Voltage-clamp analysis of gap junctions between embryonic muscles in *Drosophila*

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1. Intercellular communication between embryonic muscle fibres was examined in *Drosophila melanogaster*.

2. Injection of fluorescent dye revealed extensive coupling between muscle fibres which form a uniform communicating arrangement of cells without restriction at the segmental borders.

3. Dye transfer was blocked by octanol and membrane depolarization suggesting that it is mediated by gap junctions.

4. Double voltage-clamp experiments from cell pairs *in situ* showed that the ionic coupling is sensitive to the voltage difference between the cytoplasm and the extracellular space (transmembrane voltage, V_{i-o}) as well as between the cells (transjunctional voltage, V_j).

5. In steady-state conditions, the gap conductance (g_j) was maximal for hyperpolarized V_{i-o} and decreased progressively to near zero as V_{i-o} became more positive than −50 mV.

6. Gap conductance decreased from a maximal value as V_j increased either in the positive or negative direction (by depolarizing or hyperpolarizing, respectively, one of the cells from a holding potential of −60 mV). In both cases, g_j asymptotically approached a non-zero residual value which was different for negative and positive V_j (about 20% of the maximal conductance for negative transmembrane potentials and 10% for positive values).

7. Application of octanol (1 mM) resulted in an almost complete and reversible block of g_j.

Gap junctions are distinct domains of the plasma membrane containing arrays of specialized membrane-spanning channels that facilitate the exchange of ions and molecules between adjacent cells. Each channel is formed by a pair of multimeric hemichannels (connexons) arranged in tandem and linked at the extracellular domain. Each connexon is an oligomer composed of six monomers arranged such that an aqueous pore lies at the centre. The large dimensions of the pore allow not only ions (ionic coupling) but also molecules up to 1·5 kDa (chemical coupling) to cross from one cell to another (for review see Bennett, Barrio, Bargiello, Spray, Hertzberg & Sáez, 1991; Finbow & Pitts, 1993).

Intercellular communication mediated by gap junctions is extensively observed in embryonic tissue where it may be important in the transfer of signals having positional, differentiational or growth value to the cells. It is common to find groups of cells that are tightly coupled among themselves, but not at all, or poorly coupled to cells outside the group. These groups have been termed communication compartments and may reflect domains with similar developmental potentialities (Lo & Gilula, 1979). In *Drosophila* and other Diptera, communication compartments have been observed in imaginal discs as well as in epidermis (Warner & Lawrence, 1982; Ruangvoravat & Lo, 1992). In the latter, compartments are restricted to a subfield in each thoracic or abdominal segment.

Drosophila melanogaster is a useful model for studies aimed at understanding the role of gap junctions in development. This organism offers advantages in terms of the availability of diverse physiological, genetic and molecular tools (see Rubin, 1988). As a first step towards a future multidisciplinary approach, the present study is focused on the analysis of gap junctions during myogenesis in wild-type embryos.

Intercellular communication mediated by gap junctions has been demonstrated by injecting fluorescent dyes into one cell and observing their diffusion into adjacent cells. Also, dual microelectrode impalements have been used to

characterize the intercellular flow of ions (junctional current) between the pair of cells recorded. Both chemical and ionic coupling are regulated by a variety of factors including second messengers, pH, calcium ions and voltage (Bennett *et al.* 1991).

Gap junction conductances (g_j) having little or no voltage dependence have been observed in adult tissues such as crayfish septate axons (Johnston & Ramon, 1982), rat lacrimal gland cells (Neyton & Trautmann, 1985), rat ventricular myocytes (White, Spray, de Carvalho, Wittenberg & Bennett, 1985) and earthworm septate axons (Verselis & Brink, 1984).

In other preparations, in which the gap junction conductance is voltage dependent, g_j is modulated by either the transjunctional voltage (V_j; i.e. the voltage difference between both cells), the transmembrane voltage (V_{i-o}; i.e. the voltage difference between the cytoplasm and the extracellular space), or both. In most gap junctions sensitive to V_{i-o}, g_j decreases with depolarized membrane potentials. This is the case for gap junctions from Diptera such as *Chironomus thummi* (Obaid, Socolar & Rose, 1983), *Aedes albopictus* (Bukauskas, Kempf & Weingart, 1992*a*), from salivary glands of *Drosophila melanogaster* (Verselis, Bennett & Bargiello, 1991) and from epidermal cells of *Tenebrio molitor* (Churchill & Caveney, 1993).

The situation is more variable concerning the sensitivity of gap junctions to V_j. The relationship between g_j and V_j may exhibit a clear cut asymmetric rectification as in crayfish (Giaume, Kado & Korn, 1987), leech (Nicholls & Purves, 1972), *Limulus* (Smith & Bauman, 1969) and lamprey (Ringham, 1975). For example, in the crayfish giant motor synapse, current flows more readily from a depolarized axon terminal into the muscle than in the reverse direction (Furshpan & Potter, 1959; Giaume *et al.* 1987). Alternatively, the relationship between g_j and V_j may be non-linear and symmetric as described in blastomeres from embryos of *Ambystoma mexicanum*, *Xenopus laevis* and *Rana pipiens* (Spray, Harris & Bennett, 1981), as well as in all tissues from insects so far studied; gap junctions from this latter group are also sensitive to V_{i-o} (see references above).

In *Drosophila* embryos, the formation of mature larval muscles takes about 12 h. During this period, myoblasts fuse into myotubes that insert at specific sites over the internal face of the cuticle in a stereotyped pattern. Later, myotubes differentiate into functional, contractile larval muscles (Johansen, Halpern & Keshishian, 1989; Bate, 1990). During the early stages of myogenesis (first 5 h) the myotubes are dye coupled; later, this coupling abruptly disappears and each myotube remains a separate entity for the rest of the myogenesis (Johansen *et al.* 1989; Bate, 1990). The uncoupling process appears to coincide with synaptic development and muscle electrogenesis (Broadie & Bate, 1993*b*).

The present article shows that dye coupling between embryonic muscle fibres of *Drosophila* is mediated by gap junctions and that they do not form discrete communication compartments restricted to each segment. Furthermore, the ionic current through these gap junctions is voltage dependent and its conductance is not only a function of the transjunctional potential but also of the absolute transmembrane potential between the coupled cell and the external saline.

METHODS

Drosophila embryos

The wild-type Canton-Special strain was used throughout. Breeding flies were maintained at 25 °C and allowed to lay eggs on traditional agar plates overnight. All experiments were performed on embryos at early stage 16, 12 h 45 min–12 h 55 min after egg laying (Campos-Ortega & Hartenstein, 1985; Broadie & Bate, 1993*b*). In order to determine the embryonic stage, the eggs were dechorionated in commercial bleach and observed with a standard binocular microscope. Embryos at stage 16 were identified by the presence of characteristic disc-like contractions of the gut (see Merritt, Hawken & Whitington, 1993).

Dissection

The flat-embryo preparation was prepared as described previously (Ashburner, 1989). Briefly, one embryo was stuck to double-sided tape under normal saline and extracted from the vitelline membrane using a sharp tungsten needle before being transferred to the recording chamber where it adhered to the glass lower surface. The embryo was then opened along the dorsal mid-line and the ventral nervous system and body-wall muscles were exposed by cleaning with a gentle stream of saline.

Electrophysiological recordings

The preparation was viewed using a Nomarski-optic microscope using a water-immersion objective lens (×40). Two Sylgard-coated patch pipettes (3–5 MΩ) were used to record from pairs of adjacent muscle fibres in the whole-cell configuration. Both cells of the pair were voltage clamped at a holding potential of −60 mV using independent single-electrode voltage-clamp devices (Axon Instruments, Foster City, CA, USA). Single-electrode voltage clamping (SEVC) was used because it avoids the problems associated with electrode series resistance and provides a true measurement of the voltage across the membrane of the cell. Sylgard-coated electrodes permitted the use of a high switching frequency (around 10 kHz) which provided an acceptable resolution of junctional currents. Because the switching frequency was high compared with the value of the membrane time constant (about 3 ms), problems arising from interference between both clamp devices were not encountered. Data were collected during the first 5 min of whole-cell configuration; with longer duration recording sessions the current observed was reduced, probably due to the dialysis of the internal fluid.

To measure the junctional conductance (g_j) between two adjacent cells, a voltage step, V_1, was applied to one of the cells (driven cell), while the other (passive cell) was held at a constant potential, V_2. The junctional potential ($V_j = V_1 - V_2$) generated by the voltage step in the driven cell evoked a junctional current (I_j) measured in the clamping current of the passive cell. I_j was equal, but reversed in polarity, to the

current supplied by the clamp in the passive cell (I_2) to maintain its membrane potential at V_2 (i.e. $I_j = -I_2$). Knowing the junctional current crossing between both cells, the junctional conductance (g_j) was calculated according to the expression $g_j = I_j / V_j$. Because the cells recorded were also in electrical contact with neighbouring muscle fibres at different resting potentials, a non-null holding current was required to maintain the pair of cells at an identical membrane potential ($V_j = 0$). Consequently, when g_j *versus* V_j was analysed, I_j was measured from the holding current required to maintain the passive cell at the holding potential of −60 mV. Similarly, when g_j *versus* V_{i-o} was analysed, I_j was measured from current required to maintain the passive cell at a series of voltage values applied simultaneously to both cells.

Due to the extensive coupled network of muscle fibres, I_j recorded in the passive cell after a voltage step in the driven cell had two components: (1) the current due to changes in the junctional potential between the clamped driven and passive cell (indicated by straight arrows in Fig. 1*A*) and (2) currents generated by changes in junctional potentials between the surrounding cells and the passive cell (indicated by curved arrows in Fig. 1*A*). This latter component would depend on the changes in membrane potential of the surrounding cell caused by the voltage step in the driven cell, since the neighbouring cells are not voltage clamped. The existence of this second component introduces an overestimation in the measurement of g_j. This source of error was minimized by restricting the recordings to cell pairs from adjacent segments (homologous pairs; i.e. cell 6–cell 6) and from the same segment (heterologous pairs; i.e. cell 6–cell 7); two combinations that display a high degree of coupling. The degree of electrical coupling was estimated by calculating the ratio (r) between the junctional current in a neighbouring cell held at a constant membrane potential (−60 mV) and the total current evoked by a voltage step in the driven cell. Figure 1*B* shows that this ratio is between 0·3–0·2 for pairs of cells in direct contact with

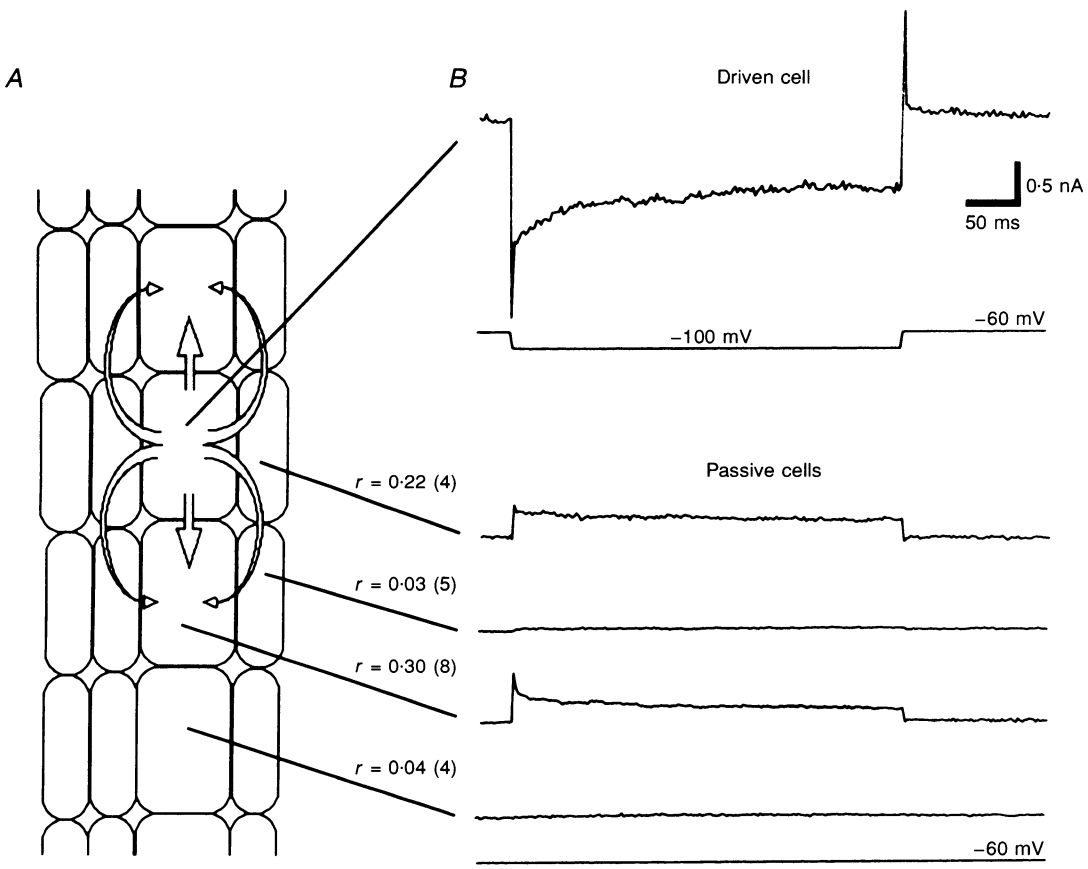

Figure 1. Ionic coupling between pairs of muscle fibres *in situ*
A, schematic diagram of the abdominal muscle layout. The arrows represent possible current flow after a voltage step into a cell. Straight arrows indicate direct current flow from one cell to an adjacent cell; curved arrows indicate indirect current flow involving neighbouring fibres. *B*, representative current records obtained in different neighbouring cells after applying a hyperpolarizing voltage step to the driven cell (to −100 mV from a holding potential of −60 mV) and holding the passive cell at −60 mV. The r value is the average ratio between the junctional current recorded in the passive cell and the total current evoked after a voltage step into the driven cell, the number of pairs recorded are shown in parentheses. Note that only a fraction of the current injected into the driven cell is recorded in the other cells and this fraction drops almost 10-fold for cells not adjacent to the driven cell. Note also that the direction of current flow in the passive cell is always opposite with respect to that in the driven cell.

the driven cell. In contrast, this ratio drops to 0·04–0·03 for cells located at least one cell away from the driven cell. The significant drop in this current ratio (almost 10-fold) in cells not adjacent to the driven cell indicates that second-order cells are only weakly coupled to the driven cell. The weak electrical coupling between secondary cells and the driven cell, plus the low input resistance of the cells (around 30 MΩ) suggests that the resting membrane potential of secondary cells will not change much during a voltage step in the driven cell. As such, no changes in junctional potential will be generated between the passive cell and the unclamped surrounding cells during the voltage step. Consequently, the spurious indirect current component in I_j can be considered negligible, suggesting that most of the junctional current recorded between two adjacent cells reflects a direct current pathway.

Voltage and current traces for each cell were analysed using pCLAMP software (Axon Instruments). Experimental data were fitted using the Boltzmann distribution applied to voltage-dependent systems to describe the equilibrium behaviour of a two-state process assuming that the energy difference between both states is a linear function of the voltage.

Solutions

All recordings were performed at room temperature (approximately 21 °C), the external saline contained (mM): NaCl, 60; sodium citrate, 20; KCl, 2; $MgCl_2$, 4; $CaCl_2$, 5·4; sucrose, 175; Hepes, 5; and the pipette solution contained (mM): KCl, 120; $MgCl_2$, 4; $CaCl_2$, 1; EGTA–KOH, 10; Hepes, 5; the pH of all solutions was adjusted to 7·3. 2-Octanol was prepared as a 100 mM stock solution in dimethylsulphoxide (DMSO), and then added to the external saline to give a final concentration of 1 mM. The resulting concentration of DMSO (1% v/v) had no effect on cell coupling.

Dyefilling

Muscle fibres were recorded with a patch pipette containing 0·05–5% Lucifer Yellow potassium salt (Sigma Chemical Co., St Louis, MO, USA) in internal saline. Lucifer Yellow was iontophoresed into the cell by applying hyperpolarizing current pulses or was allowed to diffuse passively. A high concentration of dye (5%) was used rather than a lower concentration (0·05%) since this allowed for a shorter injection time (2–3 *versus* 15 min). After the period of dye loading, the patch pipette was removed, and the pattern of dye transfer across cells was observed using epifluorescence.

RESULTS

Embryonic muscles at stage 16 are already inserted at specific sites of the epidermis, forming the typical muscle layout of the larva (Johansen *et al.* 1989; Bate, 1990). The mature muscle pattern of each abdominal hemisegment (2–7) is composed of thirty syncytial fibres which are transiently dye coupled during the first stages of myogenesis (Johansen *et al.* 1989; Bate, 1990; Broadie & Bate, 1993*b*). The analysis of cell coupling was focused on the large ventrolateral longitudinal muscle fibres, nos. 6 and 7, in the four most anterior abdominal segments. This report is based on recordings from thirty-seven cell pairs, twenty-eight pairs of no. 6 fibres from adjacent segments (homologous pairs) and nine pairs composed of fibre nos. 6 and 7 from the same segment (heterologous pairs). The fibres had a resting membrane potential ranging between −30 and −63 mV (46 ± 7 mV, mean ± S.D., $n = 51$).

Dye coupling

Dye coupling was observed in all cells recorded. After 2–3 min injections of Lucifer Yellow into a muscle fibre, several adjacent fibres were labelled suggesting extensive coupling between the cells (Fig. 2*A*). Dye transfer consistently occurred into those cells in immediate contact with the injected cell, whether they belonged to the same or an adjacent segment; and dye was also frequently transferred into cells located as far as two segments away. Lucifer Yellow spreading was also observed across the thoracic–abdominal boundary (Fig. 2*A*). Figure 2*B* depicts a schematic view of the abdominal muscle layout in which the results from twenty different dye injections have been pooled. In this figure, the degree of hatching indicates the probability of dye being transferred to a given fibre after injection into the cell indicated by an asterisk. From Fig. 2*B* it is clear that dye transfer did not occur in any preferential direction as all cells around the injected cell were equally stained. Similar results were observed when injections were performed with pipettes containing 0·5 or 0·05% instead of 5% Lucifer Yellow, ruling out the possibility that the pattern of dye transfer observed was an artifact due to the high concentration of dye used. Taken together, these results suggest that the coupling between muscle fibres does not organize the muscle layout into segmental communication compartments. Rather, the coupling is between both inter- and intrasegmentally adjacent cells.

Dye transfer is mediated by gap junctions

Dye transfer between muscle fibres can be mediated by gap junctions and/or cytoplasmic bridges. These two types of intercellular communication can be differentiated on the basis of electrophysiological and pharmacological tests. In particular cytoplasmic bridges, unlike gap junctions, never display complex voltage dependency or pharmacological blocking (Spray, Cherbas, Cherbas, Morales & Carrow, 1989). Therefore, I tested whether dye transfer is affected by octanol, a substance known to block gap junctions (Johnston, Simon & Ramon, 1980), and/or by changes in junctional voltage.

When octanol (1 mM) was applied to the bath at least 2 min prior to recording from a muscle fibre with a Lucifer Yellow-containing electrode, dye diffusion was restricted to the impaled cell (Fig. 3*A*). Bath application of octanol completely blocked dye transfer in all of six embryos tested.

In order to test whether dye transfer was blocked by transjunctional depolarization, one fibre of a cell pair was voltage clamped at −10 mV, prior to injection of the

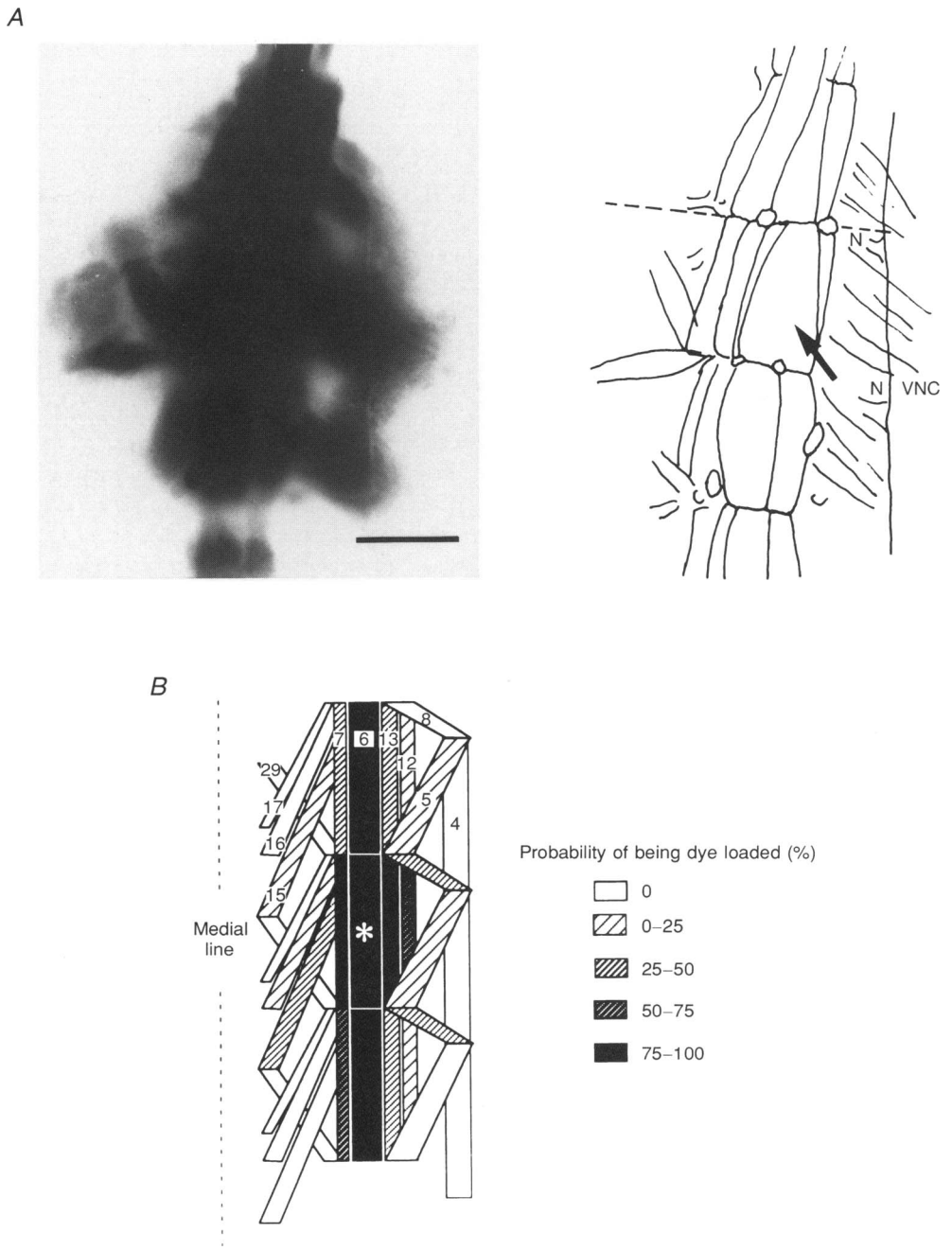

Figure 2. Dye transfer occurs uniformly around a cell injected with Lucifer Yellow
A, microphotograph (left) and schematic diagram (right) of the ventral muscle layout extending across five segmental boundaries from a stage 16 embryo (VNC, ventral nerve cord; N, segmental nerves; the dashed line indicates the thoracic–abdominal boundary; anterior side up in this and next figure). Muscle fibre no. 31 in the first abdominal segment, indicated by an arrow, was injected with dye. Note the extensive dye transfer to muscles of the same segment as well as to those of thoracic and abdominal adjacent segments. Scale bar, 10 μm. *B*, schematic diagram of the ventral and pleural abdominal muscle fibres (fibre types are identified by numbers) summarizing data from twenty different dye-injection experiments. The cell injected with Lucifer Yellow is indicated by an asterisk. The degree of hatching represents the probability of a given cell in the vicinity of the one injected being dye loaded. Note that dye transfer does not occur in any preferential direction and that all the fibres directly in contact with the injected cell were nearly always labelled.

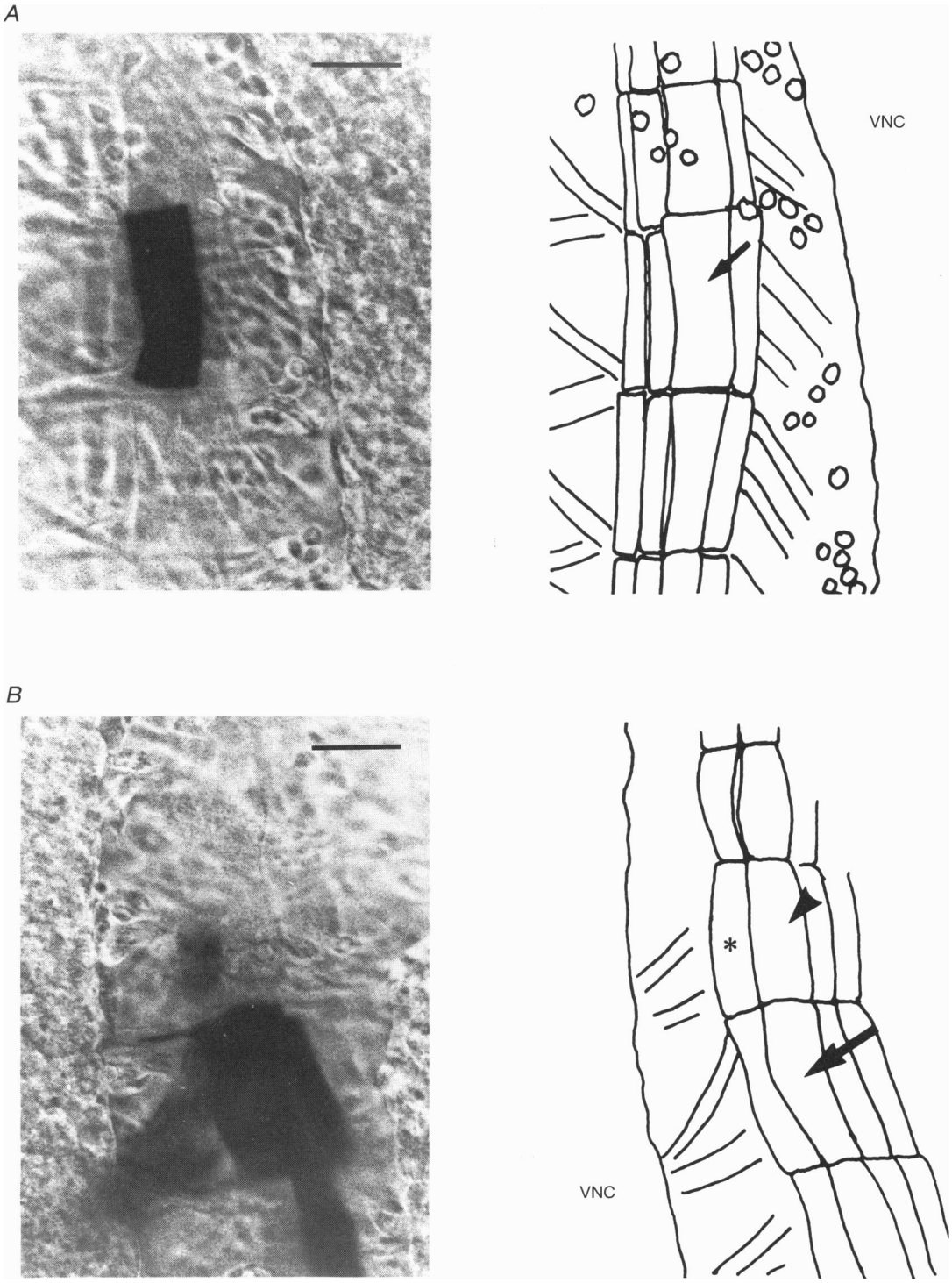

Figure 3. Dye transfer is mediated by gap junctions
Microphotograph (left) and schematic diagram (right) of ventral muscle fibres from an embryo at stage 16. *A*, a no. 6 muscle fibre (arrow) was injected with Lucifer Yellow in the presence of 1 mM octanol. Under these conditions the dye was restricted to the injected fibre suggesting that octanol blocked dye transfer into adjacent cells. *B*, a no. 6 muscle fibre (arrow) was dye injected while a neighbouring fibre from the adjacent segment (arrowhead) was clamped at −10 mV. Note that the depolarized fibre remains unlabelled even though fibre no. 7 from the same segment (asterisk) was passively dye loaded. VNC, ventral nerve cord. Scale bars, 10 μm.

second cell with Lucifer Yellow. Under these conditions, dye transfer occurred normally into all muscle fibres surrounding the cell injected, except for the fibre clamped at a depolarized potential (Fig. 3*B*, arrowhead). The same result was obtained consistently in five similar experiments suggesting that dye transfer was prevented by membrane depolarization. This was confirmed by the observation that dye transfer resumed after the cell returned to a normal resting potential of −60 mV (data not shown).

As dye transfer can be prevented by procedures known to affect gap junctions, these results strongly suggest that gap junctions mediate cellular communication between embryonic muscle fibres of *Drosophila*.

Electrical coupling

In order to analyse the electrical properties of the gap junctions present in embryonic muscle fibres, the ionic-coupling between cells was studied by double recording from adjoining muscle fibres. Both cells were voltage

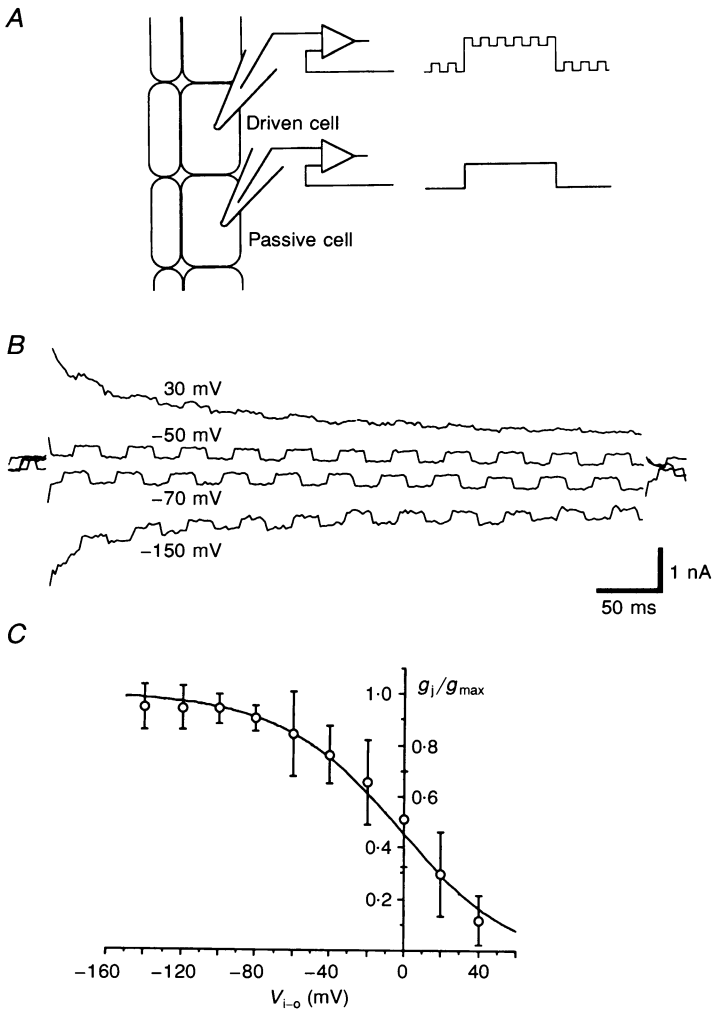

Figure 4. Junctional conductance (g_j) depends on transmembrane voltage (V_{i-o})
A, schematic diagram of the experimental protocol. Identical voltage steps were applied simultaneously to a pair of adjacent cells. To assay g_j, brief voltage pulses were superimposed on the voltage applied to a cell (the driven cell) and the junctional current (I_j) flowing between both cells was measured in the other cell (the passive cell). *B*, junctional current traces recorded in response to four different voltage steps whose values are indicated on the left. The magnitude of the pulse-like current deflections, elicited by the brief voltage pulses applied to the driven cell, are directly related to g_j. Note that hyperpolarizing voltage steps did not produce noticeable changes in g_j, whereas depolarization progressively reduced and nearly blocked g_j. *C*, steady-state g_j–V_{i-o} relationship pooled from three different cell pairs (each point represents the mean ± S.D.). g_j for each cell was normalized to its respective maximal conductance (g_{max}). The data were well fitted by a Boltzmann relation with $A = 0{\cdot}028\ mV^{-1}$ and $V_0 = 26$ mV (see eqn (1)).

clamped at different potentials and the I_j from one cell to the other was measured. Previous studies have shown that voltage-dependent ionic currents are not yet functional at the developmental stage studied (Broadie & Bate, 1993*a*). Under the given experimental conditions, the results obtained in the present study support these findings (see below). This observation suggests that all the current supplied by the clamp system after a voltage command is only due to the intrinsic leak current and the junctional currents to the adjacent fibres.

Dependency on the transmembrane potential, V_{i-o}

The sensitivity of g_j to V_{i-o} was examined by applying a series of pulses ranging from −140 to +40 mV simultaneously to immediately adjacent cells. A pulse duration of 450 ms was sufficient for g_j to reach a steady state. Junctional conductance was calculated by superimposing small, brief depolarizing steps (10 mV, 25 ms at 20 Hz) on the voltage applied to the driven cell (Fig. 4*A*), and measuring the resultant I_j in the other cell, the passive cell. Junctional conductance for each V_{i-o} was calculated by the expression: $g_j = I_j/10$ mV. The brief voltage steps applied to the driven cell were small enough that the contribution of the transjunctional potential to g_j can be considered negligible.

The voltage commands applied to both cells generated transjunctional potentials with adjacent cells of the muscle layout. This potential difference induced in each cell a transient current (Fig. 4*B*) that will be described in detail in the next section. Superimposed on this current, brief current deflections were observed in the passive cell in response to the 10 mV voltage steps applied to the driven cell (Fig. 4*B*). The amplitude of these current deflections (I_j) was directly related to the junctional conductance between both cells. From Fig. 4*B*, it is clear that I_j was reduced when both cells were simultaneously depolarized but was not significantly affected by hyperpolarized V_{i-o} values. The asymmetric relationship between junctional conductance and V_{i-o} is shown in Fig. 4*C*. Data from two homologous and one heterologous cell pair, analysed in detail, have been pooled and normalized to the maximal conductance. At negative V_{i-o} values below −70 mV, g_j remained almost constant, whereas at V_{i-o} values positive to −50 mV, the conductance progressively decreased towards zero. To model the falling phase of the g_j–V_j relationship, two assumptions were made. First, that

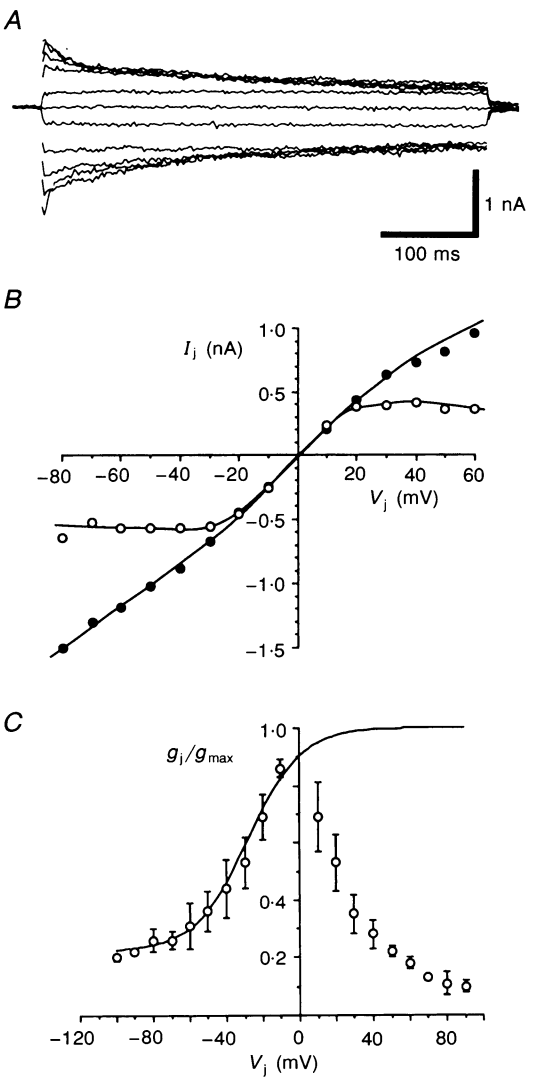

Figure 5. Junctional conductance (g_j) depends on transjunctional voltage (V_j)
Both cells of a coupled pair were held at −60 mV, one cell was stepped to various potentials and the evoked I_j values were measured in the other cell. *A*, superimposed I_j traces evoked by V_j steps from 90 to −90 mV in 20 mV increments reading from the uppermost trace downwards. Note that I_j, was constant for small V_j but inactivated to a steady-state value in response to large depolarizing or hyperpolarizing V_j. *B*, I_j–V_j relationship for the experiment shown in *A*. Both the early (instantaneous; ●) and late (steady-state; ○) current amplitudes are plotted *versus* V_j. The instantaneous values follow an almost linear relationship while the steady-state values closely follow the instantaneous plot for small V_j, but deviate substantially for large V_j, revealing a strong voltage dependency. *C*, steady-state g_j–V_j relationship. Each measurement represents the mean ± S.D. from six different experiments. g_j for each cell pair was normalized to its respective maximal conductance (g_{max}). Note that the residual conductance is lower for depolarizing than for hyperpolarizing V_j. The values obtained at hyperpolarizing V_j were well fitted by a Boltzmann relation with $A = -0{\cdot}07$ mV^{-1} and $V_0 = -28$ mV (see eqn (2)).

coupling is mediated by gap junction channels composed of two similar and independent hemichannels disposed in series, each having voltage-sensitive elements. Second, that in steady-state conditions, the hemichannel can display either a high or a low conductance state and the energy controlling this transition is a linear function of V_{i-o}. Under these conditions, the relationship between g_j and V_{i-o} would follow the Boltzmann equation given by Verselis *et al.* 1991:

$$\frac{g_j}{g_{max}} = \frac{1}{\{1 + \exp[A(V_{i-o} - V_0)]\}^2}, \quad (1)$$

where g_{max} is the maximum conductance; V_0 is a voltage reference equal to the voltage at which g_j is 25% of g_{max} and A is a constant related to the degree of voltage sensitivity. Maximum conductance was estimated from the maximum current obtained at negative V_{i-o}. The continuous line in Fig. 4*C* corresponds to the best-fit curve predicted from eqn (1) using values for parameters A and V_0 of 0·028 mV^{-1} and 26 mV, respectively. These parameters were obtained after linearization of eqn (1), assuming a g_{max} value of 26 and 11 nS for the two homologous pairs and 9 nS for the heterologous pair.

Dependency on the transjunctional potential, V_j

In order to study the sensitivity of g_j to V_j both cells were clamped at a holding potential of −60 mV. Junctional currents were then evoked by delivering voltage steps to the driven cell (V_1) while maintaining the passive cell at the holding potential. Figure 5*A* shows superimposed junctional currents evoked by a series of hyperpolarizing and depolarizing V_j. Regardless of the polarity, I_j exhibited an initial, almost instantaneous peak, that slowly decayed to a residual steady-state level. This time-dependent relaxation was a function of the transjunctional potential and was nearly absent for V_j within ±20 mV. The voltage and time dependency of g_j is observed in the current–voltage plots of Fig. 5*B* where the instant and steady-state current amplitudes were plotted *versus* V_j. For voltage steps between 0 and ±20 mV, both the instantaneous and steady-state current amplitudes were similar suggesting that in this range there was no time-dependent relaxation. However, over ±20 mV, the instantaneous current followed an approximately linear ohmic relationship with respect to V_j while the steady-state current reached a plateau, showing the development of a time-dependent relaxation. Moreover, the steady-state I–V relationship was not symmetric since I_js elicited by depolarizing and hyperpolarizing steps of equivalent amplitude were different in magnitude.

Comparable voltage dependency of the junctional current was obtained when the role of the cells was reversed, i.e. when voltage steps were applied to the formerly passive cell and the formerly driven cell was held at a constant membrane potential. These results show that, with respect to electrical coupling, no. 6 muscle fibres are indistinguishable from each other. This was also the case for electrical coupling between heterologous pairs of muscle fibres.

Figure 5*C* shows g_j, measured at the end of the pulse (steady state), as a function of V_j for six cell pairs in which detailed analyses were performed (4 homologous and 2 heterologous pairs), g_j was maximal when V_j was near zero (i.e. when both cells were maintained at the same holding potential) and the steady-state conductance decreased sigmoidally as the membrane potential of the driven cell moved in the depolarizing or hyperpolarizing direction from the resting membrane potential (−60 mV), positive and negative V_j, respectively. At resting membrane potential, the maximal conductance in the six cells studied was: 29, 18, 11 and 8 nS for the homologous pairs, and 50 and 15 nS for the heterologous pairs. In both cases, g_j asymptotically approached a non-zero value that was approximately 20% of g_{max} for negative V_j and 10% for positive V_j. The g_j–V_j relationship was therefore not strictly symmetric around zero, probably as a consequence of an interaction between V_{i-o} and V_j on the voltage sensitivity of the gap channels. Depolarization of one cell not only affects g_j due to the increase in V_j but also due to V_{i-o}. Because of a possible interaction between V_j and V_{i-o} on g_j when the driven cell was depolarized, I have only attempted to model the conductance data obtained at negative V_j. It was assumed that the distribution of open channels as a function of V_j also follows a Boltzmann relationship. The conductance values were fitted using the following expression (Spray *et al.* 1981):

$$g_j = \left\{\frac{g_{max} - g_{min}}{1 + \exp[-A(V_j - V_0)]}\right\} + g_{min}, \quad (2)$$

in which g_{min} and g_{max} are the minimum and maximum conductances, respectively. V_0 is a voltage reference equal to the voltage at which g_j is half of g_{max} and A is defined as in eqn (1). Maximum conductance was estimated by successive approximation (see Spray *et al.* 1981). The continuous line in Fig. 5*C* corresponds to the best-fit curve for the g_j–V_j relationship predicted from eqn (2). The optimal values of the parameters A and V_0 were −0·069 mV^{-1} and −27·8 mV, respectively, assuming $g_{min}/g_{max} = 0{\cdot}22$. Similar parameters were obtained when both cells were held at a more hyperpolarized holding potential (−80 mV; data not shown).

Octanol blocks electrical coupling

In many cell types, halothane and several higher alcohols including heptanol and octanol reversibly block gap junction-mediated ion coupling (Johnston *et al.* 1980; Burt & Spray, 1989). As octanol proved effective in blocking dye transfer (Fig. 3), it was of interest to determine whether this effect was accompanied by inhibition of electrical coupling. Pairs of adjacent muscle fibres were voltage

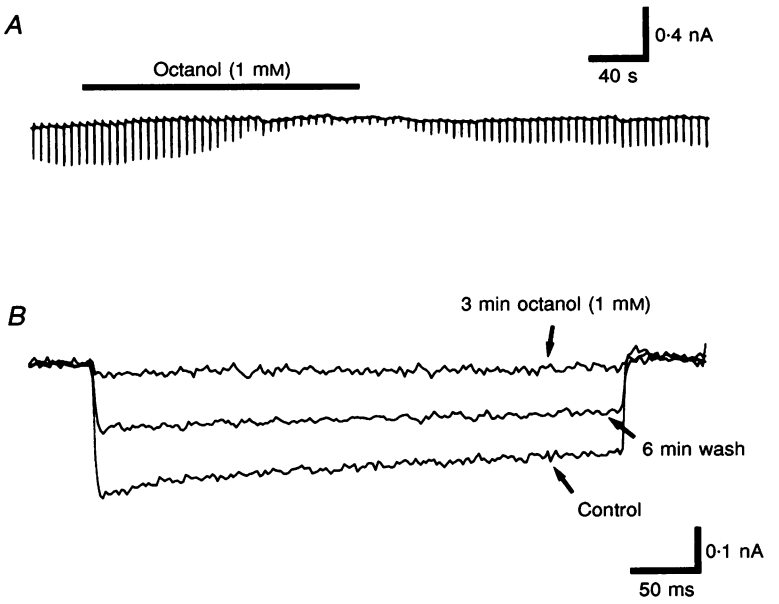

Figure 6. Octanol reversibly blocks ionic coupling
A, hyperpolarizing voltage steps, −40 mV in amplitude, were applied repetitively to one cell and the associated junctional currents were recorded in the second cell before, during and after bath application of octanol (1 mM for 3 min). *B*, representative traces from the same experiment as shown in *A* displayed with a faster time base. Note that the effect of octanol was only partially reversible.

clamped at −60 mV; repetitive hyperpolarizing voltage commands, −40 mV in amplitude, were applied to one cell and I_j was recorded in the other cell (Fig. 6). After a 3 min bath application of octanol (1 mM) I_j was reduced in all four cell pairs tested. With brief applications of octanol, as demonstrated in Fig. 6, this observed reduction was reversed after 10 min in control solution; the fact that the recovery was only partial is probably due to a run-down of the I_j amplitude, commonly observed in long duration experiments. Longer applications or higher concentrations of octanol (2 mM) induced a complete, but irreversible, blockade of junctional currents. Under these conditions, the current–voltage relationship obtained from either the driven or the passive cell was linear, i.e. no voltage-dependent currents were apparent (not shown).

DISCUSSION

The results presented here show that *Drosophila* embryonic muscle fibres are electrically and dye coupled in a voltage-dependent manner. Dye and electrical coupling appear to be mediated by the same molecular structure as they were abolished by the same pharmacological and electrophysiological manipulations. Both gap junctions and cytoplasmic bridges can mediate intercellular communication. These intercellular communication pathways were originally defined based on morphological criteria. However, it is now possible to differentiate between them using electrophysiological procedures (Bukauskas *et al.* 1992*a*; Bukauskas, Kempf & Weingart, 1992*b*). In the muscular system of *Drosophila*, the coupling seems to be mediated by gap junctions. In fact, only complex devices such as channels could account for the pharmacological and complex voltage-dependent properties observed in this system. Cytoplasmic bridges, the presence of which has been documented in undifferentiated cell lines (Flagg-Newton, Dahl & Loewenstein, 1981; Spray *et al.* 1989), are not blocked by octanol or other related compounds and never display non-linear voltage-dependent characteristics unlike gap junctions. Moreover, similar voltage-dependent currents have been observed when isolated gap junctions have been expressed in *Xenopus* oocytes or incorporated into artificial membranes (Barrio *et al.* 1991; Mazet, Jarry, Gros & Mazet, 1992). Thus, the evidence strongly suggests that gap junctions are present in embryonic muscle fibres of *Drosophila* and that they mediate dye and electrical coupling between these cells. Further electronmicroscopic studies and serial reconstructions of the insertion points of the fibres are necessary to reconcile information on coupling pathways and the attachment of the muscle fibres to the intersegmental cuticular apodeme.

Cell–cell communication through gap junctions is common in embryonic tissue (Guthrie & Gilula, 1989; Warner, 1992; Dermietzel & Spray, 1993); where temporal and spatial variations in the degree of coupling frequently

segregate the tissue into communication compartments (Lo & Gilula, 1979). In *Drosophila*, communication compartments have been described both in wing imaginal discs and epidermis of larvae (Warner & Lawrence, 1982; Ruangvoravat & Lo, 1992). Since no preferential direction of dye transfer was ever observed, it would appear that the muscular system is not arranged into communication compartments. Rather, the muscle fibres form a continuous lattice of electrically and dye-coupled cells until stage 16. However, it is not known whether uncoupling, which occurs shortly after the embryonic stage studied (Johansen *et al.* 1989; Bate, 1990; Broadie & Bate, 1993*a*), follows some preferential direction that might provide a certain degree of compartmentalization. In any case, the rapidity of the uncoupling process (i.e. minutes), makes it unlikely that such compartmentalization, if it exists, would play any important role in the muscle development.

Previous studies (Johansen *et al.* 1989; Bate, 1990; Broadie & Bate, 1993*a*) have not reported the extensive degree of dye coupling documented in this report. This inconsistency may be due to slight differences in the developmental age of the embryos used. In fact, at the embryonic stage studied, embryos can present important variations in the degree of electrical coupling since at this stage the normal uncoupling process begins. Another possibility which would explain the failure to observe extensive dye coupling in the previous reports can be found in the different methods used to inject the dye into the cells. Some studies employed classic microelectrode impalements to inject the dye (Johansen *et al.* 1989), probably depolarizing the cell and subsequently closing the gap junctions. Others utilized semi-fixed tissues (Bate, 1990), probably affecting cell uncoupling through changes in cell metabolism. It is well known that gap junctions are sensitive to pH and various second messengers (Bennett *et al.* 1991).

Electrical coupling between multiple cells is a limitation in the study of gap-junction mediated coupling in an intact system. In this particular system, there is evidence that the junctional properties reported here are similar to those expected from studies involving isolated cell pairs. For example, the membrane potential of the cells between which the indirect current flows (second-order cells) seem to be unaffected by a voltage step applied in a fibre one cell away. Therefore, the junctional current evoked in a passive cell, by a voltage step in an adjacent driven cell, will not be contaminated by spurious current generated by possible changes in the junctional potential between the passive cell and its surrounding cells.

The dependency of g_j on V_j and V_{i-o} was fitted assuming a Boltzmann model. Because of the intrinsic limitations of the preparation already described, quantitative analysis required to distinguish between the various arrangements of hemichannels proposed for the gap-junction channel structure was not attempted. Indeed, the data were fitted assuming that *Drosophila* embryonic gap channels behave as if they are composed of two similar hemichannels disposed in tandem, each of them carrying one gate sensitive to the intercellular voltage difference (V_j) and another gate sensitive to the voltage drop between the lumen of the channel and the external medium (V_{i-o}). This assumption allowed for a comparison with data obtained from other preparations.

The junctional channels in embryonic muscle fibres were sensitive to both V_{i-o} and V_j. At a steady state, the g_j, remained almost constant for V_{i-o} values more negative than the holding potential (−60 mV) but declined progressively for more positive V_{i-o} values. An almost complete blockade of the junctional current was observed at V_{i-o} of 40 mV. Moreover, g_j displays a bell-shaped and asymmetric relationship with V_j. This asymmetry could be due in part to the fact that the depolarizing steps needed to obtain positive values of V_j also increase V_{i-o}, which in turn modulates the channel sensitivity. In all cases studied, a non-zero residual current was present at the end of the V_j pulse, even with large V_j pulses. This residual conductance, about 20% of the maximal steady-state conductance, has been observed in many other gap-junctions (Spray *et al.* 1981; Verselis *et al.* 1991; Weingart & Bukauskas, 1993). The fact that I_j was almost completely blocked by octanol and large V_{i-o} pulses suggests that the residual current is not due to an alternative cell–cell communication mechanism (e.g. cytoplasmic bridges) but is an intrinsic characteristic of gap junction channels. Unitary analysis of these channels is needed to clarify this point.

Many voltage-dependent gap junctions are exclusively sensitive to V_j including gaps from crayfish neurons (Giaume *et al.* 1987); amphibian blastomeres (Spray *et al.* 1981); vertebrate cardiac muscle fibres (Rook, Jongsma & van Ginneken, 1988; Veenstra, 1990) and rat liver cells (Moreno, de Carvalho, Verselis, Eghbali & Spray, 1991). Gap junctions dependent on both V_j and V_{i-o}, such as those described in the present work, have been described in a cell line from the mosquito *Aedes albopictus*, in the salivary glands of *Drosophila melanogaster* larvae and in epidermal cells from *Tenebrio molitor* (Verselis *et al.* 1991; Bukauskas *et al.* 1992*a*; Churchill & Caveney, 1993). A comparison of the present results with those from other studies is difficult because of methodological variations, particularly with respect to the holding potential values chosen. Nevertheless, it appears that the dependency of g_j on V_j is similar in all cases, both in the degree of voltage dependency (parameter A) and in the voltage reference (V_0). The dependency of g_j on V_{i-o} is qualitatively similar in as much as positive V_{i-o} reduces gap conductance. Gap junctions from embryonic muscle fibres of *Drosophila* have a lesser degree of voltage dependence than those observed in salivary glands (0·02 *versus* 0·08 mV^{-1}). Those epidermal cells have an intermediate value of 0·05 mV^{-1}.

All the preparations exhibit different voltage references V_0 (embryonic muscle: 30 mV; salivary gland: 5 mV; and epidermal cell: −5 mV). It is unclear whether or not these differences in V_0 reflect intrinsic variations between gap junctions of the different preparations.

The function of voltage-dependent gap junctions in embryonic muscle fibres remains unknown. The dependency of g_j on transjunctional and transmembrane potentials can provide a mechanism to maintain the cells within a given range of membrane potentials. For example, if the membrane potential of one cell becomes sufficiently different from neighbouring cells by the action of intrinsic or external factors, this cell will partially uncouple, thus allowing the rest of the cells to maintain their optimal membrane potential. The dependence on transmembrane potential suggests that the cells will also uncouple if both cells depolarize in concert. The uncoupling that follows a hyper- or depolarizing stimulus is of a regenerative nature, i.e. a stimulus sufficient to reduce junctional conductance results in an increase in cell input resistance making the cell more responsive to polarization with subsequent stimulation (Harris, Spray & Bennett, 1983). The uncoupling following depolarizing V_{i-o} could allow a more rapid recovery to normal membrane potential values following a widespread depolarization. One might imagine that less metabolic energy would be necessary for a given cell to recover its optimal membrane potential when it is isolated than when it is in contact with other depolarized cells. Furthermore, after the repolarization of a substantial fraction of fibres in the muscular lattice, the repolarization of the rest of the fibres will be facilitated by current transfer from the neighbouring cells as the coupling is re-established.

Further genetic, physiological and molecular studies, using *Drosophila* as a model, should provide insight into the functional role of voltage-dependent gap junctions in embryonic and mature tissues.

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