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SHORT COMMUNICATION

ISOLATION OF EPIDERMAL CELL PAIRS FROM AN INSECT, *TENEBRIO MOLITOR*, FOR DUAL WHOLE-CELL RECORDING OF LARGE-CONDUCTANCE GAP-JUNCTIONAL CHANNELS

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The segmented insect integument, composed of an epidermal monolayer and its cuticular secretion, is a paradigm for the study of pattern formation during development (Lawrence, 1992). Epidermal activity during development may be coordinated by the transfer of cytoplasmic molecules through cell–cell gap-junctional channels (reviewed in Caveney, 1985). Gap junctions within intact epidermal segments exhibit dynamic changes in their permeability properties during the moult cycle *in vivo* (Caveney, 1978) and with exposure to the developmental hormone 20-hydroxyecdysone *in vitro* (Caveney and Blennerhassett, 1980). In addition, the row of epidermal cells at segment borders has distinct permeability properties creating developmental and communication compartments (Warner and Lawrence, 1982; Blennerhassett and Caveney, 1984). This paper documents a method for isolating epidermal cells that are suitable for dual whole-cell voltage-clamp studies of gap-junctional currents. We have identified a large-conductance gap-junctional channel in cell pairs with octanol-reduced junctional currents. This cell model may be useful for examining mechanisms of gap-junctional channel gating during development.

Abdominal segments of integument were dissected from mid-instar *Tenebrio molitor* larvae (as described by Caveney and Blennerhassett, 1980) and the effects of a series of proteolytic enzymes on the epidermis were examined. The epidermis of mid-instar *Tenebrio molitor* larvae is attached apically to the cuticle and basally to the basal lamina. Following dissection, segments were placed into *Tenebrio molitor* bath saline ('TBS'; in $mmol\,l^{-1}$: 80 NaCl, 43 KCl, 3 $CaCl_2$, 10 $MgCl_2$, 90 sucrose, 10 glucose, 20 Pipes; adjusted to pH6.7 with $1mmol\,l^{-1}$ NaOH) and incubated for 1–60min in TBS containing 0.1–0.4% elastase (type IIA), crude collagenase (type IA), trypsin (type III), pronase E (type XXV), dispase (P3417), hyaluronidase (type IV) or pancreatin (P3292) (Sigma, St Louis, Missouri; stored at $-20^{\circ}C$ as 1% stocks in TBS). The presence of basal lamina was tested for by scraping the epidermis with patch pipettes while observing the cells using phase-contrast on an inverted microscope. Scraping only damaged a narrow lane of

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cells in tissue treated with 0.4% collagenase (10–20min) or 0.1% pronase (1–5min). However, scraping damaged large regions of cells (still connected by an intact basal lamina) in tissue treated with the other enzymes. Also, gigaohm seals were only obtained on the basal plasma membranes of epidermal cells treated with collagenase or pronase. Collagenase digestion of basal lamina contrasts with earlier enzymatic studies of other insect tissues in which elastase removed the basal lamina (Levinson and Bradley, 1984; Koefoed, 1987). Though elastase had little effect on *Tenebrio molitor* epidermal basal lamina, long incubations (0.2% for 30min) loosened the epidermal sheet from the cuticle, suggesting the presence of elastin or elastin-like components in the connective tissue between the epidermis and cuticle.

Attempts to isolate mid-instar cells using combinations of elastase, collagenase, low [Ca^{2+}] and mechanical agitation were unsuccessful. To facilitate dissociation further, cells were then treated at a stage in the moult cycle when both intercellular and epidermal–cuticular contacts are reduced naturally. This occurs during a 4- to 5-day period of the pharate pupal stage just prior to pupal cuticle secretion when the epidermis detaches from the cuticle (apolysis) and a phase of mitosis and cellular rearrangement occurs.

Sternites were excised from 6–10 pharate pupae at eye stages 7–11 (staged according to Stellwaag-Kittler, 1954). Fat body was teased away and the epidermis, loosely bound to the cuticle through muscle attachments, was incubated in 1ml of 0.2–0.4% collagenase (10–20min) or 0.1% pronase (1–5min) in a 15ml Falcon tissue culture tube. This was diluted to 15ml with TBS, gently triturated 1–3 times with a siliconized Pasteur pipette (4 mm diameter opening) and centrifuged at 100 ***g*** (5min). The pellet was resuspended in 15ml of TBS, spun down again, resuspended in 1ml of TBS and plated onto the glass coverslip base of an acrylic superfusion chamber. The cuticle, now cell-free, was removed from the chamber with forceps. Cells were allowed to adhere to the glass for 30–60min and were used within 8–10h. Cell preparation and experimentation were carried out at 24–28˚C.

Small numbers (10–50) of single cells, pairs of cells and larger groups of spherical, phase-bright cells were isolated (Fig. 1A). Pronase was used for patch-clamp experiments because it yielded larger numbers of cells than collagenase. Most cells remained morphologically unchanged for several hours in primary culture; however, some cells extended fine cellular processes and others flattened. Although the cellular processes were difficult to see, cell pairs clearly separated by a small space were often electrically coupled. This isolated-cell preparation may be useful for studying epidermal cell behaviour *in vitro* since spreading and filopodia formation have been observed for epidermal cells in undissociated tissue (Wigglesworth, 1937; Locke, 1987).

Standard whole-cell voltage-clamp techniques were used on single isolated spherical cells and on pairs of cells (Hamill *et al.* 1981). Fire-polished, beeswax-coated borosilicate glass patch pipettes (resistance 5–8 MΩ) were filled with pipette solution (in mmol l^{-1}: 100 KCl, 10 NaCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 EGTA/45 KOH, 20 Pipes, 80 sucrose, 5 Na_2ATP; adjusted to pH6.7 with 1mol l^{-1} KOH) and were attached to the head stages of two EPC-7 (List, Germany) patch-clamp amplifiers. Data were displayed on a four-channel ink-pen chart recorder (Gould, Cleveland) and were also digitized by an IBM

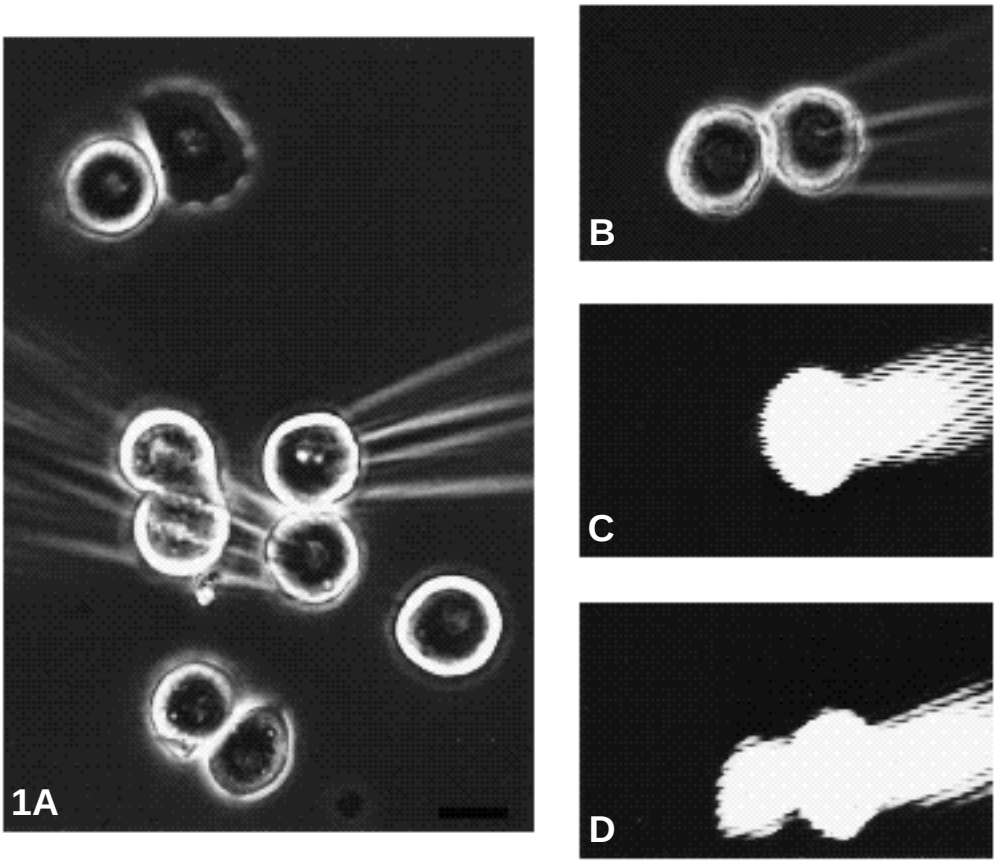

Fig. 1. (A) Phase-contrast micrograph of isolated epidermal cells. Most cells are phase-bright and spherical with large round nuclei. A few cells flatten (note cell in top left corner). Two patch pipettes are shown on a cell pair. (B–D) Phase contrast (B) and epifluorescent (C and D) micrographs of dye-transfer in a cell pair. Carboxyfluorescein (10mmoll^{-1}) was applied through a single patch pipette. (C) was taken 2s and (D) 45s following rupture of the patch of membrane in the pipette. Scale bar, 10 μm.

computer with hard disk storage for off-line analysis using Axotape or pCLAMP (Axon Instruments, Foster City, California). Voltage commands were supplied using pCLAMP for single cells or in-house software (written in Axobasic, Axon Instruments) for cell pairs. All data were filtered for analysis at 0.2 × sample frequency with an eight-pole Bessel analogue filter.

High-resistance seals greater than 10 GΩ were easily obtained on the isolated cells (success rate >90%; N>200) and could often be maintained for up to 1h. The non-junctional resistance of single whole-cell voltage-clamped cells was ohmic [mean input resistance 14±2.6 GΩ (± S.E.M.); N=29], with membrane potentials ranging between −20 and −60mV and access resistances between 15 and 40 MΩ. These high-input and seal resistances are ideal for high-resolution recording of single gap-junctional channel currents using dual whole-cell recording techniques (Neyton and Trautmann, 1985;

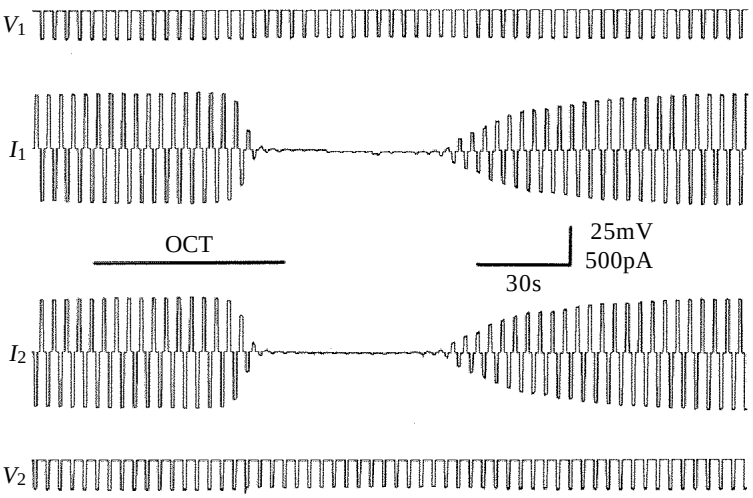

Fig. 2. Junctional currents measured in an epidermal cell pair. Voltage (V_1 and V_2) and current (I_1 and I_2) traces for both cells are shown. Junctional currents were generated by applying 1 s 20mV transjunctional voltage pulses at 0.5Hz in alternating fashion to both cells. All cells studied were voltage-clamped at −50mV. Upward current deflections in the non-pulsed cell represent junctional current while downward deflections in the pulsed cell represent junctional current plus a small non-junctional current. Non-junctional currents in this cell pair were 10–15pA (about 2% of I_J), corresponding to non-junctional membrane resistances of 1.3–2 GΩ. When the bath was perfused continuously (4–6mlmin^{-1}) with 1mmoll^{-1} octanol (OCT) during the horizontal bar, complete uncoupling occurred in 1min, leaving only a small downward non-junctional current in the pulsed cell. Coupling was completely restored in 2 min upon washout of octanol.

Veenstra and DeHaan, 1988). Isolated cell pairs were both electrically coupled and dye-coupled, showing that functional intercellular junctions survive the dissociation procedure. Carboxyfluorescein transfer (Fig. 1B–D) was detected between 44% of cell pairs studied (N=34) and junctional currents (Fig. 2) were detected in 70% of the successful recordings. The mean initial junctional conductance (G_J), calculated by dividing the junctional current by the transjunctional voltage (I_J/V_J), was 9.0±1.2nS (N=41). G_J remained high during most recordings, dropping to 50% in 30min.

Several volatile anaesthetics, lipophilic compounds known to block gap junctions in many cells (Burt and Spray, 1989), were used to demonstrate that these currents were passing through gap junctions and not cytoplasmic bridges. 1-Octanol (1mmoll^{-1}), 1-heptanol (2mmoll^{-1}) and halothane (10mmoll^{-1}) (Sigma) (added directly to TBS and shaken vigorously for 1min just prior to use) rapidly and reversibly uncoupled epidermal cell pairs when perfused into the bath (Fig. 2). The rates of uncoupling were the same for all compounds (at these concentrations) with lower concentrations not fully uncoupling the cells.

Single gap-junctional channel open–close events were recognized in cell pairs with octanol-reduced (0.5–1mmoll^{-1}) junctional currents as spontaneous step-like current transitions which were equal in magnitude but opposite in sign in both cells (Fig. 3). The magnitude of the single-channel currents (ΔI_J) was measured by visually averaging the

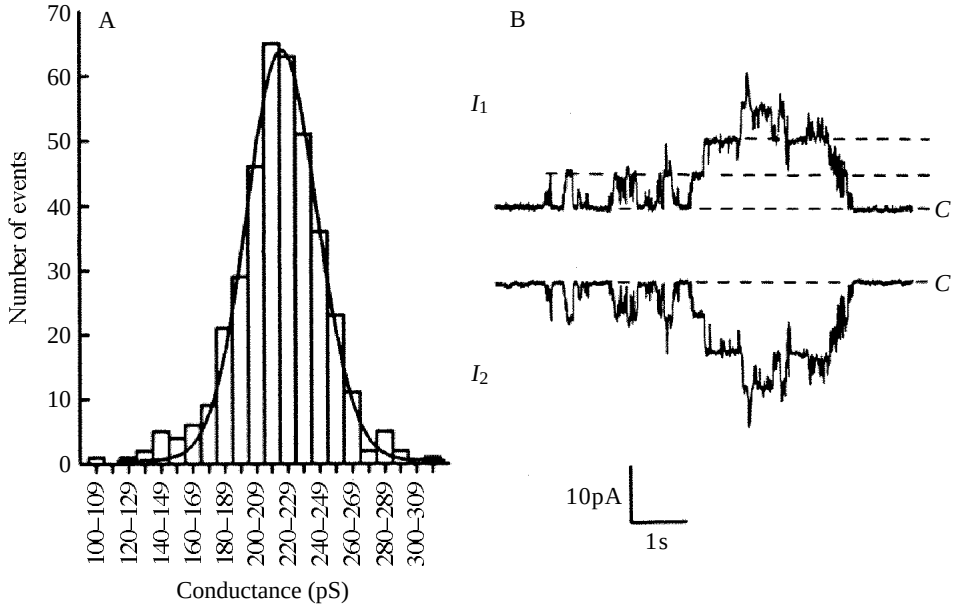

Fig. 3. Single-channel currents in octanol-uncoupled cells. Voltages in both cells were held steady between +10 to −60mV to create transjunctional voltages of 20mV. (A) Frequency histogram of single-channel events from one cell pair best-fitted with a single Gaussian distribution (mean 211.3±24.0pS, S.D.; *N*=383 events). (B) Example of typical single-channel events. Currents from both cells are shown (I_1 and I_2). The fully closed state is denoted at *C*. The dashed lines represent a single-channel conductance of 320pS. Channel flickering often occurred during channel opening or closing, as seen when these channels finally close at the end of the trace. Data for this figure were sampled at 500Hz.

plateau of each transition using the vertical cursors of Axotape (non-plateauing transitions were not included in this analysis). Single-channel conductances ($\gamma_J=\Delta I_J/V_J$) from each cell pair were plotted in frequency histograms (10pS bins) and were fitted to single Gaussian curves using the Gaussian–Newton approximation algorithm of pCLAMP. Values of γ_J (mean ± S.D.) of the best fitting curve in four cell pairs were: 198.9±22.1pS (*N*=366 events), 211.3±24.0pS (*N*=383), 303.7±16.6pS (*N*=333) and 402.9±26.7pS (*N*=209). These single-channel conductances are larger than those reported for most other cell types, which are typically 20–100pS. Conductance values greater than 200pS have only been reported for embryonic chick lens (Miller *et al.* 1992) and embryonic chick cardiac cells (Chen and DeHaan, 1992). The cell–cell variability in conductance (between 198 and 402 pS) in epidermal cells may be explained by the existence of distinct channel types with different values of γ_J. However, only a single γ_J peak occurred in each cell, suggesting that the epidermis may have just one large-conductance gap-junctional channel. Other factors, such as a variable effect of octanol on conductance or, space-clamp problems in filopodia-connected cells, may also explain cell–cell variability in conductance.

Transition times from the fully closed to fully open state for the epidermal channels are slow: in a 2min recording of one cell pair, transition times of 1.6–23.4ms (mean

8.2±0.55ms; *N*=79) were measured (sample frequency 5kHz). Channels remained in the open state from 10ms to more than 1000ms. Other flickering junctional currents with a wide range of conductances between the fully closed and fully open state were also observed. Since these flickering transitions rarely reached a current plateau and often occurred during the opening or closing of the main channel (see Fig. 3B), they may represent a gating behaviour of the main channel (i.e. incomplete openings) and not the existence of many other smaller-conductance channels. A detailed report of voltage-dependent gating and subconductance behaviour of gap-junctional channels in naturally occurring and spontaneously uncoupled epidermal cells is forthcoming (D. Churchill and S. Caveney, in preparation).

In conclusion, we have developed the first method for isolating *Tenebrio molitor* epidermal cells. Using these cells, we have obtained high-resolution single-channel recordings in whole-cell voltage-clamped cell pairs and report a large-conductance channel with slow and occasional flickering open–close transitional gating properties. This epidermal cell preparation will be useful for studying the physiological regulation of gap-junctional channel gating, possibly elucidating the role of gap junctions in patterning and development in insect tissues.

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