Intercellular communication in the early embryo of the ascidian

Ciona intestinalis

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Summary

We have studied the intercellular communication pathways in early embryos of the ascidian Ciona intestinalis. In two different series of experiments, we injected iontophoretically the dyes Lucifer Yellow and Fluorescein Complexon, and we analysed the spread of fluorescence to the neighbouring cells. We found that before the 32-cell stage no dye spread occurs between nonsister cells, whereas sister cells are dye-coupled, possibly via cytoplasmic bridges. After the 32-cell stage, dye spread occurs throughout the embryo. However, electrophysiological experiments showed that nonsister cells are ionically coupled before the 32-cell stage. We also found that at the 4-cell stage junctional conductance between nonsister cells is voltage dependent, which suggests that conductance is mediated by gap junctions in a way similar to that observed in other embryos.

Key words: ascidians, intercellular communication, junctions, Ciona intestinalis.

Introduction

Cells of most animal tissues are able to communicate through low-resistance channels known as gap junctions (Furshpan & Potter, 1959), which allow the flux of molecules up to 2 nm in diameter (Pitts & Simms, 1977; Simpson et al. 1977; Schwarzmann et al. 1981). These channels provide an intercellular pathway for the exchange of ions and metabolites needed for the coordination of cellular activities (Pitts, 1980; Loewenstein, 1981).

Gap-junctional communication has also been described in embryos of many vertebrates and invertebrates (see Caveney, 1985 for review). Gap junctions occur very early in development, suggesting that junctional communication mediates the cell-to-cell transfer of regulatory signals involved in the control of development (Potter et al. 1966). Furthermore, in the amphibian and mouse embryos it has been demonstrated that morphogenesis can be severely disturbed when intercellular communication is prevented by injection of antibodies to gap-junctional protein (Warner et al. 1984; Warner, 1987).

In the mouse embryo, analysis of intercellular communication by dye transfer and electrical coupling has shown that the blastomeres become coupled shortly after the embryo has reached the 8-cell stage (Lo & Gilula, 1979a). After implantation, the cells of the inner cell mass do not transfer dye to the trophectoderm cells, although they are still electrically coupled (Lo & Gilula, 1979b). These experiments demonstrate that intercellular communication is strictly regulated during development. It has been suggested that such a regulation may be important in embryos with 'regulative' development (Lo, 1985). However, recently it has been reported that intercellular communication may also be significant for the development of mosaic systems like the molluscs Patella (Serras et al. 1985) and Lymnaea (Serras & Van den Biggelaar, 1987).

In the present study, we have investigated intercellular communication in the early ascidian embryo, which displays a highly mosaic development. Ascidian embryos are well suited for the study of morphogenesis and differentiation because there is an obvious relationship between the segregation of

cytoplasmic determinants and the differentiation of specific cell lines (Reverberi, 1971; Whittaker, 1979). Moreover, the rigidly determinative cleavage pattern has permitted detailed cell lineage work which allows the origin of different adult or larval structures to be traced back (Conklin, 1905; Reverberi, 1971; Ortolani, 1955; Nishida & Satoh, 1983; Nishida & Satoh, 1985; Zalokar & Sardet, 1984). Ionic coupling up to the 8-cell stage has been reported in *Ciona intestinalis* and *Ascidia malaca* (Dale *et al.* 1982), but changes of cell coupling through early development have not been documented so far. In this paper, we report on intercellular communication in embryos of the ascidian *Ciona intestinalis* between the 2- and 32-cell stages, using both electrotonic and dye-coupling techniques.

Materials and methods

All experiments were performed at the Station Biologique in Roscoff. Adult animals of *Ciona intestinalis* were obtained from Morgat and Roscoff (Bretagne, France), and kept in tanks with running sea water. Fertilization was done artificially. Adult animals were opened and sperm and eggs sucked from gono- and spermioduct with a Pasteur pipette. For each fertilization, freshly collected sperm and eggs from three different animals were mixed. Sperm was used directly from the spermioduct without any previous dilution; no sign of polyspermy was ever detected. Under these conditions, 90–95 % of the eggs were successfully fertilized and developed normally.

In order to remove the mucus around the follicular cells, protease (0·05 %) was applied during 30–45 s. This treatment did not affect normal development and facilitated the manipulation of the embryos. All impalements for dye-injection and most for electrical recordings were performed without removing the follicle and test cells. Since these cells are transparent, orientation of the embryos and identification of the cells were easy. The test cells are normally not pigmented. In some cases, they showed a slight autofluorescence (see Fig. 2F), which, however, could not be confused with the fluorescence of the dye inside the blastomeres.

Dye iontophoresis

Microelectrodes were made from glass capillaries containing a microfilament (GC150-TF, Clark Electromedical Instruments, Pangbourne, UK). They had a resistance of about 10 MΩ when filled with 3 M-KCl. The tip was backfilled with a 3 % aqueous solution of the lithium salt of Lucifer Yellow CH (Sigma, St Louis, MO). The remainder of the microelectrode was filled with 3 M-LiCl. In a second series of experiments, the dye Fluorescein Complexon (Eastman Kodak Co., Rochester, NY) was used in a 3 % aqueous solution; the rest of the microelectrode was then filled with 1 M-KCl.

The embryos were positioned under a stereomicroscope; the dye-filled microelectrode was brought to the surface of the embryo by a micromanipulator (Microcontrole, Saint

Guenault, Evry, France) and impaled into the desired blastomere. Rectangular hyperpolarizing current pulses (duration 0·2–0·3 s; amplitude 5 nA) were applied at intervals of 2–4 s during 90 s. In the cases where a restricted diffusion of dye in the embryo was observed (the stages before dye spread begins), the time of iontophoresis was increased up to 5 min, in order to ensure that restriction of dye spread was not due to an insufficient amount of dye in the injected cell. After iontophoresis, the electrode was removed from the cell and the embryo was transferred to an epiluminescence fluorescence microscope (excitation: 490–510 nm; emission: 520 nm; Olympus, BH2, Japan). Microphotographs were taken on Kodak Ektachrome 400 ASA.

Since our equipment did not allow simultaneous injection and fluorescence microscopy, it was not possible to estimate the time between the start of iontophoresis and the moment at which the diffusion of dye became apparent. The average time needed to transfer the embryo to the fluorescence microscope was 2 min. From then on, subsequent observations were made every 5 min, during 30 min. As the duration of one cell cycle at 20–22°C is approximately 20 min, each dye-injected embryo was observed at least until it reached the next division.

Ionic coupling

Embryos at different stages of development were transferred to the recording chamber in which a slow perfusion of artificial sea water was maintained. Experiments were performed at room temperature (20–22°C). In a few experiments, follicle and test cells were manually removed before impalement but, in most cases, embryos with intact envelopes were used. The embryos were impaled and observed under a stereomicroscope. Two electrode holders were mounted on micromanipulators (Prior and Co., Herts, UK). Microelectrodes were pulled from glass capillaries with filament (Clark GC 150 TF) on a horizontal puller (BB-CH Mecanex, Geneva, Switzerland) and had a resistance of 10–20 MΩ when filled with 3 M-KCl. Grounding was *via* an Ag/AgCl wire positioned in the chamber. We used two different amplifiers connected to each electrode. Both amplifiers could measure the potential and inject a current through a single electrode. One amplifier (M707, WPI, New Haven, CT) had a bridge circuit to cancel the electrode resistance whereas the second one (RK400, Biologic, Grenoble, France) did not have such a bridge circuit.

The use of the same microelectrode for injection as well as for measurement of the potential may lead to errors in cell-resistance measurements, because the electrode resistance can change to an unknown degree during the experiment. However, this is not a serious problem when working with embryos of *Ciona intestinalis*, for two reasons. First, microelectrodes with a relatively low resistance can be used, which are less subject to clogging by cellular particles; second, the input resistance of the embryonic

cells is high (20–100 MΩ) and the time constant of the membrane potential response to a square current pulse is long (more than 100 ms) as compared to the time constant of the electrode response (less than 3 ms). Therefore, the two responses can be easily distinguished and the cell input resistance accurately measured with a single current-injecting microelectrode. Fig. 1 shows the equivalent electrical circuit, together with a typical set of data from a 16-cell embryo. The right trace in Fig. 1B illustrates the two different time constants.
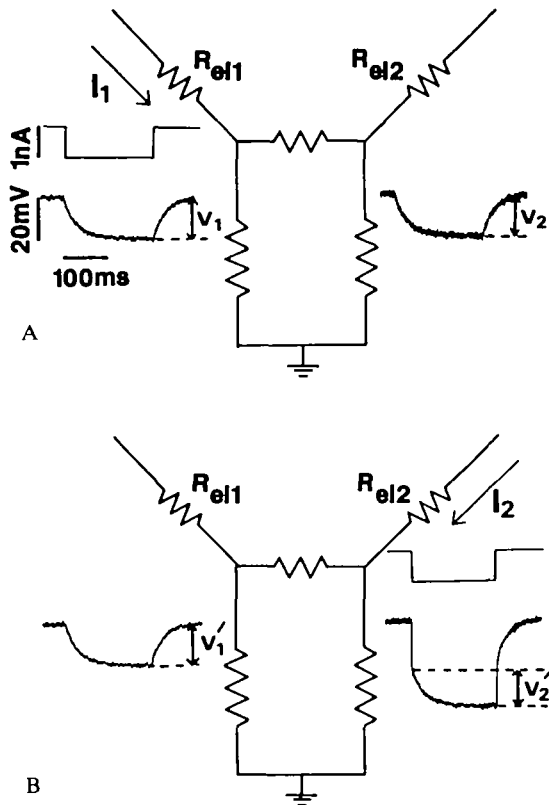

Fig. 1. Schematic representation of the experimental set up for electrotonic coupling measurements. The left and right insets represent the potential changes recorded in both cells. In A, a 1 nA current pulse is injected through the electrode on the left side (electrode 1, with resistance R_{el1}) into the cell. The current may divide between the impaled cell and the junctional resistance into the second cell. The left inset shows the transient voltage (V_1) recorded by electrode 1. The electrode resistance has been electronically cancelled. The right inset shows the simultaneous voltage shift (V_2) recorded in cell 2. In B, the current pulse is injected through electrode 2 (with resistance R_{el2}). Here, the amplifier does not allow electrode resistance compensation. Therefore, the transient voltage recorded by electrode 2 on the right first shows a rapid transient due to electrode resistance, and then a slower transient (V'_2) due to the membrane resistance. The left inset shows the transient (V'_1) recorded by electrode 1. These recordings are from a 16-cell-stage embryo in which the two cells are perfectly coupled. The four traces are drawn on the same scale.

With the parameters defined in the legend of Fig. 1, two coupling ratios were calculated:

$$C_1 = V_2/V_1$$
$$C_2 = V'_1/V'_2.$$

Input resistances were also calculated as:

$$R_1 = V_1/I_1$$
$$R_2 = V'_2/I_2.$$

Results

In *Ciona* embryos, the first cleavage takes place about 50 min after fertilization. The plane of cleavage is meridional and coincides with the plane of bilateral symmetry of the larva. The blastomeres are of equal size and according to Conklin's (1905) nomenclature they are termed AB2 and *AB2*. The second plane of cleavage is perpendicular to the first and the resulting four blastomeres (A3, *A3*, B3, *B3*) have the same size. The third cleavage plane is equatorial and gives rise to four animal blastomeres (a4.2, *a4.2*, b4.2, *b4.2*) and four vegetal blastomeres (A4.1, *A4.1*, B4.1, *B4.1*). After the fourth and fifth cleavages, the blastomeres are positioned symmetrically to the right and left of the first plane of segmentation so that the embryo consists of two symmetrical halves.

Dye coupling

In the course of 18 experiments, a total of 85 embryos (Table 1) were successfully impaled in a single cell and dye iontophoresed.

At the 2-cell stage, when injection was performed during the first 5–10 min after the first cleavage, Lucifer Yellow CH spread from the impaled cell to the other one (Fig. 2A). In contrast, when the injection was performed at the end of the 2-cell stage, no transfer of dye was detected, as illustrated in Fig. 2B.

Dye injection in 4-, 8- and 16-cell-stage embryos gave the same results: when Lucifer Yellow was iontophoresed early after cell division, the sister cell of the injected cell was also labelled. However, no spread of Lucifer Yellow CH was detected to nonsister cells. This is illustrated in Fig. 2D–F for an 8-cell stage. Like at the 2-cell stage, only one cell was labelled if the injection was performed at the end of a given stage as illustrated in Fig. 2C for a 4-cell stage.

These results suggest that after each cleavage, cytoplasmic bridges remain between sister cells; after about 10 min, these bridges disappear and diffusion of Lucifer Yellow to neighbouring cells can no longer be detected. This is similar to what has been described in mouse embryos up to the 8-cell stage (Lo & Gilula, 1979a).

It should be noticed that at the 16-cell stage, in two embryos out of ten, dye spread was found to neighbouring cells. In one of these two cases (Fig. 3B) in

Table 1. *Summary of dye-injection experiments*

Dye	Stage	Cases of no spread or spread to sister cell only	Cases of spread to nonsister cells
Lucifer Yellow			
	2	10	–
	4	15	–
	8	14	–
	16	8	2
	32	–	22
Fluorescein Complexion			
	2	2	–
	4	2	–
	8	2	–
	16	3	–
	32	–	5

which the dye had been injected in a cell at the vegetal side of the embryo (cell B5.1), spread of dye was detected to three cells on the same half of the plane of bilateral symmetry (cells A5.1, A5.2, B5.2) and to one cell of the other half (*B5.1*). In the other case, the cell injected (b5.4) belonged to the animal side and the dye spread to the symmetrical cell *b5.4* and to other cells on the same half as the injected cell (not illustrated). However, other embryos injected into the same two cells (B5.1 or b5.4) showed no dye spread.

After the fifth cleavage (32-cell stage and further), spread of dye to nonsister cells occurred in all embryos tested. Fig. 3D–F shows examples of dye spread to ten or more neighbouring cells. An extensive dye spread was observed when the dye was injected at the 32-cell stage. However, with the same dye–iontophoresis conditions, when a single cell was injected at the 16-cell stage and observed at the 32-cell stage, there was no or very little dye spread to adjacent blastomeres (Fig. 3C). This may be because the dye may be freely diffusible for a limited period only and it may gradually bind to cytoplasmic constituents (Stewart, 1978) as has been suggested in other developing embryos (De Laat *et al.* 1980; Dorresteijn *et al.* 1983).

Dye injections were performed into different animal and vegetal cells. In contrast to the amphibian embryo (Guthrie, 1984), in the 32-cell-stage *Ciona* embryo, the dye always spreads to neighbouring cells independently of the position of the injected cell. In some cases, dye spread seemed to be limited to one half of the embryo, on the injected side (see for instance Fig. 3F). However, later observations of these same embryos showed that the dye also spread towards the other half.

It has been reported recently that dyes with different chemical properties could show distinct dye-spread patterns (Fraser & Bryant, 1985). Therefore,

we also used the dye Fluorescein Complexon (Kodak; MW 618 D) to analyse the patterns of dye coupling in early *Ciona* development. We found exactly the same pattern as with Lucifer Yellow: no dye transfer before the 32-cell stage and extensive transfer after this stage (not illustrated).

Ionic coupling

Embryos at different stages of development, between the 2- and 32-cell stage, were impaled with two microelectrodes in two different cells. Current pulses were given alternately through one of the two electrodes and the voltage transients similar to those shown in Fig. 1 were recorded from both cells for a few minutes. For the sake of consistency, we measured all parameters within the first 10 min of impalement since, in some cases, embryos impaled for long periods of time divided abnormally. One reason is probably the osmotic modification brought about by KCl leaking out of the electrodes into the cells.

For each embryo, input resistances (R1 and R2) and coupling ratios (C1 and C2) were calculated as described in the Materials and methods section. In Fig. 4 data from 28 pairs of cells are plotted *versus* the number of cells in the embryo. At the 2-cell stage, coupling ratios vary from one to almost zero. At the 4-cell stage, the ratio falls into two groups, one around 0·3 and another at 1. It is likely that a coupling ratio of 1 is due to cytoplasmic bridges remaining between sister cells at the end of the division. Embryos at the 16- and 32-cell stages always had a coupling ratio close to 1, although we chose to impale only nonsister or nonadjacent pairs of cells.

The input resistances measured by each microelectrode in all cases are given in Table 2. At the 2-, 4- and 8-cell stages, the input resistances were $49\,M\Omega \pm 32$ (n = 26, $\pm$s.d.); at the 16 and 32-cell stages, they were $32\,M\Omega \pm 25$ (n = 18, $\pm$s.d.).
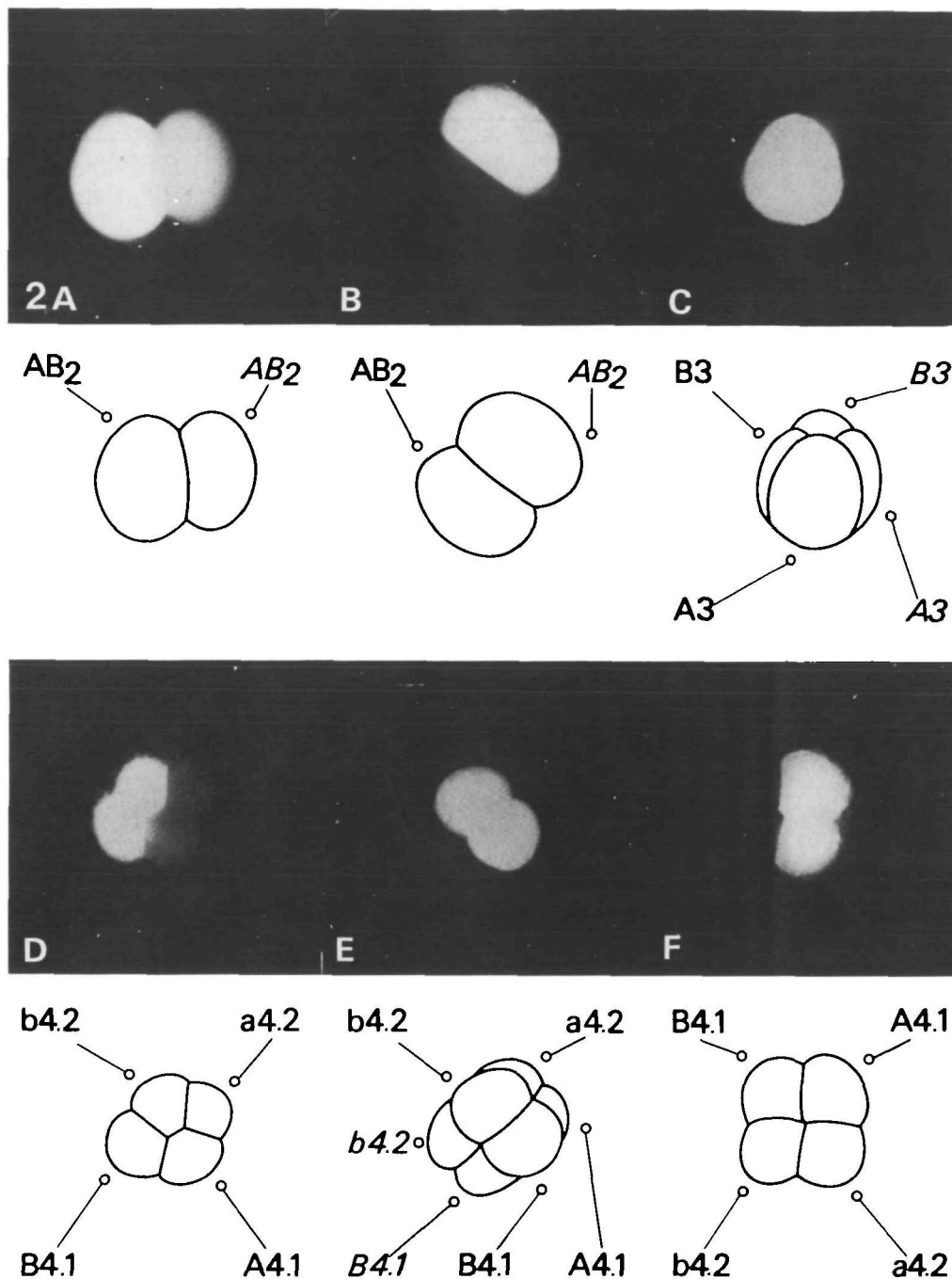
Fig. 2. Micrographs of embryos injected with Lucifer Yellow. (A) 2-cell-stage embryo injected just after first cleavage. (B) 2-cell-stage embryo injected at the end of the same stage. (C) Lateral view of a 4-cell-stage embryo injected in a A3 blastomere. (D–F) 8-cell-stage embryos injected in blastomere B4.1 (D,E, same embryo from different sides) and in blastomere A4.1 (F), showing dye spread to the sister cell only. Scale bar, 50 μm.

The question then arises whether at early stages the non-dye-permeant junctions are really *gap junctions*. One physiological criterion for gap-junctional communication is its voltage dependence (Spray *et al.* 1981; Harris *et al.* 1981, 1983; White *et al.* 1982; Knier *et al.* 1986). Therefore, we looked for a possible voltage dependence of junctions in the early *Ciona* embryo. When the coupling ratio was close to 1, no

rectification was observed in the voltage traces (see for instance Fig. 1), suggesting that the nonjunctional membrane did not have obvious rectifying properties. However, in the cases where the coupling ratio was small, a rectification was observed in the traces. Fig. 5 shows one example from a 4-cell-stage embryo with a coupling ratio of 0·2 in one direction and 0·8 in the other. Under these conditions, it was possible to
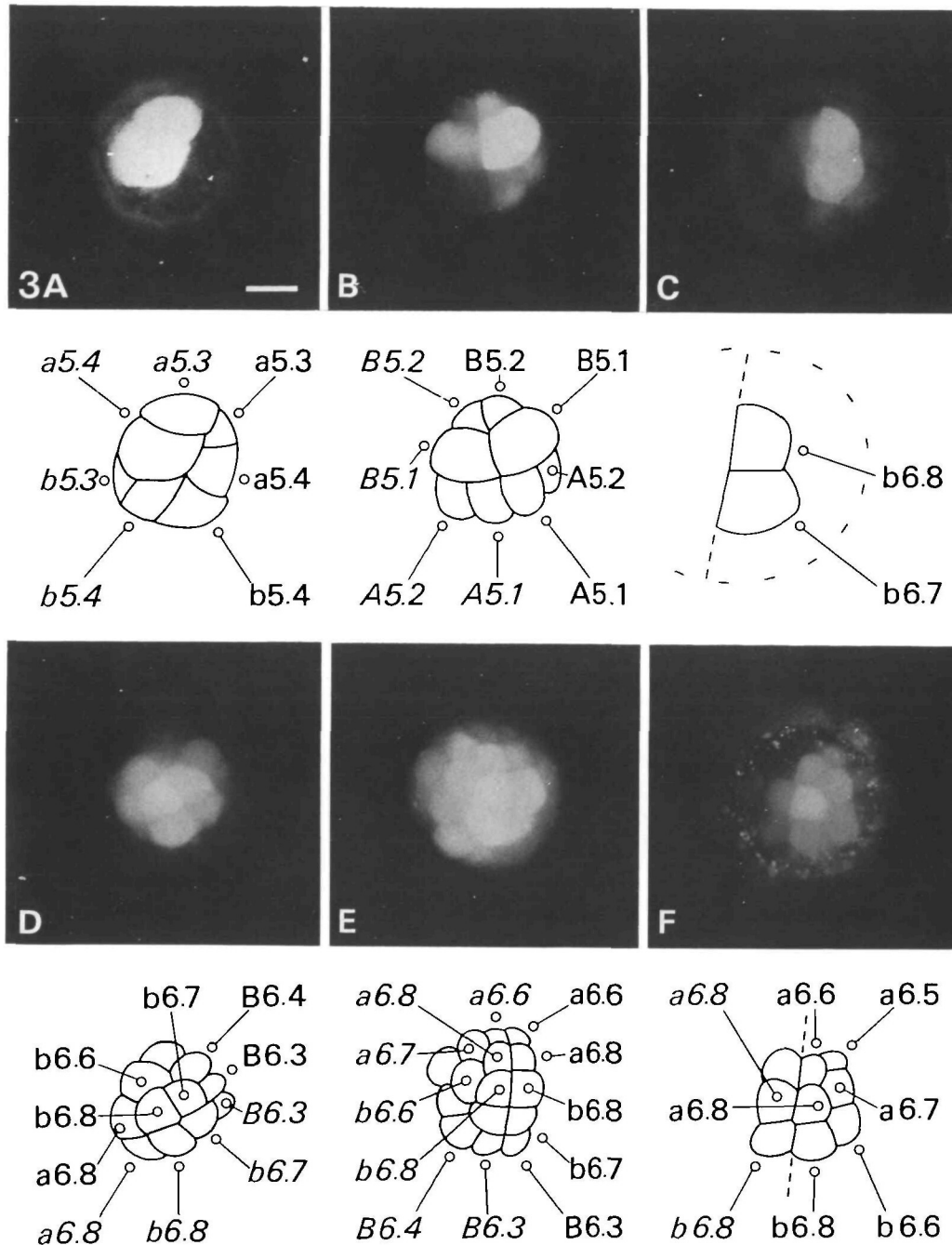
Fig. 3. Photomicrographs of 16- and 32-cell-stage embryos, injected with Lucifer Yellow. (A) 16-cell-stage embryo injected in cell a5.4; the dye spreads only to the sister cell a5·3. (B) 16-cell-stage embryo injected in B5.1 cell; the dye spreads to cells B5.2, A5.1, A5.2 of the same embryonic half and to cell B5.1 of the other half. (C) This 32-cell embryo has been injected in b5.4 at the 16-cell stage. The two sister cells are highly labelled, whereas little dye coupling to neighbouring cells is detected in contrast to D–F. (D,E) Embryos injected at the 32-cell stage. The dye spread to all neighbouring cells. These photomicrographs were taken 10 min after injection. (F) Embryo injected at the 32-cell stage and photographed 5 min after injection. Dye spread to the cells of one half seems to be stronger. The injected cell is at the border of the plane of bilateral symmetry. Scale bar, 50 μm.

produce a large membrane potential difference between the two impaled cells. The upper trace is from the injected cell, the lower trace is from the other cell. The coupling changed within the 300 ms pulse from 0·2 to almost 0, when the voltage difference reached about 30 mV. Since such a rectification was observed only in the cases of small coupling ratio, this is a clear indication that the junctions themselves are voltage dependent.
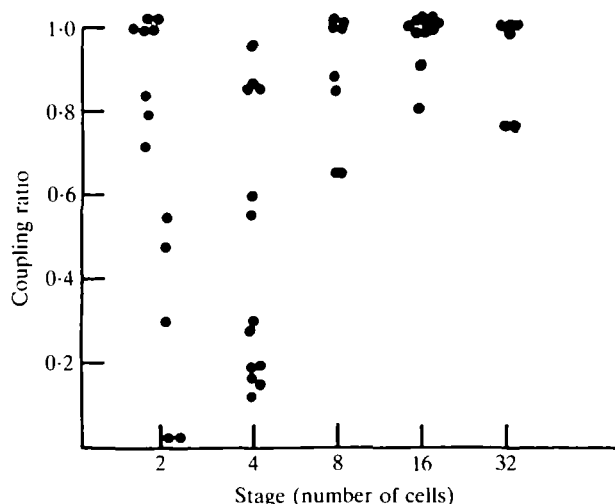
Fig. 4. Coupling ratios measured between 28 pairs of cells plotted against the stage of development. For each pair of cells, both coupling ratios are plotted, since they may be slightly different.

Fig. 5. Evidence for voltage dependence of the junctions in a 4-cell-stage embryo. Upper trace: potential change in the cell in which a 0·5 nA hyperpolarizing current is injected; middle trace: potential change in the other cell. Notice the difference in scale between the two traces. Lower trace: current pulse. The current pulse produces first an hyperpolarization of cell 1 of about 30 mV; cell 2 is hyperpolarized by only 10 mV; consequently, a voltage difference of about 20 mV is generated between the two cells. Then, cell 1 hyperpolarizes more whereas cell 2 depolarizes, indicating the closure of the junction. At the end of the pulse, the opposite effect can be observed: the potential in cell 1 goes back to the resting level and when the difference with cell 2 reaches 20 mV, the junction reopens. During a short period of time the potential spreads into cell 2, creating a little hump in the potential of cell 2.

Table 2. *Electrical characteristics of pairs impaled cells at different stages of development*

Stage	R1 (MΩ)	R2 (MΩ)	C1	C2
2	50	55	0·04	0·036
2	25	52	0·72	0·3
2	36	90	1·0	1·0
2	26	–	0·89	–
2	13	18	1·0	0·55
2	19	40	0·78	0·47
4	56	80	0·96	0·85
4	–	40	–	0·56
4	25	70	0·28	0·12
4	14	40	0·85	0·2
4	–	20	–	0·3
4	–	70	–	0·16
8	65	80	1·0	1·0
8	25	22	0·88	1·0
8	100	150	0·67	0·66
16	52	70	1·0	1·0
16	18	25	1·0	1·0
16	14	14	0·85	1·0
16	22	14	0·56	0·81
16	17	17	1·0	1·0
16	99	60	1·0	1·0
32	50	50	1·0	1·0
32	11	10	0·77	0·77
32	17	13	1·0	1·0

Discussion

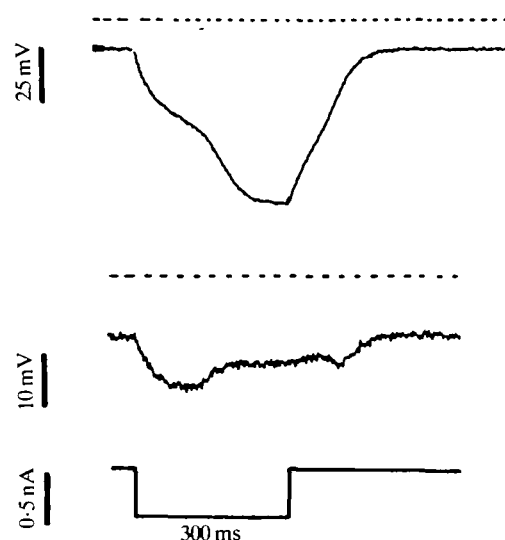In most of the embryos studied so far, cells are connected *via* gap junctions from early stages of development (Caveney, 1985). However, after each cleavage, pairs of sister cells are transiently coupled by cytoplasmic bridges resulting from mitosis with incomplete cleavage (Ducibella *et al.* 1975; Lo & Gilula, 1979a). In *Ciona* also, cytoplasmic bridges remain for up to approximately 10 min after cleavage, as shown by spread of dye between sister cells.

Dye-coupling experiments showed that after complete cytokinesis there is no transfer between non-sister cells in the 2-, 4-, 8- and 16-cell-stage embryos. However, extensive dye spread can be observed after the beginning of the 32-cell stage. This indicates that a striking change in cell communication occurs after the fifth division and it is likely that at this stage, junctional channels become capable of transferring small molecules.

Electrophysiological experiments give additional information about intercellular communication in *Ciona* development. It has been reported that, at the late blastula and gastrula stages in other ascidian species, cells are highly coupled (Miyazaki *et al.* 1974; Takahashi & Yoshii, 1981; Merritt *et al.* 1986). We found that, at early stages when no dye-coupling is

detectable, the blastomeres are already electrically coupled. Our findings partially resemble the results reported by Dale *et al.* (1982). We measured many coupling ratios smaller than 1 at the earliest stages of development, whereas Dale *et al.* (1982) did not mention finding coupling ratios smaller than 1. This discrepancy might be explained by the fact that they were following the cell after each division and did not consider the possibility of transient cytoplasmic bridges; furthermore, they did not record ratios between nonadjacent cells. Another reason might be that they reported a higher input resistance than we report here (200–500 MΩ against 20–100 MΩ). Under their conditions, it is to be expected that generally the coupling ratio will be higher. A possible reason for their better electrical characteristics is that they removed the follicle cells before impalement, whereas we, in order to create the same conditions as in our dye-injection experiments, did not remove the follicle cells.

Our electrical measurements indicate that the coupling ratio increases as development proceeds from the 8-cell stage onwards. This is consistent with the data from dye injection. However, since our measurements were made in intact embryos, several factors may contribute to this apparent increase in coupling ratio with development. First, in an intact embryo, the number of cells in contact increases at each division, therefore the number of electrical pathways between two cells increases. Second, part of the coupling may be due to current flowing out of the injected cell into an intercellular space and then to ground *via* a relatively high electrical resistance. Although in *Ciona* there does not seem to be an identifiable blastocoele before the 32-cell stage, this possibility cannot be ruled out. Another possibility is that each junctional conductance between two cells increases as development proceeds. At this point, we do not know which of these possibilities contributes most to the increase in ionic coupling between the 8- and 32-cell stages.

Intercellular communication at the early stages of *Ciona* development before dye coupling appears, is probably mediated in part by junctional channels because these junctions show voltage dependency as described for gap junctions of other embryos. Thus, taken together, data from dye-injection and electri-cal-coupling indicate that during development from 2- to 32-cell stage a transition of the junctions from low to high conductance occurs. It remains to be elucidated whether these changes of permeability in the early development of *Ciona* are due to a quantitative increase of the number of junctional plaques or to a qualitative change of the gap-junctional configuration.

The *Ciona* embryo differs from the mouse embryo in that in the latter, ionic and dye coupling seem to occur at the same time during the 8-cell stage (Lo & Gilula, 1979*a*; McLachlin *et al.* 1983; Goodall & Johnson, 1984). However, differences in dye diffusion rate indicate that there is a gradual increase of the junctional conductance throughout compaction, which may be due to an increase of the junctional channels between cells as development proceeds (McLachlin & Kidder, 1986). Therefore, it is possible that, in both embryos, the same phenomenon takes place, although in *Ciona* it extends from the 4-cell stage to the 32-cell stage whereas it is limited to the 8-cell stage in mouse.

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