cells, but electrotonic spread at the border is unimpeded. Since uncoupling of arthropod cells is believed to be due to closure of the central pore of the membrane channel (Peracchia & Peracchia, 1978), it is more likely that dye uncoupling would occur before a decrease in electrotonic conductance.

In CA from *D. punctata*, if the large reduction in electrotonic potential during days 3–8 (Fig. 9) was primarily due to a partial 'gate' closure at the junctional membrane, then significant dye-decoupling should have been apparent. Data presented here suggest that other factors are involved which contribute to the measured decrease in the electrotonic potential. Possible factors that are discussed below include cell membrane resistance, cell size, cell number and cAMP concentration.

The CA of mated female *D. punctata* increase in volume as the rate of JH biosynthesis increases during the first five days of adult life (Engelmann, 1959; Szibbo & Tobe, 1981). This increase in size is accounted for by a 50 % increase in cell number by day 5 (Szibbo & Tobe, 1981) and by an increase in the cytoplasmic volume of single cells (Johnson *et al.* 1985). The surface area of single cells increases nearly two-fold as volume increases during this period (Johnson *et al.* 1985). Thus, the input resistance of single cells will decrease, assuming that the specific membrane resistance does not change. This decrease in resistance and the addition of more cells to the network of electrically coupled cells would be expected to decrease the input resistance of the system. Hence, the decrease in electrotonic potential observed during days 0–3 in the present study may be in part due to these factors.

It is possible that the intraglandular JH content is responsible for part of the decrease in electrotonic potential observed during days 3–5 (Fig. 9). The JH content of biosynthetically active CA can be as high as 35 pmol per gland (Tobe & Stay, 1977). The lipophilic nature of the JH molecule renders it soluble in cell membranes and hence, during periods of high JH biosynthesis, the CA cell membranes may have a significant JH component. The great difficulty in achieving adequate fixation for electron microscopy during periods of high JH synthesis suggests that this may be the case (Johnson *et al.* 1985). It has been shown that JH raises the conductance of cell membranes in salivary glands of *Galleria mellonella* (Baumann, 1968). Thus, the JH molecule itself may act to increase membrane conductance and decrease the electrotonic potential. However, this effect is probably not very large, if present at all, because the electrotonic potential remains low (days 6–8) as JH synthesis decreases from maximal levels following the completion of vitellogenesis (Tobe & Stay, 1977). CA cell size and cell number decline during pregnancy (Szibbo & Tobe, 1981) and it is likely that it is this decrease in total CA cell membrane which accounts for much of the increase in electrotonic potential observed by day 11.

The observation that exposure of CA to forskolin and IBMX increases the electrotonic potential and decreases JH release suggests that changes in intracellular cAMP content may account in part for the changes in electrotonic potential which

occur throughout the gonadotrophic cycle in *D. punctata*. At present, the relationship between electrotonic potential, cell coupling and JH release remains undefined although there is a clear correlation between increased electrotonic potential and reduced JH release, and its modulation by cAMP. The levels of forskolin and IBMX used in the present study greatly elevate the intraglandular cAMP content (Meller *et al.* 1985) and cAMP has been shown to increase cell-to-cell coupling in other systems (De Mello, 1983, 1984; Estape-Wainwright & De Mello, 1983; Hax, van Venrooij & Vossenberg, 1974; in't Veld, Schmit & Pipleers, 1985). In the salivary glands of larval *Drosophila hydei*, cAMP increases the permeability of low-resistance junctions between cells (Hax *et al.* 1974). In addition, by fitting their experimental observations to a mathematical model, the latter authors calculated that cAMP induces a considerable increase in the resistance of the non-junctional membrane. We cannot apply this model to the CA because of the large number and three-dimensional arrangement of cells. As a result we cannot determine whether the increase in electrotonic potential brought about by exposure to forskolin and IBMX is due primarily to an increase in junctional conductance or to a decrease in non-junctional conductance, or both.

It is interesting to note that cAMP hyperpolarizes salivary gland cells of *D. hydei* in conjunction with changes in junctional and non-junctional conductance. cAMP-induced hyperpolarization has been reported in other cell types (Drummond, Benson & Levitan, 1980; Hennessey, 1985) but in some cases is associated with a decrease in membrane resistance (Drummond *et al.* 1980).

One additional factor which would be expected to influence the size of the electrotonic potential in CA is the intracellular concentration of calcium ions. The permeability of cell-to-cell junctions has been shown to depend on the local cytoplasmic calcium activity (Rose & Loewenstein, 1975, 1976) and JH production by CA is extremely sensitive to changes in calcium ion concentration (S. Kikukawa, S. S. Tobe, S. Solowiej, S. M. Rankin & B. Stay, in preparation). Thus, changes in intracellular calcium ion activity may be occurring throughout the gonadotrophic cycle of *D. punctata*.

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