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Inhibition of Dye-Coupling in *Patella* (Mollusca) Embryos by Microinjection of Antiserum against Nephrops (Arthropoda) Gap Junctions

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Antiserum raised against *Nephrops* gap junctions was injected into single cells of the 2-, 4-, 8-, 16-, and 32-cell stage of the *Patella vulgata* embryos. The pattern of junctional communication by iontophoresis of Lucifer Yellow CH was tested at the 32-cell stage. The results show that the normal pattern of dye-coupling at the 32-cell stage is disrupted in greater than 65% of embryos previously injected with antisera. In contrast, less than 15% of embryos injected with preimmune serum exhibited disrupted patterns of dye-coupling. Up to the late 32-cell stage no effect of the antiserum on the pattern of cleavage was detected. This antiserum may provide a powerful tool to investigate the role of junctional communication in later stages of development of *Patella* embryos.

The cells in most tissues of metazoans are joined by gap junctions. These junctions contain channels that are freely permeable to small ions and molecules and so provide pathways for direct cell-to-cell communication (see Ref. [1] for review). It has been proposed that junctional communication is important for developmental control [2]. Many embryonic systems show reduction of junctional communication between compartments with different developmental programmes [3, 4]. Junctional communication is also markedly reduced at the intersegmental boundaries of the epidermis of insect larvae [5] and at the boundaries of developmental compartments in the *Drosophila* imaginal wing disk [6–8].

Various agents and treatments such as increasing intracellular free Ca^{2+} [9], decrease of intracellular pH [10], 12-*O*-tetradecanoyl-phorbol 13-acetate [11], retinoic acid [12], and octanol [13] inhibit junctional communication. Unfortunately, none of these is sufficiently specific to allow them to be used as a means of studying the potential role of gap junctions in embryogenesis. Antibodies raised against gap junctions or junctional proteins can interfere with junctional permeability when injected into cells and offer the prospect of more specific inhibitors for developmental studies [14, 15]. The first attempt to use this approach [14] produced striking results. *Xenopus* embryos injected in one blastomere at the 8-cell stage with antibodies to a 27 K protein from preparations of rat

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liver gap junctions gave rise to a proportion of tadpoles with patterning defects in the region derived from the injected cell. The long delay between the treatment and the observed developmental disturbances makes it difficult to analyze the direct effect of blocking gap junctional communication. Therefore, in a developmental system where specific patterning can be identified at a much earlier stage, it should be possible, using the same strategy of antibody injection, to assess if junctional communication is necessary for early pattern formation.

Development in the molluscan embryo follows a well-defined and characterized course. In *Patella* embryos individual cells and their precise lineages can be identified from fertilization. Because of the constancy of the geometric relations between successive generations of cells, the four quadrants can be continuously distinguished [16, 17]. Furthermore, detailed studies have mapped the distribution of gap junctions seen by electron microscopy and junctional communication seen by dye injection [18, 19].

No counterpart to the 27K protein in rat liver gap junction preparations has yet been found in mollusks, but recent studies have shown a 19K protein in junctional preparations from octopus [20] which cross-reacts immunologically with the 18K protein of gap junctions of the arthropod *Nephrops norvegicus* and with the 16K protein of gap junctions isolated from mouse, rat, and chicken [21].

An antiserum raised in rabbits against *Nephrops* gap junctions (described in Ref. [22]) is shown in this study to block junctional communication between cells in *Patella* embryos while having no effect on the pattern of development up to the late 32-cell stage.

MATERIALS AND METHODS

Embryos. Adult specimens of the marine gastropod *Patella vulgata* were collected on the French Atlantic coast at Roscoff and Dieppe. They were kept at 15°C in recirculating, filtered seawater. Embryos were obtained by *in vitro* fertilization at 20°C as described in Ref. [16]. Experiments were performed at 20°C. Diagrams of the embryos at the 4-cell and 32-cell stages are shown in Figs. 1 and 2. The large vegetal cross furrow (CFM) and non-cross furrow macromeres (NCFM) have been marked.

Serum injection. Synchronously developing embryos were selected and washed in acidified Millipore filtered seawater (pH 4.0) for 2 min to remove the gelatinous egg capsule which develops after the follicle cells have been stripped off. Embryos treated this way continued to develop normally [19]. The present study was confined to the CFM of the vegetal pole because the large size of the cells makes them easy to identify as well as minimizing potential damage caused by the microinjections. Preimmune and immune serum were labeled with tetramethyl rhodamine isothiocyanate (Sigma Chemical Co.), using the procedure of Haaijman [23], and clarified by centrifugation. A thermal expansion pressure system [24] was used to inject single cells. Serum (5–10 p*l*) was delivered through micropipets with tip diameter smaller than 0.1 μm. Injection of larger volumes sometimes resulted in cell damage and cytoplasmic extrusion or in abnormal cleavages. Injections were followed visually under an epiluminescence fluorescence microscope.

Dye injection. Lucifer Yellow CH (Sigma Chemical Co, Li salt, 3% in H_2O) was injected iontophoretically (rectangular hyperpolarizing current pulses of 5 nA for 0.4 s at intervals of 5 s for 5 min) into single cells of control or serum-injected embryos. The spread of fluorescence was followed visually during injection under a stereomicroscope equipped with an epifluorescent excitation beam at 488 nm (air cooled argon ion laser 162 A, Spectra-Physics, Mountain View, CA). The resting potential (−60 to −70 mV) of the impaled cells was recorded during injection to monitor the condition of the cell and the stability of the penetration. After iontophoresis, embryos were examined in a fluorescence microscope for distribution of serum (tetramethylrhodamine; excitation wavelength, 590 nm) and Lucifer Yellow CH (excitation wavelength, 540 nm) and photographed on Kodak Ektachrome film (ASA 400).

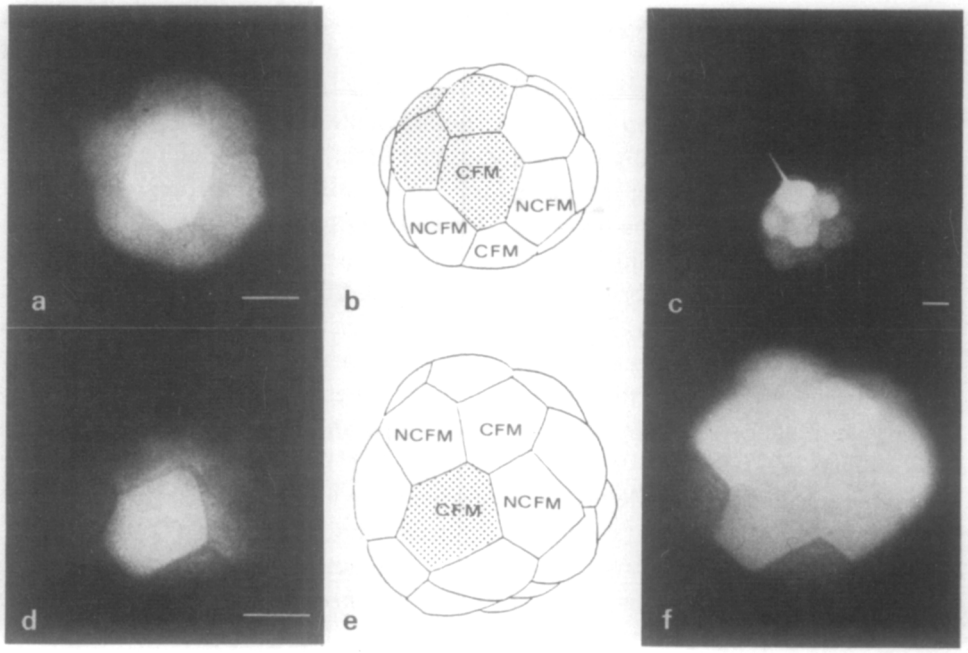

Fig. 1. Pattern of dye coupling in the 32-cell stage embryo after injection with preimmune serum. The micrographs and drawing in (*a*), (*b*), and (*c*) show an experiment where a cross furrow macromere (CFM) was injected with preimmune serum at the 8-cell stage. The four descendent serum-containing cells, seen by their TRITC fluorescence, are shown by the stippled areas in the diagram in (*b*). One of these cells (stippled CFM in (*b*)) was injected with Lucifer Yellow CH and junctional spread of the fluorescent dye to surrounding cells is shown during the Lucifer Yellow CH injection (*c*) and in higher magnification at the end of the injection (*a*). A similar experiment is shown in (*d*), (*e*) and (*f*) except that a CFM (stippled CFM in (*e*)) was injected with preimmune serum at the early 32-cell stage and 70 min later a neighboring CFM (nonstippled CFM in (*e*)) was injected with Lucifer Yellow CH. The fluorescence due to the TRITC-labeled serum is shown in (*d*) and fluorescence due to Lucifer Yellow CH is shown in (*f*) at the end of the 5-min injection.

Sera. The antiserum was prepared in rabbits against purified preparation of gap junctions from *Nephrops norvegicus* hepatopancreas. The gap junction preparation and the antiserum have been described elsewhere [22, 25]. The preimmune serum was collected from the same rabbit up to one week before the first injection of antigen.

RESULTS

All experiments followed a similar schedule. Serum was injected into one cell at the 2-cell stage or into a single CFM at the 4-, 8-, 16-, or early 32-cell stage. The embryos were left to develop to the late 32-cell stage when junctional communication between all the cells in control embryos has been fully established [19]. Lucifer Yellow CH injections were carried out during the last 45 min of the 32-cell stage to avoid dye transfer through cytoplasmic bridges which persist for a short time after cleavage. In some experiments, Lucifer Yellow CH was injected into serum containing CFMs, recognized by their TRITC fluorescence, and in other the Lucifer Yellow CH was injected into a contacting non-serum-containing neighbor.

TABLE 1

Table of the incidence of dye-coupling patterns in preimmune serum-injected embryos

Injection preimmune		Dye-coupling pattern		
Cell stage	*n*	a	b	c
2	3	3	–	–
4	2	2	–	–
8	8	8	–	–
16	7	4	1	2
32	4	4	–	–
Total	24	21	1	2

Note. a, Immediate dye-spread to all cells around the iontophoresed one. b, Retarded dye-spread, and to less of the six primary contacting cells. c, No dye-coupling detected.

Control experiments show that junctional transfer of Lucifer Yellow CH from the injected cell to first-order neighbor cells occurs within seconds after the start of the injection. By the end of the injection period (5 min), dye has spread to all first-order neighbors (6 cells). Dye spread to second-order cells of the embryo can often be seen but is sometimes difficult to detect due to the large size of the cells and the binding of Lucifer Yellow CH to cellular components. Therefore the analysis of the extent of junctional communication in serum-injected embryos was confined to examination of primary cells.

After injection of the preimmune serum, 21 out of 24 cases showed a similar extent of dye spread as found in control experiments (Fig. 1, Table 1). In the other three embryos injected with preimmune serum at the 16-cell stage, dye spread was retarded in one of them, and not detected in the other two. It is

TABLE 2

Dye coupling in embryos injected with polyclonal antiserum against Nephrops gap junctions

Injection antiserum		Dye-coupling pattern		
Cell stage	*n*	a	b	c
2	2	–	2	–
4	8	2	3	3
8	5	1	1	3
16	9	5	3	1
32	4	1	1	2
Total	28	29	10	9

Note. a, Immediate dye-spread to all cells around the iontophoresed one. b, Retarded dye-spread, and to less of the six primary contacting cells. c, No dye-coupling detected.

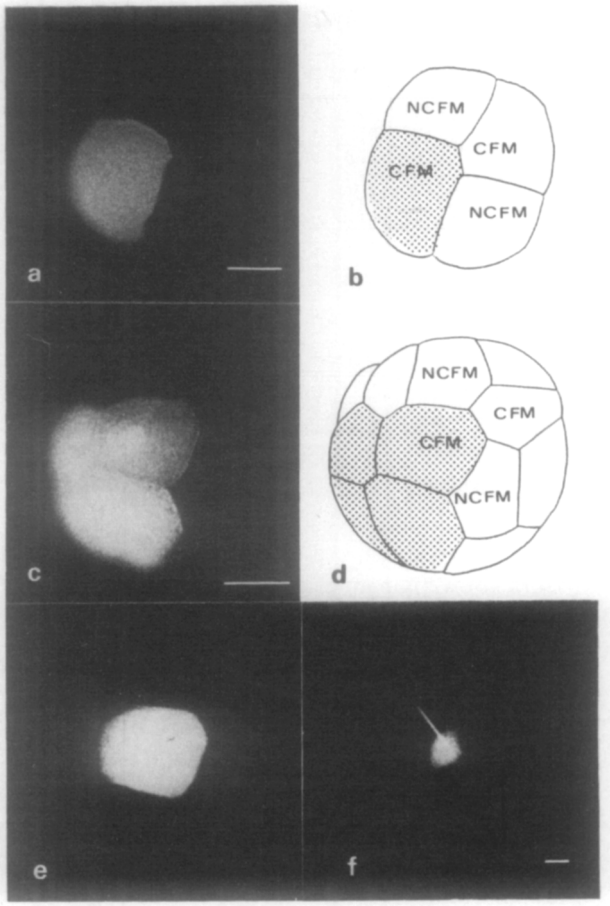

Fig. 2. Pattern of dye coupling in the 32-cell stage embryo after injection with antiserum raised against *Nephrops* gap junctions. A CFM was injected at the 4-cell stage with antiserum against *Nephrops* gap junctions shown by the TRITC fluorescence in (*a*). The embryo was incubated until the 32-cell stage. (*c*) The TRITC fluorescence due to the presence of the antiserum at the 32-cell stage. In this orientation four of the eight TRITC fluorescent CFMs can be seen. Lucifer yellow CH was injected into the antiserum containing CFM (stippled CFM in (*d*)). (*f*) The Lucifer Yellow CH fluorescence during the injection and (*e*) the Lucifer yellow CH fluorescence at the end of the injection.

unlikely that in these three cases the cell was damaged, since the resting potentials were normal throughout the dye-injection.

Injection of the antiserum to *Nephrops* gap junctions resulted in a marked effect on dye-coupling in 19 out of 28 cases (Figs. 2, Table 2). In 9 of these embryos there was no detectable dye spread to or from the antibody containing CFMs. Of these 9 embryos 7 had Lucifer Yellow CH injected into an antiserum containing CFM and in the other 2 embryos, the Lucifer Yellow CH was injected into a non-antiserum-containing CFM. In the remaining 10 embryos, dye spread could be seen at the end of the injection (5 min) but was greatly reduced. Of these 10 embryos, 5 of them had the Lucifer Yellow CH injected into an antiserum-

containing cell. In all 5 embryos, less than 6 of the potentially communicating primary cells were seen to be dye-coupled. The intensity of Lucifer Yellow fluorescence in these primary cells was also greatly reduced. In the other 5 instances, Lucifer Yellow CH was injected into non-antiserum-containing CFMs. In all 5 embryos, the level of fluorescence in the antiserum-containing CFM was much reduced in comparison to other primary cells not containing antiserum.

These results show that injection of antiserum against *Nephrops* gap junctions can disrupt the normal pattern of dye-coupling at the 32-cell stage after it has been injected into a blastomere at the same or preceding cell stages. None of the antiserum-injected embryos showed any abnormality with respect to their cleavage pattern.

DISCUSSION

Earlier studies have shown that while gap junctions first appear at the 2-cell stage of *Patella* embryos, dye-coupling becomes detectable only at the 32-cell stage [18, 19]. Thus, these stages provide an opportunity to examine possible non-junctional-dependent toxicity of antibodies against gap junctional proteins while testing the efficacy of these antibodies to impair junctional communication. Injection of the antiserum raised against *Nephrops* gap junctions into cells of early *Patella* embryos reduces junctional communication between the antiserum-injected cells and serum-free blastomeres at the 32-cell stage. However, the normal cleavage pattern of antiserum-injected cells up to this stage is not disturbed.

The ability of the antiserum raised against arthropod gap junctions to impair gap junctional communication in mollusks indicates that the junctional proteins are immunologically cross-reactive. This cross phyletic conservation is consistent with recent peptide mapping studies of the *Xenopus* 16K protein and the *Nephrops* 18K protein showing comigrating tryptic peptides [22]. This antiserum also blocks junctional communication between mammalian BRL cells (rat liver parenchymal cell line) after injection [20].

The efficiency of blocking varies from embryo to embryo (Table 2). This could be due to differences in the amount of antiserum injected or possibly the stage at which the embryos were injected. However, there is no obvious stage-specific difference except at the 16-cell stage where the results obtained with preimmune serum are similar. All three of the control embryos (n=24) injected with preimmune serum which had reduced junctional communication come from the 16-cell stage (n=7). The number of examples is insufficient to draw any conclusion. Increased efficiency in blocking was found by Hertzberg *et al.* [15] using affinity-purified antibodies against the liver 27K protein although the antibody preparation also contained low levels of antibodies against the 16K protein, the vertebrate equivalent of the *Nephrops* 18K protein. It is possible that the anti-27K and anti-16K antibodies acted in a synergistic manner to give a greater efficiency of blocking. They also used pairs of coupled cells where junctional communication is easier to monitor. However, the efficiency of blocking in our study compares favorably to that found by Warner *et al.* [14] using *Xenopus* embryos. The

antibodies may interfere with permeability by binding to the cytoplasmic faces of a proportion of the junctional channels and reducing dye transfer. Alternatively, the antibodies might selectively recognize the closed conformation and hold a proportion of the junctional channels in a closed state.

The patterns of junctional communication which occur in later stages of molluskan embryogenesis suggest a role of this form of communication in development [4]. The ability to selectively interfere with junctional communication by microinjection at these later stages of antibodies to gap junctional proteins may provide insights into the developmental significance of gap junctional communication.

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