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SHORT COMMUNICATION

CALCIUM-DEPENDENT ACTION POTENTIALS IN THE PROTHORACIC GLAND OF *MANDUCA SEXTA*

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The physiological changes which occur during insect moulting are brought about by the steroid hormone 20-hydroxyecdysone (Riddiford & Truman, 1978). The prohormone ecdysone is secreted by the bilaterally paired prothoracic glands (PGs) in response to prothoracicotropic hormone (PTTH) released from the brain (Bollenbacher, Agui, Granger & Gilbert, 1979). Recently, Smith, Gilbert & Bollenbacher (1985) demonstrated that Ca^{2+} is required for PTTH-stimulated ecdysone synthesis and, consequently, proposed the existence of a hormone-dependent calcium channel associated with the PTTH receptor. In the present study, we show for the first time the existence of voltage-dependent calcium channels in PG cells. Furthermore, we have observed electrical coupling between the cells which appears sufficiently strong to permit the propagation of an action potential throughout the gland in response to stimulation at a single location.

Larvae of the tobacco hornworm, *Manduca sexta*, were reared individually on an artificial diet (Bell & Joachim, 1978) at 26–27°C in a 17:7 L:D photoperiod. Both males and females were used. Specimens were gathered 3 days after the moult into the fifth instar, weighed, and anaesthetized by submersion in water. Individual glands, which appear as Y-shaped sheets of loosely attached translucent cells, were removed and placed in modified Weever's saline (in mmol l^{-1}): NaCl, 6·5; KCl, 33·5; $CaCl_2$, 13·6; $MgCl_2$, 7·6; dextrose, 166·5; $KHCO_3$, 1·25; KH_2PO_4, 1·25 (Weeks & Truman, 1984). Sodium-free saline was made by replacing NaCl with an equimolar amount of choline chloride. Calcium-free saline was made by substituting Mg^{2+} for Ca^{2+}. The gland was pinned out on a Sylgard platform in a perfusion chamber with a solution volume of 0·5 ml. Microelectrodes were pulled from thin-walled, 1·5 mm o.d. capillary tubes and filled with 3 mol l^{-1} KCl. Recordings were made by implanting two electrodes, one for current passing and the other for recording, at various distances from each other. The current-passing microelectrode was broken to a resistance of 5–10 MΩ, while the voltage-recording electrode was broken to a resistance of 10–20 MΩ. The cells were impaled using a combination of negative capacitance oscillation and light tapping. Recordings were displayed on a digital oscilloscope and recorded on FM magnetic tape.

Key words: prothoracic gland, steroid secretion, Ca^{2+} channels, electrophysiology.

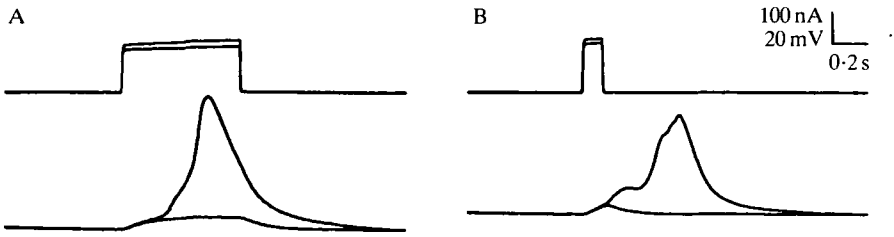

Fig. 1. Resting potential and electrical responses in prothoracic gland cells. (A) With the electrodes separated by approximately 15 cells, responses were elicited by 600 ms current injections of 120 nA and 140 nA. (B) In the same cell, shorter duration (100 ms) current pulses 125 nA and 140 nA in amplitude caused a passive response and a post-stimulation action potential. The small bumps on the main spike are probably due to the asynchronous firing of different cell populations which is discussed in the text.

After an initial period of experimentation with different electrode resistances and tip configurations, resting potentials of −60 to −80 mV were obtained. The preparation was allowed to rest for 5–10 min after electrode insertion to ensure the stability of the recording. With intermittent saline flow, such conditions were maintained for up to 4 h. It became more difficult to obtain stable resting potentials as the distance between impaled cells decreased, probably because of mechanical coupling between the cells.

Action potentials were consistently seen in the gland cells in response to depolarizing current pulses (Fig. 1). The amplitude and duration of the active response showed considerable variability from one preparation to another but, as discussed below, much of this can probably be accounted for by the cable properties of the system and variable placement of the two electrodes. Spike amplitude ranged from 55 to 70 mV (peak voltage: −5 to +8 mV), and duration from 600 to 800 ms. Maximum rate of rise was of the order of 1 mV ms^{-1}. It was notable that spontaneous action potentials were never seen after the initial impalement, nor was it possible to elicit repetitive action potentials in response to prolonged depolarizations. Fatigue of the active response occurred if repetitive depolarizing current pulses were delivered at intervals shorter than approximately 5 s. The apparent input resistance of the cell shown in Fig. 1A was $5 \times 10^4\ \Omega$, but such analyses were of little value since the cable properties of the intact gland make it very difficult to quantify the dispersal of applied current into cells surrounding the stimulating electrode.

To determine which ions were involved in the action potential, experiments were performed using Ca^{2+}-free and Na^+-free solutions. Action potentials were blocked by a brief (4 min) superfusion of the gland with Ca^{2+}-free solution (Fig. 2A), but were virtually unaffected by even prolonged (25 min) exposure to Na^+-free external solution (Fig. 2B). Both Na^+-free and Ca^{2+}-free external solutions caused a moderate (approx. 25 %) reduction in the apparent input resistance of the preparation. If this effect is in fact a result of a decrease in membrane specific resistance, we probably overestimated its magnitude due to the additional effect of increased cable attenuation of signals between the two microelectrodes. This loss of input resistance itself is unlikely to affect action potential waveform substantially

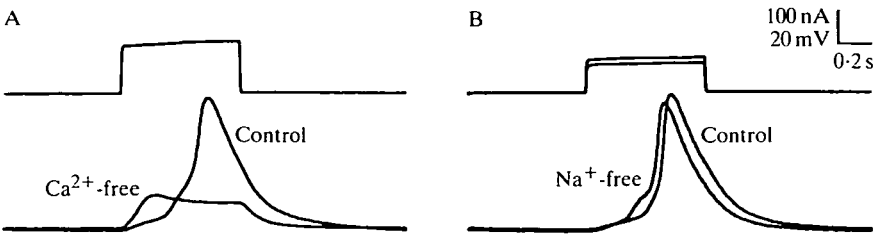

Fig. 2. Ionic dependence of the active response in prothoracic gland cells. (A) In normal saline, a spike was elicited by the injection of 135 nA into a nearby cell. After 4 min in Ca^{2+}-free saline the cell was depolarized above original threshold by current pulses greater than 300 nA, yet no active response was seen. The current trace for the largest pulse in Ca^{2+}-free saline is not displayed here. (B) In a different preparation, 60 nA of current was required to cause an action potential in normal saline. After a 25-min application of Na^{+}-free saline, the cell fired in response to 70 nA of injected current. The magnitude of the active response had decreased 4 mV in the Na^{+}-free solution.

because (1) both Na^{+}-free and Ca^{2+}-free solutions caused the decrease in resistance, while only Ca^{2+}-free solutions blocked the spike generation, and (2) the decrease in input resistance in Ca^{2+}-free solutions was not reversible, whereas full action potential amplitude was restored upon return to normal saline. We conclude from these experiments that most, if not all, of the inward current underlying the action potential in these cells is carried by Ca^{2+}.

To further investigate electrical coupling and action potential propagation between cells, we performed experiments in which the recording electrode was moved progressively closer to a fixed stimulating electrode. Responses were not recorded at the site of stimulation because with our combination of electrode resistance and current amplitude, the bridge circuit was unreliable. In Fig. 3A, depolarizing current pulses were injected into a cell while membrane potential was being recorded five cells distant. The membrane in the cell penetrated with the recording electrode was depolarized by +10 mV during the passive response and +67 mV during the active phase. Fig. 3B depicts subthreshold and active responses in the same gland with the electrodes approximately 20 cells apart. The passive response in the distant

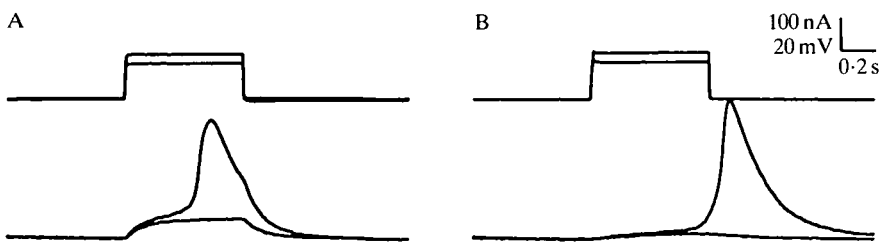

Fig. 3. Demonstration of electrical coupling in prothoracic gland cells. (A) The appearance of both active and passive responses in a cell five cells away from the point of stimulation was seen after current injections of 100 nA and 140 nA. (B) The recording electrode was then implanted 20 cells from the stimulating electrode and pulses of 95 nA and 115 nA caused subthreshold and active responses. Spike amplitude remained constant, but the passive response decreased from +10 mV to +2 mV as electrode separation increased.

cell decreased in comparison to the depolarization seen in the cell nearer the stimulus (+2 mV *vs* +10 mV), whereas the amplitude of the active response was independent of the distance between stimulating and recording sites. Thus, an all-or-none action potential appears to be able to propagate over the entire gland in response to stimulation at a single point. From a number of such experiments, we can get a rough idea of the cable properties of the gland: the steady-state passive response to injected current decreases e-fold for about every nine cells of distance over the gland. Occasionally, we observed mixed spike populations as shown in Fig. 4, apparently the results of asynchrony in the firing of different populations of cells. Based upon chronological appearance, we suggest that the smaller spike is the activation of channels in the stimulated cell, or in a limited area surrounding the point of stimulation, while the larger spike results from concerted firing of the entire gland.

Our results show that a majority, if not all, of the action potential seen in the PG can be attributed to the entry of Ca^{2+} through voltage-activated calcium channels and that the electrical coupling between cells allows the calcium influx to propagate across many, if not all, of the cells in the gland in response to a depolarizing point stimulus.

The presence of calcium-dependent action potentials suggests a possible role for voltage-dependent calcium influx in the PG. The most likely possibility is that calcium entry during electrical activity triggers the exocytotic release of ecdysone, given evidence that the steroid is transported out of PG cells by a vesicular mechanism (Sedlak, Marchione, Devorkin & Davino, 1983; Birkenbeil, 1983). In mammalian endocrine cells, a number of studies have demonstrated a relationship between depolarization, electrical activity and steroid release (e.g. see Lymangrover, Matthews & Saffran, 1982; Higuchi, Kaneko, Abel & Niswender, 1976). Another possibility is that calcium entry stimulates ecdysone synthesis. The calcium dependence of steroid synthesis has been demonstrated in the *Manduca* PG (Smith *et al.* 1985) as well in a variety of vertebrate cells (e.g. adrenal cortex, Lymangrover *et al.* 1982; ovarian cells, Veldhuis & Klase, 1982; Leydig cells, Moger, 1983).

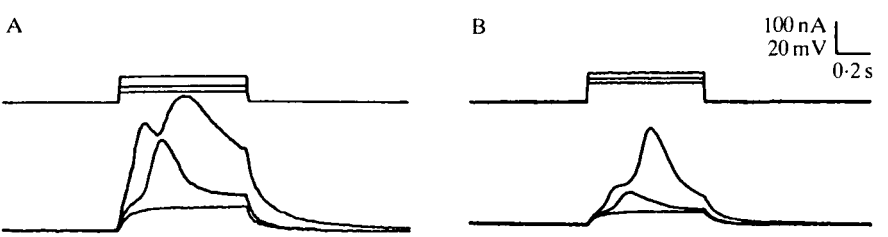

Fig. 4. Appearance of mixed spike populations in electrically stimulated prothoracic gland cells. (A) A lower threshold localized spike is shown with a larger action potential elicited by increased depolarization. The smaller spike was generated by 35 nA of current, while the larger spike occurred after the same cell was stimulated with 60 nA. (B) A similar pulse paradigm in another preparation produced active responses to current injections of 50 nA and 75 nA.

At least two possible mechanisms may operate to depolarize the PG *in vivo*. First, the gland may depolarize in response to PTTH, similar to the vertebrate ACTH–adrenocortical cell system (Lymangrover *et al.* 1982). A second possible effector of depolarization may be nervous stimulation. Although innervation of the PG in *Manduca* has not been demonstrated, neuronal connections have been reported in the PGs of other species (Singh, 1975; Birkenbeil & Agricola, 1980). Recently, Richter & Gersch (1983) presented electrophysiological evidence for nervous control in the PG in *Periplaneta americana* by comparing nervous activity in the prothoracic ganglion with ecdysteroid titres.

We are at present investigating the correlation between electrical activity and steroid release in the PG both *in vivo* and *in vitro*, to try to assess the physiological role of the voltage-dependent calcium channel.

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