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FURA-2 MEASUREMENT OF CYTOSOLIC FREE Ca^{2+} CONCENTRATION IN CORPUS ALLATUM CELLS OF LARVAL *MANDUCA SEXTA*

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Summary

Cytosolic free Ca^{2+} has been implicated in the regulation of the larval corpus allatum (CA) of the tobacco hornworm *Manduca sexta* since agents presumed to cause changes in intracellular Ca^{2+} concentrations affect both basal and neuropeptide-modulated synthesis/release of the juvenile hormones. To determine whether differences in cytosolic free $[Ca^{2+}]$ in CA cells were associated with different levels of gland activity, methods were developed for dissociating CA cells with the retention of biosynthetic activity and for uptake by the dissociated cells of the fluorescent Ca^{2+} indicator Fura-2. Digitized fluorescence microscopy of the Fura-2-loaded cells enabled measurement of intracellular Ca^{2+} levels in individual cells. Intracellular free Ca^{2+} levels were measured in CA cells from selected days during the fifth larval stadium and were found to be highest when the CA were biosynthetically active. Treatment of CA cells from day 6 with the Ca^{2+} ionophore ionomycin elevated the intracellular Ca^{2+} level, corroborating the involvement of elevated intracellular $[Ca^{2+}]$ in ionophore effects on juvenile hormone (JH) and JH acid synthesis. The results of the study are considered in relation to our knowledge of the role of Ca^{2+} in the function of endocrine gland cells.

Introduction

Cytosolic free Ca^{2+} has been implicated as an intracellular second messenger in a number of vertebrate endocrine glands, including the adrenal cortex and medulla, the anterior pituitary and the pancreatic islets (see Godfraind *et al.* 1986, for a review). The binding of a ligand to a receptor on the external surface of the cells of these glands leads to the opening or closing of Ca^{2+}-selective channels residing in the plasma membrane (Meldolesi and Pozzan, 1987; Carafoli, 1987) or the release of intracellular stored Ca^{2+}, resulting in changes in the concentration of cytosolic free Ca^{2+}.

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It has been proposed that calcium plays a pivotal role in the regulation of two insect endocrine glands, the prothoracic glands (PGs) of the tobacco hornworm *Manduca sexta* and the corpus allatum (CA) of the cockroach *Diploptera punctata* and of *Manduca sexta*. *Manduca sexta* PGs are activated by a cerebral neuropeptide, the prothoracicotropic hormone (PTTH), which apparently binds to a Ca^{2+} channel, increasing intracellular free [Ca^{2+}]. This activates a Ca^{2+}/calmodulin (CaM)-dependent adenylate cyclase, and the resulting rise in cyclic AMP concentration triggers a cascade of phosphorylation events culminating in the up-regulation of ecdysteroid biosynthesis (Smith *et al.* 1985; Gilbert *et al.* 1988; Meller *et al.* 1988). For *Diploptera punctata* CA, optimal concentrations of extracellular Ca^{2+} are required for maximal JH biosynthesis in the adult female, and there is considerable evidence for the existence of Ca^{2+} channels in the cell membranes of these glands (Kikukawa *et al.* 1987; Aucoin *et al.* 1987). Thus, Ca^{2+} may play an important role in the regulation of JH biosynthesis and release in this species. Changes in Ca^{2+} concentration also differentially affect the biosynthetic activity of *Manduca sexta* CA during larval–pupal development (Allen *et al.* 1992). Glands prior to the time of pupal commitment during the last larval stadium require an optimal concentration of extracellular Ca^{2+} for maximal JH/JH acid biosynthesis, while CA after pupal commitment do not. Furthermore, altering free [Ca^{2+}] within the CA by various pharmacological manipulations, such as the use of Ca^{2+} ionophores and Ca^{2+} channel blockers or antagonists, affects both JH and JH acid biosynthesis, but a more pronounced effect is observed with glands prior to commitment. Thus, it appears that the role of Ca^{2+} in the regulation of JH/JH acid biosynthesis by the CA of *Manduca sexta* undergoes a change during development.

To understand the basis for the differential response of the CA to [Ca^{2+}] during larval–pupal development and to confirm that the pharmacological treatments eliciting these responses altered cytosolic free [Ca^{2+}], a procedure was developed to dissociate the CA into individual cells. The free Ca^{2+} content of these cells was then measured with the Ca^{2+}-sensitive fluorophore Fura-2 (Tsien, 1989), using fluorescence microscopy and video image analysis (DiGuiseppi *et al.* 1985; Roe *et al.* 1990). The results confirm that resting cytosolic Ca^{2+} levels are in the nanomolar range but demonstrate significant differences in the levels of Ca^{2+} between larval and pupally committed CA. Biosynthetically active glands of either type have high intracellular Ca^{2+} concentrations. In addition, direct measurements of intracellular [Ca^{2+}] confirm the effects of agents altering Ca^{2+} flux at the level of the single cell and permit a comparison with the pharmacological effects of these agents on JH/JH acid synthesis.

Materials and methods

Animals

Larvae of *Manduca sexta* (Linnaeus) (Lepidoptera: Sphingidae) were reared on an artificial diet (Bell and Joachim, 1976) at 26°C, high humidity (60–70 %) and a

non-diapausing photoperiod (L:D 16 h:8 h) (Vince and Gilbert, 1977). For this study, CA were dissected from various stages of the fifth stadium. To synchronize the development of these larvae, animals were selected within a 6–8 h period at the time of the moult to the fourth larval stadium. From this group, pharate fifth instars were chosen on the morning of the third day, and those moulting between 14:00 h and 16:00 h were designated as day 0, fifth-stadium larvae. More than 80 % of these larvae were gate 2, as judged by their further development. Animals selected in this manner were gated again by weight at 14:00 h on day 2, using previously established weight curves for the different gates, and were re-gated at wandering. Gate 2 fifth-stadium larvae wander on day 5 and undergo pupal ecdysis on day 10.

Dissection of corpora allata

Larvae were anaesthetized in water and dissected in Grace's lepidopteran tissue culture medium (J. R. Scientific, Woodland, CA). Isolated CA were placed in standing drops of Grace's medium, cleaned of any remaining tracheae, fat body and corpora cardiaca, and then removed to a large drop of Grace's medium for rinsing.

Dissociation of corpora allata

Up to 22 CA were dissected from 12 or more larvae, rinsed and transferred to a 180 μl drop of Grace's calcium-free medium (GIBCO, Grand Island, NY) containing 50 units of elastase (porcine type IV, Sigma, St Louis, MO; 1 unit solubilizes 1 mg of elastase in 20 min at pH 8.8 and 37 °C) and 2 % collagenase D (Boehringer/Mannheim, Indianapolis, IN). Digestion of the basal lamina and intercellular matrix was allowed to proceed for 50–70 min at 37 °C. The enzyme solution was then drawn off and the CA were carefully rinsed three times with 200 μl of Grace's medium. The CA were transferred to a culture well constructed of a coverslip mounted in an aluminium frame containing Grace's medium, pH 6.5, with 0.1 mmol l^{-1} $CaCl_2$. The coverslip was coated with Celltak (Collaborative Research, Inc., Bedford MA) and dried prior to mounting it in the frame. Dissociation of the glands into individual cells and small clusters of cells was accomplished by repeated aspiration with a polyethylene glycol (PEG)-coated Pasteur pipette, with an orifice drawn out to an inner diameter of 80–100 μm. After dissociation, the cells were allowed to settle and attach to the coverslip for at least 20 min before further treatment. Dissociated cells for measurement of intracellular Ca^{2+} were prepared three times for day 0, twice for day 6 and once for the other stages.

Scanning electron microscopy

Corpora allata were dissected from day 6 fifth-stadium larvae, rinsed in Grace's medium and individually placed on the surface of Celltak-coated coverslips (immediate adhesion of the gland occurs to the substratum surface). The coverslips were then immersed in 2.0 % glutaraldehyde in 75 mmol l^{-1} phosphate

buffer containing 0.1 mmol l^{-1} $CaCl_2$ (pH 7.0, approx. 300 mosmol l^{-1}) and the glands were fixed for 24–48 h. Following fixation, the preparations were rinsed three times in 0.1 mol l^{-1} phosphate buffer (pH 7.0) and postfixed for 60 min with 2.0 % osmium tetroxide in 0.1 mol l^{-1} phosphate buffer (pH 7.2). The glands were then rinsed in 0.1 mol l^{-1} phosphate buffer (pH 7.2), followed by two rinses in distilled water and dehydration in a graded series of ethanol concentrations. The tissue was then critical-point dried with liquid CO_2, mounted on stubs and sputter-coated with gold:palladium (Hayat, 1978; Y. Tanaka, personal communication). Specimens were examined with a Joel JSM-820 scanning electron microscope and were photographed with Tri-X pan negative film.

JH acid biosynthesis by dissociated CA cells

To assess the physiological competence of the dissociated cells, their production of JH acid was measured by radioimmunoassay (RIA). CA from day 6 of the fifth stadium were dissociated according to the method described above, but the entire procedure was carried out in a PEG-coated glass well containing standard medium for the incubation of CA (Grace's medium plus 0.1 % bovine serum albumin, pH 6.5, containing 0.1 mmol l^{-1} $CaCl_2$). Following the dissociation, the volume of medium in the well was adjusted to 210 μl, and the cells were incubated at 26 °C for 2 h. During the incubation, the CA cells settled on the bottom of the well, allowing the careful withdrawal of medium alone for assay with a JH I RIA, according to a well-established protocol (Granger *et al.* 1979; Granger and Goodman, 1983).

Fura-2 loading of CA cells

Fura-2-acetoxymethyl ester (Molecular Probes, Eugene, OR) was diluted in Grace's medium (pH 6.5) containing 0.1 mmol l^{-1} $CaCl_2$ and carefully added to the culture well containing the CA cells attached to a coverslip. A final concentration of 5 μmol l^{-1} was used to load the cells. Incorporation of Fura-2 was allowed to proceed for 30 min at 26 °C in the dark, after which the label was removed and the cells were washed three times with Grace's medium.

The possibility of selective sequestration of Fura-2 within cellular organelles such as vesicles was addressed by exposure of the corpus allatum cells to 20 μmol l^{-1} digitonin, which permeabilizes the cell membrane to Fura-2 and allows cytosolic Fura-2 to diffuse from loaded cells into the extracellular medium (Roe *et al.* 1990).

Fluorescence microscopy and image analysis

The coverslip with attached Fura-2-loaded CA cells was transferred within its aluminium frame to the stage of an inverted Zeiss IM-35 phase/fluorescence microscope with a 40× Nikon UVF oil-immersion phase objective for epifluorescence illumination. The optics and computerized image analysis system for the measurement of intracellular $[Ca^{2+}]$ have been previously discussed in detail (see DiGuiseppi *et al.* 1985) and will only be briefly described here. Appropriate narrow-band interference filters and a range of neutral density filters, controlled

by separate stepping motors, were positioned between the light source and the objective turret of the microscope to enable excitation at 340, 365 and 380 nm. The microscope was attached to a Dage-MTI (Michigan City, IN) Isit video camera, and the analogue signal from the camera was digitized by a Minvideo board (Datacube, Peabody, NA) installed in a Sun Microsystems (Mountain View, CA) 3/110 computer. The computer automatically adjusted the filters and could store and average 64–256 frames per image, depending on the rate of image acquisition.

In a typical experiment, the Fura-2-loaded specimen was exposed sequentially to excitation wavelengths of 340 and 380 nm, and the individual fluorescence images were stored in separate frame buffer memories on the video board. Background fluorescence in the preparation of dissociated cells was obtained by measuring the fluorescence of the surface of a Celltak-coated coverslip in an area free of cells. Unlabelled cells incubated in an identical fashion were illuminated under experimental conditions and autofluorescence was found to be approximately 0.1 % of the minimal signal observed from Fura-2-labelled cells. Background fluorescence of the system, including camera dark noise and fluorescence of the optical components, had previously been determined for each excitation wavelength and was subtracted on a pixel by pixel basis from experimental images. The raw 340 nm and 380 nm images were then thresholded, and the emission intensity values for the 340 nm images (calcium-dependent fluorescence) were divided by those for the 380 nm images (calcium-independent fluorescence) on a pixel by pixel basis, resulting in a ratio image.

The ratio image displays the spatial distribution of cytosolic free Ca^{2+} within the field as a range of grey values, from 0 to 255, with 0 being black and 255 white. Concentrations of Ca^{2+} are correlated to the grey values by means of a standard curve, derived from the 340 nm/380 nm ratio fluorescence intensity of EGTA-buffered Ca^{2+}/Fura-2 ($5K^+$ salt, Molecular Probes) standard solutions (see Roe *et al.* 1990). The computer system can then overlay a colour spectrum by assigning appropriate colours to the grey level intensity values of individual pixels. A pseudo-coloured image is thus generated, which makes identification of the relative differences in the spatial distribution of free Ca^{2+} within the cell easier to see.

Results

Dissociation procedure

The corpus allatum of *Manduca sexta* is made up of only 150–200 cells; thus, use of CA cell preparations for spectrophotometric measurements of total cytosolic free Ca^{2+} using a fluorescent Ca^{2+} probe was not feasible. However, measurements of [Ca^{2+}] in individual cells were possible once the appropriate conditions for the dissociation of the gland had been established. The procedure, modified from those of Satmary and Bradley (1984) and Levinson and Bradley (1984), employed collagenase to digest the basal lamina and elastase to digest the intercellular matrix, resulting in the dissociation of the gland into individual cells

or small clusters of cells. Use of collagenase alone removed the basal lamina but did not result in the dissociation of individual cells.

Following the dissociation procedure, cells were judged to be viable both from their appearance and from their cytosolic free [Ca^{2+}]. Non-viable cells contained swollen nuclei with pyknotic contents, showed reduced cytoplasmic volume and had large, clear, fluid-filled blebs of plasma membrane on their external surface. Intracellular free Ca^{2+} concentrations in these cells were 2–10 times higher than in healthy cells. For example, healthy cells from day 0 pupal CA contained $22\pm1.5\,nmol\,l^{-1}$ Ca^{2+} (mean±S.E.M.; $N=22$), while the cells judged to be non-viable by morphological criteria contained $191.4\pm11.6\,nmol\,l^{-1}$ Ca^{2+} ($N=7$).

Glands from the different stages dissociated with differing degrees of difficulty, as judged by the duration of exposure to the enzyme solution required to accomplish the dissociation and by the percentage of viable cells obtained after the dissociation. The most difficult glands to dissociate were those from day 4 fifth-instar and day 0 pupae, times when the gland is relatively inactive biosynthetically.

Fig. 1A is a scanning electron micrograph of an intact day 6 CA with the corpus cardiacum still attached; Fig. 1B shows a gland without attached corpus cardiacum, following a 1 h collagenase/elastase treatment but prior to dissociation. With the basal lamina removed, the protrusion of individual CA cells on the surface of the gland can be seen, as can a profusion of fibrous processes, some of which may represent neurosecretory cell axons (Agui *et al.* 1980; O'Brien *et al.* 1988). Fig. 1C is a scanning electron micrograph from a dissociated day 6 CA, demonstrating the morphological difference between an endocrine cell (40–60 μm in diameter) and two contaminating non-endocrine cells, undoubtedly haemocytes (5–20 μm in diameter). The surfaces of the CA cells were generally characterized by a profusion of filopodia, microblebs and other processes. The longer the CA cells remained attached to the coverslip prior to fixation, the longer and more numerous the surface processes became. Cells judged to be non-viable had a noticeably smoother surface.

As a means of assessing the viability of the dissociated cells, the production of JH I acid by dissociated cells from day 6 CA was measured by RIA and compared to the rate of synthesis by intact CA. Two pairs of intact CA produced 0.09 ng of JH I in 2 h, while the dissociated cells from two pairs of CA produced 0.05 ng of JH I in the same time. Prior to the removal of samples of incubation medium for RIA, the total number of cells per incubation of dissociated CA was counted. About half the total number of cells were recovered following dissociation, based on an average of 150 cells per CA, with the loss primarily due to adhesion of cells to the wall of the Pasteur pipette used for dissociation and to breakage of cells from shear stress. Thus, the percentage decrease in synthesis level was approximately equal to the percentage loss of cells.

Basal levels of cytosolic free Ca^{2+} during the fifth stadium

Fig. 2 summarizes the results of measurements of intracellular [Ca^{2+}] in CA cells on selected days during the fifth larval stadium. Interestingly, the two highest

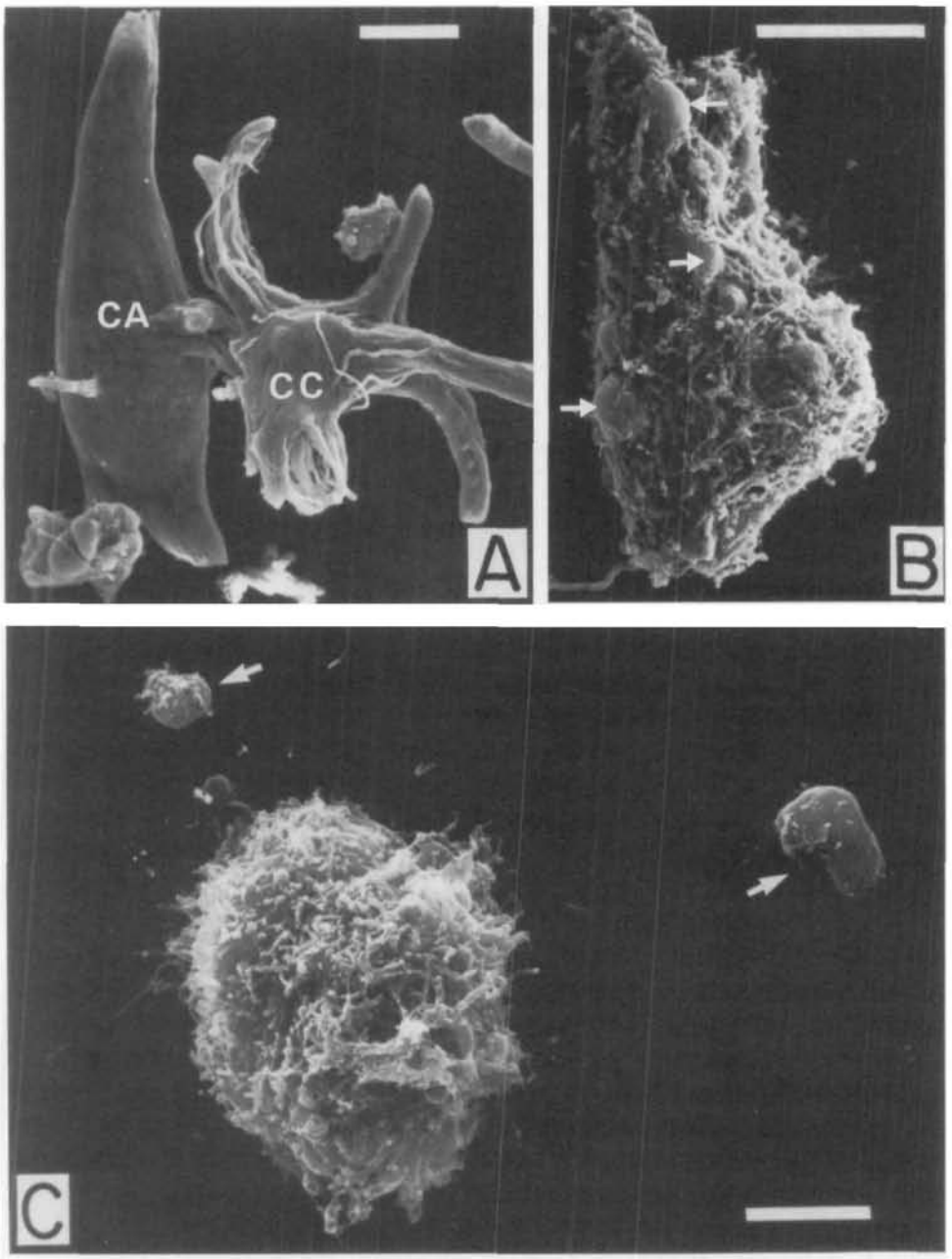


Fig. 1. Scanning electron micrographs of corpus allatum and corpus allatum cells from day 6 fifth-stadium *Manduca sexta* larvae at different steps in the dissociation procedure. (A) Intact retrocerebral complex of corpus allatum (CA) and corpus cardiacum (CC) prior to dissociation. Scale bar, 100 μm. (B) The corpus allatum following collagenase/elastase treatment and rinsing. The basal lamina has been removed and the intercellular matrix has been partially digested, but the gland cells are not dissociated. Arrows, protruding gland cells. Scale bar, 100 μm. (C) Dissociated corpus allatum cell. Cell size range 40–50 μm. Arrows point to pleomorphic haemocytes with a size range of 5–20 μm. Scale bar, 10 μm.

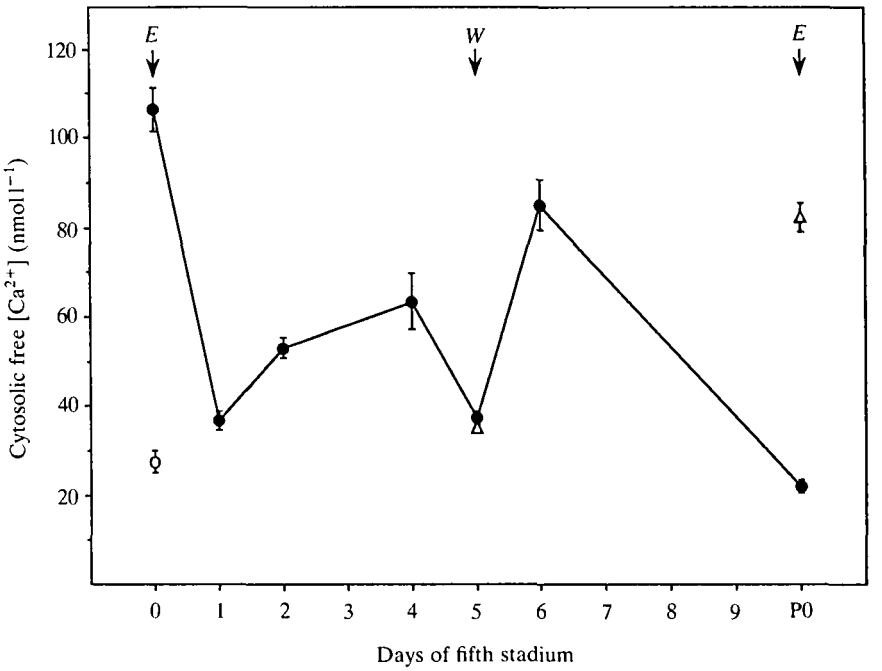


Fig. 2. Basal cytosolic free Ca^{2+} concentrations measured with Fura-2 in corpus allatum cells from *Manduca sexta* larvae on different days during the fifth stadium and at the time of the pupal moult (P0). Filled circles are measurements made with the cells maintained in Grace's medium containing 0.1 mmol l^{-1} Ca^{2+}; the open circle is a measurement made in calcium-free Grace's medium containing 0.1 mmol l^{-1} EGTA; the triangle denotes measurements made 5 h after the initial measurements. *E*, ecdysis; *W*, wandering. Each point is the mean±S.E.M. of values for 13–56 cells.

levels of intracellular Ca^{2+} occur on days 0 and 6, when JH/JH acid biosynthesis by the CA is most active. Nevertheless, there is a statistically significant difference in the concentrations of cytosolic free Ca^{2+} at these two times, with levels at the very beginning of the stadium being higher. The validity of using Fura-2 as a specific indicator for $[Ca^{2+}]$ is demonstrated by the measurement of $[Ca^{2+}]$ in day 0 cells incubated with Fura-2-acetoxymethyl ester plus EGTA, a Ca^{2+}-specific chelator (Fig. 2, open circle). The concentration of cytosolic free Ca^{2+} in these cells in the presence of 0.1 mmol l^{-1} external Ca^{2+} was 106.2±5.0 nmol l^{-1}, and the addition of EGTA decreased $[Ca^{2+}]$ by 74 % to 28±2.4 nmol l^{-1}. The effect of digitonin on the corpus allatum cells indicated a general diffusion of Fura-2 throughout the cells. When gland cells were incubated with 20 μmol l^{-1} digitonin, more than 95 % of the Fura-2 fluorescence was released and no localized fluorescence was observed within the cells, indicating a lack of organellar sequestration.

It is interesting that on day 1, by which time the JH haemolymph titres have begun to decline, cytosolic free $[Ca^{2+}]$ in the CA cells had decreased to nearly the same level obtained by adding EGTA on day 0, 36.8±2.1 nmol l^{-1}. Although the

decrease in intracellular free [Ca^{2+}] on this day is not correlated to a significant decrease in the biosynthetic activity of the gland *in vitro*, the decrease at the end of the stadium (P0, day 0 pupa; 22.0±1.5 nmol l^{-1}) is (Granger *et al.* 1982; Janzen *et al.* 1991).

In an attempt to determine the stability of intracellular free Ca^{2+} levels in the dissociated cells, Ca^{2+} concentrations were determined 5 h after the initial measurements for CA cells from day 5 fifth instars and day 0 pupae (Fig. 2, open triangles). The initial measurements were made between 0.75 and 2.5 h after dissociation, so some of the final determinations were made as long as 7.5 h following dissociation. Clearly, the intracellular [Ca^{2+}] of the day 5 cells was much more stable post-dissociation than that of day 0 pupal cells, which showed an approximately fourfold increase over 5 h, from 22.0±1.5 to 82.2+3.3 nmol l^{-1} (N=18). This may reflect an artefact of the dissociation protocol, since glands from day 0 pupae were among the most difficult to dissociate.

Distribution of cytosolic free Ca^{2+} within the cell

Some differences in the distribution of free Ca^{2+} or in the accessibility of free Ca^{2+} to Fura-2 were noted in cells from different stages, as revealed by the pseudocoloured computer-generated images of untreated CA cells (Fig. 3A–F). Furthermore, these differences appeared to be related to the length of time after a moult. On day 0 of the fifth stadium, when a significant proportion of the cell volume is occupied by the nucleus (Fig. 3A) and cytosolic free [Ca^{2+}] is high, nuclear [Ca^{2+}] is somewhat higher (but less than 50 nmol l^{-1}) than that of the cytosol. On day 1, when the average intracellular free [Ca^{2+}] has dropped significantly, the nuclear concentration remains higher than the cytosolic concentration (Fig. 3B). However, the difference between the two is reduced, and this trend continues on day 2 (Fig. 3C). By day 4, the distribution of free Ca^{2+} is more or less uniform across the cell (Fig. 3D), and this persists through day 5 (not shown) and day 6 (Fig. 3E), when the cytosolic [Ca^{2+}] is again elevated. After pupal ecdysis, the differential between nuclear and cytosolic free Ca^{2+} is once again established (Fig. 3F), with higher nuclear [Ca^{2+}] (again a difference of less than 50 nmol l^{-1}).

The effect of ionomycin on [Ca^{2+}] in cells of the corpus allatum

Treatment of CA cells from day 6 of the fifth stadium with 2 μmol l^{-1} ionomycin revealed an influx of Ca^{2+} within 20 s of exposure to the ionophore (Fig. 4A,B). The subsequent responses of the ionomycin-treated cells fell into one of two categories. In the first, inflowing Ca^{2+} slowly equilibrated across the cytoplasm (Fig. 4C,D), reaching a stable maximum concentration by 5–14 min, which in this case was more than six times the pretreatment levels (50.5 *vs* 370.6 nmol l^{-1}) (Fig. 4). This concentration was still three orders of magnitude below the external [Ca^{2+}] of 0.1 mmol l^{-1}. After 5 min of exposure, [Ca^{2+}] in the nucleus was frequently observed to increase above cytoplasmic levels, with a continued increase for at least 8 min (Fig. 4C,D). A second type of response to ionomycin

Fig. 3. Pseudocoloured ratio images of corpus allatum cells from the fifth larval stadium and early pupal period loaded with Fura-2 and incubated in Grace's medium containing 0.1 mmol l^{-1} Ca^{2+}. (A) Day 0, fifth stadium; (B) day 1; (C) day 2; (D) day 4; (E) day 6; (F) day 0 pupa. The colour spectrum in A corresponds to the concentration range (nmol l^{-1}) of Ca^{2+}. Scale bars, 10 μm.

observed in day 6 CA (not shown) was a three- to fourfold increase in the level of cytosolic free Ca^{2+} within about 2 min, followed by a return to resting levels within another 2 min.

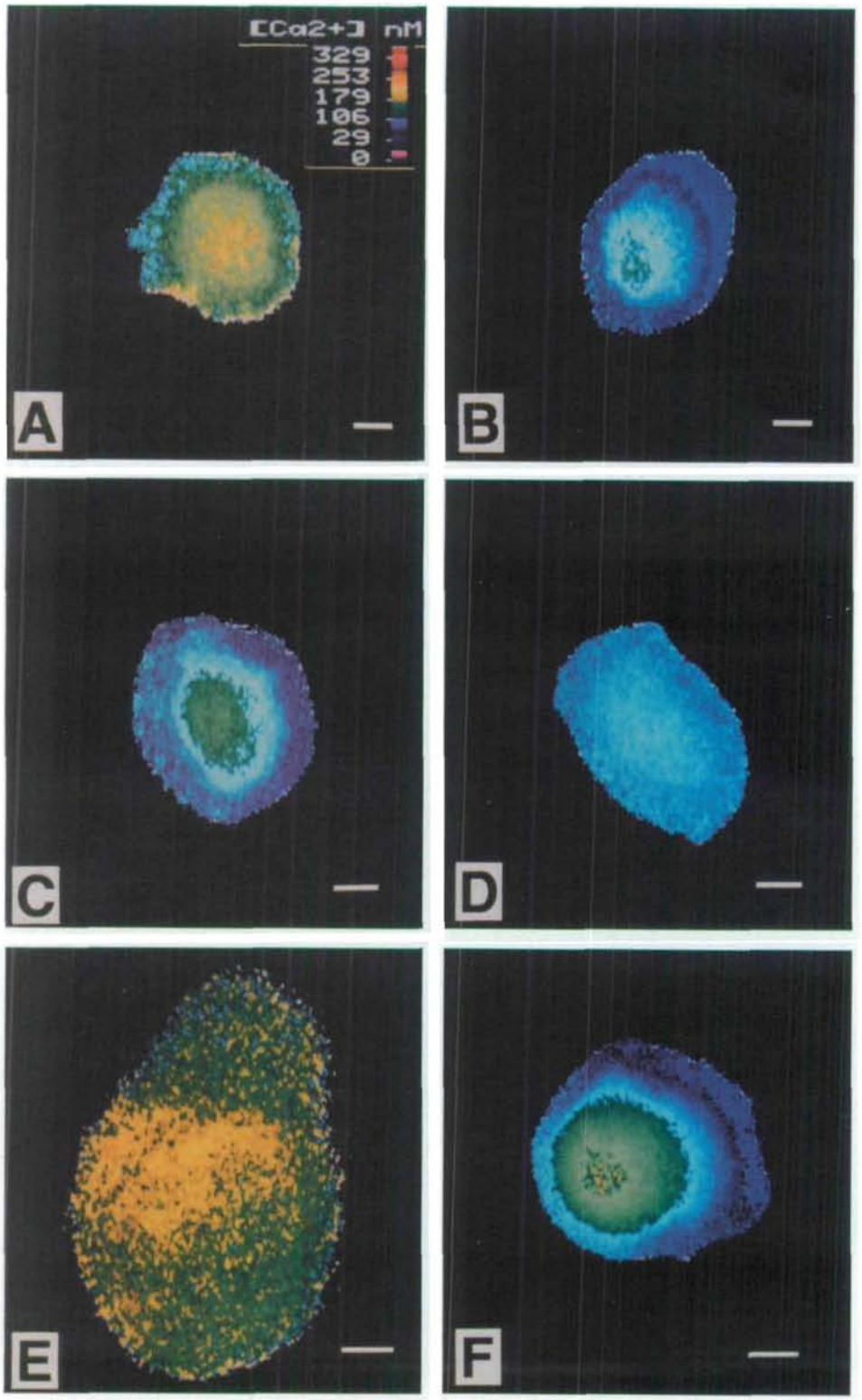
Discussion

Intracellular ionized calcium (Ca^{2+}) has been recognized for many years as an important regulator of a variety of cell functions (Abdel-Latif, 1986; Rasmussen and Rasmussen, 1990), including the production of JH and JH acid by the cells of the insect corpus allatum (Kikukawa *et al.* 1987; Aucoin *et al.* 1987; Allen *et al.* 1992). The corpus allatum of *Manduca sexta* displays a markedly different sensitivity to external Ca^{2+} before and after the time of pupal commitment during the last larval stadium, and it has been proposed that differences in intracellular free [Ca^{2+}] could be the contributing factor (Allen *et al.* 1992).

Evaluation of this possibility required the quantitative measurement of cytosolic free Ca^{2+} concentrations. Since a *Manduca sexta* corpus allatum is made up of fewer than 200 cells, measurements of cytosolic [Ca^{2+}] were only feasible in individual cells, and digitized video microscopy (DVM) provided the capability for such measurements. Corpus allatum cells also offered a particular advantage for this approach since their viability after dissociation can be assessed by measuring their production of JH/JH acid. It was estimated that approximately half of the cells in the enzymatically treated glands were lost during dissociation, and a number of cells theoretically equivalent to that in four glands produced 56 % of the amount of JH I acid produced by the intact glands. This suggests that those cells remaining were, for the most part, viable. These results are thus comparable to those of Chiang *et al.* (1991), who found that their dissociation procedure did not affect the synthesis of JH by *Blatella germanica* CA cells in suspension.

Before the results of the measurements of intracellular calcium levels, $[Ca^{2+}]_i$, can be discussed, a consideration of their validity is necessary; a number of observations support the idea that the quantified Fura-2 fluorescence does in fact represent $[Ca^{2+}]_i$. First, the values for $[Ca^{2+}]_i$ in the corpus allatum cells fall in the range of those for other cells, i.e. nanomolar concentrations (Meldolesi and Pozzan, 1987; Carafoli, 1987). Second, the effect of EGTA is consistent with the fact that $[Ca^{2+}]_i$ is being measured: chelation of extracellular calcium, $[Ca^{2+}]_o$, results in a precipitous reduction in $[Ca^{2+}]_i$. Finally, exposure of the cells to digitonin allows Fura-2 to diffuse uniformly out of the cells, indicating no organellar sequestration.

Measurements of cytosolic free [Ca^{2+}] in corpus allatum cells on different days


[Ca2+] nM
329
253
179
106
29
0
A
B
C
D
E
F

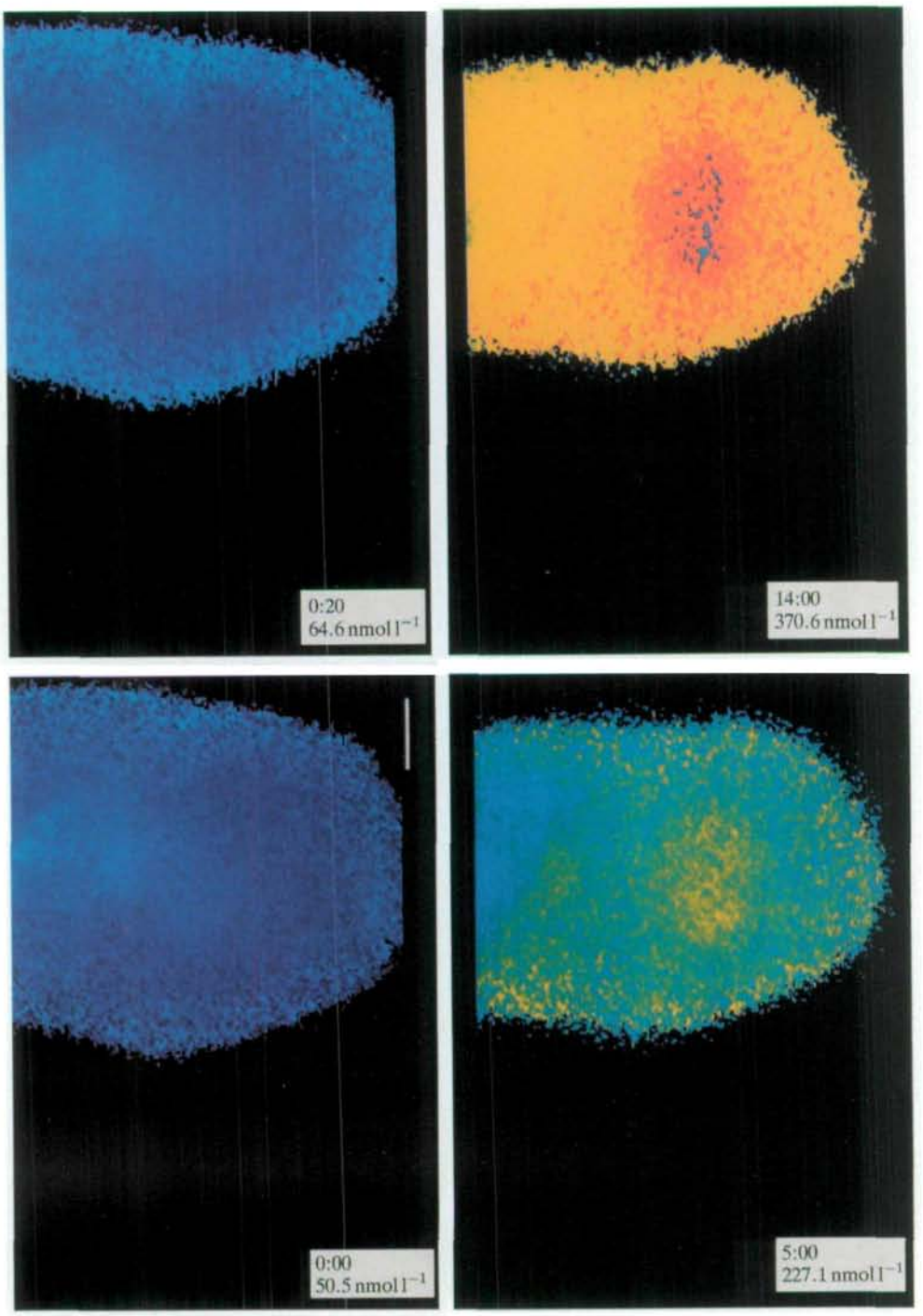

0:20
64.6 nmol l^{-1}
14:00
370.6 nmol l^{-1}
0:00
50.5 nmol l^{-1}
5:00
227.1 nmol l^{-1}

Fig. 4. Pseudocoloured ratio images of a day 6, fifth-stadium corpus allatum cell loaded with Fura-2 and incubated in Grace's medium containing 0.1 mmol l^{-1} Ca^{2+}, with the addition at time zero of 2 μmol l^{-1} ionomycin. Measurements were made at time 0 (0:00), 20 s (0:20), 5 min (5:00) and 14 min (14:00). Areas of lowest free [Ca^{2+}] are blue to purple, with increasing intracellular [Ca^{2+}] represented by changes through the colour spectrum to red. Areas with concentrations exceeding those represented by red are turquoise. Concentrations (nmol l^{-1}) at each time are an average value for the whole cell. Scale bar, 10 μm.

during the last larval stadium reveal that $[Ca^{2+}]_i$ is relatively high for cells from both day 0 and day 6 glands, with the value on day 6 being approximately 80 % of that on day 0. Although the difference between the values for these two days is statistically significant ($P<0.01$), it does not provide compelling evidence that the differential sensitivity of day 0 and day 6 corpora allata to external calcium is due to differences in their cytosolic free [Ca^{2+}]. The difference may be in the types and numbers of calcium channels in the corpus allatum cell membrane or in the accessibility of stored calcium for cellular processes.

A comparison of this titre with that for haemolymph free [Ca^{2+}] during this same period (Allen *et al.* 1992) reveals two other points of interest. First, the 10 000-fold differential between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ maintained by vertebrate cells (Meldolesi and Pozzan, 1987) is also found for these invertebrate cells. Second, changes in $[Ca^{2+}]_i$ during the course of the fifth stadium loosely follow changes in gland activity (Granger *et al.* 1982; Janzen *et al.* 1991), with $[Ca^{2+}]_i$ being highest when the glands are biosynthetically active.

This latter observation suggests that calcium could be a second messenger in the stimulation of gland activity, and indeed early in the last larval stadium (day 0), there is an optimal concentration of 10^{-3}–10^{4} mol l^{-1} extracellular free Ca^{2+} necessary for maximal gland activity (Allen *et al.* 1992). Thus, $[Ca^{2+}]_i$ on day 0 of the last stadium may be optimal for JH synthesis, and the increases in $[Ca^{2+}]_i$ produced by micromolar concentrations of ionophore would sharply decrease synthesis by the day 0 glands. In contrast, corpora allata on day 6, which are insensitive to changes in $[Ca^{2+}]_o$, have a suboptimal $[Ca^{2+}]_i$ and respond to 1 μmol l^{-1} ionomycin with a twofold increase in the rate of JH acid synthesis (Allen *et al.* 1992).

Corresponding with times of high biosynthetic activity and high $[Ca^{2+}]_i$ in corpus allatum cells are high titres of ecdysteroids in the haemolymph of *Manduca sexta* (Bollenbacher, 1988). The steroid hormone progesterone has been shown to stimulate calcium influx in *Xenopus laevis* oocytes (Wasserman *et al.* 1980) and human sperm (Blackmore *et al.* 1990). Thus, it is possible that the higher intracellular Ca^{2+} concentrations on days 0 and 6 are elicited by the ecdysteroid titre and that the ecdysteroid titre could affect JH/JH acid biosynthesis *via* this mechanism.

Several cerebral peptides have been shown to affect *Manduca sexta* corpus allatum activity (Granger and Janzen, 1987; Kataoka *et al.* 1989; Bhaskaran *et al.*

1990). A preliminary examination of the effect on $[Ca^{2+}]_i$ of an HPLC-purified allatostatin, which inhibits JH I/JH I acid synthesis by larval glands (Granger and Janzen, 1987), revealed a slow but steady twofold increase in $[Ca^{2+}]_i$ of day 1 corpus allatum cells over 22 min of observation (C. U. Allen, unpublished data). A comparison of this response to the several-fold, immediate increase that occurs in response to a calcium-mediated signal, such as in porcine smooth muscle cells exposed to platelet-derived growth factor (PDGF) (Herman *et al.* 1987), suggests that Ca^{2+} is not the primary mediator of the allatostatin effect. However, the response to allatostatin is very similar to that of renal epithelial cells to parathyroid hormone, which activates dihydropyridine-sensitive channels responsible for Ca^{2+} entry by recruiting latent channels (Bacskai and Friedman, 1990). The corpus allatum is of epidermal origin and appears to have dihydropyridine-sensitive Ca^{2+} channels (Allen *et al.* 1992); thus, a direct role for calcium in the effect of allatostatin cannot be ruled out.

The concentration of free Ca^{2+} in the nucleus of the corpus allatum cell appears to be somewhat higher than that in the cytosol at the time of a moult, either larval or pupal. Higher levels of free Ca^{2+} have also been found using Fura-2 in the nucleus of enzymatically disaggregated smooth muscle cells (Williams *et al.* 1985). Although not all cell types examined with Fura-2 exhibit a higher $[Ca^{2+}]$ in the nucleus (Williams *et al.* 1985; Herman *et al.* 1987), intranuclear $[Ca^{2+}]$ is usually different from $[Ca^{2+}]$ in the cytoplasm. Furthermore, exposure of cells to a signal molecule, such as PDGF with porcine smooth muscle cells (Herman *et al.* 1987), can elicit changes in intranuclear $[Ca^{2+}]$ that are not necessarily coordinated with those in the cytoplasm. These results suggest that intranuclear free $[Ca^{2+}]$ is regulated by nuclear-membrane-dependent processes and that this may be a general property of all cells. The changes in intranuclear $[Ca^{2+}]$ in the corpus allatum cells as development proceeds suggest that this could be the case here.

In summary, this is the first study to utilize a fluorescent probe in combination with digitized video microscopy for the measurement of $[Ca^{2+}]$ in insect endocrine cells. The determinations of $[Ca^{2+}]_i$ at selected times during the last larval stadium have revealed that the intracellular concentration of calcium is maintained at a level considerably below the calcium concentration in the haemolymph bathing the gland. The highest values for $[Ca^{2+}]_i$ occur on days when the corpora allata are most biosynthetically active. Coupled with the results of a previous study (Allen *et al.* 1992), these findings suggest that, at least early in the last larval stadium, Ca^{2+} alone could act as a second messenger. However, it is generally recognized that the insect corpus allatum engages in a complex and hierarchical array of communications involving both neuropeptides and neurotransmitters (Tobe and Stay, 1985; Thomsen *et al.* 1990), and there are undoubtedly present in the gland several second-messenger systems that could either operate discretely at different times or act coordinatedly at the same time (see Berridge, 1985). Furthermore, calcium is integrally associated with the function of the other second-messenger systems (Abdel-Latif, 1986; Rasmussen and Rasmussen, 1990), as well as with a wide variety of cell processes. Thus, these results lay the groundwork for further

studies on the relationship of calcium to second-messenger systems and cell-specific processes in the corpus allatum.

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