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DYE COUPLING IN THE MUSCLES CONTROLLING SQUID CHROMATOPHORE EXPANSION

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Summary

Dye coupling between the cone-shaped radial muscle fibres, which control the expansion and closing of a squid chromatophore organ, was investigated in the squid *Loligo vulgaris*. Particular attention was paid to the role of the myomuscular junctions located between the muscle fibres. Lucifer Yellow was injected ionophoretically into single muscle fibres under normal artificial sea water (ASW) and under various concentrations of calcium in ASW. Under ASW, 44 % of muscle fibres examined were dye-coupled, 82 % were coupled under calcium-free sea water and 67 % were coupled under sea water containing high concentrations of calcium. Dye transfer was blocked by octanol. Muscle fibres were never seen to link adjacent chromatophore organs. Results are discussed in terms of the role of the myomuscular junctions in the regulation of chromatophore expansion in the living animal.

Key words: dye coupling, Lucifer Yellow, chromatophore muscles, squid, *Loligo vulgaris*.

Introduction

Body patterning and colour changes in squid skin are brought about by the expansion and closing of numerous chromatophore organs. Opening of the pigment sac is achieved by contraction of 10–24 cone-shaped radial muscle fibres, joined at their widest point to form a complete ring around the pigment sac (Fig. 1). Electron microscopy carried out on the skin of *Loligo opalescens* (Cloney and Florey, 1968) has revealed that these radial chromatophore muscles are connected by myomuscular junctions at their widest point. The areas where myomuscular junctions occur are extensively folded, with gaps of around 3 nm. They are described by Mirow (1972) as being like a ball and socket, and they resemble the gap junctions described by Gilula (1973).

It was once thought that in *Loligo* and *Sepia* the entire chromatophore population and its muscles form a complete syncytium (Hofmann, 1907) because in some dead or ageing preparations whole populations of chromatophores are seen to pulsate, their muscles appearing to contract synchronously. Froesch-Gaetzi and Froesch (1977) suggested that such synchronously acting chromatophores may be connected horizontally by their muscles in the squids *Loligo*, *Sepia* and *Eledone*. Bozler (1928, 1931) had shown clearly, however, that individual radial chromatophore muscles in the squid *Loligo vulgaris* could be activated independently through selective stimulation of their nerve supply, and he concluded from this that the muscles do not form a syncytium. Florey (1966) confirmed Bozler's (1931) observations with intracellular recordings from the skin of *Loligo opalescens*. He showed that non-pulsating chromatophore muscle fibres respond to nerve stimulation with only local excitatory postsynaptic potentials (EPSPs) and that spikes could not be initiated by them, but that spikes were generated in synchronously pulsating chromatophores. It appeared that chromatophore muscle fibres were capable of two types of contraction: fast, twitch-like contractions elicited by nerve impulses, and spontaneously arising tonic contractions that occurred in the absence of nerve impulses. It was found that muscles function as independent units when responding with EPSPs, but synchronously when spikes are generated (Florey and Kriebel, 1969).

Florey and Kriebel (1969) found that, in the five muscle fibres they examined, neighbouring muscle fibres around a pigment sac were electrically coupled through low-resistance pathways.

In this study, dye coupling was used to confirm Florey and Kriebel's (1969) findings that chromatophore muscle fibres are coupled through myomuscular junctions. The role of the myomuscular junctions in permitting spread of excitation from one muscle fibre to the next was also investigated using this technique. Spread of excitation determines whether chromatophore muscles contract synchronously or independently to give step-wise opening of a chromatophore pigment sac.

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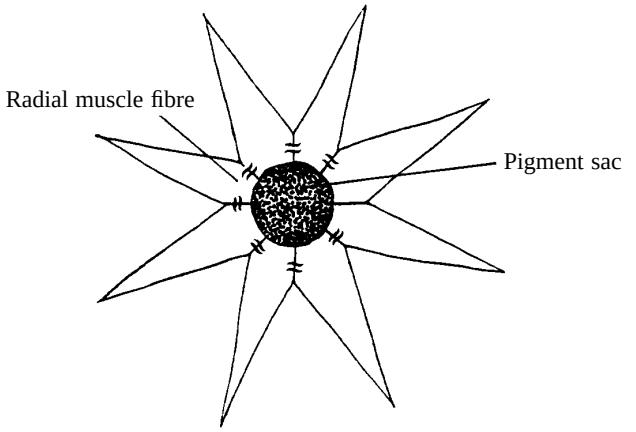

Fig. 1. Schematic diagram of a squid chromatophore organ showing the position of the myomuscular junctions (≈) in the radial muscle fibres around a pigment sac.

Materials and methods

Small pieces of skin about 2 cm^2 were removed from the dorsal mantle of *Loligo vulgaris* caught off the coast of Plymouth, UK. The skin pieces were pinned out in a Petri dish with a Sylgard base and covered with normal artificial sea water (ASW) containing (in mmol l^{-1}) 470 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, 10 Hepes buffer at pH 7.4. The epidermis and the first layer of the dermis containing the small yellow chromatophores were peeled away to expose the muscle fibres of the large brown and red chromatophores. Glass microelectrodes with filaments (Clark Electrochemical Instruments; 1.5 mm o.d. × 0.86 mm) were employed. The electrode tip was filled with a 3% solution of Lucifer Yellow-CH (Stewart, 1978) (Sigma) and then back-filled with a solution of 3 mol l^{-1} lithium chloride. These electrodes had resistances of 30–90 MΩ. The Petri dish was slotted into the stage of the microscope and impalements were made from the widest portion of the muscle (nearest the pigment sac). Resting potential was recorded during electrode insertion, before switching to current injection. Injection of hyperpolarising currents (1–5 nA) resulted in a rapid movement of Lucifer Yellow into the muscle. Dye-filling was considered to be complete after 5 min or when no more dye was seen to flow into the muscle. Longer filling times seemed to have no effect on the number of muscles filled. Dye-filled muscles were photographed under an epifluorescent microscope using colour slide film (1600 ASA). The pieces of skin were then fixed in 10% paraformaldehyde, rinsed, dehydrated and mounted in Histomount.

Results

In all, 214 muscle fibres were injected ionophoretically with Lucifer Yellow in 40 preparations. Eighty dye fills were carried out in normal artificial sea water and the remaining 134 dye fills were carried out in various other external solutions.

Recorded resting potential ranged from −7 mV to −45 mV (*N*=80, mean ± S.D. −27±8.47 mV. It is not clear whether low values are the result of damage to the cells on impalement or during preparation, but the values are similar to those recorded in previous studies on chromatophore muscle fibres (Florey, 1966). There is no significant difference between the mean resting potentials for coupled and uncoupled muscle fibres (t=9.31, d.f.=72, P>0.05). The cells were highly depolarised in calcium-free sea water and octanol, but slightly hyperpolarised in sea water containing high concentrations of calcium.

Table 1. *Dye coupling results for chromatophore muscles under normal artificial sea water and other external solutions*

	Normal ASW	Calcium-free ASW	High-calcium ASW	Octanol
Number of dye filled cells	80	28	15	20
Percentage of cells coupled	44	82	67	0
Percentage of cells coupled in one direction only	38	29	13	0
Percentage of cells coupled to more than two muscles	19	25	27	0
Mean membrane potential (mV)	–27±8.47	–19±6.13	–28±8.15	–18±5.78

ASW, artificial sea water.
Values for mean muscle potential are given ± S.D.

Results are summarised in Table 1. Of the 80 muscle fibres dye-filled in normal artificial sea water, 35 (44%) were dye-coupled (Fig. 2) and 45 muscle fibres showed no dye-coupling (Fig. 3). In 38% of coupled cells, dye passed to only one side of the impaled muscle fibre and in 19% of coupled fibres, dye passed to three or more of the neighbouring cells on both sides of the impaled fibre. When octanol, an anaesthetic known to uncouple gap junctions, was applied to the skin, all the filled muscle fibres were uncoupled (*N*=20).

In calcium-free sea water, 82% of muscle fibres (*N*=28) were dye-coupled and in high-calcium sea water (30 mmol l^{-1} calcium chloride), 67% were dye-coupled (*N*=15).

Leaving the skin preparation in ASW overnight at 4 °C resulted in 46% of muscle fibres (*N*=26) being dye-coupled, indicating that time of incubation had no significant effect on the degree of coupling of the muscle fibres.

In a number of different preparations, muscle fibres from the same chromatophore were filled at intervals around the pigment sac and a mixture of coupled and uncoupled muscle fibres was seen.

Although dye spread to the left, to the right or to both sides of an injected muscle fibre from the same chromatophore organ, dye was never seen to spread to the muscles fibres of adjacent chromatophore organs.

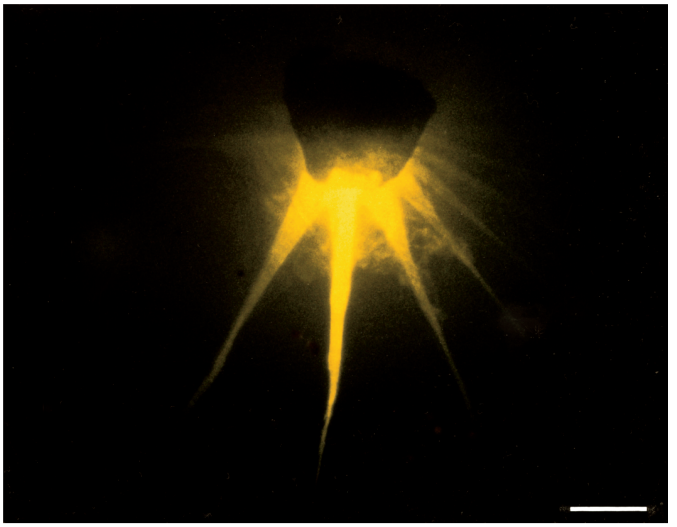

Fig. 2. Dye-coupled chromatophore muscles filled with Lucifer Yellow. Note that the sheath cells around the chromatophore organ have also been filled. The broadest parts of the filled muscle fibres lie against the pigment sac. Scale bar, 0.2 mm.

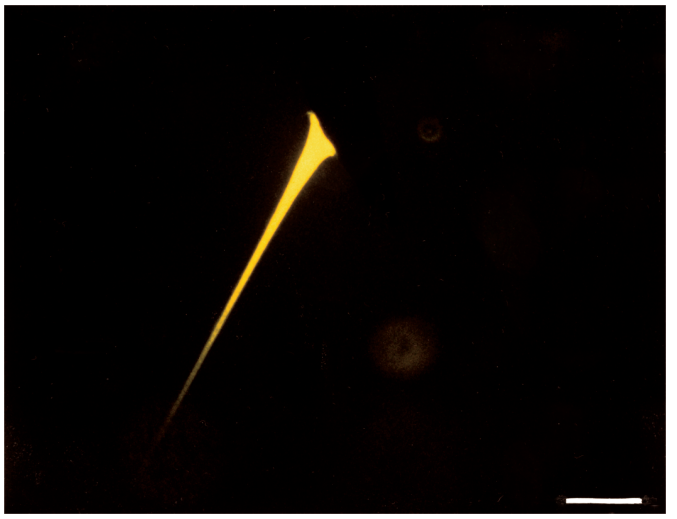

Fig. 3. Uncoupled chromatophore muscle stained with Lucifer Yellow. The broadest part of the filled muscle fibres lies against the pigment sac. Scale bar, 0.2 mm.

Discussion

Dye coupling was found to exist between some neighbouring muscle fibres around a chromatophore pigment sac. This finding confirms Florey and Kriebel's (1969) previous discovery that these muscle fibres are coupled and these results, together with the observation that application of octanol to the external solution completely blocks transfer of dye, support the hypothesis that chromatophore muscle fibres are indeed connected by myomuscular junctions or gap junctions.

The results from this investigation are not consistent with the view that adjacent chromatophore organs share muscle fibres (Froesch-Gaetzi and Froesch, 1977; Packard, 1995). In over 200 dye-fills in 40 preparations, muscle fibres were never seen to form such links between chromatophore organs. The connections could be so fine that they are impossible to visualise, but this is unlikely since Lucifer Yellow stains fine dendrites in cephalopod nerve cells (Miyan and Messenger, 1995). It is more probable that, although the muscle fibres between adjacent chromatophores *appear* to be linked, they are not linked but instead pass over each other.

It is not clear why fewer than half of the chromatophore muscle fibres are dye-coupled in normal sea water. In order to solve this problem, the opening and closing of chromatophores should be considered. Chromatophore muscle fibres from the same chromatophore can be activated independently (Bozler, 1928, 1931; Florey, 1966; Florey and Kriebel, 1969), leading to varying degrees of steady or fluctuating opening of the pigment sac; however, all the chromatophore muscle fibres around a single pigment sac can be seen to contract synchronously during pulsating activity (in dead or ageing skin).

There may therefore be a mechanism for uncoupling/coupling of the chromatophore muscle fibres, through opening and closing of the gap junctions. This would enable the squid to expand the chromatophores fully or partially according to need and, rather than all the muscles contracting synchronously in the same direction, individual muscle fibres (uncoupled) or sets of muscles fibres (coupled) would pull the pigment sac out in different directions for fine control of body patterning/colour change. Such fine control has been observed in the squid *Loligo opalescens* (Florey, 1966) and also in *Eledone cirrhosa* (Dubas and Boyle, 1985), which has been shown to progress from a light, translucent body pattern to a dark one, not only by a change in the numbers of chromatophores activated but by a change in the degree to which each chromatophore is expanded (Hanlon, 1982). This hypothesis is supported by the observation that in 38 % of all coupled muscle fibres, dye passed to only one side of the impaled muscle, and also by the observation that coupled and uncoupled muscles were seen around the pigment sac of a single chromatophore.

All the chromatophore muscle fibres contract synchronously during pulsating activity and this implies that all the gap junctions are functional in this type of chromatophore behaviour. Coupling between chromatophores thus appears to be highly variable, presumably as a result of the modulatory effects of endogenous neurotransmitters, particularly on intracellular calcium level which is known to gate gap junctions (Spray and Bennett, 1985). In the present study, muscles showed an incidence of coupling of 82 % under calcium-free sea water, which suggests that high levels of calcium cause uncoupling of the muscles, as found in previous studies on invertebrate preparations (Rose and Lowenstein, 1975; Lowenstein, 1976; Detwiler and Sarthy, 1981; Spray and Bennett, 1985). Muscles filled in high concentrations of calcium also showed an increased incidence of coupling, which is unexpected but suggests that, as explained in previous studies (Spray and Bennett, 1985), calcium has very different effects on the

opening of gap junctions according to whether it is acting intracellularly (involved in gating) or extracellularly (perhaps involved in intercellular adhesion).

The implications of such a low incidence of dye-coupled muscles in normal sea water and the effects of different external solutions are obviously complicated. The findings are possibly related to the fine control of chromatophore expansion, but it is clear that further electrophysiological investigations, together with an investigation into the effects of the putative neurotransmitters on coupling, should be carried out in order to shed more light on this complex system.

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