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Intercellular dye-coupling in intestinal smooth muscle. Are gap junctions required for intercellular coupling?

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Summary. The dye Lucifer Yellow was injected into single smooth muscle cells in the guinea pig small intestine in order to study intercellular coupling. Dye-coupling was observed in both the circular and longitudinal muscle layers and was markedly reduced when the intercellular pH was lowered. These results suggest the presence of gap junctions among intestinal muscle cells, but are inconsistent with previous ultrastructural studies that failed to demonstrate such junctions in the longitudinal muscle.
Key words. Gap junctions; dye-coupling; Lucifer Yellow; intestine; smooth muscle.

In most types of smooth muscle, adjacent cells are connected by gap junctions which allow the passage of ions and small molecules. Gap junction proteins (termed 'connexins') have been cloned in both heart and uterus muscle[1]. Gap junctions are believed to be the basis for electrical propagation in smooth muscles and for maintaining coordinated mechanical activity[2,3]. Gap junctions have been demonstrated ultrastructurally in intestinal circular muscle of various mammals. However, they have not been found in the longitudinal muscle layer of the same intestinal segments[4,5]. These observations raise the question as to the mechanism of propagation of electrical activity in the longitudinal muscle layer. In the present study we have examined the coupling of intestinal smooth muscle cells by intracellular injection of Lucifer Yellow, a dye that crosses gap junctions[6]. This technique has been previously used to demonstrate coupling among aortic smooth muscle cells in culture and the results were correlated with the presence of gap junctions and a low resistance intercellular pathway[7].

Materials and methods

Adult guinea pigs of either sex, weighing 400–800 g were used. Detailed description of the dissection and the electrophysiological methods has been published[8–10]. The preparation consisted of the longitudinal muscle layer from the guinea pig small intestine (dudenum and ileum)

with the attached myenteric plexus. The circular muscle was left intact in small portions of the preparation. The tissue was pinned out in a Sylgard covered dish with the circular muscle side facing upward and was superfused with Krebs solution of the following composition (mM): NaCl 120.7; KCl 5.9; $MgSO_4$ 1.2; $NaHCO_3$ 25; $CaCl_2$ 2.5; NaH_2PO_4 1.5 and glucose 11.5. The solution was gassed with 95% O_2/5% CO_2, pH 7.3. The temperature was kept relatively low (23–25 °C) in order to minimize spontaneous muscle movements. The dish was placed on a stage of an inverted microscope (Zeiss Invertoscope D). Transmembrane currents were passed through the electrode by means of a bridge circuit of an electrometer (WPI 707). The dye solution consisted of 4% Lucifer Yellow CH (LY) and 0.1 M lithium acetate in distilled water. LY was intracellularly injected into muscle cells from glass micropipettes by passing hyperpolarizing current pulses (3 nA, 100 ms duration at 5 Hz) for 3 to 6 min. After the injections the preparation was fixed overnight in 4% paraformaldehyde, buffered with 0.1 M phosphate buffer (pH 7.2). The tissue was dehydrated with alcohols, cleared with xylene and mounted whole in oil. The preparations were observed with a Zeiss microscope equipped with epifluorescence optics. For intracellular injection of horseradish peroxidase (HRP), the electrodes contained 3% HRP (Sigma type IV), KCl 1 M and Tris 50 mM, pH 8.6. The enzyme was injected by depolarizing current pulses (0.5–1 nA, 100 ms duration at 5 Hz) for 3 to 6 min. The tissue was fixed for 15 min in 1.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and processed using the diaminobenzidine method [8]. After dehydration and clearing the tissue was mounted in DPX.

Results and discussion

A total of 475 muscle cells were injected with LY and 149 (36 circular and 113 longitudinal) were recovered. The figure (a and b) gives examples of LY-injected muscle cells in the circular and longitudinal layers. In each instance, only a single cell was injected and dye has apparently spread into several adjacent cells. Since only one cell was injected in this area, and since injection of the dye into the extracellular space did not produce staining, it can be concluded that the dye has spread through some sort of intercellular junctions. In the following, a 'coupled cell' or 'dye-coupled cell' is defined as a cell which was stained as a result of passage of dye from an injected cell. Thus, when the number of coupled cells is given, it denotes how many cells were stained with LY as a result of the passage from a single injected cell.

The number of dye-coupled cells varied and was in the range of 1–10. Stained cells were mainly those in direct contact with the injected cell. However, in several instances, dye seemed to spread from a dye-coupled cell to cells that were second or third neighbors of the injected cell. There was no significant difference between dye-coupling in the circular and longitudinal muscles (17 of 22 [77.3%] in the circular layer, vs 50 of 55 [90.9%] in the longitudinal layer).

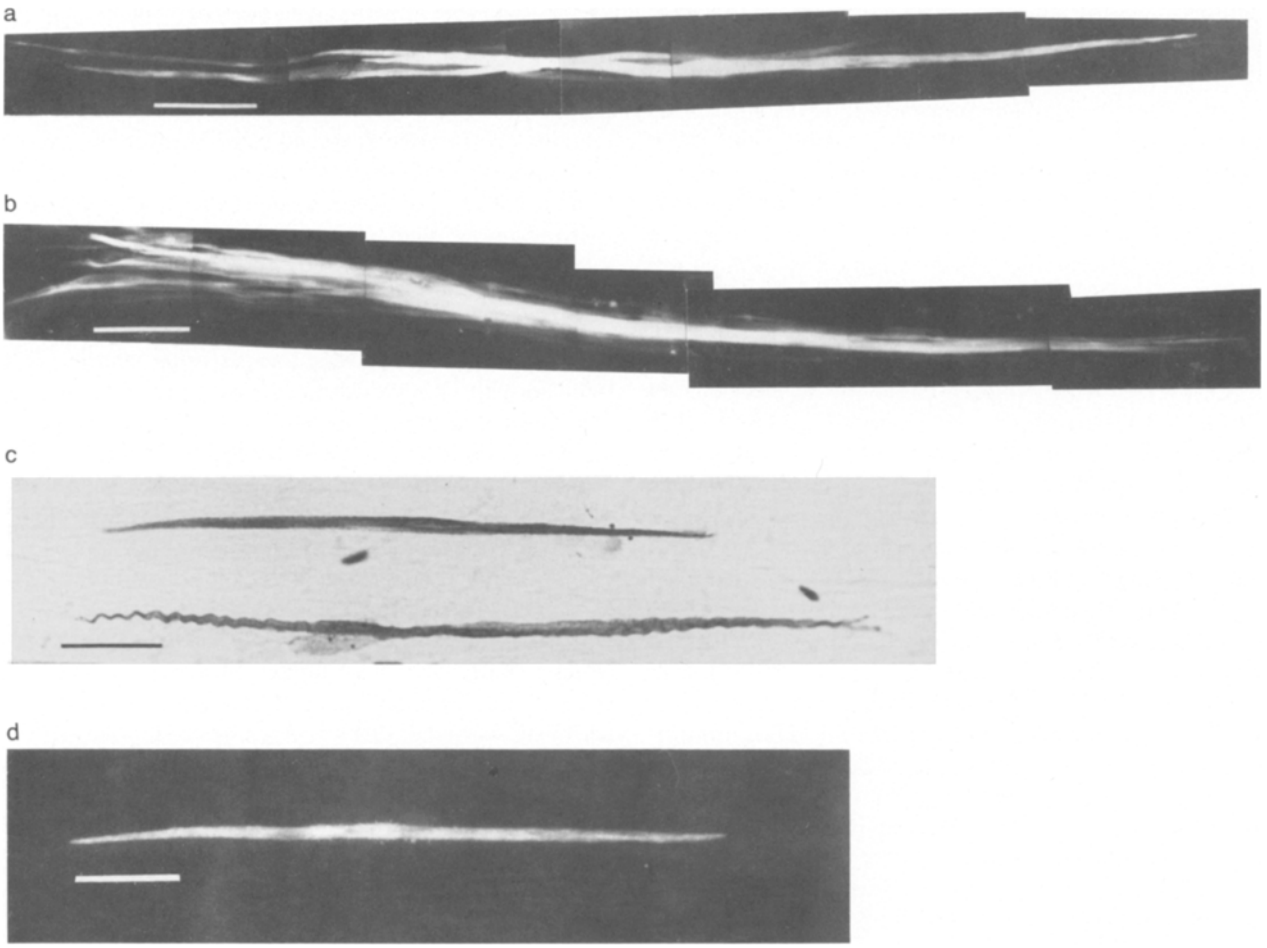
We also injected muscle cells with HRP, a protein with a molecular weight of 40,000, which does not cross gap junctions [11]. In none of the 40 cells that were recovered (36 longitudinal and four circular), could HRP transfer to adjacent cells be demonstrated (fig. c). This indicates that the intercellular channels possess a certain degree of selectivity, presumably according to molecular weight.

Intracellular pH was found to be an important factor regulating gap junction permeability [11]. To study the effect of pH on dye transfer the perfusing solution was bubbled with CO_2, which produced a pH of 6.4–6.8 in the solution. Under these conditions intracellular pH is known to be lowered [12] and indeed the degree of dye transfer was markedly reduced. An example of a longitudinal muscle cell injected with LY in the presence of high CO_2 concentration is shown in the figure (d). When the preparation was superfused with solution of normal pH (7.3), 87.0% of the injected cells showed dye-coupling, whereas only 34.8% (16 out of 46) of the cells injected under low pH were dye-coupled. Moreover, even in the cases where dye-coupling was observed, the mean number of dye-coupled cells was much lower in low pH; 1.8 in acidic pH vs 3.6 in the control experiments. This difference is highly significant ($p < 0.0001$, two-tailed t-test, $n = 67$ for normal pH, $n = 16$ for acidic pH). The effect of low pH was observed in both longitudinal and circular muscle cells. These results are summarized in the table. The effects of CO_2 were reversed on washing with normal pH solution.

We next tested the possibility that the effects described above are due not to pH lowering, but specific to CO_2. The pH was lowered to 6.5 by adding HCl to the Krebs solution and under these conditions dye-coupling was also found to be reduced (see table). It can be therefore concluded that this effect is due to changes in pH.

Alkanols such as octanol and heptanol were found to lower gap junction permeability in several tissues [11]. We examined the action of octanol at a concentration of 1.2 mM on dye-coupling in intestinal longitudinal smooth muscle cells and found that the average number of dye-coupled cells was 2.4 as compared with 3.6 in the controls (see table). This reduction in dye coupling is smaller than the effect seen with CO_2 and was not statistically significant ($p < 0.1$). The reason for the small effect of octanol is not clear. It should be mentioned, however, that in contrast to the universal action of low pH on gap junction permeability, in at least two systems octanol failed to uncouple gap junctions [13, 14].

Previous studies that employed freeze fracture and transmission electronmicroscopy failed to demonstrate gap junctions in intestinal longitudinal muscle [3, 4]. The present findings clearly show that both longitudinal and circular intestinal muscle cells are dye-coupled. The sites of dye transfer are still not known, but they appear to share certain similarities with gap junctions: 1) They only allow



Photomicrographs of dye-injected smooth muscle cells in the guinea pig duodenum. *a* Injection of the fluorescent dye Lucifer Yellow into a single longitudinal muscle cell resulted in the staining of six adjacent cells. Similar observations were made in circular muscle cells (*b*). *c* Longitudinal muscle cells injected with horseradish peroxidase do not show dye-coupling. *d* A longitudinal muscle cell injected with LY in acidic pH. Dye coupling was greatly reduced under these conditions and in this case dye spread was completely prevented. Calibration bars for *a* and *b* 100 μm, for *c* and *d* 50 μm.

The effects of various treatments on dye-coupling in intestinal smooth muscle cells

	No. of cells*	No. of dye-coupled cells				Total dye-coupling	
		0	1–2	3–4	> 4	No. of** cells (%)	Mean***
Control	77	10	24	25	18	57 (85.1)	3.6
Low pH:							
CO_2	46	30	12	4	0	16 (34.8)	1.8
HCl	14	8	3	3	0	6 (42.9)	2.5
Octanol (1.2 mM)	12	1	5	5	0	11 (91.7)	2.4
Total	149						

*The number of cells that were directly injected and successfully stained. **Total injected cells displaying dye-coupling. ***Mean number of dye-coupled cells in the cases where dye-coupling was observed.

the passage of low molecular weight compounds such as LY (molecular weight 457), and 2) their permeability is diminished by low intracellular pH.

Dye-coupling in the absence of demonstrable gap junctions has been reported for embryonic heart cell aggregates [15]. It was suggested that the intercellular channels in this preparation are functional but are not visible ultrastructurally because they are not organized into nexuses. An alternative explanation is that a different and as yet unknown structure is responsible for the coupling among the cells. Our findings on the intestinal smooth muscle suggest the presence of gap junctions and are consistent with the former hypothesis.

The dye injection method appears to be of considerable potential for smooth muscle research. For example, there is a controversy on the origin and mode of propagation

of slow waves in the intestine[16-18]. Some authors have suggested that longitudinal propagation of slow waves requires electrical coupling between the longitudinal and circular muscle layers[16,17]. Our results show that each of the muscle layers has the machinery needed for the propagation of current. Also, dye-coupling between the two muscle layers could not be demonstrated in our experiments. It appears, therefore, that an alternative explanation to the question of slow wave generation and propagation has to be sought (see also Szurszewski[18]).

Experiments using the dye injection technique in smooth muscles have been previously performed only in cultured aortic muscle cells[7]. In the present study freshly dissected tissue was used, preserving the original organization of the cells. This experimental approach may open new possibilities for studying intercellular coupling in smooth muscles.

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Functions of the testicular gland of blenniid fish: Structural and histochemical investigations

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Summary. We investigated the functions of the testicular glands of blenniid fishes by fine-structural and histochemical methods. These glands function in the differentiation and nutrition of germ cells, in the secretion of sialomucins, in phagocytosis of germ cells, and in lipid storage.
Key words. Testicular gland; ultrastructure; histochemistry; blenniid fish; function.

Since its discovery 150 years ago[1], the testicular gland of blenniid fish has been the subject of many structural and functional investigations[2-6]. The gland, which is located adjacent to the testis, was found to store lipids, to produce steroids and acid mucopolysaccharides, and to have lysomatic functions[7,8]. However, the exact role of the testicular gland in the reproduction of blenniid fish is uncertain.

The testicular gland of five species of adult, male Mediterranean blennies (8 *Salaria pavo*; 8 *Lipophrys dalmatinus*; 5 *Lipophrys adriaticus*; and 6 *Aidablennius sphynx*) was investigated with routine transmission electron microscopy (fixation: 4.5% paraformaldehyde, 2.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.5); postfixation: 1% OsO_4 in 0.1 M cacodylate buffer) and with histochemical techniques. Glycogen was demonstrated on paraffin sections (7 µm) by periodic-acid-Schiff (PAS) staining (McManus)[9] and Best's glycogen detection[9]. Sulfated and non-sulfated mucosubstances were stained with alcian blue at pH 1 and pH 2.5[9] on 7-µm-thick paraffin sections and on 10-µm-thick cryostat sections. The non-sulfated mucosubstances were shown to be sialomucins by the method of neuraminidase extraction[9] (neuraminidase from *Clostridium perfrigens*, activity: 0.5–1.3 units/mg NAN-lactose). Lysosomal activity in the testicular gland was demonstrated with Gomori's lead phosphatase method[9] and steroid production with the method of 3β-steroid dehydrogenase activity (substrate: dehydroepiandrosteron)[10]. Both reactions were carried out on 10-µm-thick frozen sections.

Most Mediterranean blenniid fish have an annual spawning season lasting from the beginning of June until the end of July. The mature testes of blennies do not contain any spermatozoa; spermiogenesis in the testis progresses only up to the stage of spermatidal development. Spermatids are then released into the testicular gland (figs 1 and 2). During the spawning period the cells of this gland are characterized by an abundance of smooth endoplas-