Rapid de novo formation of gap junctions between insect hemocytes in vitro: a freeze-fracture, dye-transfer and patch-clamp study

Dennis Churchill, Sheppy Coodin, Richard R. Shivers and Stanley Caveney*
Department of Zoology, University of Western Ontario, London, Ontario, Canada, N6A 5B7
*Author for correspondence

SUMMARY

Gap junctions form between insect hemocytes (blood cells) when they encapsulate foreign objects in the hemocoel (body cavity). In this study we show that hemocytes from cockroach (Periplaneta americana) form gap-junctions rapidly in vitro. Freeze-fracture replicas of hemocyte aggregates fixed 5 minutes after bleeding contain gap-junctional plaques. Dye passage was detected between carboxyfluorescein diacetate-labelled and unlabelled hemocytes within 3 minutes of bleeding, when the cells made contact as they flattened rapidly onto coverslips. When double whole-cell voltage-clamp was used to measure gap-junction formation between cells which were pushed together, electrical coupling was detected within one second of cell-cell contact. To prevent extensive flattening, cells were plated onto lipophorin-coated coverslips. Junctional conductance increased in staircase fashion with steps corresponding to an average single channel conductance of 345 pS. Assuming all channels to have this conductance, the maximal accumulation rate of channels to the growing junction was one channel per second. Junctional currents and dye-coupling were detected in the absence of Ca\(^{2+}\), indicating that involvement of Ca\(^{2+}\)-dependent adhesion molecules is not a prerequisite for gap-junction formation in hemocytes. Hemocytes from distantly related insects (cockroach and moth) form functional gap junctions with each other, suggesting sequence homology among gap-junction proteins in insects. The function of rapid gap-junction formation between hemocytes during encapsulation and wound healing in vivo are discussed.

Key words: gap junctions, patch-clamp, hemocytes, single channels, encapsulation

INTRODUCTION

Insect hemocytes (blood cells) normally circulate freely in the hemocoel. During the cellular immune response to parasites or other large foreign objects in the hemocoel, hemocytes encapsulate the invader by adhering and flattening onto them and onto one another, forming a capsule many cell layers thick (Grimstone et al., 1967; and reviewed by Götz, 1986). Hemocytes form various intercellular junctions during encapsulation, including gap junctions (GJs) (Baerwald, 1975; Norton and Vinson, 1977; Han and Gupta, 1989) which are plasma membrane structures composed of a lattice of cell-to-cell channels that directly connect the cytoplasm of cells within most metazoan tissues. GJs are considered to be involved in electrical and metabolic coupling, and direct intercellular signalling (for reviews, see Loewenstein, 1981; Bennett et al., 1991).

In Periplaneta americana, GJal plaques were present on freeze-fracture replicas of 48-hour-old hemocyte capsules that formed around synthetic implants removed from the hemocoel (Baerwald, 1975), and in a 72-hour-old capsule, hemocytes were shown to be strongly dye- and electrically-coupled (Caveney and Berdan, 1982). If GJs form between hemocytes when they flatten rapidly onto surfaces in vitro then this would be a model system for studying GJ formation. In most other studies of formation, cells with fully-formed GJs have been dispersed and then re-aggregated to allow GJs to re-form (e.g. Preus et al., 1981; Chow and Young, 1987). Hemocytes, however, form GJs de novo when these normally monodispersed cells aggregate.

In this paper we present both structural and functional evidence showing that hemocytes form GJs rapidly, if not instantaneously, in vitro. We show that E-face GJal plaques are present in freeze-fractured hemocyte aggregates soon after bleeding. Furthermore, the formation of functional GJs is confirmed using sensitive assays with excellent time-resolution. A simple carboxyfluorescein diacetate (CFDA) dye-transfer assay was used to demonstrate dye passage between newly contacting hemocytes, within 3 minutes of bleeding. The double whole-cell patch-clamp technique was used to detect clear step-like transitions in the junctional current measurable within 1 second of manipulating hemocytes into contact. These steps, all similar in size, reflect the accretion of single channels as the nascent GJ grows in size.
MATERIALS AND METHODS

Insects
Periplaneta americana (Coodin and Caveney, 1992) and Calpodes ethlius (Locke, 1970) were reared as previously described. Other insects were kept at 25°C and fed on lettuce (Gryllus pennsylvaniae vaticus), ground dog chow mixed with refined sugar (Leucophaea maderae) or cockroaches and mealworms (Mantis religiosa), and water ad libitum.

Freeze-fracture
Cockroaches (Periplaneta americana) were bled through cut antennae into Carlson’s saline (Mitsuhashi, 1982). Hemocytes immediately formed large clumps which were easily manipulated. After the indicated time, cells were fixed in 2.5% glutaraldehyde/0.1 M sodium cacodylate (2 × 30 min), rinsed in 0.1 M sodium cacodylate (× 2) and soaked in 30% glycerol/0.1 M sodium cacodylate (2 × 30 min). Hemocytes were then frozen in liquid nitrogen-cooled Freon-22. Platinum-carbon replicas were made in a Balzers BAF 301 Freeze-Etch Unit (Balzers, Liechtenstein) according to the procedure of Shivers and Brightman (1976). Replicas were examined in a Philips 201 electron microscope operating at an accelerating voltage of 60 kV.

CFDA-assay
In the CFDA-assay, hemocytes were incubated in the nonfluorescent, non-polar reagent CFDA (Molecular Probes, Junction City, Oregon), which enters cells freely. Within the hemocytes CFDA is cleaved by esterases to form the hydrophilic dye, carboxyfluorescein, which is trapped within the cells. Unlabelled hemocytes bled from a second insect were then added and dye transfer between labelled and unlabelled cells monitored. Cockroaches were punctured at the base of a mesothoracic leg and 1 µl of hemolymph was quickly collected into a glass micropipet containing 5 µl of SAL I (125 mM NaCl, 13 mM KCl, 17 mM EDTA-2Na, 10 mM Hepes, 1 mM NaHCO3, adjusted to pH 6.8 with NaOH). This was immediately added to 500 µl of SAL I and spun at 372 g for 30 s. The pellet of cells was resuspended in 500 µl of Perisal (150 mM NaCl, 13 mM KCl, 10 mM Hepes, 1 mM NaHCO3, 2 mM MgCl2, 10 mM EGTA/45 mM KOH (added from a 50 mM EGTA/225 mM KOH stock solution), 10 mM Hepes, pH 7.2 (adjusted with 1 M HCl) and then coated with beeswax. These had resistances of 1-10 MΩ (mean, 3.7 MΩ) and were attached to two independent List L/M EPC-7 patch-clamp amplifiers (Medical Systems Inc., New York). Analogue signals were recorded on a 4-channel, ink-pen chart recorder (model RS 3400; Gould, Cleveland, Ohio) and digitized signals were stored to VCR tape using a pulse code modulator with a sampling frequency (fs) of 22 kHz (model PCM 4/8; Medical Systems, New York), and to the hard disk of an IBM-compatible 386 computer using a 125 kHz TL-1 DMA AD/DA interface (Axon Instruments, Foster City, California). In-house software for double patch-clamp experiments (written in Axobasic; Axon Instruments) was used to control the applied voltages and current acquisition. Data (on playback) were filtered at 0.2 × fs using an 8-pole, low-pass Besel filter (Frequency Devices, Haverhill, Massachusetts).

Preparation of cells for patch-clamp
Glass coverslips were attached to tissue culture dishes that had holes drilled in the bottom, and were coated with 50 µl of SAL II (Perisal without 2 mM CaCl2) containing 50 µg lipophorin /ml for 30 min, and then aspirated dry. Lipophorin was purified as described by Coodin and Caveney (1992).

Cockroach hemolymph (5-10 µl) was collected into a micropipet containing 10 µl of SAL I, and pipetted into 500 µl of SAL I. To remove proteins present in insect plasma which interfere with gigaohm-seal (GG-seal) formation during patch-clamp, hemocytes were pelleted (372 g for 30 s) and the supernatant discarded. Cells were immediately resuspended in 100 µl of SAL I + 100 µl of SAL II, and plated onto lipophorin-coated coverslip dishes containing 1.8 ml Perisal. In experiments in which the effect of the absence of Ca2+ on coupling was examined, hemocyte pellets were resuspended in 200 µl of SAL I and plated onto lipophorin-coated coverslip dishes containing 1.8 ml of SAL I. Cells were kept at 25°C and used within 5-140 min.

Whole-cell recording
Whole-cell patch-clamp methods have been described in detail (Hamill et al., 1981). Borosilicate glass patch-pipets were filled with a solution containing 100 mM KCl, 10 mM NaCl, 1 mM CaCl2, 2 mM MgCl2, 10 mM EGTA/45 mM KOH (added from a 50 mM EGTA/225 mM KOH stock solution), 10 mM Hepes, pH 7.2 (adjusted with 1 M HCl) and then coated with beeswax. These had resistances of 1-10 MΩ (mean, 3.7 MΩ) and were attached to two independent List L/M EPC-7 patch-clamp amplifiers (Medical Systems Inc., New York). Analogue signals were recorded on a 4-channel, ink-pen chart recorder (model RS 3400; Gould, Cleveland, Ohio) and digitized signals were stored to VCR tape using a pulse code modulator with a sampling frequency (fs) of 22 kHz (model PCM 4/8; Medical Systems, New York), and to the hard disk of an IBM-compatible 386 computer using a 125 kHz TL-1 DMA AD/DA interface (Axon Instruments, Foster City, California). In-house software for double patch-clamp experiments (written in Axobasic; Axon Instruments) was used to control the applied voltages and current acquisition. Data (on playback) were filtered at 0.2 × fs using an 8-pole, low-pass Besel filter (Frequency Devices, Haverhill, Massachusetts).

GG-seals were obtained (with mouth suction) on both unflattened cells of a pair 60% of the time (mean seal, 9.5 ± 0.7 GΩ, n = 131; all means expressed as mean ± s.e.m.). Rupture of the patch of membrane in the pipet with suction (break-in) to begin whole-cell recording was successful in both cells 50% of the time. Right after break-in, an example of the capacitive current (measured using −10 mV voltage pulses applied from −30 to −50 mV) was simultaneously recorded from both cells (fs = 42 kHz) for analysis using Clampfit (Axon Instruments). Due to the short time we could record from the cells (mean, 5 ± 0.8 min, n = 67) capacitance and series resistance compensation circuitry was not used. The mean passive membrane properties calculated from the capacitative currents of 38 cells were: whole-cell capacitance, 6.9 ± 0.3 pF; series resistance, 27.8 ± 1.3 MΩ; and non-junctional membrane resistance, 12.5 ± 3.6 GΩ. The mean zero current membrane voltage just after break-in in 58 cells was −33.0 ± 0.8 mV.

Junctional current measurement, begun within a minute of break-in, was performed as described elsewhere (Neyton and Trautmann, 1985; Rook et al., 1988; Veenstra and DeHaan, 1988). For well-coupled cells (many active channels), each cell was held at the same holding potential (−30 to −50 mV) and 1 s, −20 mV voltage pulses were supplied alternately to either cell every 3 s (see Fig. 4) thus creating transjunctional voltages (Vj) alternating in sign. If the junctional current (Ij) is much larger than the non-junctional current of the pulsed-cell (as in these cells) the current...
in the pulsed-cell entering the non-pulsed cell is equal in magnitude but opposite in sign to $I_J$. The junctional conductance ($G_J$) may be approximated as $I_J / V_J$. Due to the series resistance ($R_S$) through the recording pipet, significant errors in the measurement of $G_J$ may arise. Given typical experimental values these errors can be estimated with the equation:

$$R_J^* = R_J - R_{S1} - R_{S2},$$

where $R_J^*$ is the actual junctional resistance, $R_J$ the measured junctional resistance ($R_J = 1 / G_J$), and $R_{S1}$ and $R_{S2}$ the measured series resistance into each cell (Neyton and Trautmann, 1985; Rook et al., 1988; Veenstra and DeHaan, 1988; Cooper et al., 1989). Using typical experimental values for $R_S$ (0.033 GΩ) and $R_J$ (0.033 GΩ for well-coupled hemocytes and 3.33 GΩ for single channels), these errors can be as large as 100% for measurements in well-coupled cells and <2% for measurements of single channel conductance.

High gain measurement of single channel activity during the initial stages of GJ formation or following spontaneous uncoupling was done as above except that the transjunctional voltage was held steady by clamping both cells at constant but different voltages. The non-junctional current noise was smaller at holding voltages negative to $-30$ to $-40$ mV, therefore cells were held between $-30$ mV and $-60$ mV to create transjunctional voltages of $-15$ mV or $-20$ mV. Open-close events of single GJal channels were recognized as spontaneous step-like transitions in the current signals of both cells which were equal in magnitude but opposite in sign. Single channel conductances ($\gamma_J$) were measured for the trace with the larger signal-to-noise ratio. The magnitude of the single channel current ($\Delta I_J$) was measured by visually averaging the plateau of the current transitions using the vertical cursors of Axotape and taking the difference between the baseline and the plateau of one open channel event or between summing events (Axon Instruments). The calculated conductances ($\gamma_J = \Delta I_J / V_J$) of all step-like transitions (events) from all cells were grouped into 10 pS bins and plotted in a frequency histogram which was fitted to a single Gaussian curve using the Gaussian-Newton approximation algorithm of Pstat (Axon Instruments). Data were acquired from tape to disk for analysis by sampling at 500 Hz and filtering at 100 Hz.

RESULTS

Freeze-fracture

Aggregated, freshly-bled, cockroach hemocytes were incubated for various periods of time (0, 5, 10, 20, 60 min) in saline, fixed and then freeze-fractured to determine if GJs would form in vitro, and if so, how rapidly. Loose aggregates of E-face intramembranous particles, representing...
incipient GJal plaques, were visible on freeze-fracture replicas of hemocytes fixed 5 min post-bleeding (Fig. 1A). Larger GJal plaques with more-densely packed intramembranous particles formed within 10 min post-bleeding (Fig. 1B). Occasionally clusters of P-face pits corresponding to E-face plaques were observed (not shown). Clusters of particles, either on the P- or E-fracture face, were not present on hemocytes bled directly into fixative.

**CFDA-assay**

A dye-transfer assay was utilized to demonstrate that hemocytes could form functional GJs in vitro \( (n > 50\) replicate experiments). Typically, dye-transfer was apparent within 9 min of adding the unlabelled cells (Fig. 2). Occasionally, dye-transfer was observed as quickly as 3 min after the addition of unlabelled cells. Often groups of 10 or more unlabelled cells formed around a labelled cell. Dye passed

![Image](image-url)

**Fig. 2.** CFDA-assay demonstrates the rapid de novo formation of GJs between *Periplaneta* hemocytes. Phase contrast (A,B,C) and fluorescence (D,E,F) micrographs were taken 6 min (A and D), 9 min (B and E) and 12 min (C and F) after the addition of unlabelled hemocytes to already-flattened, fluorescently-labelled hemocytes. Transfer of dye from labelled (arrows) to unlabelled (arrowheads) cells is visible within 9 min of adding unlabelled cells. Bar, 10 µm.
to all the cells within such groups, including cells not directly in contact with the originally labelled cell. Small areas of contacting plasma membrane are sufficient for GJs to form as dye-transfer in some runs was observed between cells which were connected by only fine filopodia. On occasion, we observed dye-transfer between cells which subsequently migrated away from one another so that they were no longer in contact, indicating that hemocytes are capable of both forming and breaking GJs in vitro. In the absence of Ca\(^{2+}\) (i.e. in SAL I), dye was also observed to pass between labelled and unlabelled cells \((n = 3\) replicate experiments\), and occurred in under 6 min.

Even though dye-transfer was detected within 3 min of adding unlabelled cells (in Perisal), it was apparent that the actual time required for GJ formation to take place was considerably shorter than could be measured by this assay. This is due to the time required for (1) the unlabelled hemocytes to settle onto the coverslip (0-10 min) and (2) sufficient dye to pass to an unlabelled cell to make it visibly fluorescent. We have used double whole-cell patch-clamp to obtain a more accurate measurement of the time between hemocyte contact and GJ formation.

The CFDA-assay was also used to demonstrate that Periplaneta americana hemocytes can form GJs with hemocytes derived from other insects in vitro. Dye-coupling was detected in under 15 min between labelled P. americana (order Dictyoptera) hemocytes and unlabelled hemocytes from Calpodes ethlius (Lepidoptera) \((n = 4\) replicate experiments\), Leucophaea maderae (Dictyoptera) \((n = 1)\), Gryllus pennsylvaticus (Orthoptera) \((n = 1)\) and Mantis religiosa (Mantodea) \((n = 1)\). P. americana is an excellent insect to use for the CFDA-assay since (1) it has a relatively high blood volume and cell density and (2) its hemocytes retain the dye when labelled with CFDA. Some insects were difficult or impossible to use for this assay since their hemocytes did not retain the dye well (Leucophaea maderae) or hemocytes could not be obtained in sufficient numbers (Drosophila melanogaster).

**Patch-clamping of hemocytes plated on lipophorin-coated coverslips**

Within several minutes of settling out of solution, Periplaneta americana hemocytes adhere and flatten onto uncoated glass coverslips in vitro, either in the presence or absence of Ca\(^{2+}\) (Coodin and Caveney, 1992). Initial attempts to patch-clamp these highly flattened cells proved unsuccessful due to the extreme difficulty of obtaining GΩ-seals and break-ins on the cells. In order to reduce hemocyte flattening, we tried adding 5 µg lipophorin/ml to the bath solution since lipophorin in solution has been shown to inhibit hemocyte adhesion and flattening (Coodin and Caveney, 1992). Although the cells had a more rounded morphology in the presence of lipophorin, it was still difficult to obtain GΩ-seals, possibly due to lipophorin adhering to the patch-pipet. We therefore tried coating coverslips for 30 min with 50 µg lipophorin/ml, and were able to delay hemocyte flattening so that GΩ-seals could be obtained (Fig. 3). Hemocytes remained rounded and loosely adherent for approximately 30 min or more on lipophorin-coated coverslips. Under these conditions it became possible to obtain seals and break-ins for up to 45 min and occasionally up to 1–2 h after plating the cells.

On recording from cell pairs that had already formed contacts, we found that the cells were electrically coupled, confirming that they could form functional GJs in vitro. In 13 such cells G\(_t\) ranged from 2 to 75 nS immediately after break-in (mean, 32.6 ± 23.6 nS). Fig. 4 shows an example of macroscopic coupling in a well-coupled pair of cells, in which the rapid decrease in the junctional current following break-in is evident. This spontaneous uncoupling measured in 7 of these 13 cells (the other 6 cells were lost soon after break-in) had a time to half-maximal conductance of 25–100 s (mean, 45.6 ± 9.5 s) and was presumably due to the washout of cytoplasm that occurs in the whole-cell recording mode (Marty and Neher, 1983). Also, it was difficult to record from the cells for long periods of
time since the cells often migrated away from the pipet or tried to engulf it, thereby rupturing the seal.

Cells were also whole-cell voltage-clamped in the absence of Ca\(^{2+}\) (i.e. in SAL 1). Although these cells were not suitable for high resolution recording of single GJal channel activity since the input resistance was very low (< 25 M\(\Omega\)), estimated junctional conductances of 10-25 nS were measurable between 5 of 8 cell pairs.

To study GJ formation, single cells were whole-cell voltage-clamped, teased with the pipets from their loose substrate attachments and manipulated into contact. In initial attempts, discoid cells were used (Fig. 3B, arrowheads). These cells were difficult to get break-ins on; however in three successful attempts pairs of discoid cells failed to couple \((n = 3)\). In subsequent attempts using spherical, phase-bright cells (Fig. 3B, arrows) break-ins were easily obtained and the GΩ-seal usually remained intact during cell manipulation, thereby allowing the precise moment of contact (within ± 1 s) to be determined. Coupling was detected within seconds of bringing two spherical hemocytes together (Fig. 5A). Before contact the current traces were steady, with relatively low noise. Eight seconds following the moment of contact (arrow), coupling was evident as a series of step-like transitions in both current signals that were similar in magnitude and opposite in sign. These current traces presumably represent the opening of individual GJ channels as successive pairs of hemi-channels link up to form complete intercellular channels. Three representative traces of rapid GJ formation are depicted in Fig. 5B. Trace #1 shows the most rapid formation seen, with one channel forming about 1 sec after cell contact and conductance plateauing at 7.5 nS in about 20 s. Coupling was detected in 8 of 32 such cell pairs. Of the negative results, 9 recordings lasted less than 60 s after cell-cell contact. The mean time for the first event after contact was 16.6 ± 5.3 s (range, 1-55 s, \(n = 8\)). In 6 of these coupled pairs, junctional conductance plateaued in 20–150 s at 1.25–7.8 nS (mean, 3.5 ± 0.9 nS). Junctional conductance then decreased in 80–400 s in the three of these pairs in which the recording lasted long enough such that single channel open-close events could again be resolved (< 1 nS). The junctional conductance in cells brought into contact were never as great as those in cells which had already formed junctions before patch-clamping was performed. On two occasions following formation, cells were successfully pulled apart, to re-establish a steady baseline current, and then manipulated back together and coupling restored.

The mean single channel conductance (\(\gamma_i\)) from 10 cell pairs was 338.7 ± 49.3 pS \((n = 141\) events). This small number of events was due to the short amount of time available for recording single channel events (< 1 min per cell pair) and the long open time of each event (40 msec to 6.4 sec). The frequency histogram for this data is displayed in Fig. 6 and has been best-fit with a single Gaussian distribution of mean \(\gamma_i\), 345.7 pS, and s.d., 47.2.

**DISCUSSION**

In this study we have examined GJ formation in an in vitro model of encapsulation and have found that hemocytes are capable of forming functional GJs very rapidly in vitro. E-faced GJal plaques were seen in freeze-fracture replicas of hemocyte aggregates within 5 min of bleeding. The formation of functional GJs was demonstrated using sensitive assays that detect coupling between cell pairs. Carboxyfluorescein passage was detected between hemocytes within 3 min of being bled and, using the double whole-cell patch-clamp technique, clear step-like transitions in the junctional current were measurable within one second of the cells being placed in contact with one another. These steps, all similar in size, reflect the accretion of single channels as the nascent GJ grows in size.

**CFDA-assay**

CFDA has been used by others to study GJal coupling between mouse blastomeres (Goodall and Johnson, 1982; Kidder et al., 1987). There are several advantages of using CFDA over dye-injection: (1) it is mechanically noninvasive, (2) it allows for an accurate measurement of the time to onset of coupling and (3) many cells can be simultaneously labelled and observed within a single field of view in the microscope. Using CFDA we have demonstrated that hemocytes form functional GJs in vitro within 3 min post-bleeding.

Although hemocyte classification is controversial (Rowley and Ratcliffe, 1981; Brehélin and Zachary, 1986; Gupta, 1991b), there is growing evidence that different hemocyte types interact during cellular immune reactions in insects (Ratcliffe et al., 1984; Huxham and Lackie, 1988; Anggraeni and Ratcliffe, 1991). Dye passage to all cells within large groups of Periplaneta hemocytes during
CFDA-assays suggests that all (or nearly all) types of hemocytes are capable of forming GJs. Epstein and Gilula (1977) demonstrated in dye-injection experiments that functional GJs can form between cells taken from related insect species, but not from species in different orders, in co-culture. We have extended these findings to show that hemocytes from distantly related insect orders (Dictyoptera (cockroach) and Lepidoptera (moth)) form functional GJs. Although no insect GJ gene has yet been sequenced, our results indicate that there is sufficient conservation of (at least one) GJ protein among insects to enable inter-order coupling to occur.

**Fig. 5.** Recordings of single channel activity during GJ formation in cells manipulated into contact. (A) Current traces ($I_1$ and $I_2$) from both cells are shown. Cells held at constant holding potentials of $-55$ and $-40$ mV ($V_J = -15$ mV). Step-like current transitions are evident, starting 8 s after the cells make contact (arrow). One channel opens and then closes and then many channels open as the steps sum on top of each other. The steps are equal in magnitude and opposite in polarity in both cells, as evidence that these current patterns represent GJ channel openings. After about 10 transitions the traces become too noisy to discern distinct steps. The first channel opening had a conductance of about 350 pS (dotted lines on upper trace). Some of the subsequent transitions clearly had smaller conductances: of 7 easily measured events $\gamma_J$ ranged from 180 to 347 pS (mean, 282.9 ± 23.3 pS). $I_2$ appears noisier than $I_1$, probably due to non-junctional channel activity. ($f_S = 50$ Hz). (B) Representative current traces of GJ formation in 3 different cell pairs. The trace from each pair with the best signal-to-noise ratio is shown with current converted to conductance for direct comparison. Holding voltages for each pair were: #1 and #3, $-40$ and $-60$ mV; #2, $-40$ and $-55$ mV. #2 is the upper trace shown in (A). Dots joined by dotted lines are measurements taken from junctional current pulses in response to alternating transjunctional voltage jumps. Pulses were used to monitor junctional current after 20–30 s so that the baseline current of the cells could be monitored. ($f_S = 25$ or 30 Hz).

**Fig. 6.** Frequency histogram of single channel events for 10 cell pairs. Weighted mean conductance was 342.9 ± 10.9 pS ($n = 10$). Mean conductance for each pair (in pS) was: 314.9 ± 6.7 ($n = 27$); 380.0 ± 3.5 ($n = 2$); 333 ($n = 1$); 319.6 ± 8.3 ($n = 13$); 343.1 ± 10.3 ($n = 26$); 383.3 ± 13.6 ($n = 3$); 394.5 ± 6.0 ($n = 15$); 333.3 ± 5.1 ($n = 15$); 282.9 ± 21.6 ($n = 7$); 344 ± 8.8 ($n = 35$). The distribution, which appeared to approximate a normal distribution, was best-fit with a Gaussian curve of mean, $\gamma_J$ 345.7 pS, and s.d., 47.17.

**The electrophysiology of rapid GJ formation**

Several studies have examined GJ formation by measuring electrical coupling between cell pairs manipulated into con-
tact. Characteristically, the onset of GJ formation occurs soon after contact (0.5–15 min) and additional channels are added to the growing junction, one channel at a time (Loewenstein et al., 1978; Chow and Young, 1987; Rook et al., 1988). We have used double whole-cell voltage-clamp to follow de novo formation and have measured the onset of electrical coupling within seconds of hemocyte-hemocyte contact. This explosive increase in junctional conductance (7.5 nS in 20 s) corresponds to an accretion rate of one channel added every second during the early stages of coupling (assuming a channel conductance of 0.35 nS).

The washout of cytoplasm by the pipet solution which occurs following break-in is reported to uncouple (within 20–30 min of break-in) vertebrate cell pairs (e.g. Somogyi and Kolb, 1988). Washout is likely responsible for the rapid drop in conductance (within seconds of break-in) between already-coupled hemocytes, and probably limits whole-cell measurements of GJ formation. The peak conductance seen during formation plateaued at levels well below that seen in already-coupled cells and thereafter declined rapidly. Whether washout-induced uncoupling represents an inhibition of formation per se, or just of channel gating to the open state, may be investigated by adding experimental components to the pipet solution.

The 345 pS single channel conductance in the hemocyte GJ is the largest for any cell type studied to date. Of over 30 vertebrate cell types for which single channel data have been collected, more than 80% of the reported conductances are below 100 pS (e.g. Neyton and Trautmann, 1985; Burt and Spray, 1988; Somogyi and Kolb, 1988; Giaume et al., 1991), with only two in the 200–300 pS range (Chen and DeHaan, 1992; Miller et al., 1992). For other insect tissues 200–400 pS has been found in beetle epidermal cells (Churchill and Caveney, 1993) and 133 pS in a mosquito cell-line (Bukauskas et al., 1991). Insects have generally larger single channel conductances than vertebrate cells, consistent with the larger pore sizes found in insects (Schwarzmann et al., 1981).

Given the broad range of single channel conductances observed (note different step sizes in Fig. 5A), the possibility exists that there are distinct subconductances of a channel, or alternatively, multiple channels with different conductances. Both of these possibilities have been suggested to explain multiple conductances found in GJ channels in other cells (Neyton and Trautmann, 1985; Veenstra and DeHaan, 1988; Somogyi and Kolb, 1988).

**Mechanism of GJ formation**

Hemocytes circulating in vivo probably contain a pool of GJ protein, since hemocyte GJ formation occurs in vitro within 3 min post-bleeding (CFDA experiments). GJ precursors or complete hemi-channels might be dispersed in the plasma membrane of circulating hemocytes and/or stored in the membranes of cytoplasmic vesicles which rapidly fuse with the plasma membrane upon hemocyte activation.

What activates circulating discoid hemocytes to become mutually adhesive and capable of flattening and forming GJs rapidly? In our in vitro model, hemocyte activation might be initiated by wound factors released during bleeding from damaged epidermal cells at the puncture site (Cherbas, 1973). Activation may involve hemocyte aggregation, with sustained cell-to-cell contact sufficient to induce GJ formation. We, however, did not see GJ formation when discoid hemocytes were brought into contact - discoid being the morphology of inactivated circulating cells (Lackie et al., 1985) - suggesting that more than cell-to-cell contact is required to induce GJ formation. Similarly in mammalian lymphocytes, cell contact alone was not sufficient to induce GJ formation; the cells first had to be activated by a soluble mitogen (Hülser and Peters, 1972; Oliveira-Castro et al., 1973).

It has been proposed that in order for the plasma membranes of two cells to come into close enough contact for GJal hemi-channels to dock, other specific cell contacts must form first (Edelman, 1988). The necessity for the binding of Ca²⁺-dependent cell adhesion molecules (cadherins) between cells prior to GJ formation has been demonstrated for mouse epidermal cells (Jongen et al., 1991) and mouse S180 sarcoma cells (Mege et al., 1988), but not for mouse blastomeres (Goodall et al., 1986). Hemocyte GJ formation in the absence of Ca²⁺ does not support the proposal that cadherin-like binding is a prerequisite for GJ formation during hemocyte encapsulation (Gupta, 1991a). The involvement of Ca²⁺-independent cell adhesion molecules in hemocyte adhesion and/or GJ formation, however, cannot be ruled out.

**What role(s) might hemocyte GJ formation play in vivo?**

Extensive GJal coupling between hemocytes within capsules has been demonstrated in both structural (Baerwald, 1975; Norton and Vinson, 1977; Han and Gupta, 1989) and functional studies (Caveney and Berdan, 1982). Since only cells on the outer surface of a capsule have direct access to the hemolymph, junctional coupling could allow for the transfer of required metabolites to hemocytes in the inner layers of a capsule, a function for GJs that has been proposed for other tissues (e.g. Gilula et al., 1978).

GJs may also be involved in the regulation of capsule thickness (Norton and Vinson, 1977). This is supported by three pieces of evidence: (1) soon after new hemocytes contact a capsule they form GJs with it (Baerwald, 1975), (2) the outermost hemocytes of a capsule are able to detach and re-enter the circulation (Grimstone et al., 1967; Götz, 1986) and (3) during CFDA experiments, hemocytes were seen to form GJs and then detach as they moved apart from one another, minutes later. Soon after joining a capsule, hemocytes form GJs through which they could receive a signal responsible for regulating capsule thickness.

Although hemocyte encapsulation is known to occur in many invertebrates, including arthropods, molluscs and annelids (Götz, 1986), only in insects has GJ formation during encapsulation been demonstrated. Determining how widespread a phenomenon hemocyte GJ formation is may not only tell us when the ability for hemocytes to form GJs evolved, but also support or detract from the roles we have proposed for hemocyte GJs.

Hemocytes are also intimately involved in the repair of various insect tissues, including epidermis, Malpighian tubules, nervous system and gut following experimental
wounding (reviewed by Lackie, 1988). During epidermal wound repair, hemocytes form a sheath over the wound upon which epidermal cells then migrate to re-establish, within 24 hours of wounding, a complete monolayer (Rowley and Ratcliffe, 1978). This suggests that cell-to-cell signalling between hemocytes and other tissues might be important during the wound healing process. Since GJs might participate in inter-tissue signalling, we are examining the ability of hemocytes to form GJs with cells of other tissues, such as the epidermis.

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