Manipulation of gap junctional communication during compaction of the mouse early embryo

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SUMMARY

Three treatments that prevent cell flattening during compaction of the mouse preimplantation embryo were assessed for their effects on the onset of gap junctional communication. Medium low in calcium (LCM) and an antiserum to an embryonal carcinoma cell line (anti-EC; Johnson *et al.* 1979) both prevented the establishment of coupling between blastomeres of the 8-cell embryo as assessed by transmission of carboxyfluorescein or by ionic coupling. Since neither of these agents prevents the contact-mediated induction of cell polarity that occurs at this stage, it is concluded that the induction of this process is not signalled via gap junctions. A monoclonal antibody (ECCD-1; Yoshida-Noro, Suzuki & Takeichi, 1984), that recognizes more specific components of the calcium-dependent cell adhesion system, failed to prevent the onset of junctional coupling. This suggests that the onset of junctional coupling is not dependent upon extensive cell apposition and that the requirement for extracellular Ca^{2+} resides at a level other than that of cell adhesion. Moreover, neither LCM nor anti-EC could reverse cell coupling once it had become established despite their complete reversal of cell flattening.

INTRODUCTION

In the mouse preimplantation embryo, intercellular communication via gap junctions appears first at the 8-cell stage as the process of compaction begins (Lo & Gilula, 1979a; Goodall & Johnson, 1982, 1984; McLachlin, Caveney & Kidder, 1983). During this process the blastomeres begin also to flatten on one another (Ziomek & Johnson, 1980; Lehtonen, 1980) and become polarized. Zones rich in microvilli become localized only on the outer surface of the embryo and sub-cellular components are reorganized in a radially polarized array (Ducibella & Anderson, 1975; Lehtonen & Badley, 1980; Reeve, 1981; Reeve & Ziomek, 1981; Reeve & Kelly, 1983; Johnson & Maro, 1984; Fleming & Pickering, 1985; Maro, Johnson, Pickering & Louvard, 1985). Elements of this polarity are stable and are conserved through division to the 16-cell stage resulting in the formation of inner and outer cells with characteristic properties (Johnson & Ziomek, 1981a; Randle, 1982). It has been proposed that these cells are destined to become the inner cell mass and trophectoderm cells, respectively, of the blastocyst (Johnson, 1985).

It seems that both the induction of polarity and the maintenance of the divergent cell lineages are central events in the development of the blastocyst. Cell interactions appear to occupy a key role in these processes as shown by the

Key words: gap junctions, cell flattening, morula, mouse, monoclonal antibody.

dependence of the axis of polarity on the extent and geometry of cell contacts and by the stage specificity of the signal that induces polarity (Ziomek & Johnson, 1980; Johnson & Ziomek, 1983). Since the induction of cell polarity (Johnson & Ziomek, 1981b) and the ability of blastomeres to form junctional channels (Goodall & Johnson, 1982, 1984) both develop during the first 2–5 h after division to the 8-cell stage, junctional coupling presents itself as a potential regulatory mechanism for mediating the induction process (Lo, 1982). The process of cell flattening itself, that occurs at this time, also offers a potential regulatory influence on the onset of junction formation, an apparent correlation between the two having been noted previously (McLachlin et al. 1983; Goodall & Johnson, 1984).

An opportunity to dissect out these events is provided by the ability of various agents to inhibit cell flattening whilst allowing the development of cell polarity. Three of these agents were chosen for this study: a rabbit antiserum (anti-EC) raised against an undifferentiated embryonal carcinoma cell line (Johnson et al. 1979) and shown to allow development of polar organization (Pratt et al. 1982); low-Ca^{2+} medium (LCM), which interferes with a major cell–cell adhesion system (Yoshida & Takeichi, 1982; Shirayoshi, Okada & Takeichi, 1983) but allows development of a polar organization (Pratt, Ziomek, Reeve & Johnson, 1982); finally, a monoclonal antibody (ECCD-1) directed against a major component of the calcium-dependent cell adhesion system (Shirayoshi et al. 1983; Yoshida-Noro et al. 1984) and which has also been shown to allow development of polarity (Johnson, 1985; M. H. Johnson, B. Maro & M. Takeichi, submitted for publication). The extent of junctional coupling was monitored by ionophoretic injection of electric current and fluorescent dye (carboxyfluorescein) in conditions where cell flattening was prevented or reversed by these agents.

MATERIALS AND METHODS

(1) Collection and culture of embryos

Female mice of both CFLP (Hacking and Churchill Ltd, Alconbury, U.K.) and MFI strain (Olac Ltd, Bicester, U.K.) were superovulated by injection of 5 i.u. Pregnant Mare Serum gonadotrophin (PMS, Folligon, Intervet), followed 44–48 h later by 5 i.u. human chorionic gonadotrophin (hCG, Chorulon, Intervet) and mated with CFLP males. The presence of a vaginal plug indicated successful mating. Mice were killed by cervical dislocation 48–50 h (CFLP) or 52–55 h (MF1) following injection of hCG and the oviducts were flushed with Medium 2 (Medium 16 modified for atmosphere culture by replacement of most of the bicarbonate with HEPES; Fulton & Whittingham, 1978) containing $4 \, mg \, ml^{-1}$ bovine serum albumin (M2+BSA). The early 4-cell embryos recovered were placed either in drops of Medium $16+4 \, mg \, ml^{-1}$ BSA (M16+BSA, Whittingham, 1971) or in drops of the appropriate experimental medium at 37°C under oil in an atmosphere containing 5 % CO_2 for 18–22 h. During this time the majority of control embryos in M16+BSA had reached the compacted 8-cell stage whilst those in the experimental media had undergone cleavage at the appropriate time but were non-compact. In some experiments, late 4-cell embryos were selected hourly as they divided to 8-cells (approximately 14–16 h after the initial collection) and were then exposed to the various culture conditions for a period of no more than 6 h before use. There was no significant difference between results gained from these embryos and those gained by culture from the early 4-cell stage. Embryos were assayed for junctional coupling soon after compaction was underway in the controls (approximately 8–10 h after division from 4-cells) so that the

down-regulation of coupling that occurs during mitosis (Goodall & Maro, 1986) would not be encountered. To reverse cell flattening, newly compacted embryos were transferred from microdrops of M16+BSA to drops containing the various agents and cultured for approximately 2 h.

(2) *Agents used to inhibit or reverse cell flattening*

(a) Rabbit antiserum (anti-EC) to mouse LS 5770 embryonal carcinoma cells as described by Johnson *et al.* (1979) was diluted 1:40 in M16.

(b) Low calcium medium (LCM) was prepared by replacing the Ca^{2+} salt in M16 with the isotonic equivalent of NaCl, thus raising the NaCl concentration from 94 mM to 97 mM. The BSA concentration was elevated to 6 mg ml^{-1} (Pratt *et al.* 1982).

(c) ECCD-1, a monoclonal antibody raised against embryonal carcinoma cell line F9 and recognizing cadherin, a major component of the calcium-dependent cell adhesion system (Yoshida-Noro *et al.* 1984), was diluted 1/50 in M16+BSA.

(3) *Preparation of embryos for electrophysiology*

Approximately 0·2 ml of a solution of poly-L-lysine (PLL, Sigma, U.K.) at a concentration of 1 mg ml^{-1} was applied to 35 mm plastic cell culture dishes (Falcon plastics U.K.) in which slots had been cut to allow for the horizontal entry of microelectrodes. The PLL was spread evenly over the surface with a glass rod, dried at 37 °C and the dishes stored over desiccant at 4 °C until required. Immediately before use, approximately 1 ml of a solution containing 100 μg ml^{-1} Concanavalin A (Miles-Yeda Ltd, Illinois, USA, 3× crystallized, lyoph.) in phosphate-buffered saline was spread over the dish, left for 5–10 min and washed with two rinses of distilled water followed by two rinses of protein-free M2. Embryos, still within their zonae pellucidae, were pipetted directly into the M2 on the dish, whereupon they adhered firmly to the treated surface. The medium was then aspirated and replaced with M2+4 mg ml^{-1} BSA containing the appropriate agent. Under these conditions the embryos remained adherent. Embryos treated with LCM were subjected to M2+BSA containing Ca^{2+} during the microelectrode impalements due to initial difficulties in obtaining a good seal in low Ca^{2+} conditions. However, subsequent investigations using microelectrodes of resistance 80 Megohms or greater showed this not to be necessary. Results were identical with either medium. The preparation was then maintained at 25 °C on a water-heated microscope stage.

(4) *Microscopy*

The dishes holding the affixed embryos were viewed under a modified Leitz Ortholux microscope with combined incidence and transmission capability. Light at a wavelength of 488 nm was provided by a 2 W Argon ion laser (Spectra-Physics, St Albans, U.K.) and reflected into the limb of the microscope via a Pol-vertical illuminator attachment, housing an achromatic beam-splitter suitable for transmission of fluorescein emission. Fluorescence issuing from the specimen was passed further through a Zeiss No. 50 barrier filter which allowed precise cut-off of transmission below 500 nm.

(5) *Electrophysiology*

A Neurolog system (Digitimer, Welwyn Garden City, UK) comprising NL303 period generator, NL403 delay-width module and two NL102 amplifier modules, was used. In the majority of experiments, a current monitor (Purves, 1981) was incorporated to measure the actual current that passed through the preparation. Hyperpolarizing current pulses of 2 nA were applied for 0·5 s every second during a period of 10 min via a glass electrode made from 1·5 mm 'Kwik-Fil' tubing (Clarke Electro-medical Instruments, Pangbourne, UK) and pulled to a resistance of 50–80 Megohms when filled with 0·2 M-KCl. The electrode tip was filled with a solution containing 5 mM-carboxyfluorescein (CF) in 0·2 M-KCl initially dissolved at a pH of 10·0 and reduced subsequently to 7·0 (Socolar & Loewenstein, 1979); the shaft was filled with 0·2 M-KCl. In a similar recording electrode, both tip and shaft were filled with 0·2 M-KCl. Entry

through the cell membrane was achieved by the brief use of maximum capacitance compensation, and stable resting potentials were recorded from embryos cultured in either control or experimental media. The preamplifiers holding the electrodes were themselves held by Leitz micromanipulators. Two silver/silver chloride/0·2 M-KCl/2 % agar bridges completed the circuit, separating the functions of current sink and measurement (Purves, 1981). The three traces comprising injected current, signal from the injected cell and signal from a remote cell were displayed on a Tektronix 5lll storage oscilloscope. Traces were photographed using a Tektronix C5C camera on Polaroid 667 film. For the reasons outlined in Goodall & Johnson (1984), no attempt was made to cancel that component of the signal from the injecting electrode arising from the microelectrode resistance. Consequently, this trace has no quantitative significance but was used to monitor the resting potential in the injected cell.

(6) Assessment of gap junctional communication

In the mouse early embryo, the conventional indicators of gap junctional communication, rapid dye passage and ionic continuity, often are found only between adjacent blastomeres rather than through the whole embryo. Indeed, in the 8-cell embryo it is usual to find ionic coupling among quartets of blastomeres where no gap junctions have yet formed (Goodall & Johnson, 1984). This localized form of coupling is mediated by cytoplasmic bridges (midbodies) that persist from previous mitotic cleavages. It is clear, therefore, that care must be exercised in the interpretation of such data. In the present study, analysis of coupling was carried out in three stages. Firstly, a broad assessment of the extent of cell coupling was made by injection of CF into one blastomere and observing the spread of dye over a period of 10 min. Secondly, if an experimental treatment resulted in a majority of embryos showing only limited dye spread, the extent of ionic coupling was then estimated. Impalement of one blastomere with the injecting electrode was followed by serial impalement of the recording electrode into at least four other blastomeres and the presence or absence of ionic coupling to each was recorded. Where five or more blastomeres in total were so coupled, gap junctional coupling was assumed to be responsible. If four or fewer blastomeres only were coupled, midbodies were assumed to be responsible. Thirdly, an independent measure of midbody-mediated coupling was achieved by injection and visualization of horseradish peroxidase, which traverses midbodies but not gap junctions (Lo & Gilula, 1979a,b; Bałakier & Pedersen, 1982). This procedure identifies virtually all midbodies from the immediately previous mitotic division but does so with lesser efficiency for midbodies persisting from earlier divisions (Goodall & Johnson, 1984).

(7) Assessment of midbody communication

The procedure for assessment of the extent of coupling due to midbodies was adapted from Lo & Gilula (1979a) and Bałakier & Pedersen (1982), as described in Goodall & Johnson (1984). Single glass electrodes were pulled from 1·5 mm 'Kwik-Fil' tubing (Clarke Electromedical Instruments, Pangbourne, U.K.), to a resistance of 30–50 Megohms when filled with a solution of 5 mg ml^{-1} horseradish peroxidase (HRP-Type VI; Sigma UK) in 0·2 M-KCl. Groups of 5–10 embryos in their zonae pellucidae were mounted in M2+BSA with or without the appropriate cell-flattening inhibitor. The electrode was inserted into one blastomere as described in the last section and, after ensuring that a resting potential of at least −10 mV was recorded, depolarizing pulses of 5 nA magnitude and 5 s duration at 6 s intervals were applied for a total of 2 min. The current trace was inspected closely during this time, since electrode blockage and reduction of the injected current to 1–2 nA resulted invariably in failure of HRP to enter the blastomere. After sequential injection of the embryos, all were removed carefully and returned to the appropriate culture medium for 3 h, after which they were fixed with a solution of 2·5 % glutaraldehyde in 0·1 M-phosphate buffer at pH 7·2 for 30 min. The embryos were then washed in buffer alone.

Visualization of HRP was achieved by a modification of the method of Graham & Karnovsky (1966), as described previously (Goodall & Johnson, 1984). The fixed and washed embryos were rinsed in 0·05 M-Tris-HCl, pH 7·4, for 5 min and then incubated for 30 min at room temperature in 0·5 M-Tris-HCl containing 1 mg ml^{-1} diaminobenzidine tetrahydrochloride (DAB; Sigma, U.K.) and 0·002 % fresh hydrogen peroxide (Sigma, U.K.). Embryos were then washed in

buffer alone. Since DAB is a suspected carcinogen, great care was exercised in its use and handling.

The embryos were scored for HRP activity by bright-field stereomicroscopy (Wild M5) at a magnification of ×100. Blastomeres were scored as darkly-, lightly-, or very lightly-labelled. For reasons described previously (Goodall & Johnson, 1984), those with very lightly stained blastomeres alone were discarded. Uninjected control embryos were treated similarly and in some, HRP was injected into the subzonal space to control for endocytosis of HRP leaked from the electrode. No artifactual staining was seen in these circumstances.

(8) Photography

Photographs were taken on Tri-X film (Kodak, U.K.) with a Leica M3 camera, using 1/60 s or 1/125 s exposures for bright-field images and 1 s for fluorescence images. The laser output was maintained at 200 mW for the duration of the exposure.

RESULTS

The mean resting potential measured in control embryos was $-19 \cdot 6 \pm 6 \cdot 6$ mV (mean ± S.D.; n = 44). ECCD-1 treated embryos differed slightly from this value at $-22 \cdot 9 \pm 7 \cdot 8$ mV (n = 77), this small hyperpolarization being significant using the non-parametric Mann-Whitney U-test ($P \leqslant 0 \cdot 01$). However, significant depolarization of the measured resting potentials was seen in embryos cultured in anti-EC or LCM, the means being $-15 \cdot 9 \pm 4 \cdot 2$ mV (n = 40; $P \leqslant 0 \cdot 02$) and $-14 \cdot 3 \pm 3 \cdot 6$ mV (n = 42; $P \leqslant 0 \cdot 001$) respectively. Almost certainly, the variation in resting potentials was due to differences in the ease of effecting the initial microelectrode seal since all of the agents have a major influence on the cell surface. In each case, however, the resting potential was maintained throughout the period of injection indicating that the seal was effective.

The effects of each of these treatments on the spread of ionophoretically injected CF were characteristic, are illustrated in Fig. 1 and analysed in Fig. 2. After a 10-min injection period dye was found to have permeated in 1, 2, 4 or all 8 blastomeres, intermediate values being seldom observed, presumably due to the geometry of cell contacts across which the dye diffuses. The fluorescence image seen in Fig. 1E appears to reveal just such an intermediate condition but this was in fact due to a small degree of bleaching by the inspection beam. It must be emphasized that the distribution of CF observed may not be final, only representing the progress of the dye after 10 min. Thus if little spread of dye is observed after this time, communication junctions may either be absent or of lower transmission capability. Among compacted controls (Figs 1A–C, 2A), it was rare to find dye confined to 1, 2 or 4 blastomeres only; by far the majority of control embryos (76%) showed dye transmission to all blastomeres during the injection period, this taking place over $6 \pm 2 \cdot 5$ min (mean ± S.D.; n = 26).

Although ECCD-1 prevented the onset of cell flattening (Fig. 1D) it did not have a major effect on the distribution of dye transmission (Fig. 2B). Sixty-two percent of embryos showed dye passage to all cells and the overall pattern of dye distribution was similar to that in the control population. In two of the embryos that failed to show complete dye spread, serial impalement of each blastomere by
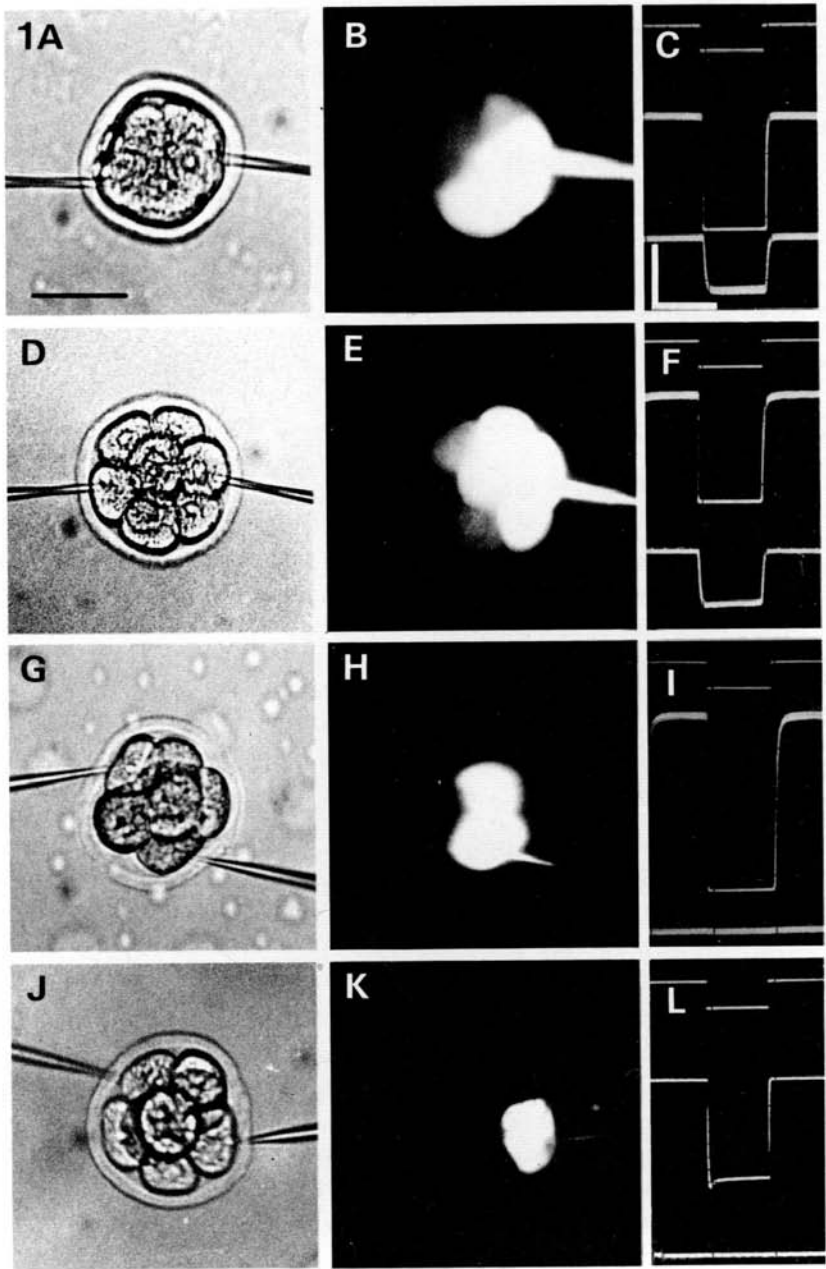
a second recording electrode showed ionic coupling between 7 out of 7, and 8 out of 8 blastomeres probed respectively, indicating a reduction of coupling in degree only within these embryos. In contrast, anti-EC (Figs 1G,H, 2C) and LCM (Figs 1J,K, 2D) prevented both cell flattening and dye transmission beyond the injected cell in all but 4 % of anti-EC treated embryos in which dye spread only to
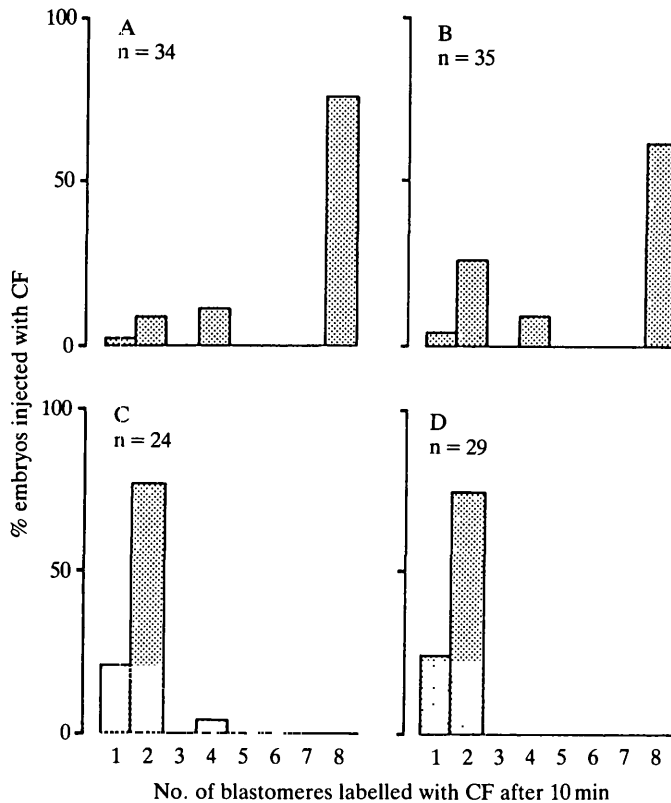
Fig. 2. Extent of CF transmission among blastomeres of 8-cell mouse embryos cultured under the conditions described in Fig. 1. Under control conditions (A) the majority of embryos cultured from the early 4- or 8-cell stage to the late 8-cell stage showed transmission of the dye to all 8 cells as did those that were cultured in ECCD-1 (B). Embryos cultured in anti-EC (C) or LCM (D) however showed little or no transmission of dye beyond the injected cell and its midbody-coupled sister during the 10-min injection period. The injection schedule is described in the legend to Fig. 1.

Fig. 1. Effect of cell-flattening inhibitors on the appearance of junctional coupling in mouse embryos by the late 8-cell stage. Under control conditions, most of the embryos showed extensive cell apposition (A) and complete transmission of ionophoretically injected carboxyfluorescein (5 mM in 0·2 M-KCl) (B) and ionic coupling (C) within 10 min. Prevention of cell flattening by the monoclonal antibody ECCD-1 (D) nevertheless allowed extensive dye transmission (E) and ionic coupling (F). In the polyclonal antiserum, anti-EC (G), cell flattening was inhibited but neither dye transmission (H) nor ionic coupling (I) was observed over the 10-min injection period. Similarly, low Ca^{2+} medium prevented cell flattening (J) but allowed neither dye transmission (K) nor ionic coupling (L). Embryos were cultured from the early 4- or early 8-cell stage (see text) until the majority of control embryos were compacted. The oscilloscope traces (C,F,I,L) indicate: upper trace – hyperpolarizing current pulse (0·5 s every 1 s); middle trace – combined signal from electrode and injected cell (this was limited at the lower end of its excursion in C,F and I and was used only for inspection of the resting potential in that cell (see text for explanation); lower trace – perceived signal from remote cell. In the bright-field and fluorescence photographs, horizontal bar = 50 μm. In the oscilloscope traces the horizontal bar = 0·5 s and the vertical bar for the current signal = 5 nA or, for the potential traces, 10 mV (C,F,I) or 50 mV (L).

two further cells. Furthermore, the majority of embryos subjected to anti-EC or LCM also lacked extensive ionic coupling (Table 1), indicating that the establishment of junctional coupling was very difficult under these conditions. The possibility that anti-EC and LCM could have interfered with the electrode seal sufficiently to shunt the majority of the current, leading to artifactual lack of coupling, is rendered unlikely by two observations. Firstly, neither of these treatments prevented the detection of ionic coupling in postcompaction embryos (see end of this section) and secondly, whilst the measured resting potentials were reduced in these treatments, they remained stable (see earlier).

The failure of dye to spread beyond the injected cell in 21–24 % of embryos cultured in anti-EC and LCM (Fig. 2C,D) also raises the possibility that these agents may have had some influence on midbody connections between mitotic sister blastomeres. It has been shown previously that injected HRP, which may pass only through these channels but not through gap junctions, fails to do so in only around 10–15 % of normal 8-cell embryos (Goodall & Johnson, 1984). The results of such HRP injections into embryos treated with ECCD-1, anti-EC and LCM (Fig. 3) are compared with control embryos in Table 2. Failure to transmit HRP to any other blastomere was only slightly more common in cells treated with ECCD-1 than in controls and indeed was less common among those cultured in LCM. However, persistence of midbodies from the earlier mitotic division, illustrated by passage of HRP to three or four cells (Goodall & Johnson, 1984), was depressed by all three treatments to less than half the control incidence.

Finally, although both anti-EC and LCM were able to prevent the establishment of coupling, neither could reverse junctional coupling significantly between blasto-meres of embryos that had already formed their junctions (Figs 4, 5). Despite the capacity of these agents to reverse cell flattening, and to prevent the establishment of junctional coupling, very little change from the control distribution of dye transmission was seen following postcompaction culture in anti-EC (Fig. 5B) and only a minor reduction in the incidence of dye transmission results from such culture in LCM (Fig. 5C).

Table 1. *The extent of ionic coupling in embryos cultured in anti-EC or LCM from the early 4- or 8-cell stage to the mid–late 8-cell stage (approx. 8–10 h postdivision from 4 cells)*

Treatment	No. of embryos probed	No. of embryos in which ionic continuity was recorded between		
		0–2 blastomeres	3–4 blastomeres	>4 blastomeres
anti-EC	17	16	1	0
LCM	19	16	2	1

A minimum of four blastomeres was impaled serially by a glass electrode to record electrical continuity with the injected blastomere, into which a 2 nA current was passed for 0·5 s at 1 s intervals. Ionic continuity between more than four blastomeres is interpreted as being due to gap junctional coupling: control embryos were not amenable to this experimental regime but showed complete dye spread throughout all blastomeres in the majority of cases (Figs 2, 5).
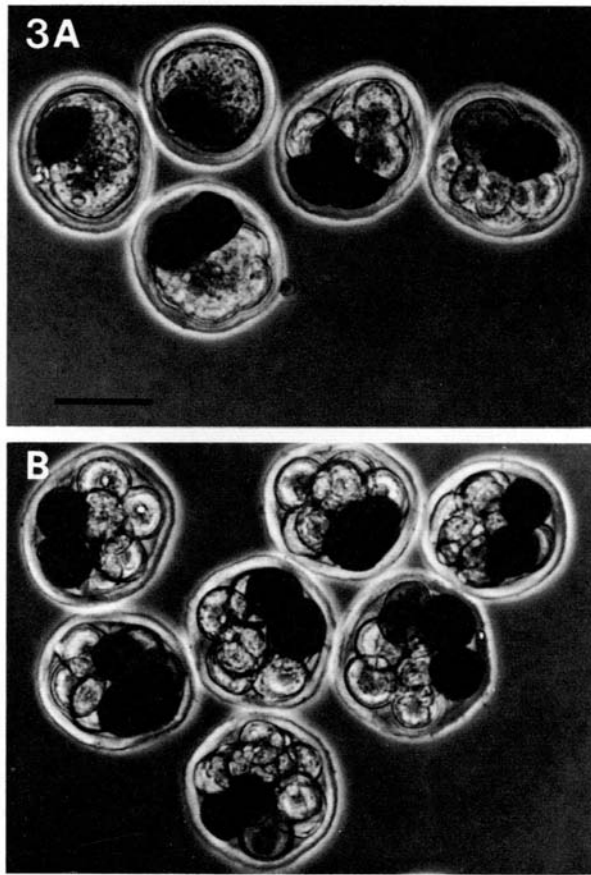
Fig. 3. Distribution of ionophoretically injected horseradish peroxidase (HRP) among blastomeres of late 8-cell mouse embryos 3 h after injection. Embryos in (A) had been cultured in control medium and in (B) in LCM from the early 4- to the mid 8-cell stage. 1, 2, 3 or 4 blastomeres are seen to be labelled in both conditions due to the persistence of midbody channels. Bar, 50 μm.

DISCUSSION

The hypothesis that cell interactions might be mediated by direct cytoplasmic transmission of signals through gap junctions is suggested by observations from many developing and differentiated systems (Warner & Lawrence, 1982; Pitts & Finbow, 1982; Weir & Lo, 1982, 1984). However, the evidence presented here suggests that despite a good temporal correlation between the emergence of functional gap junctions and the induction of contact-mediated cell polarity in the mouse 8-cell embryo (Goodall & Johnson, 1984), transmission of the inducing signal is not dependent upon such junctions. Thus, despite the ability of blastomeres in the 8-cell embryo to become polarized in the presence of LCM or anti-EC (Pratt *et al.* 1982) no junctional communication could be detected by injection of dye or ionic current. Independent evidence in support of this conclusion has also been presented. Thus blastomeres from 2- and 4-cell stages, known to be able to cue polarity in a significant proportion of disaggregated 8-cell blastomeres

(Johnson & Ziomek, 1981a) were unable to form channels of communication with them (Goodall & Johnson, 1984). It appears therefore that the functional gap junction is but one item of cellular architecture that emerges at this stage. Since gap junction precursors are already present (McLachlin *et al.* 1983), it is probably the extensive cell surface reorganization taking place during compaction that allows the establishment of gap junctions during the early stages of polarization. The newly formed channels may then serve to link together the two quartets of blastomeres already coupled by persistent midbodies (Goodall & Johnson, 1984) as an integrative step for further development.

Table 2. *Distribution of HRP, 3 h after injection into single blastomeres of late 8-cell mouse embryos (approximately 8–10 h after division from 4 cells) that were cultured in M16 (controls), ECCD-1, anti-EC or LCM from the early 4-cell stage*

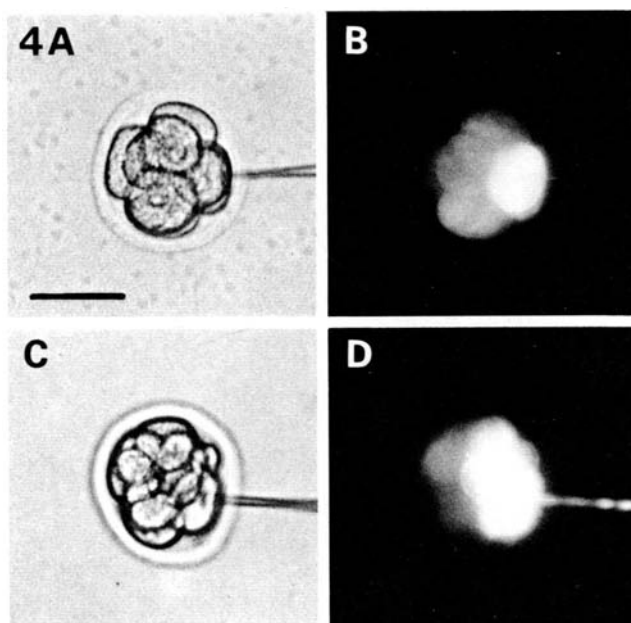| Culture condition	No. of embryos scored	% of embryos with HRP in		
		1 blastomere	2 blastomeres	3–4 blastomeres
Control	38	8	68	24
ECCD-1	21	14	76	10
anti-EC	26	8	81	11
LCM	24	0	91	9

Fig. 4. Fully compacted embryos cultured for 2 h in anti-EC (A,B) or LCM (C,D) showed extensive spread of injected CF despite complete reversal of cell flattening. Fluorescence photographs (B,D) were taken 10 min after the start of injection, which consisted of hyperpolarizing current pulses of 2 nA for 0·5 s every 1 s. Bar, 50 μm.
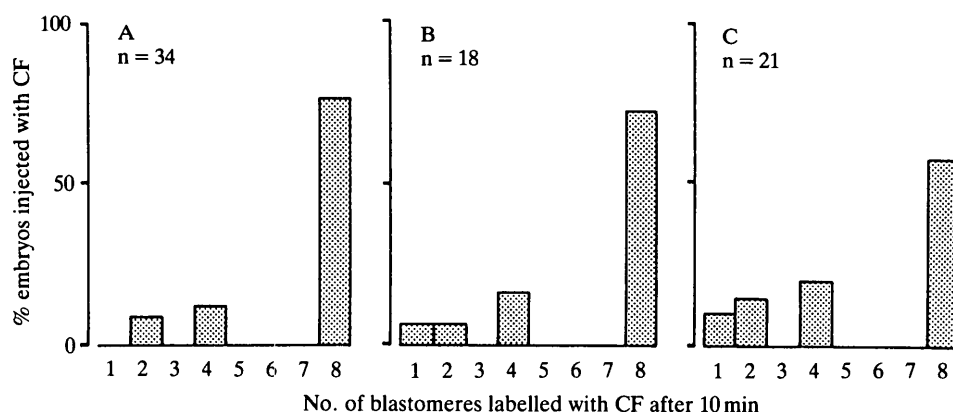
Fig. 5. Extent of CF transmission through 8-cell mouse embryos that were (A) fully compacted before reversal of cell flattening by anti-EC (B) or LCM (C). Culture and injection conditions were as described in the legend to Fig. 4.

The nature and effects of the various agents used here may provide some insight into the conditions that are required for gap junction formation. Coupling could not develop in the presence of LCM suggesting that extracellular Ca^{2+} is required either to allow junctions to assemble or to facilitate the opening of their channels. The internal Ca^{2+} concentration has been shown to be one, and possibly the major, regulator of junctional coupling in many types of cell (Loewenstein, 1981) and the manipulation of the external Ca^{2+} concentration may perturb this process (Rose & Loewenstein, 1976). However, if the internal Ca^{2+} concentration could be so perturbed, the culture of embryos in LCM after establishment of coupling should lead to uncoupling; this does not occur. The likely conclusion is that external Ca^{2+} at relatively high concentrations (LCM still contains residual un-chelated Ca^{2+}) is required in itself for the physical formation of coupling junctions. This may be a feature common to other aspects of intercellular organization since the assembly of another type of junctional complex, the desmosome, has been shown to require extracellular calcium (Watt, Mattey & Garrod, 1984). The simultaneous prevention of cell flattening and junctional coupling in anti-EC or LCM is consistent with the association that appears to exist between them as the process of compaction begins (McLachlin *et al.* 1983; Goodall & Johnson, 1984). However, the inability of the monoclonal antibody ECCD-1 to prevent the onset of cell coupling despite a highly effective inhibition of cell flattening argues against a regulatory role for cell flattening in gap junction formation. Furthermore, the inability of either anti-EC or LCM to reverse junctional coupling following its inception, despite extensive removal of close cell apposition, also argues against such a role.

Although the extent of cell apposition may not be of primary importance in this context, there is evidence from other cell systems that the quality of cell contact may exert a regulatory influence upon junctional coupling. An antiserum of similar derivation to anti-EC has been shown to block the onset of metabolic

communication between reaggregated teratocarcinoma cells (Nicolas, Kemler & Jacob, 1981) and is known to recognize a major cell surface glycoprotein, uvomorulin, an important component of the Ca^{2+}-dependent cell–cell adhesion system in the embryo and in teratocarcinoma cells (Hyafil, Morello, Babinet & Jacob, 1980; Hyafil, Babinet & Jacob, 1981). Anti-EC also appears to have activity against this or a similar adhesion system (P. Stern – personal communication) which would be consistent with the prevention of coupling seen here in the mouse embryo. However, the inability of ECCD-1 to block junctional coupling in the present study argues against the direct involvement of the calcium-dependent cell adhesion system in gap junction formation, since ECCD-1 recognizes, more specifically, what is probably the same component of this system, known alternatively as cadherin (Yoshida-Noro et al. 1984). Nevertheless, it has been reported recently that ECCD-1 inhibits junctional coupling in PCC3 teratocarcinoma cells, (Kanno et al. 1984) although this study was conducted only in the context of dye transmission, ionic coupling not being measured. Possibly anti-EC may have some activity directed against gap junctions themselves although this has not been investigated here.

None of the treatments used here had a significant effect on the survival of midbodies from the third cleavage division (4- to 8-cell transition), presumably reflecting the stability of these structures. However, the longer-lived midbodies from the second cleavage division are known to be of lower transmission capability than the more recently formed structures and therefore more tenuous (Goodall & Johnson, 1984). It is likely therefore that their reduced survival under these experimental conditions is a direct mechanical consequence of preventing the cells flattening upon one another.

As yet, no agent has been found that affects gap junction formation without impinging on other aspects of compaction although antibodies to gap junctions have now begun to appear with the capacity to block junctional coupling (e.g. Warner, Guthrie & Gilula, 1984). Nevertheless, the associations described here allow some insight into the role and regulation of these junctions in the early mouse embryo. Their formation requires extracellular Ca^{2+} but not extensive cell apposition; they are not required, however, for the signalling system that results in the generation of the first two distinct cell subpopulations in the embryo. A role for gap junctions in the subsequent maintenance of cell differences between these subpopulations, during the ensuing period of development, is yet to be established but is suggested by the observation of distinctly reduced dye passage across the boundary between these cells during the early postimplantation stages (Lo & Gilula, 1979b).

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