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VOLTAGE-DEPENDENT DYE COUPLING AT A RECTIFYING ELECTROTONIC SYNAPSE OF THE CRAYFISH

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SUMMARY

1. At the crayfish giant motor synapse, the lateral giant axon (l.g.a.) and the giant motor fibre (g.m.f.) form an electrotonic junction which exhibits two states of ionic coupling (Furshpan & Potter, 1959*a*; Giaume & Korn, 1983). Junctional conductance is low at resting membrane potentials (i.e. with lateral axon more negative than the motor fibre) and high when the polarity of the voltage difference (ΔV) across the synapse is reversed.

2. For these two states of conductance, junctional permeability was investigated using the intercellular tracer Lucifer Yellow. The dye was ionophoretically injected into either the presynaptic (l.g.a.) or the post-synaptic (g.m.f.) cell.

3. In the high conductance state ($\Delta V > 0$), fluorescence was detected in both neurones whether Lucifer Yellow had been injected pre- or post-synaptically.

4. By contrast, at the resting junctional polarization ($\Delta V < 0$) Lucifer Yellow spread from the giant axon to the g.m.f., but not from the g.m.f. to the giant axons.

5. These data demonstrate that dye transfer at the giant motor synapse, like ionic coupling, is sensitive to junctional polarization and is more marked in the high conductance state. Possible explanations for the asymmetry observed in the low conductance state are discussed.

INTRODUCTION

Gap junctions and their associated intercytoplasmic bridging structures (Revel & Karnovsky, 1967) are not only related to electrotonic coupling between neurones (reference in Bennett, 1977) but are also involved in metabolic (or chemical) coupling (reference in Loewenstein, 1981). Studies performed to investigate cytoplasmic exchanges of probe molecules have helped to define properties of these channels, as for example, the size limit for permeant molecules (Simpson, Rose & Loewenstein, 1977), the influence of the charge on tracer movements (Flagg-Newton, Simpson & Loewenstein, 1979), and the presence of fixed charges within the channel (Brink & Dewey, 1980).

Although electrotonic transmission at gap junctions is most often described as being bidirectional, in various systems synaptic 'rectification' has been demonstrated.

Evidence for this concept was first obtained at the crayfish giant motor synapse (g.m.s.) where action potentials and/or large depolarizing currents are unidirectionally transmitted from the giant axons to the motor fibre, while passage of large hyperpolarizing currents is allowed in the opposite direction (Furshpan & Potter, 1959*a*). Similar observations have been made in several other preparations (Arvanitaki & Chalazonitis, 1959; Auerbach & Bennett, 1969; Smith & Baumann, 1969; Nicholls & Purves, 1970; Ringham, 1975; Roberts, Krasne, Hagiwara, Wine & Kramer, 1982). The permeability characteristics and the voltage dependence of these peculiar synapses remain however poorly understood. In particular, it is not clear whether the rectifying behaviour of the synapse as a global entity is linked with a rectification of the individual junctional channels.

In a previous work we have demonstrated that electrotonic coupling at the g.m.s. is in fact symmetrical if the resting junctional polarization is reversed (Giaume & Korn, 1983; see also Margiotta & Walcott, 1983). This finding prompted us to investigate whether the permeability of the synapse for molecules larger than the main current carrying ions would also be sensitive to junctional polarization. For this purpose, we have used the fluorescent and low molecular weight (457) tracer, Lucifer Yellow (Stewart, 1978, 1981), which has allowed other authors to relate 'dye coupling' between neurones with electrotonic transmission (see Stewart, 1981) despite occasional failures to demonstrate dye coupling at electrotonically coupled cells (Audesirk, Audesirk & Bowsher, 1982; Kater & Hadley, 1982).

We here report that, indeed, passage of Lucifer Yellow at the g.m.s. is sensitive to junctional polarization. As the junction is placed in a high conductance state dye coupling is bidirectional, while it appears unidirectional in the low conductance state. The reasons for this apparent asymmetry will be discussed.

METHODS

Experiments were performed on crayfish (*Procambarus clarkii*) supplied from the Monterey Bay Hydroculture Farms (Soquel, CA) and kept in running water at 17 °C. The abdominal nerve cord, first cooled to 5 °C over a period of about 30 min, was dissected as described by Furshpan & Potter (1959*a*). After removal of the exoskeleton, the abdomen was fixed, dorsal side up, in a chamber continuously perfused with a Van Harreveld solution containing (in mM): NaCl, 207·5; KCl, 5·4; $CaCl_2$, 13·5; $MgCl_2$, 2·6 (Van Harreveld, 1936) and fixed at pH 7·4 with 10 mM-Trisma Maleate. The nerve cord was then separated; special care was taken with the third ganglionic roots which were cut as near as possible to the flexor muscles. The isolated ganglionic chain was pinned dorsally in a lucite chamber and observed under dark-field illumination. The synaptic area, defined by the presynaptic elements (the lateral giant axon, l.g.a., and both medial giant axons, m.g.a.) and the post-synaptic giant motor fibre (g.m.f.) was prepared for micro-electrode impalement by removing the sheath which encloses the cord.

Our investigations were restricted to the first and second abdominal ganglions, and dye coupling was mainly studied at the giant synapse between the l.g.a. and the g.m.f. (Pl. 1*A*).

All experiments were conducted with four intracellular micro-electrodes. A voltage and a current electrode were located on each side of the synapse, for injection of the tracer and for application of polarizing currents. Voltage and current micro-electrodes were filled with 3 M-KCl for the l.g.a. but with 0·6 M-K_2SO_4 for the motor fibre which is subjected to a massive and spontaneously active Cl^--dependent inhibitory input (Furshpan & Potter, 1959*b*). The resistance of the electrodes ranged from 10 to 15 MΩ. Both synaptic elements could be stimulated by extracellular bipolar electrodes placed on the nerve cord itself and on the third root extension.

Dye-coupling experiments were achieved using micro-electrodes filled with Lucifer Yellow CH

(Sigma, No. L0259, diluted to 5% in distilled water) having a resistance of 30–50 MΩ. Neurones were filled by passing at most 50 nA hyperpolarizing currents of 1 s duration every 2 s, during about 45 min. The time allowed for diffusion of Lucifer Yellow before fixation ranged from 1 to 20 h after the end of the injection period (see Table 1). Preparations were gradually dehydrated in alcohol (50–100%), and then cleared in xylene and mounted in Entellan (Merck, No. 7960). Fluorescence photomicrographs were taken on Kodak Tri X Pan films through a Zeiss microscope, using a dry dark-field condenser and Neofluar optics.

Intracellular injections of horseradish peroxidase were performed as described by Triller & Korn (1981).

RESULTS

The g.m.s. conductance is critically dependent upon junctional polarization, i.e. upon the difference ($\Delta V = V_{pre} - V_{post}$) between the membrane potentials of the presynaptic cell (V_{pre}) and the post-synaptic axon (V_{post}) (Giaume & Korn, 1983). In the present experiments ΔV, at rest, averaged -14 mV (S.D. $= \pm 7$, $n = 25$) since the presynaptic l.g.a. and the g.m.f. had membrane potentials of -89 mV (S.D. $= \pm 3$, $n = 26$) and -75 mV (S.D. $= \pm 7$, $n = 25$) respectively. These simply derived values are consistent with those previously reported. As explained below, during Lucifer Yellow injections in the g.m.f., a more complicated procedure was necessary to compute ΔV (which must be shifted to about $+40$ mV in order to make the g.m.s. a bidirectionally transmitting synapse for large intracellular polarizing currents: Giaume & Korn, 1983).

Plots of the relation between the pre- and post-synaptic potential changes (V_{pre} and V_{post}) produced by intracellular current pulses permitted us to assess the degree of electrotonic transmission and to compare its values from one experiment to another. An example of such a transfer function is illustrated in Fig. 1. For small post-synaptic hyperpolarizations, no response was detected in the presynaptic element. As the intensity of the applied current was augmented a presynaptic hyperpolarization was observed which progressively increased until it became linearly related to V_{post}. The coupling parameter was defined at the beginning of each experiment as the slope of the linear segment of this curve. The mean value of this parameter was 0·29 (S.D. $= \pm 0{\cdot}04$, $n = 21$).

The V_{post}/V_{pre} curve was also used to estimate ΔV when the membrane potentials could not be directly measured throughout the experiment especially when the post-synaptic neurone was simultaneously hyperpolarized and dye injected. In this case, both membrane potentials were measured before the beginning of Lucifer Yellow injections; the voltage-transfer function was then established in relation to the absolute potentials of the two synaptic elements. Later, during post-synaptic dye injections as shown in Fig. 1, for a given presynaptic potential, the junctional polarization ΔV was obtained by measuring the difference between the V_{post}/V_{pre} relation and the plot of junctional isopotentiality. In the example illustrated here, when the V_{pre} was at -101 mV, the transjunctional voltage was calculated to be $+32$ mV.

Asymmetry of dye coupling at rest

Fifteen experiments have been carried out in cells exhibiting a mean ΔV of -15 mV (S.D. $= \pm 11$, $n = 15$) and a mean coupling slope of 0·29 (S.D. $= \pm 0{\cdot}05$, $n = 10$) in the

absence of polarizing currents (Table 1). As stated below, the intensity of injecting currents did not modify significantly these values (see Discussion).

(*a*) *Injections in the presynaptic fibre.* In five experiments, Lucifer Yellow was injected in the l.g.a. (Expts. 1–5). In four trials the dye spread into the motor fibre and in only one case (injection No. 3) fluorescence was not detected in the post-synaptic neurone. Fig. 2*C* and Pl. 1*B*, which are from the same experiment, illustrate the dye

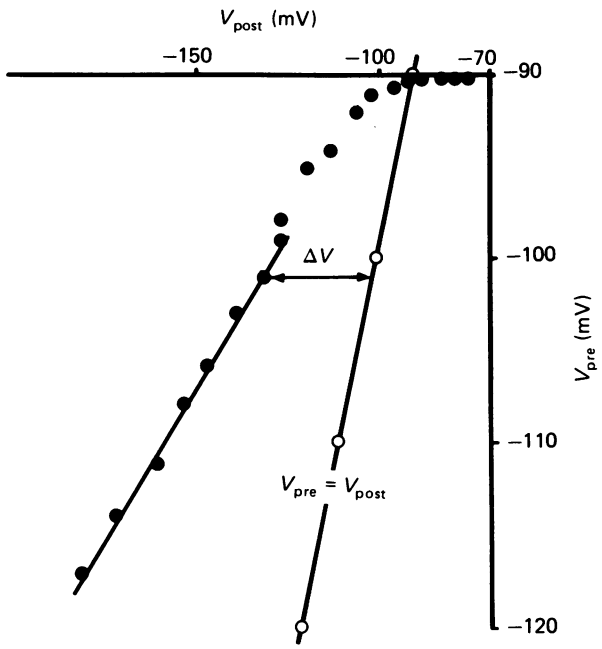

Fig. 1. Voltage-transfer function at the rectifying synapse. Relation between post-synaptic (abscissa) and presynaptic (ordinate) voltages when a hyperpolarizing current is applied in the post-synaptic fibre (circles). Both potentials are expressed in absolute values which take into account the initial membrane potentials (−70 and −90 mV respectively). The slope of the linear part of this relation (0·33 in this case), gives an estimation of the degree of electrotonic coupling between cells and also permits the evaluation of the transjunctional potential ΔV when V_{post} cannot be directly measured, due to maintained hyperpolarizations applied through the recording post-synaptic micro-electrode (Expt. 13 of Table 1). For a fixed V_{pre}, ΔV is obtained by making the difference between the point given by the voltage-transfer function established at the beginning of the experiment and the corresponding value on the traced $V_{pre} = V_{post}$ relationship (○).

transfer from the l.g.a. to the g.m.f. Rectifying behaviour of the junction is shown by Fig. 2*A*, *Ba* and *Bb*. The l.g.a. was injected during 40 min with a subsequent diffusion time for the dye of about 1 h. The micrographic reconstruction of Fig. 2*C* shows that Lucifer Yellow completely filled both the l.g.a. and the g.m.f. The fluorescence intensity was the same in the pre- and post-synaptic elements, the latter being stained up to its soma. As a convention in the drawings, neurones in black indicate the cell where the marker has been directly applied, while the ones stained

by dye coupling through the gap junctions are represented in grey. The Figure further shows that the marker had also crossed the septate junction between the injected axon and the adjacent l.g.a. running to the following ganglia presumably because of the high symmetrical coupling which exists at this level (Watanabe & Grundfest, 1961).

(*b*) *Injections in the post-synaptic axon.* Injections of Lucifer Yellow into the g.m.f. were never followed, at rest, by obvious migration of the dye into the l.g.a. (Expts.

TABLE 1. Permeability of the g.m.s. for Lucifer Yellow at resting membrane potential. Comparison of junctional permeability, depending upon the site of injection of the marker. Positive signs indicate that fluorescence was detected at each side of the junction and that the tracer had spread transsynaptically. Negative signs indicate that Lucifer Yellow was only present in the injected cell

Expt. No.	Resting membrane potential (mV)		Resting ΔV (mV)	Coupling (limiting slope)	Cells injected	Time allowed for diffusion (h)
	Pre.	Post.				
1	−85	−45	−40	0·21	L.g.a.	1
2	−87	−80	−7	0·27	L.g.a.	1
3	−93	−78	−15	0·32	L.g.a.	1
4	−95	−78	−10	0·29	L.g.a.	1
5	−89	−75	−14	0·30	L.g.a.	1
6	−87	−77	−10	0·30	G.m.f.	1
7	−87	−79	−8	0·29	G.m.f.	1
8	−89	−75	−14	0·21	G.m.f.	1
9	—	—	—	0·37	G.m.f.	1
10	—	—	—	0·30	G.m.f.	1
11	−90	−77	−13	—	G.m.f.	5
12	−90	−80	−10	—	G.m.f.	4
13	−86	−67	−19	—	G.m.f.	4
14	−93	−78	−15	—	G.m.f.	5·5
15	−85	−66	−19	—	G.m.f.	20

6–15). Fig. 3 and Pl. 2*A* illustrate this finding; the two synapses studied here were electrically rectifying, as shown by the intracellular recordings from one of them (Fig. 3*Aa–c*). Fluorescence was not detected after fixation, either in the ipsilateral l.g.a. or in the coupled m.g.a. (Fig. 3*Ad* and *B*). All the results of a set of similar experiments in which the preparation was fixed 45–60 min after the end of dye ionophoresis were identical (Table 1, Expts. 6–10). The same observations were made in a second experimental series (Table 1, Expts. 11–14) in which the preparation was kept in physiological medium for considerably longer periods of time, during which transmission was tested at regular intervals to confirm that rectification and voltage dependency were present, until fixation. Even in the case of Expt. 15 during which the preparation was kept in the perfusing chamber for 20 h, no trace of fluorescence was detected in the presynaptic element. The profile of the g.m.s. was identical (Pl. 3*A* and *B*) when the motor fibre was stained at rest by Lucifer Yellow or with horseradish peroxidase (HRP), which does not cross gap junctions (Bennett, Feder, Reese & Stewart, 1973).

The difference in shape and dimensions of the two fibres illustrated in Fig. 3 is due

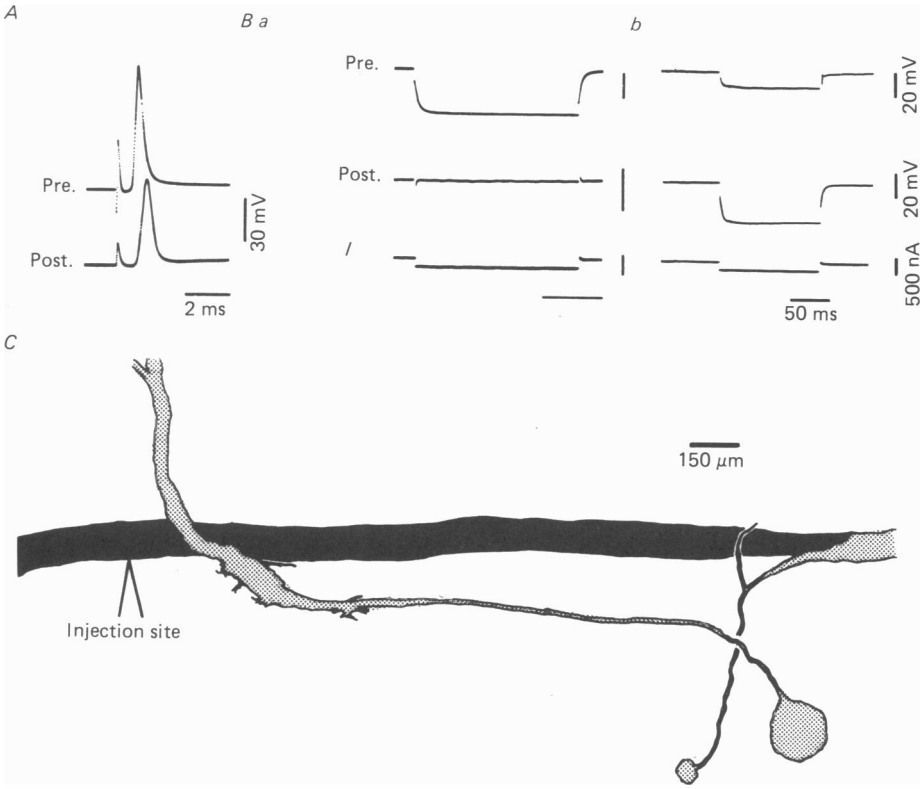

Fig. 2. Orthodromic spread of Lucifer Yellow at resting membrane potentials. *A*, *Ba* and *Bb*, electrophysiological demonstration of the rectifying properties of synaptic transmission at the g.m.s. *A*, action potentials generated by extracellular stimulation of the abdominal nerve cord were transmitted from the l.g.a. (pre.) to the g.m.f. (post.). *Ba* and *Bb*, upper and middle traces: hyperpolarizing currents injected in the prefibre were not transmitted to the post-synaptic axon (*Ba*) but crossed the g.m.s. from the g.m.f. to the l.g.a. *C*, drawing after microscopic photograph reconstruction of the stained cells. Lucifer Yellow injected in the l.g.a. (black) also filled the motor fibre (grey). In this experiment which was performed at the second abdominal ganglion, the dye also crossed the septate junction and coloured the soma of the adjacent lateral giant axon (grey) (Expt. 2, Table 1). The black and grey do not refer to a difference in fluorescence intensity (see Pl. 1 *B*) but only show a distinction between the cell in which the dye was injected (black) and the ones to which the dye diffused (grey).

to the fact that one was from the first nerve cord ganglion (*Ad*) while the other was from the second ganglion (*B*). Since electrotonic junctions are localized to the dendrites of the motor fibre in the axonal region (Mittenthal & Wine, 1973; Hanna, Keeter & Pappas, 1978) it can be concluded that the contact zone between the pre- and post-synaptic elements is larger at the g.m.s. of the first than at that of the second ganglion. The reduction in synaptic size from the thoracic to the abdominal level (Margiotta & Walcott, 1983) is further pronounced more caudally.

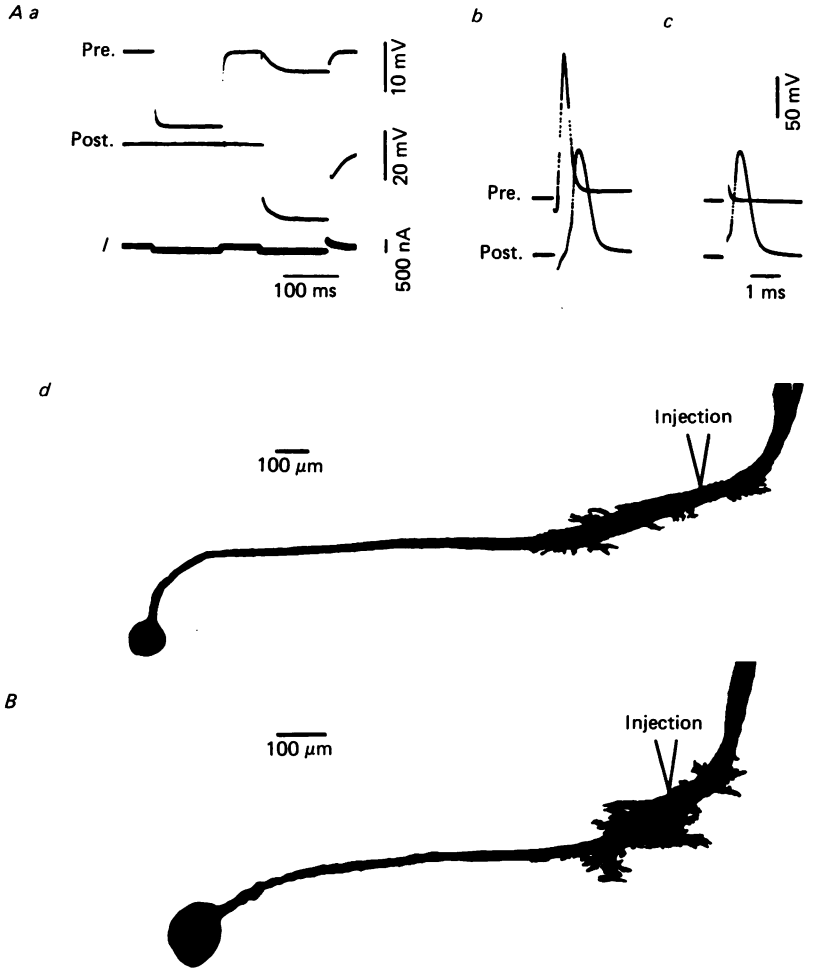

Fig. 3. Failure of Lucifer Yellow to cross the g.m.f. in the antidromic direction at rest. *Aa*, *b* and *c*, tests for transmission of electrotonic potentials in resting conditions. *Aa*, upper, middle and lower traces: same as for Fig. 2*Ba–b*. Hyperpolarizing currents did not spread in the orthodromic direction, they only crossed the synapse from the giant axon to the l.g.a. *Ab–c*, action potentials evoked in the l.g.a. by nerve cord stimulation (upper trace) were reliably transmitted to the motor fibre (lower trace, *Ab*) whereas a spike in the post-synaptic axon failed to depolarize the l.g.a. (*Ac*). *Ad*, Lucifer Yellow injected in the post-synaptic neurone of this synapse remained localized in the motor fibre; no fluorescence was detected in the adjacent l.g.a. *B*, drawing of another stained motor fibre with Lucifer Yellow strictly localized in this neurone (same experiment as for Pl. 2*A*). Reconstructions from mountings obtained at first (*Ad*) and second (*B*) abdominal ganglia. Note the greater number of processes and the longer length of contact areas in *Ad* (from Expts. 7 and 8 of Table 1).

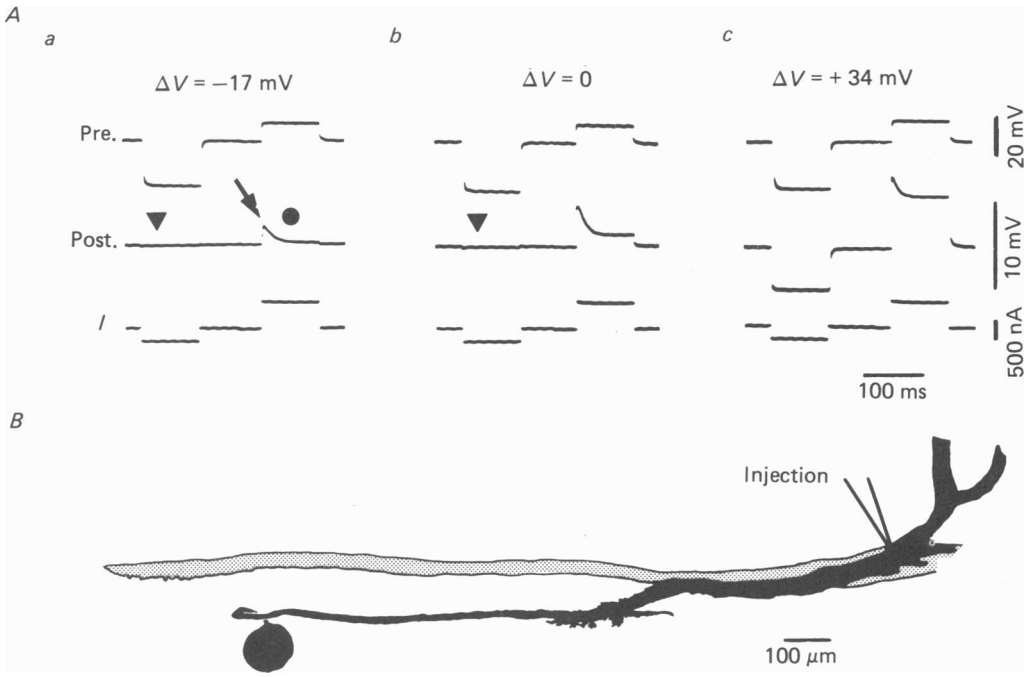

Fig. 4. Antidromic dye coupling produced by inversion of transjunctional polarization. *Aa*, *b* and *c*, voltage dependency of electrotonic transmission at the g.m.s. Upper and middle traces: potentials intracellularly recorded from the l.g.a. (pre.) and the g.m.f. (post.); lower sweep: current trace. *Aa*, at rest ($\Delta V = -17$ mV) depolarizing currents applied in the l.g.a. produce a depolarizing voltage drop in the g.m.f. (●), but hyperpolarizations are not transmitted (▼). The depolarization was sufficient to generate a pre- and post-synaptic action potential (not illustrated here) which was followed in the g.m.f. by a slow post-synaptic potential (arrow). *Ab*, as $\Delta V = 0$ the transmission of a positive pulse is increased but hyperpolarizations are still blocked (▼). *Ac*, when ΔV is inverted and approximates +34 mV, currents of both polarities are transmitted symmetrically and post-synaptic responses are of equal amplitudes. *B*, Lucifer Yellow injected in the motor fibre (black) crossed the synapse in conditions of maintained post-synaptic hyperpolarization and filled the lateral axon (grey) up to the septum (from Expt. 18, Table 2).

Symmetric spread of Lucifer Yellow under conditions of reversed junctional polarization

At rest the g.m.s. shows rectification for movements of the marker as well as for large ionic currents. Thus, to determine if the permeability of the dye is voltage sensitive, the conductance of the junctional channels was increased and electrical transmission was made bidirectional. For this purpose, prolonged hyperpolarizations were imposed to the motor fibre while Lucifer Yellow was injected into the same cell by a second intracellular electrode. As shown previously (Giaume & Korn, 1983), electrotonic coupling becomes symmetrical at the g.m.s. when the transjunctional voltage difference is inverted either by presynaptic depolarization or, conversely, by hyperpolarization of the motor fibre. This property is illustrated in Fig. 4 *Aa–c*. In

this experiment, the spread of polarizing currents injected into the lateral axon was tested at different values of ΔV.

The degree of electrotonic coupling between two cells was evaluated by calculating the coupling coefficient k as the ratio between the voltage change recorded on the non-injected side of the junction (V_2) and the one measured in the directly polarized element (V_1) (Bennett, 1966). Thus, the coupling coefficient from cell 1 to cell 2 is given by $k = V_2/V_1$ and ranges from 0 to 1 according to the degree of junctional conductance.

TABLE 2. Bidirectionality of dye coupling when junctional polarization is reversed. Increasing number of positive signs indicate larger areas of fluorescence in the non-injected cell. Negative signs stand for failure of Lucifer Yellow to migrate post-synaptically

Expt. No.	Resting membrane potential (mV) Pre.	Post.	Resting ΔV (mV)	Coupling (limiting slope)	Cell injected	Estimated imposed ΔV (mV)	Duration of imposed ΔV (min)	Dye coupling
16	−89	−78	−11	0·27	G.m.f.	+49	64	+++
17	−88	−78	−10	0·30	G.m.f.	+31	38	+++
18	−90	−70	−20	0·32	G.m.f.	+42	35	+++
19	−88	−71	−17	0·19	G.m.f.	+37	29	++
20	−87	−74	−13	0·35	G.m.f.	+36	20	++
21	−89	−80	−9	—	G.m.f.	+34	23	+
22	−87	−76	−13	0·29	G.m.f.	+39	16	+
23	−94	−75	−19	0·29	G.m.f.	+43	4	+
24	−88	−80	−8	—	G.m.f.	+36	17	+
25	−90	—	—	0·30	G.m.f.	+30	7	−
26	−90	−75	−15	0·28	G.m.f.	+35	4	−
27	−86	−73	−13	0·33	L.g.a.	+23	20	+++
28	−89	−77	−12	0·30	L.g.a.	+24	19	+++

At resting membrane potentials ($\Delta V = -17$ mV), only depolarizing currents were transmitted, with a coupling coefficient k of about 0·1, while hyperpolarizing pulses were blocked when applied in the l.g.a. (Fig. 4*Aa*). When the g.m.f. was hyperpolarized until ΔV was zero, k increased to 0·25 for depolarizations (Fig. 4*Ab*) but hyperpolarizing currents still only spread orthodromically. When ΔV was inverted and brought to +37 mV (Fig. 4*Ac*), the coupling ratio became maximum, and had a similar value for either polarity (0·52 (+) and 0·54 (−), respectively) (Giaume & Korn, 1983).

An example of a successful antidromic dye coupling which was obtained under such conditions is shown in Fig. 4*B* and Pl. 2*B*. In this case, the initial ΔV was −20 mV and the coupling limiting slope was 0·32. After 35 min of a −65 mV imposed hyperpolarization of the g.m.f. (ΔV calculated: +42 mV), the tracer was well detected in the l.g.a., up to the level of the septate junction.

Table 2 summarizes the results of the experiments in which Lucifer Yellow was injected in the g.m.f. while ΔV was maintained at positive values. In nine preparations (from Expts. 16–26), the marker crossed the junction and could be visualized in the l.g.a. within variable distances. In only two experiments (25 and 26), the Lucifer

Yellow failed to pass the synapse, and in one more (Expt. 24) only small (a few) traces of fluorescence were perceptible in the adjacent non-injected cell.

A simple evaluation of the Lucifer Yellow transfer was carried out by distinguishing cases in which only a few spots of fluorescence were detected near the synaptic contact (+), in which a larger fraction but not all the l.g.a. axon was stained (++), and in which the latter was entirely fluorescent (+++). A comparison of the dye extension suggests that the spread of the tracer was dependent upon the duration of the imposed high conductance state. The three experiments in which transfer of Lucifer Yellow had been massive were those with longer times of imposed transjunctional voltage inversion (64, 38 and 35 min, respectively). In these experiments the values of ΔV and the V_{post}/V_{pre} slope were not markedly different from those measured in other experiments. By contrast, negative cases were those of shorter duration (except for Expt. 18 which was held at a ΔV as large as +43 mV).

In two additional experiments (27 and 28, Table 2) the l.g.a.s were injected during about 20 min while the motor fibres were hyperpolarized with maintained ΔVs of +24 mV, but the preparations were fixed immediately, i.e. at most 1 min after the end of this procedure. Both g.m.f.s were well stained without noticeable differences from experiments made at resting membrane potentials (Table 1). These results show that currents flowing from the g.m.f. to the l.g.a. did not prevent the spread of the negatively charged probe molecule in the reverse direction. Thus it is unlikely that the dye is carried solely by the ionophoretic currents when dye coupling occurs in the antidromic direction.

Distribution of electrotonic coupling between the g.m.f. and adjacent giant axons

The synapses between the m.g.a.s and the motor fibre are also rectifying and voltage sensitive (Giaume & Korn, 1983). However, when the dye was injected at high conductance in the g.m.f., it was only visualized in the l.g.a. and fluorescence was not detected in the two m.g.a.s. The relative ratio of electrotonic coupling between the g.m.f. and the three giant axons was therefore evaluated in order to determine if differences in coupling ratios could account for this selectivity.

In Fig. 5, the diagram on the left illustrates the current–voltage (I–V) curves obtained in the g.m.f. and the l.g.a. after injections of currents in the post-synaptic cell. The plot for the motor fibre is not linear: there is an inflexion point at 40 mV below resting potential and the points are well fitted by two regression lines with respective slopes of 0·4 and 0·1 MΩ. The change in slope was nearly concomitant with the appearance of an electrotonic response in the l.g.a. Note that the second value is in fact only an apparent input resistance of the fibre, because in this case a noticeable part of the injected current flows through the electrotonic junctions. The right-hand diagram of Fig. 5 further shows that when the presynaptic membrane potential was displaced by a constant current to about −110 mV, the electrotonic response in the l.g.a. appeared only with a −150 mV post-synaptic hyperpolarization. This higher threshold of coupling was correlated with a significant change in the shape of the motor fibre I–V curve which now was fitted by three regression lines, with slopes of 0·4, 0·2 and 0·1 MΩ, respectively. The first of the two inflexion points occurred for the same current intensities as those obtained in resting conditions, while the second one was associated with the appearance of coupling to the l.g.a. A simple explanation

for this finding is that the two apparent changes of g.m.f. input resistance are the consequence of shunts introduced by the opening of gap junction channels at the level of the synapses established between the motor fibres and the medial axons first, then with the lateral axon.

The equivalent circuit of Fig. 6*A* illustrates the above hypothesis in which currents injected in the g.m.f. can flow through three distinct rectifying synapses. To estimate quantitatively the redistribution of current flow, we have recorded simultaneously

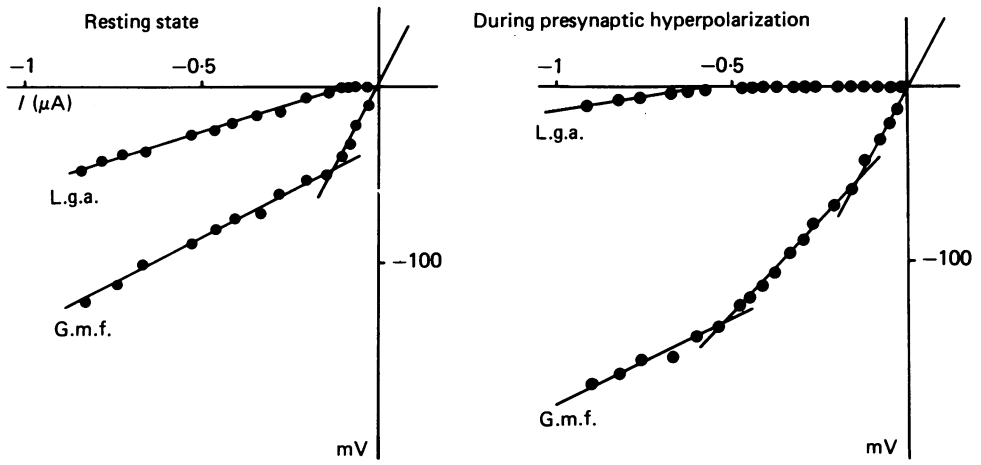

Fig. 5. *I–V* relationship of the pre- and post-synaptic neurones. Diagram on the left: *I–V* curves of the g.m.f. and l.g.a. obtained with hyperpolarizing pulses of increasing intensities applied in the g.m.f.; note that the inflexion point of the former occurs when a response appears in the l.g.a. Diagram on the right (same experiment): a maintained hyperpolarization of 60 mV of the l.g.a. delayed the appearance of the electrotonic response in the l.g.a. and two inflexion points were manifest in the post-synaptic *I–V* curve, the slope of the *I–V* curve being presumably reduced when part of the injected current in the post-synaptic cell was diverted when junctional channels were opened at synapses between the motor fibre and the m.g.a.s (first inflexion point) and at junctions between the motor fibre and the lateral axon (second inflexion point). All curves are regression lines obtained from experimental points.

from the post-synaptic fibre and two presynaptic cells, the l.g.a. and m.g.a. (Fig. 6*B*). Plots of the *I–V* curves of these three neurones for hyperpolarizing currents are shown in Fig. 6*C*. Each of them could be fitted by regression lines which are labelled $g.m.f._1$ and $g.m.f._2$ for the post-synaptic fibre (before and after the inflexion point, respectively), l.g.a. and m.g.a. for the presynaptic giant axons. The last two lines have a common point of origin which indicates the same threshold for initiation of an electrotonic response. An evaluation of coupling distribution can be made by comparing the amplitude of the responses in the giant axons for the same post-synaptic current application, as one can consider that the input impedances of the l.g.a. and the m.g.a. are in the same order of magnitude (Watanabe & Grundfest, 1961). The difference in the slopes of the l.g.a. and m.g.a. lines suggests that the coupling ratio

is larger at the junction with the lateral axon than at that with the medial one. More precisely, the respective fractions of the electrotonic coupling at the three synapses with the post-synaptic fibre are around 60% for the l.g.a. and 20% for each of the m.g.a.s (100% being the total electrotonic response generated in the three giant axons).

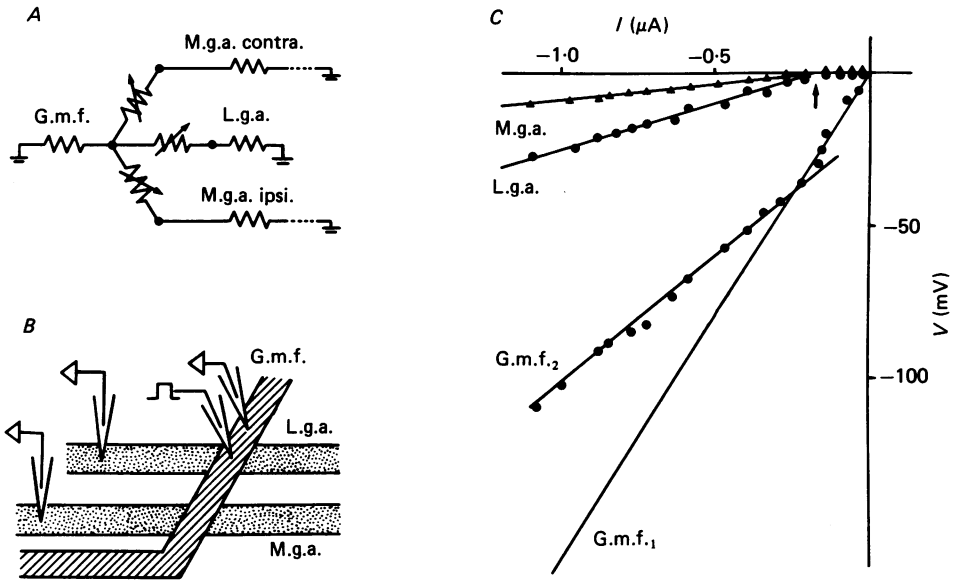

Fig. 6. Electrotonic coupling distribution at the g.m.s. *A*, equivalent circuit of synapses connecting the g.m.f. to adjacent giant fibres. Resistances with arrows represent voltage-dependent junctions and single resistors are symbols for cell's input resistance (abbreviations: m.g.a. contra. and ipsi.: contralateral and ipsilateral medial giant axons). *B*, diagram of the experimental set up. Three cells were simultaneously recorded with intracellular micro-electrodes and hyperpolarizing currents of increasing intensities were applied in the g.m.f. *C*, plots of the different *I–V* relations at the g.m.s. and other synaptic elements. Currents in the g.m.f. produced voltage changes which were fitted by two regression lines, $g.m.f._1$ and $g.m.f._2$ respectively, with an inflexion point between them at −170 nA. The electrotonic responses in the coupled neurones are represented by lines labelled l.g.a. and m.g.a. which exhibit a common point of origin (arrow).

DISCUSSION

The results reported in this paper clearly demonstrate that at the g.m.s., dye coupling is voltage dependent. At low conductance state, Lucifer Yellow crosses the synapse solely from the presynaptic to the post-synaptic neurone; however, the marker spreads in both directions when the resting junctional polarity is reversed. Such a correlation between electrical and dye coupling as a function of the voltage described here for excitable cells, completes the observations made on voltage-sensitive gap junctions between amphibian embryonic cells (Spray, Harris & Bennett, 1979) and salivary glands of *Chironomus* (Zimmerman & Rose, 1983), although the characteristics of their voltage dependency are clearly different.

In a recent study (Margiotta & Walcott, 1983) it was concluded that Lucifer Yellow crosses the crayfish rectifying synapse at low as well as at high synaptic conductance states. However, in this investigation, the site of dye injection was not taken into account: at negative ΔVs, spread of Lucifer Yellow was solely tested in the orthodromic direction, while at positive ΔVs it was only tested from the g.m.f. to the l.g.a. No comparison was available for the two other situations, i.e. for antidromic dye passage from the g.m.f. to the l.g.a. at low conductance and for orthodromic permeability during inversion of the junctional polarity.

Validity of the experimental procedure

Lucifer Yellow was injected intracellularly by ionophoresis rather than by means of micropressure pulses to exclude damage of gap junctions, as observed in the same preparation with the latter technique (Remler, Selverston & Kennedy, 1968). Ionophoretic currents were limited to 50 nA to exclude additional potential changes at these voltage-sensitive junctions. Hyperpolarizing pulses used to fill the l.g.a. did not modify significantly the coupling ratio of the synapse (Expts. 1–5). The possibility that the bidirectional transfer was the consequence of ionophoretic effects appears to be excluded by the experiments in which Lucifer Yellow was injected into one cell during simultaneous application of negative current to 'clamp' the neighbouring element immediately followed by fixation of the preparation. Under these conditions, the orthodromic spread of Lucifer Yellow was unaltered as expected if the changes in permeability are solely dependent upon voltage gradients.

Our results were obtained from fixed and dehydrated material, that is under conditions which are claimed to increase the threshold of Lucifer Yellow visualization (Margiotta & Walcott, 1983). Several points argue for the reliability of our procedure and suggest that the failure to detect dye coupling at rest, in the antidromic direction, was not artifactual: (i) we never found that fixation prevented visualization of Lucifer Yellow transfer whether the dye was injected in the l.g.a. (except in Expt. 3) or, in the high conductance state, into either side (except in Expts. 25 and 26 but note that, in these experiments, ΔV was maintained positive for short periods of time); (ii) if only a limited amount of dye had crossed the junction antidromically at rest it would probably have been visible since, at the commissural junction, the weak but symmetrical coupling between paired l.g.a.s (Watanabe & Grundfest, 1961) neither prevents the spread nor the detection of the marker; (iii) even when a long time was allowed for its diffusion Lucifer Yellow was not present in the l.g.a. when it was injected in the g.m.f. (Expts. 6–15); (iv) despite the large volume of the giant axons weak antidromic dye coupling would have led to enough fluorescence of the l.g.a., if the passage of the probe molecule is unrestricted within the channel, as shown by the calculations of time diffusion presented in the Appendix.

The failure to detect spread of the marker from the g.m.f. to the m.g.a.s, even when the synapses are in a high conductance state, appears surprising at first. The important length constant of the motor fibre (Furshpan & Potter, 1959*a*) guarantees that, at their level, ΔV can be inverted by a strong post-synaptic remote hyperpolarization and, accordingly, induce a bidirectional transmission. In confirmation, the analysis of the I–V relationship of the motor fibre and of its associated responses in the presynaptic cells has shown changes in the g.m.f. I–V relation which are

consistent with the hypothesis that shunts are introduced by voltage-dependent openings of the channels present between the motor fibre and the giant axons. However, the analysis of these diagrams indicates that the coupling coefficient between the g.m.f. and the m.g.a. is much lower (see Fig. 6) than between the g.m.f. and the l.g.a. It is thus conceivable that Lucifer Yellow injected post-synaptically equilibrates its concentration gradient much more readily with the l.g.a. than with the m.g.a.s.

Possible interpretations of the asymmetric permeability at rest

Dye-coupling properties, and in particular the asymmetric permeability observed at rest, i.e. when ΔV was negative, can be interpreted in two ways. One possibility is that junctional channels, once open, are permeable to the tracer in both directions. In this case, an excessive cytoplasmic dilution is the reason for which fluorescence is undetected in the l.g.a. when the injection is performed into the post-fibre. According to this hypothesis, a 'simple' gating mechanism for junctional channels, similar to that described at voltage-dependent ionic channels distributed in excitable membranes (reference in Finkelstein & Mauro, 1977) can account for the data obtained from both electrophysiological and permeability studies at the g.m.s. The electrical field across the synapse which commands the aperture and closure of the junctional channels would allow ions and probe molecules to cross in both directions, as suggested by the linearity of the $I-V$ relation of the g.m.s. for small current intensities (Furshpan & Potter, 1959*a*).

The second possibility is that, in addition to the voltage-dependent gating necessary to explain the difference between high and low conductance states, the channels possess an intrinsic asymmetry which restricts the passage of the probe molecule in the antidromic direction, i.e. that they behave as 'chemical rectifiers', using the terminology introduced by others (see Loewenstein, 1981). Such a conclusion does not mean that this rectification is absolute and that, at rest, a small amount of the dye cannot antidromically cross the junction, in quantities insufficient however to reach to threshold of detection under our experimental conditions. According to this, it is conceivable that a charge molecule like Lucifer Yellow, which tends to equilibrate its concentration through the junction, could meet a higher energy barrier when injected into the g.m.f. than when applied into the l.g.a. Such an asymmetry of the junctional permeability for a probe molecule resembles the chemical rectification described at intercytoplasmic channels between heterologous junctions of mammalian cell culture (Flagg-Newton & Loewenstein, 1980). These investigators suggested that the directional selectivity was the consequence of a saturation phenomenon of passive diffusion. The tested molecules were in this case assumed to interact with an asymmetrical channel site with kinetics such that their flux through the channel saturates at different concentrations depending on the direction of the flux (Loewenstein, 1981). However, the chemical rectification studied at heterologous junctions is not modified by electrical fields (Flagg-Newton & Loewenstein, 1980) which distinguished it from the one reported here.

APPENDIX

Equilibration time of Lucifer Yellow

Bennett, Spira & Spray (1978) have shown that it is possible to calculate the time constant for dye equilibration across gap junctions if one makes the simplifying assumption that the diffusional resistance d is the same for the probe molecule and the main current carrying ions. In their scheme, the system is modelled as a circuit composed by two capacitors connected by a resistance and d is deduced from the measurement of ionic coupling. When the dye is injected in one compartment the chemical coupling coefficient for a molecule which crosses the gap junctions (but not the non-junctional membrane) is unity while ionic coupling ranges from 0 to 1 (Harris, Spray & Bennett, 1983).

Under these conditions, for two cells of equal volume, the diffusional time constant t_d will be:

$$t_d = 1/2V \times d \quad \text{(Bennett } et\ al. \text{ 1978)},$$

where V is the cellular volume and d the diffusional resistance. For two compartments of different volumes, respectively V_1 and V_2, this relation is:

$$t_d = \frac{V_1 V_2}{V_1 + V_2} \times d.$$

Estimation of cell volumes leads to a value of about 55×10^{-6} cm^3 for the l.g.a. (assuming a constant diameter of 100 μm, and a length of 7 mm from the first to the second abdominal ganglion) and to 2×10^{-6} cm^3 for the g.m.f. (considering a cylinder of 50 μm diameter and 1 mm long). The diffusional resistance d was calculated by using the same values as those given by Bennett *et al.* (1978), except that the electrical resistance of the junction was measured for the g.m.s., at the low and high conductance states (Giaume & Korn, 1983). In these conditions, the parameter d was respectively $1{\cdot}5 \times 10^9$ and $0{\cdot}1 \times 10^9$ s/cm^3, and finally gave 50 and 4 min for Lucifer Yellow equilibration time constant across the rectifying electrotonic synapse.

The comparison of these calculations with our experimental conditions (Tables 1 and 2) indicates that if the initial hypothesis is correct (i.e. that d is the same for ions and the probe molecule) the dye would have been detected in the non-injected cells in all the trials that we made. This is not the case and therefore we must conclude that either d is in fact lower for probe than for ions, which argues for the dilution hypothesis at low conductance state, or d has not the same value in both directions and chemical rectification is real. In these two cases, the results suggest that there is an interaction between the molecule of Lucifer Yellow and the junctional channel.

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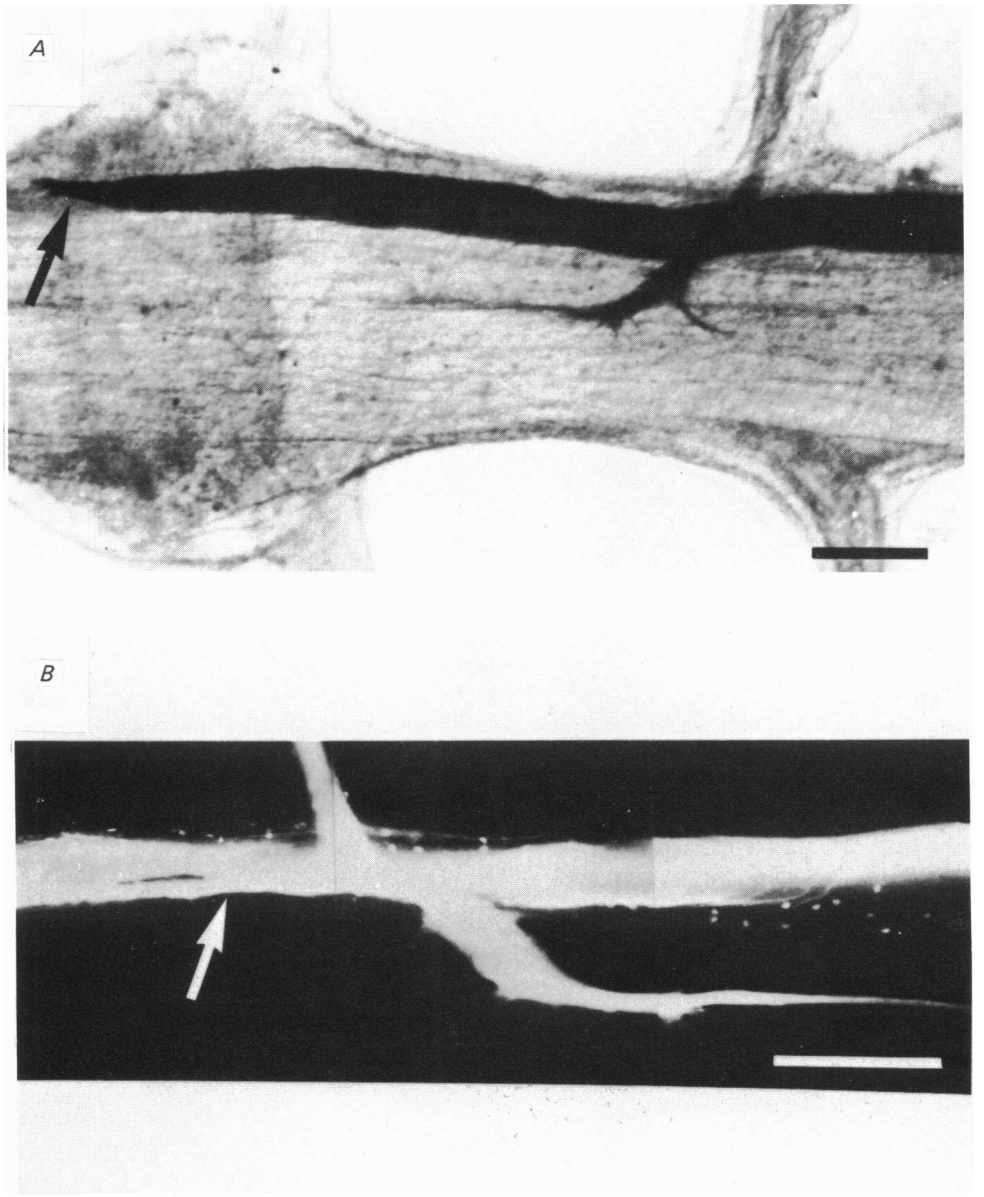

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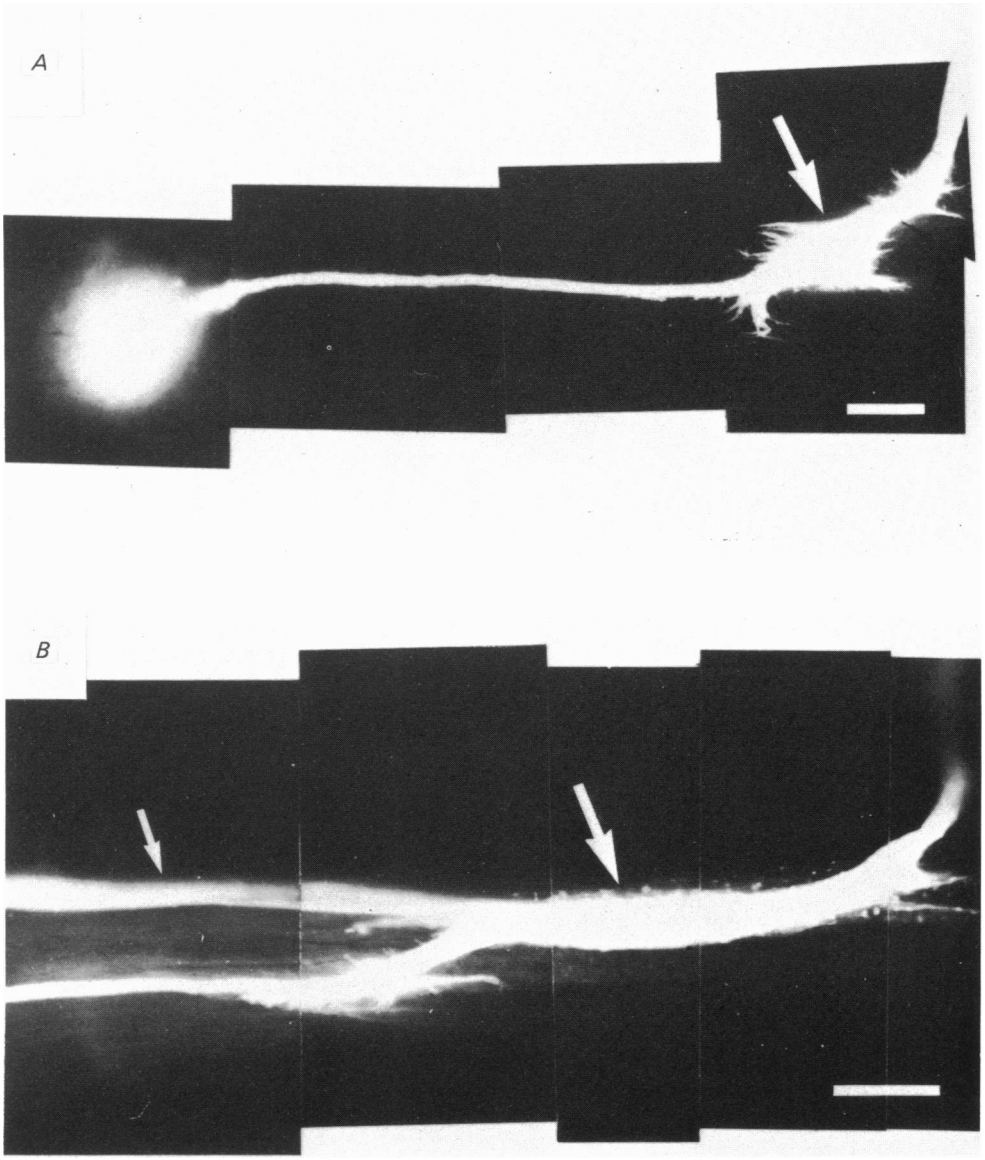
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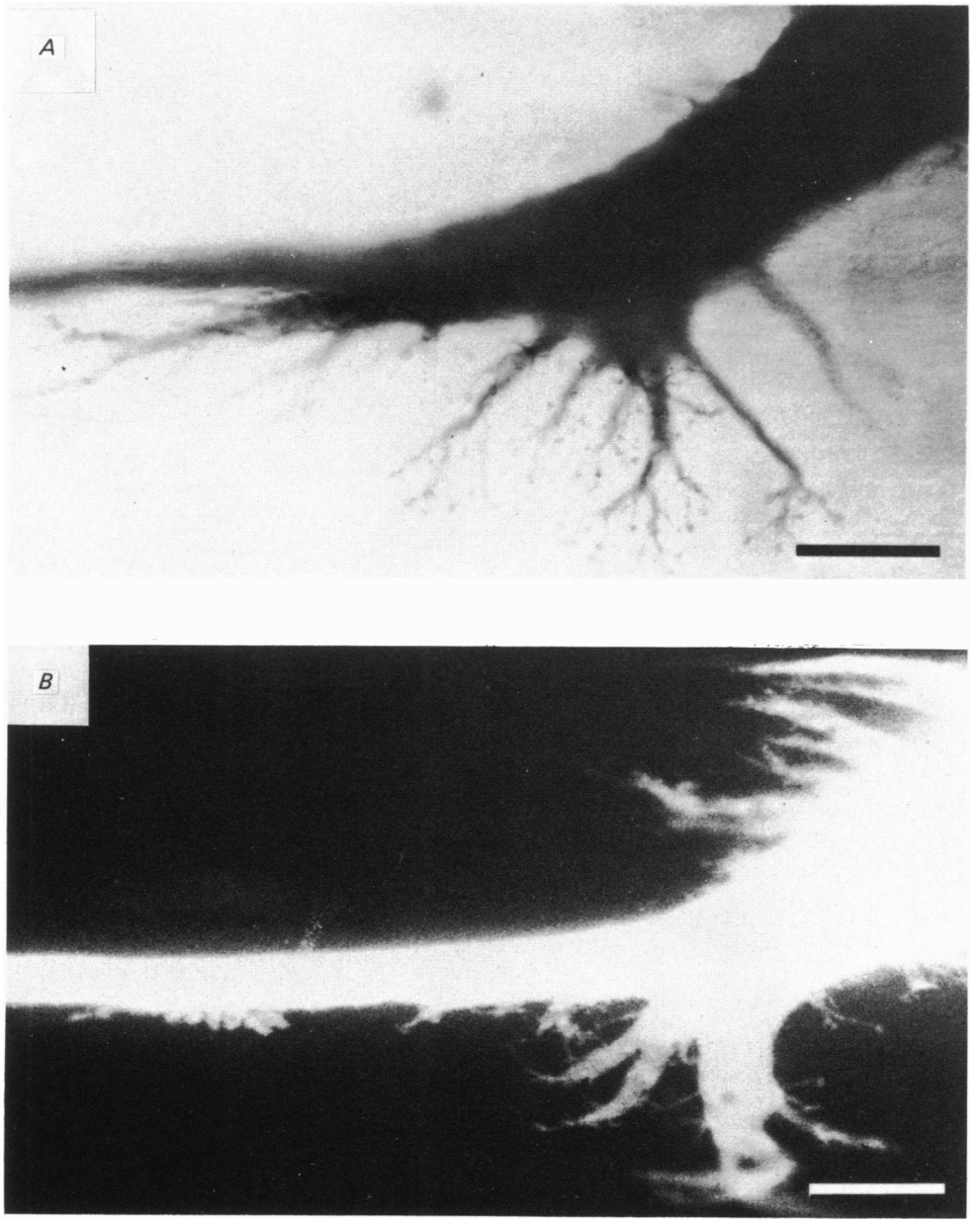
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EXPLANATION OF PLATES

PLATE 1

A, l.g.a. and g.m.f. separately marked intracellularly with HRP. Note the difference of cellular volumes between the two elements. The enzyme did not cross gap junctions at the level of the septum (arrow). Calibration bar, 250 μm. *B*, evidence for orthodromic permeability of the g.m.s. at rest. Lucifer Yellow was injected in the presynaptical cell (site of injection indicated by arrow) migrated transsynaptically in the motor fibre (same experiment as in Fig. 2). Calibration bar, 250 μm.

PLATE 2

A, lack of permeability of the g.m.s. in the antidromic direction at rest. Lucifer Yellow was injected in the g.m.f. at the level of the second abdominal ganglion (arrow) but the tracer was not visualized in the presynaptic cells, fluorescence remaining restricted to the g.m.f. which is entirely stained (same experiment as in Fig. 3*B*). Calibration bar, 50 μm. *B*, evidence for voltage-dependent antidromic migration of dye injected in the motor fibre spread to the l.g.a. (small arrow) when the voltage difference across the junction was inverted by a steady hyperpolarization of the motor fibre and fixed at the value of +42 mV (same experiment as in Fig. 4). Calibration bar, 250 μm.

PLATE 3

Cell-to-cell contact zone at the g.m.s. High magnitude of fibre processes stained by HRP in *A* and by Lucifer Yellow in *B* (from Pls. 1*A* and 2*A*, respectively). The profuse short dendrites which protrude from the post-synaptic motor fibre define the limits of the g.m.s., and represent the site of electrotonic and dye coupling. Calibration bars, 50 μm.