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The role of muscarinic receptors and intracellular Ca^{2+} in the spectral reflectivity changes of squid iridophores

Lydia M. Mäthger[1,2,*], Toby F. T. Collins[1,2,†] and Pedro A. Lima[1,‡]

[1]*The Marine Biological Association of the United Kingdom, The Laboratory, Citadel Hill, Plymouth PL1 2PB, UK* and [2]*Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK*

*Author for correspondence at present address: Vision, Touch and Hearing Research Centre, University of Queensland, Brisbane, Queensland 4072, Australia (e-mail: l.mathger@uq.edu.au)

†Present address: University Department of Pharmacology, Mansfield Road, Oxford OX1 3QT, UK

‡Present address: Department of Pharmacology, University Walk, University of Bristol, Bristol BS8 1TD, UK

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Summary

In this paper we describe changes in spectral reflectivity of the light reflectors (iridophores) of the squid *Alloteuthis subulata*. The spectral changes that can be seen in living squid, can also be brought about by superfusing whole skin preparations with acetylcholine (ACh) (20 μmol l^{-1}) and muscarine (30 μmol l^{-1}) but not nicotine (up to 50 mmol l^{-1}), suggesting that cholinergic muscarinic receptors are involved. Changing the osmolarity of the external solution had no effect on spectral reflectivity. To study the iridophores at the cellular level, iridophores were isolated enzymatically. Lucifer Yellow filled the iridophores uniformly, showing cellular individuality. Isolated iridophore cells were loaded with Fura-2 AM and cytoplasmic Ca^{2+} was recorded ratiometrically. Intracellular Ca^{2+} (resting concentration at 66.16 nmol l^{-1}) increased transiently after addition of ACh (50 μmol l^{-1}), muscarine (25 μmol l^{-1}), but not nicotine (up to 5 mmol l^{-1}). Ca^{2+} also increased when superfused with potassium chloride (10 mmol l^{-1}) and caffeine (2.5 mmol l^{-1}). Hypo- and hyperosmotic solutions had no effects on the cytoplasmic Ca^{2+}. By presenting direct evidence that iridophores are polarised cellular structures containing Ca^{2+} stores and that they are activated *via* cholinergic muscarinic receptors, we demonstrate that Ca^{2+} is involved in the reflectivity changes of the iridophores of *A. subulata*. Specimens were prepared for transmission electron microscopy. It was found that the orientations of the plates with respect to the skin surface are in good agreement with the expected orientations based on the prediction that the iridophores act as multilayer reflectors.

Key words: squid, *Alloteuthis subulata*, invertebrate, multilayer reflector, reflectivity, iridophore, calcium, acetylcholine, muscarine receptor.

Introduction

Squid can change the colour of their skin for camouflage and signalling. This is done by using two distinct systems within the skin: pigment-containing organs (chromatophores) and light reflecting cells (iridophores). There are many accounts in the literature describing the mechanisms of chromatophore colour change and how these changes relate to the behaviour of the animal (for a review, see Hanlon and Messenger, 1996). Much less, however, is known about the role of iridophores in the colour change of squid. Iridophores are found in distinct stripes in a layer beneath the chromatophores. Within the iridophores there are separate structures known as iridosomes, which contain the reflective plates that produce iridescence (Fig. 1A) (Williams, 1909; Hanlon, 1982, 1988; Hanlon et al., 1994, 1999; Mirow, 1972; Cloney and Brocco, 1983; Cornwell et al., 1997). It was at first thought that cephalopod iridophores were static structures, whose optical appearance depended only on the ambient light and that they were not under any control by the animal (Schäfer, 1937; Parker, 1948; Mirow, 1972). Subsequently, it was discovered that squid iridophores could be observed in a variety of reflective states, between non-reflective and highly reflective, suggesting that there may be some degree of control by the animal (Hanlon, 1982; Cooper and Hanlon, 1986; Hanlon et al., 1990, 1999; Cooper et al., 1990). These reports show that the optical properties of some iridophores change following topical applications of acetylcholine (ACh). In *Lolliguncula brevis*, Cooper and Hanlon (1986) noticed that the reflected wavelengths shifted from the long (red) end of the visible spectrum to the shorter (blue/UV) end with increasing concentrations of ACh. Two hypotheses were subsequently proposed by Cooper et al. (1990) to explain the mechanisms underlying iridophore activity in *L. brevis*. (1) Iridophore plates may be made of a labile proteinaceous material, rather than chitin, as proposed by Denton and Land (1971) (for recent evidence that reflective plates are indeed composed of protein, see Crookes et al., 2004). Cooper et al. (1990) argue that the protein within the

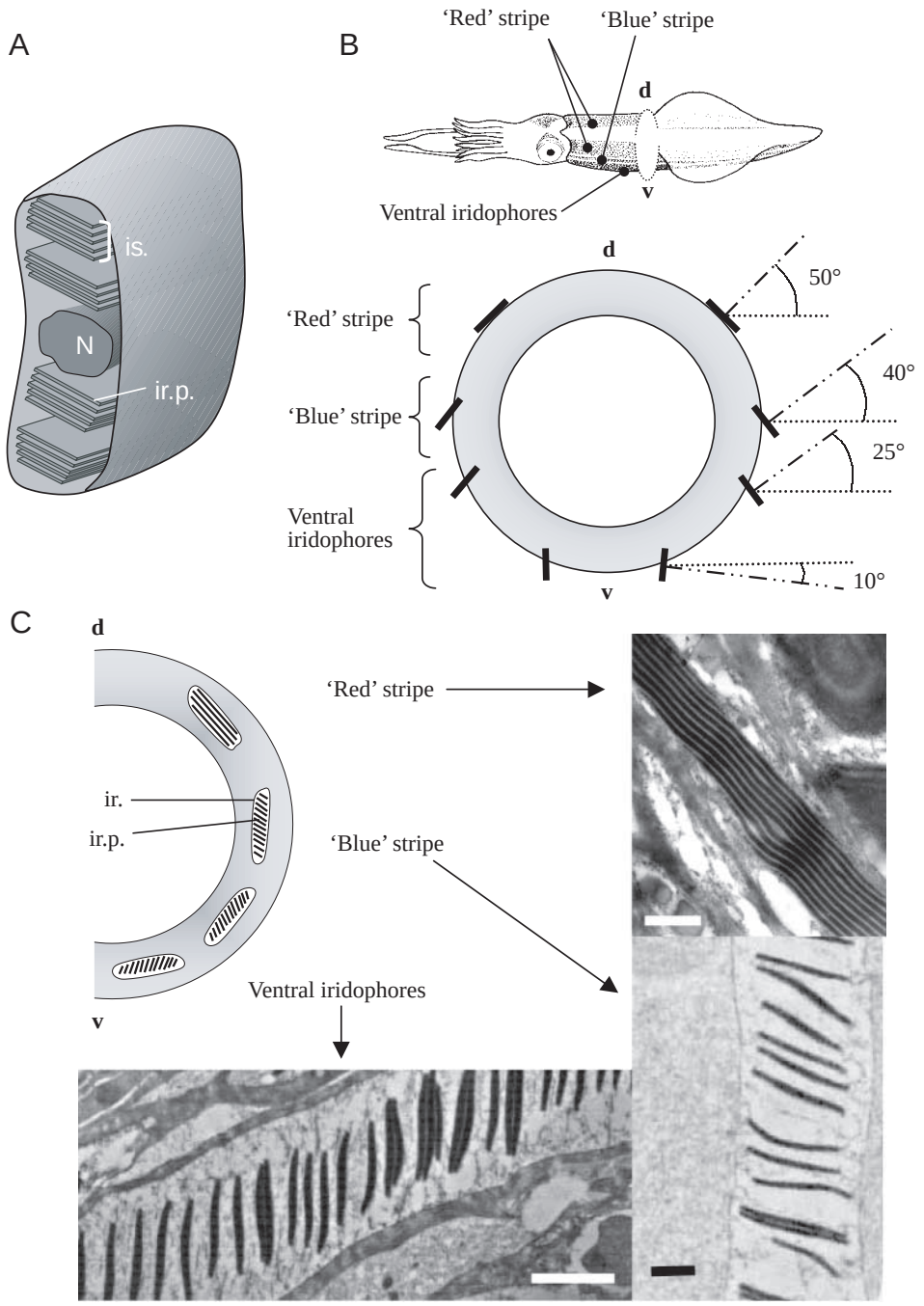

Fig. 1. (A) Diagram of a squid iridophore, showing iridosomes (is.), which are made up of groups of iridophore plates (ir.p.); N, nucleus (drawn using information given in Cloney and Brocco, 1983; Mirow, 1972). The iridophore cells are oriented with their long axes parallel to the plane of the skin, whereas the iridophore plates within the iridosomes can be oriented at a variety of angles (see Mirow, 1972). (B) Iridophore stripes of the squid *Alloteuthis subulata*. The names are given according to the colour reflected when the stripes are viewed in white light at near-normal incidence. The figure also shows a cross-section perpendicular to the long axis of a squid's mantle, in the region of the fins (d, dorsal; v, ventral). For each stripe, solid lines indicate the iridophores; dot-dashed lines, normal incidence to the iridophores; dotted lines, horizontal (adapted from Mäthger and Denton, 2001). (C) Diagram showing in cross-section the location in an iridophore of the different reflective stripes and the orientation of their reflective plates (not to scale). ir., iridophore; ir.p., iridophore plates. Shown also are electron micrographs of the iridophore plates with the respective stripes shown in diagram. The electron micrographs are oriented in the figure so that the skin surface in each micrograph (off scale, not shown) coincides with the orientation of the skin surface in the diagram. Scale bars, 1 μm.

plates may change in viscosity during iridophore activity. This would result in a change in refractive index and consequently a change in the reflected wavelengths. (2) Based on electron microscopical investigations, Cooper et al. (1990) suggest that the iridophore plates of reflective iridophores may change in thickness, with plates becoming thinner as the reflected wavelengths move from the longer to the shorter end of the spectrum. They propose that while the change from non-reflectivity to reflectivity may be caused by changes in viscosity of the plate, the plate thicknesses may have effects on the reflected wavelengths, and fluid movement within the plates could cause the dimensional changes in platelet thickness. In these studies, active iridophores were found only on the dorsal part of the mantle; the iridophores of the ventral side appeared to be inactive (Cooper and Hanlon, 1986; Hanlon et al., 1990; Cooper et al., 1990).

Ca^{2+} serves in many second messenger systems in a variety of cell types and triggers numerous physiological phenomena (for reviews, see Berridge, 1998; Bootman et al., 2001). To date it has, however, not been shown directly whether Ca^{2+} is involved in the functioning of animal reflectors. In this study we measured intracellular Ca^{2+} concentrations and we present data to suggest that reflectivity changes of squid iridophores are mediated by cytoplasmic Ca^{2+}. Hanlon et al. (1990)

suggested that reflectivity changes in squid may be mediated by Ca^{2+}, but were unable to substantiate these claims, as no direct measurements of Ca^{2+} were made.

Mäthger and Denton (2001) described the organisation and possible functions of the iridophores of the small squid *Alloteuthis subulata*, found off the coast of Northern Europe. They found that the reflective properties of squid iridophores resembled those of ideal quarter-wavelength multilayer reflectors. Light reflected from a reflector of this kind is almost always coloured. Multilayer reflectors are characterised by the fact that they contain thin plates of a higher refractive index than the spaces separating them. In an ideal multilayer reflector the plates and spaces both have an optical thickness (actual thickness multiplied by refractive index) of a quarter of the wavelength reflected by the stack at normal incidence. 'Ideal' here means that such a stack has the highest reflectivity in comparison with 'non-ideal' reflectors, for which the plates and spaces differ in optical thickness. It therefore becomes obvious that a change in the thicknesses of the plates and/or spaces will change the wavelengths reflected from the stack (Huxley, 1968; Land, 1972). Fig. 1B shows the location of the various reflective stripes found in *A. subulata* and the orientation of the iridophores with respect to the skin surface. Mäthger and Denton (2001) described only one reflective state of the iridophore stripes of this squid. However, the iridophores of these stripes can often be observed in varying reflective states. They can be highly reflective as well as non-reflective and they can undergo changes in spectral reflectivity. The present paper describes in detail the reflectivity changes of iridophores of the squid *A. subulata*. The aim was to study the ultrastructure of iridophores in a whole-skin preparation as well as at the cellular level, and to study the pharmacology and the Ca^{2+} dynamics of these cells.

Materials and methods

Squid

Alloteuthis subulata Lamarck (mantle length 10–15 cm) were trawled off the Plymouth coast and maintained in the closed circulating seawater system of the Marine Biological Association (UK) at approximately 12–16°C. Squid were killed by decapitation.

Transmission electron microscopy (TEM)

After decapitation the mantles of 5 squid were fixed in 2.5% glutaraldehyde in artificial sea water (ASW, in mmol l^{-1}: NaCl 470, KCl 10, $CaCl_2$ 10, $MgCl_2$ 60, Hepes 10, pH 7.8). Fixing the entire mantle ensured that the iridophores maintained their original orientations when tissue was processed for TEM. Small samples were cut out from various skin areas containing the different reflective stripes and dehydrated in a graded series of acetone. Specimens were embedded in Spurr's resin (TAAB, Aldermaston, UK). Sections were cut on a Reichert–Jung Ultracut microtome using glass knives (TAAB), stained with uranyl acetate and lead citrate and examined on a transmission electron microscope (JEOL JEM 200 CX, Welwyn Garden City, UK). Following dissociation, isolated cells were treated in the same way. However, a 15 min period was given between each dehydration step to allow cells to settle.

Whole skin preparation

The effects of different agonists and antagonists on the various iridophore stripes were studied. The shape of the mantle was maintained by inserting a cylinder made of black plastic foil into the mantle cavity. This was crucial to determine the angle of incidence at which the reflective stripes were observed. The cylinder was firmly attached inside a 100 ml perfusion chamber. Rotation of the cylinder allowed all reflective stripes to be observed. All drugs were superfused across the preparation. The responses of iridophores were observed through a dissecting microscope. Unless specified otherwise, all observations were made at near-normal incidence (approximately 20°) to the individual reflective stripes (see Fig. 2A for orientation of normal incidence). Photographs were taken on a camera (Nikon UFX-II, Kingston Upon Thames, UK) attached to the microscope. A video camera (Panasonic VHS, Osaka, Japan) was used to record changes in spectral reflectivity in living squid.

Electrical stimulation was carried out using a bipolar silver wire field electrode. The squid mantle was cut open ventrally and pinned onto the Sylgard base of a Petri dish containing ASW. While observing the spectral reflectivities of iridophores, areas, including the iridophore and chromatophore layers and the stellate ganglion, were stimulated using a Grass SD 5 Stimulator (Rhode Island, USA). Iridophores were stimulated for up to several seconds with voltages between 0.8 and 80 V, applied in pulses lasting 0.2–20 ms at frequencies of 2–20 Hz.

For some experiments a Ca^{2+} free solution was used (EGTA Ca^{2+} free ASW, in mmol l^{-1}: NaCl 470, KCl 10, $MgCl_2$ 10, Hepes 10, EGTA 10, pH 7.8).

Isolation of iridophores

The iridophore layers were dissected from 1.5–2 cm^2 pieces of skin, taken from the dorsal, lateral and ventral regions of the mantle. The iridophore layers were incubated in centrifuge tubes (1 ml volume) for 15–20 min in ASW containing 1 mg ml^{-1} trypsin (type III, Sigma Aldrich, Poole, UK) at room temperature. After washing in ASW the cells were transferred to a solution of ASW containing 3 mg ml^{-1} papain (Sigma) and 3 mg ml^{-1} collagenase P [Boehringer Mannheim (Roche Diagnostics), East Sussex, UK]) (filtered through a 2 μm filter) for 20–30 min at room temperature. The tissue was then washed thoroughly in ASW and strongly agitated in a Petri dish containing glass coverslips in ASW. The cells were allowed to settle for 10 min and were kept viable in the refrigerator for up to 4 h. In order to establish whether the dissociated cells were iridophores, some isolated material was processed for TEM.

Dye filling using whole cell patch-clamp electrodes

The coverslips holding the isolated iridophores were

transferred to the chamber of a pre-cooled stage (11–13°C) of an inverted microscope (Diaphot, Nikon, Kingston Upon Thames, UK). Cells were filled with Lucifer Yellow after establishing whole-cell configuration, by dialysing the pipette internal solution (0.3% of the dye). The bath solution used was ASW. The pipette solution contained (in mmol l^{-1}): caesium-D-aspartate, 450; $MgCl_2$, 15; EGTA-Cs, 15; MOPS-Cs, 30 and TEA-Cl, 6 (pH 7.2). Patch electrode resistance ranged from 2–4 MΩ. In this study, we used a laboratory-made amplifier with a headstage current–voltage converter, with a feedback resistance of 1 GΩ.

Ratiometric Ca^{2+} measurements

Isolated iridophore cells were ester loaded with the Ca^{2+} sensitive dye Fura-2 AM (Molecular Probes, Oregon, USA) in ASW for 30 min (final loading concentration of dye was 5 μmol l^{-1}). Fluorescence from Fura-2 loaded iridophores was measured on a pre-cooled stage (11–13°C) of a microscope (Optiphot-2, Nikon), which was equipped with a photometric analysis system (Cairn, Kent, UK). A spinning wheel with filters of 340, 360 and 380 nm was used to provide the excitation wavelengths needed to record the Ca^{2+} dynamics of Fura-2 loaded cells. The light source was a Xenon lamp (75W; AMKO, Tornesch, Germany). Fluorescence emission was measured at 450–520 nm. During the measurements, iridophores were visualised on a monitor, using an infra-red video camera.

Some experiments were done in EGTA Ca^{2+} free ASW. The solution was the same as mentioned above, only the concentration of EGTA was reduced to 3 mmol l^{-1}.

Calculations of 'resting' Ca^{2+} concentrations

The dual-wavelength fluorescence data (ratio 340 nm/380 nm) was calibrated using an adaptation of the method given by Thomas and Delaville (1991), in which cells, permeabilised by ionomycin (1 μmol l^{-1}), are bathed in EGTA Ca^{2+} free ASW to obtain minimal Ca^{2+} concentrations. Saturating Ca^{2+} concentrations are obtained by bathing cells in ASW. At the end of the calibration, 2 mmol l^{-1} $MnCl_2$ is used to determine auto/background fluorescence. This procedure is fully described and illustrated in Benech et al. (2000).

Drugs

The following drugs were prediluted in the bathing solutions (ASW and EGTA Ca^{2+} free ASW) and superfused across preparations: acetylcholine (ACh), carbachol, L-glutamate (L-glu), serotonin (5-hydroxytryptamine; 5-HT), atropine, muscarine, nicotine, caffeine and potassium chloride (KCl). All chemicals were obtained from Sigma (UK).

Results

(1) Transmission electron microscopy study

Fig. 1C shows a drawing of the orientations of the iridophore plates calculated from reflective measurements of Mäthger and Denton (2001) (see Fig. 1B). The figure also shows electron micrographs taken from sections cut from the different reflective areas. Sections were cut in the longitudinal plane, perpendicular to the skin surface.

The reflective plates of the 'red' stripe were oriented approximately parallel to the skin surface. Plates were on average 103±2.38 nm thick (mean ± S.E.M., *N*=13).

The iridophore plates of the 'blue' stripe lay at angles of 50–70° with the skin surface. This corresponds to an angle between the normals of the iridophores and the horizontal of between 50 and 70°. The plates were on average 190±2.01 nm thick (mean ± S.E.M., *N*=13).

The iridophore plates of the ventral iridophores lay at angles ranging from 60 to 70° with the skin surface. For specimens taken from the area halfway between the ventral midline and the blue stripe this corresponds to an angle between the normals of the iridophores and the horizontal of 10–20°. Plates were on average 102±3.06 nm thick (mean ± S.E.M., *N*=13). No nerve terminals were found in the iridophore layer of the mantle.

(2) Changes in spectral reflectivity

In order to describe the changes in spectral reflectivity that occurred during iridophore activity a 'resting' state of reflectivity had to be recognised. This resting state was found by observing the iridophore reflections of a freshly decapitated squid at angles around normal incidence until no further changes in spectral reflectivity took place. This occurred after approximately 15–20 min of killing the squid. These resting reflective states could also be observed in living squid.

When in their resting state, the 'red' stripe iridophores are non-reflective. The 'blue' stripe iridophores have reflectivities in the blue and the ventral iridophores reflect weakly in the red parts of the spectrum. All the experiments described in section 3 were done using iridophores in their resting state.

(3) Pharmacology of iridophores using whole skin preparations

The 'red' stripe

When 'red' stripe iridophores in their resting state (i.e. non-reflective) were superfused with ACh or carbachol in ASW they 'switched on', that is, they reflected red light (Fig. 2A). This response was observed within 10–15 s of ACh perfusion (20–50 μmol l^{-1}) (*N*=10). Maximum reflectivity was reached within 1–1.5 min. Washing for approximately 15 min in ASW reversed the effects of ACh. Assuming that the changes in reflectivity are elicited by changes in the spacing between adjacent iridophore plates, this would suggest that, when non-reflective, best reflectivity would be in the infra-red, while ACh causes the plates to become closer together, resulting in reflections of red light. There are other ways in which this colour change can be produced (see Discussion). Perfusion of muscarine (30–50 μmol l^{-1}) (*N*=5) resulted in the same spectral shifts as caused by ACh and carbachol. The effects of ACh and muscarine could be blocked by 5–10 μmol l^{-1} atropine in ASW (data not shown) (*N*=3). Superfusions of nicotine (10–50 mmol l^{-1}), caffeine (30 μmol l^{-1} to

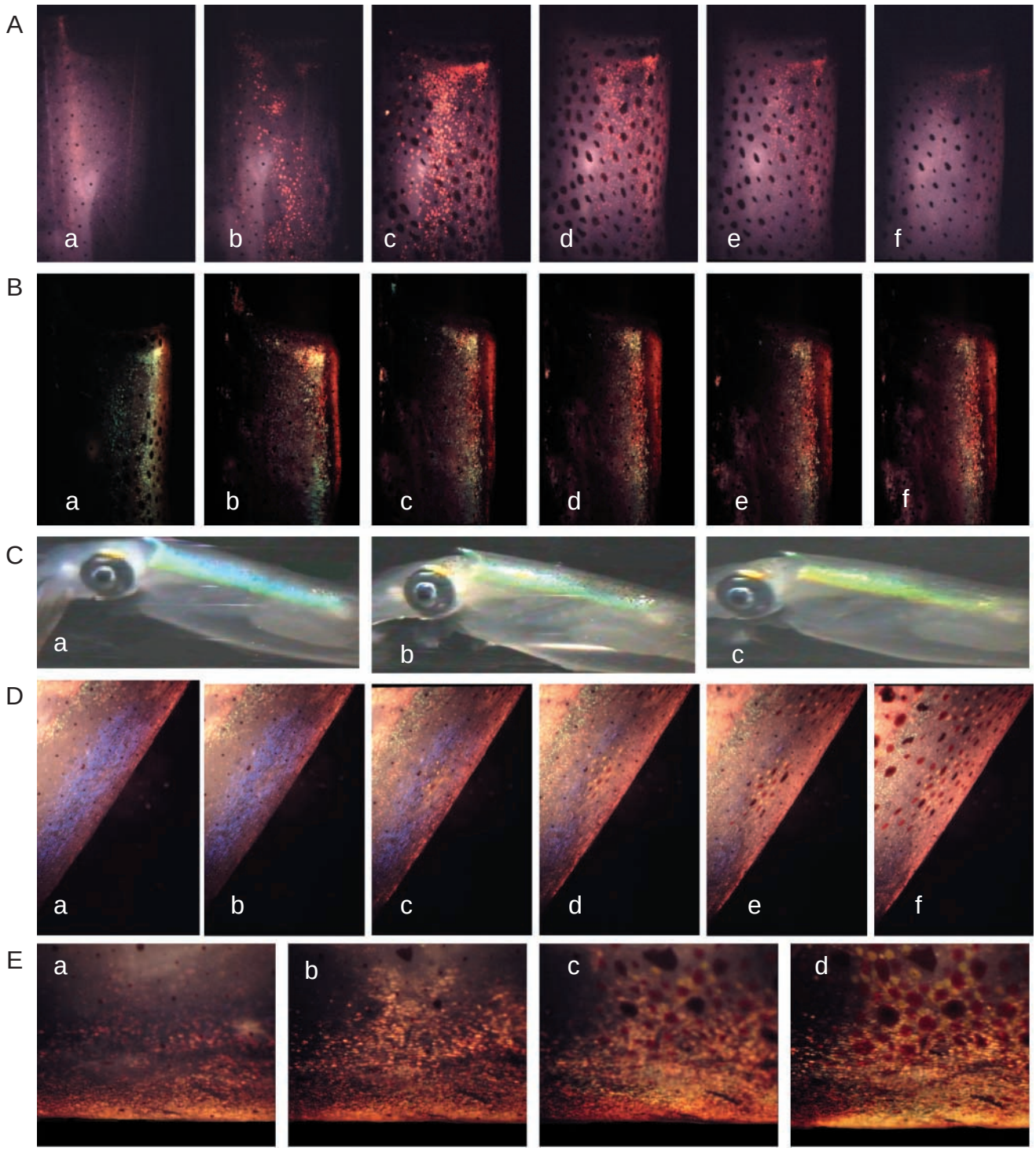

Fig. 2. (A) Iridophores of the 'red' stripe of *A. subulata* superfused with 50 μmol l^{-1} acetylcholine (ACh). (a) Before superfusion, (b) 20 s after start of superfusion, (c) after 1 min. The 'red' stripe iridophores change from non-reflective to reflective upon perfusion with ACh and become non-reflective after washing with ASW. (d) 5 min after washing, (e) 9 min after washing, (f) 12 min after washing. (B) Reflective 'red' stripe iridophores of *A. subulata* at 45° incidence. Reflectivity is best in the green parts of the spectrum. Photos are taken at 1 min intervals. Reflections gradually shift from green (a) to red (f) before switching off completely (not shown). (C) Video images of *A. subulata* at an angle of approximately 45° to the 'red' stripe. (a) Shortly after capture the 'red' stripe was strongly reflective in the blue-green. (b) After approximately 15 min the reflectance changed to green. (c) After 30 min the 'red' stripe reflected yellow-green light, with some red in the ventral parts of the stripe. (D) The 'blue' stripe of *A. subulata* superfused with 50 μmol l^{-1} ACh. (a) Before superfusion the 'blue' stripe is reflective. Following ACh superfusion it becomes gradually non-reflective. (b) After 15 s, (c) after 1 min, (d) after 1.5 min, (e) after 2 min, (f) after 3 min. (E) The iridophores of the ventral side during ACh superfusion (50 μmol l^{-1}). (a) Before superfusion the iridophores are weakly red reflective. In response to ACh, reflections shift gradually towards the orange and yellow parts of the spectrum. (b) After 1 min of perfusion, (c) after 1.5 min, (d) after 2 min.

60 mmol l^{-1}), KCl (20–100 mmol l^{-1}), L-glu and 5-HT (each 10–50 mmol l^{-1}) had no effects on spectral reflectivities (for each experiment N=5).

The effect of the absence of external Ca^{2+} was also investigated. The preparation was kept in EGTA Ca^{2+} free ASW for 15 min. This had no effect on the reflective properties of the iridophores. The preparation was then superfused with 50 μmol l^{-1} ACh in EGTA Ca^{2+} free ASW, which caused the iridophores to 'switch on' (N=5). This suggests that ACh acts *via* mechanisms independent of the presence of external Ca^{2+}.

We also investigated whether the spectral changes could be based on osmotic changes within the iridophore. This was done by perfusing the preparations with hyperosmotic ASW (from 30 to 50 mmol l^{-1} sucrose in ASW, with osmolarities of 1099–1158 mOsm kg^{-1}) and hyposmotic ASW (50–75% ASW, with osmolarities of 536–769 mOsm kg^{-1}). Even after 40 min, neither solution produced any change in spectral reflectivity (N=5). However, the iridophores remained viable, as they still responded to perfusions of ACh after these experiments.

When in a reflective state, the iridophores of the 'red' stripe reflect green light at angles of incidence around 45°. Iridophores that are observed at 45° during the process of 'switching off' reflect first yellow and then red light (Fig. 2B), before they 'switch off' completely. During the stage of red reflection at 45°, measurements of reflectivity and polarisation, using the methods described by Mäthger and Denton (2001), showed that the reflected red light is polarised (data not shown). Fig. 2C shows images taken from a video. At the beginning of filming, the 'red' stripe reflected strongly in the blue–green (Fig. 2Ca) (note that the angle of incidence to the 'red' stripe is approximately 45–50°). After approximately 30 min of filming, the iridophores began to reflect red and yellow–orange light at that angle, showing that the reflective shifts occur in a living squid.

The 'blue' stripe

The 'blue' stripe iridophores responded to perfusions of 20–50 μmol l^{-1} ACh or carbachol by 'switching off', that is the reflections became invisible to the human eye (N=10). Assuming that the activity pattern is based on a platelet spacing change, this would suggest that the reflections would shift from blue into the UV. Reflectivity changes of the 'blue' stripe took longer than those of the 'red' stripe iridophores. They set in after approximately 30 s of drug perfusion and were complete within 2–5 min (Fig. 2D). These spectral changes were also observed when superfused with 30–50 μmol l^{-1} muscarine (N=5). Atropine (5–10 μmol l^{-1}) blocked the effects of ACh and muscarine (N=3). Nicotine (10–50 mmol l^{-1}), caffeine (30 μmol l^{-1} to 60 mmol l^{-1}), KCl (20–100 mmol l^{-1}), L-glu and 5-HT (each 10–50 μmol l^{-1}) had no effects on spectral reflectivity (for each experiment, N=5). Neither the absence of external Ca^{2+} (N=5) nor hyperosmotic or hyposmotic ASW (N=5) had any visible effect on spectral reflectivity.

The ventral iridophores

It was previously reported that the iridophores of the ventral mantle of *Lolliguncula brevis* were 'inactive' (Hanlon et al., 1990; Cooper et al., 1990). This is not true of *A. subulata*. Perfusions with 20–50 μmol l^{-1} ACh, carbachol (N=10) and muscarine (N=5) resulted in the weakly red reflective ventral iridophores (Fig. 2Ea), reflecting orange-yellow light within approximately 1.5–2 min (Fig. 2Ed). These effects were blocked by 5–10 μmol l^{-1} atropine (N=3). Nicotine (10–50 mmol l^{-1}), caffeine (30 μmol l^{-1} to 60 mmol l^{-1}), KCl (20–100 mmol l^{-1}), L-glu and 5-HT (each 10–50 μmol l^{-1}) had no effects on their spectral reflectivity (for each experiment, N=5). Neither the absence of external Ca^{2+} (N=5) nor hyperosmotic or hyposmotic ASW (N=5) had any visible effect on spectral reflectivity.

(4) Electrical stimulation

Electrical stimulation of the stellate ganglion and localised stimulation of the chromatophore and iridophore layers in the skin led to contractions of the mantle and expansion of the chromatophores, but no changes were seen in spectral reflectivity of the iridophores. This supports the data reported by Hanlon et al. (1990), who stimulated the stellate ganglion, the stellar nerves and the iridophore layers of the squid *Lolliguncula brevis.*

(5) Isolated iridophore cells

Morphology of isolated iridophore cells

Fig. 3A,B shows electron micrographs of enzymatically isolated iridophore cells processed for TEM. The iridophores in these images are 50–300 μm long. Similar measurements were made in iridophores that had been enzymatically isolated (for example, see Fig. 3C). The iridosomes containing the iridophore plates can clearly be distinguished. The structures look similar to those shown for squid iridophores by, for example, Mirow (1972) and Hanlon et al. (1990; see also Fig. 1B).

Having injected a cell and filled it with Lucifer Yellow, it was clear that the dye, seen fluorescing in Fig. 3C, appeared to be taken up only within the iridosomes. Iridosomes have been shown to contain the plates and spaces that produce iridescence (Mirow, 1972; Cloney and Brocco, 1983). This was found for all iridophores filled with Lucifer Yellow (N=5). We filled iridophores from the dorsal (N=3), lateral (N=1) and ventral (N=1) regions of the skin and found no difference in the way the dye distributed within the iridosomes. It seems likely, therefore, that the iridosomes are one continuous structure and that they function as one unit during iridophore activity. The cells filled uniformly within 3–5 min, suggesting the absence of gap junctions.

Ratiometric Ca^{2+} measurements

The Ca^{2+} concentration of isolated iridophore cells under resting conditions was on average 66.16±18.71 nmol l^{-1} (mean ± S.E.M., N=4). In ASW, addition of KCl (10–50 mmol l^{-1}) evoked a transient increase in cytoplasmic Ca^{2+} (N=10)

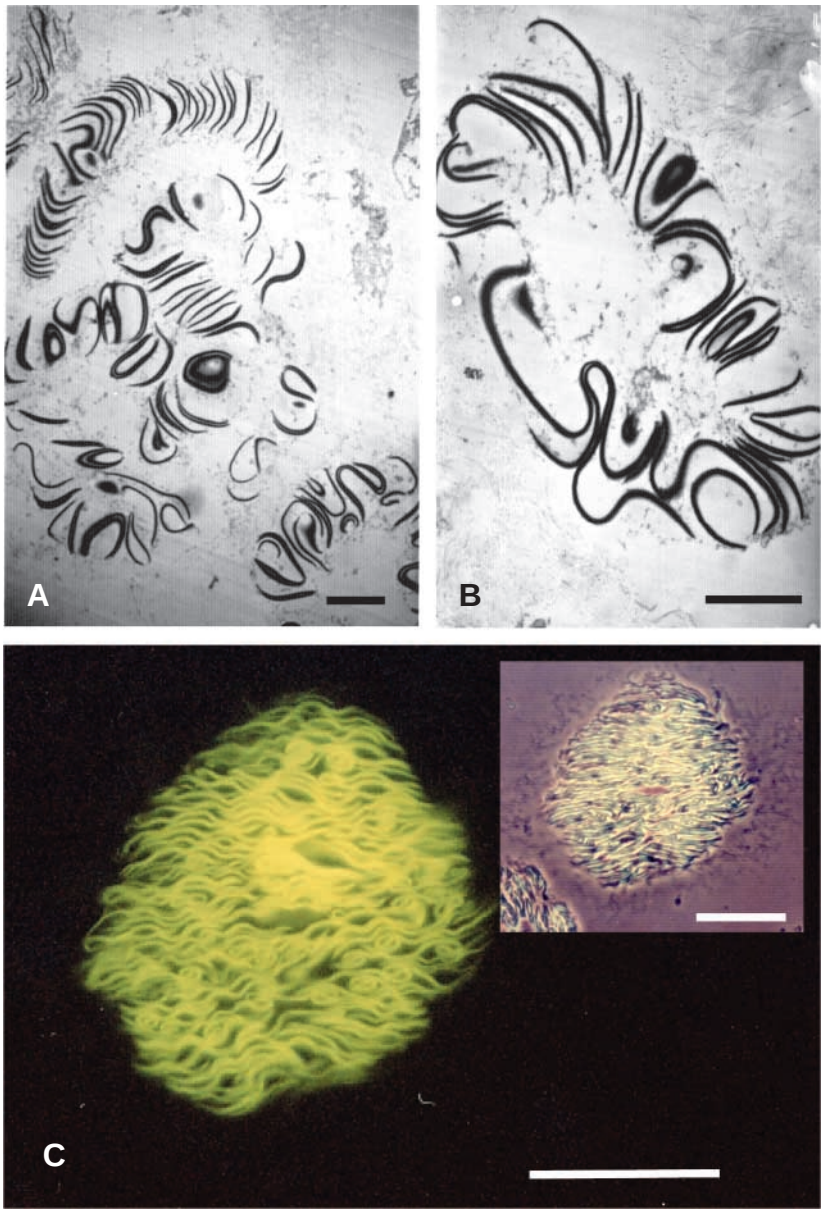

Fig. 3. (A,B) Electron micrographs of enzymatically isolated iridophore cells. Scale bars, 5 μm (A); 10 μm (B). (C) An isolated iridophore cell filled with Lucifer Yellow (scale bars, 50 μm). Inset shows the iridophore cell before being filled with Lucifer Yellow.

(Fig. 4A). In ASW, caffeine (2.5–10 mmol l^{-1}) also evoked an increase in Ca^{2+} (*N*=9) (Fig. 4B). This internal Ca^{2+} flux was observed irrespective of the Ca^{2+} concentration in the external medium (see Fig. 4E). Addition of 50–100 μmol l^{-1} ACh resulted in a transient increase in cytoplasmic Ca^{2+} (*N*=12) (Fig. 4C). This effect was also observed using similar concentrations of carbachol (*N*=6) (data not shown). Addition of 2–5 mmol l^{-1} nicotine had no effect on cytoplasmic Ca^{2+} concentrations (*N*=8) in cells for which the response to ACh was still evident (see Fig. 4C). In contrast to the nicotine response, muscarine (25–50 μmol l^{-1}) evoked a strong Ca^{2+} response (*N*=7), which could be blocked by pre-treating cells with 35–70 μmol l^{-1} atropine for 5–10 min (*N*=4). The blockage could be reversed by washing the cell with ASW for at least 10–15 min (Fig. 4D). These results suggest that the iridophore cells are activated by muscarinic acetylcholine receptors, which is further supported by the finding that neither L-glutamate (50–100 μmol l^{-1}) (*N*=4), nor 5-HT (50–100 μmol l^{-1}) (*N*=7) caused an increase in cytoplasmic Ca^{2+} (data not shown).

Experiments were conducted to investigate whether Ca^{2+} in the external medium is important to evoke the cytoplasmic increases in Ca^{2+} concentration. Cells were superfused for 10 min with EGTA Ca^{2+} free ASW before application of drugs. We found that intracellular Ca^{2+} concentration increased in response to 50 μmol l^{-1} ACh and 2 mmol l^{-1} caffeine even in the absence of external Ca^{2+} (*N*=4) (Fig. 4E).

Although the results using fresh tissue suggest that the reflective changes are not based on osmotic changes, experiments were conducted to investigate the effects of osmotic changes on the cellular level. Fura-2 loaded iridophore cells were superfused with ASW containing 50 mmol l^{-1} sorbitol (1112 mOsm kg^{-1}; s-ASW) to increase the osmolarity of the extracellular solution and 75% ASW (769 mOsm kg^{-1}) to decrease the osmolarity of the extracellular solution. Neither of these solutions had any effect on the Ca^{2+} concentration of the iridophore cells (*N*=5) (Fig. 4F). The Ca^{2+} levels remained low and subsequent addition of 50 μmol l^{-1} ACh evoked a strong Ca^{2+} response, showing that the cells were still viable.

There was no apparent difference between iridophores from the dorsal, lateral or ventral side in their intracellular Ca^{2+} response to the various drugs.

Discussion

Iridophore morphology

The results of the TEM study agree well with the orientation measurements obtained by determining the angle of reflectance at normal incidence with respect to the skin surface. The iridophore plates are oriented in such a way that thin film interference of light can occur, which suggests that squid iridophores act as multilayer reflectors. Using the plate thicknesses, obtained from the TEM study, and the refractive index of the plates, we can make theoretical predictions about the wavelengths that will be reflected. Note that a recent study by Crookes et al. (2004) has shown that the reflective plates in the iridophores of the squid *Euprymna*

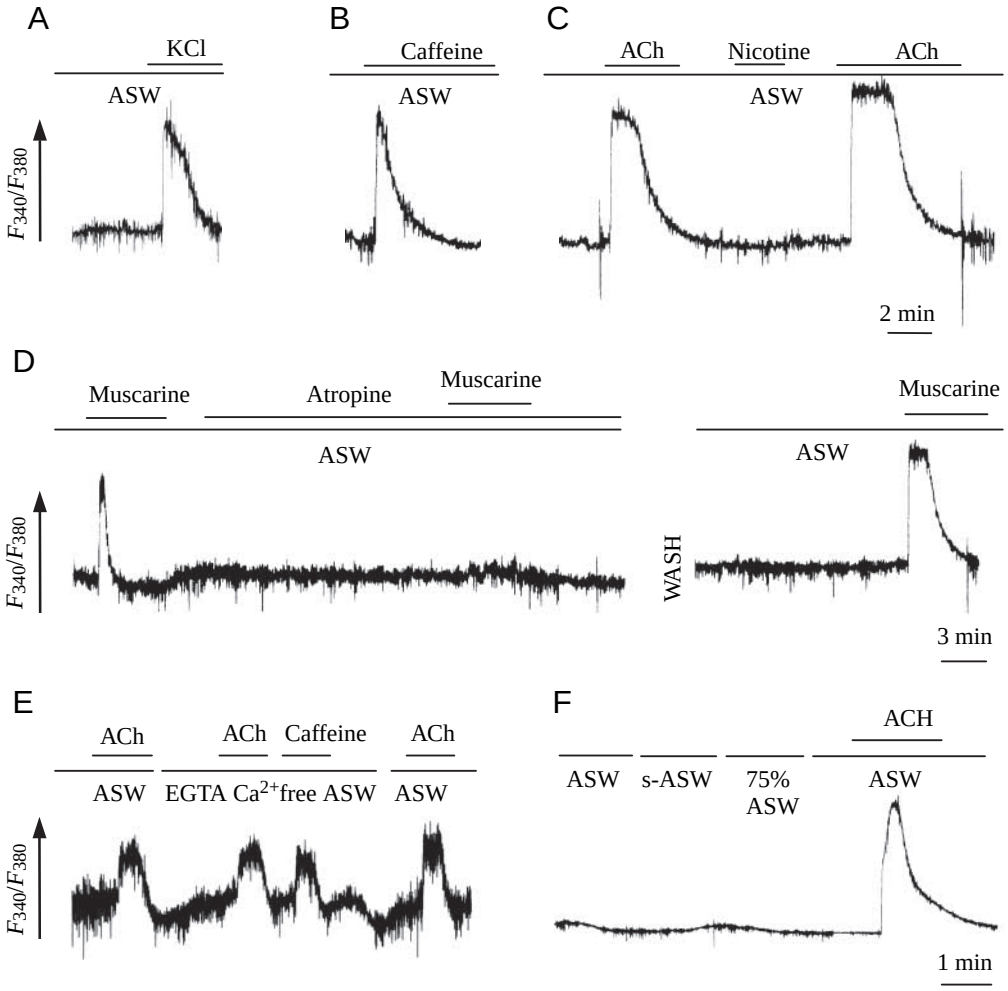

Fig. 4. Fluorescence ratio (F_{340}/F_{380}) measurements of cytoplasmic Ca^{2+} concentration of an isolated iridophore cell (concentrations of drugs given below; see text for details). Cytoplasmic [Ca^{2+}] increases transiently in response to (A) potassium chloride (KCl), (B) caffeine and (C) ACh, but not nicotine. (D) Muscarine evokes a transient increase in cytoplasmic Ca^{2+}, which is blocked by atropine. After washing for 15 min, the muscarine response is regained. (E) Ca^{2+} increases transiently in response to ACh and caffeine in the absence of Ca^{2+} in the external solution (EGTA Ca^{2+} free ASW). (F) Changing the osmolarity of the external solution has no effect on cytoplasmic Ca^{2+} concentration, although ACh evokes a strong increase in cytoplasmic [Ca^{2+}]. s-ASW, ASW + 50 mmol l^{-1} sorbitol (1112 mOsm kg^{-1}); 75% ASW, 769 mOsm kg^{-1}. Concentrations of drugs: KCl, 20 mmol l^{-1}; caffeine, 5 mmol l^{-1}; Ach, 50 μmol l^{-1}; nicotine, 2 mmol l^{-1}; muscarine, 50 μmol l^{-1}; atropine, 50 μmol l^{-1}.

scolopes are composed of proteins, which they have termed ‘reflectins’. The refractive index of these proteins is unknown at this stage, so that for the theoretical inferences below, we have assumed that squid reflective plates are made up of chitin (Denton and Land, 1971), with a refractive index (n) of 1.56.

The plate thicknesses of the ‘red’ stripes and the ventral iridophores suggest that they act as ideal quarter-wavelength (λ/4) stacks. A reflective plate of chitin with a thickness of approximately 100 nm would have best reflectivity at 624 nm ($\lambda=4nd$; Huxley, 1968; Land, 1972), where n=1.56, the refractive index for chitin, and d is the actual thickness of the plate). The plates of the ‘blue’ stripe are too thick to act as ideal λ/4 stacks. However, this could be the thickness required for the plates to act as a ¾ wavelength stack, for which $\lambda_{max}=4/3nd$. For a plate with a thickness of 200 nm, maximum reflectance would be at 416 nm.

The space thicknesses were very irregular. Assuming that the spaces between the iridophore plates are filled with cytoplasm, the dehydration of the tissue would have caused the spaces to shrink substantially. Therefore, from this study, we cannot be certain about the thicknesses of the spaces and consequently whether squid iridophores act as ideal or non-ideal multilayer reflectors. Optically, however, both ideal and non-ideal multilayer reflectors have very similar reflective properties (Land, 1972).

Changes in spectral reflectivity of iridophores

Active iridophores are found in a number of aquatic animals and are often associated with different behavioural states of the

individual. Although the anatomy and physiology underlying fish iridophores differ greatly from those of cephalopods, it is worth mentioning the blue damselfish *Chrysiptera cyanea*, whose body colouration is mainly attributed to multilayer reflectors, which can change colour. The wavelength changes are produced by a change in distance between the adjoining plates of the multilayer reflectors and it has been shown that the iridophores are under the control of the sympathetic (adrenergic) nervous system (Oshima et al., 1985a,b; Kasukawa et al., 1986, 1987). The colour change in the paradise whiptail *Pentapodus paradiseus* has also been shown to be based on this system (Mäthger et al., 2003). Colour changes mediated by active iridophores have also been reported in amphibians (e.g. Butman et al., 1979) and reptiles (e.g. Morrison et al., 1991).

When observing squid, the chromatophores are the most obvious colour changing media. The reflections from the iridophores are often inconspicuous, or even absent. If the stripes are fully reflective, however, they produce very clear reflective patterns that contribute substantially to the overall appearance of the animal. The 'red' stripes, for example, are often visible when the animal is threatened (e.g. by a hand net) or during intraspecific encounters. Similar observations were reported on *Loligo plei* by Hanlon (1982).

In the squid used in this study, the changes in spectral reflectivity are consistent with the hypothesis that these iridophores act as multilayer reflectors. The patterns of spectral changes were consistent in all stripes: ACh, muscarine and carbachol shifted the reflected wavelengths towards the shorter end of the spectrum with respect to the wavelength that they reflected in their resting state, i.e. the 'red' stripe iridophores changed from (presumably) infrared to red, the 'blue' stripe changed from blue to (presumably) UV and the ventral iridophores changed from red to yellow. Hanlon et al. (1990) also reported that in the dorsal iridophores of *L. brevis* the spectral reflections underwent a shift towards shorter wavelengths in response to topical applications of ACh. It therefore seems very likely that the distances between the plates or the thicknesses of plates and/or spaces are altered to produce these spectral changes. This hypothesis finds further support when observing the reflective changes of the 'red' stripe at 45° incidence during the process of 'switching off'. We found that the reflected light is polarised at 45° incidence, independently of which wavelength is reflected. Another hypothesis has been proposed by Cooper et al. (1990). They suggest that a change in the refractive index of the plates may also change the reflected wavelengths.

The spectral changes were also elicited using muscarine and blocked by atropine, suggesting the existence of muscarinic ACh receptors in the iridophore system. Cooper and Hanlon (1986) and Hanlon et al. (1990), who used atropine to block the effects of ACh, also suggested that a muscarinic ACh receptor type may be involved. Here we confirm their findings and also found no evidence for the presence of nicotinic receptors.

It was surprising to discover that changing the osmolarity of the external medium had no effect on spectral reflectivity of iridophores. In fish scales and in lizard skin, changes in the osmotic pressure of the external solution results in clear shifts of the reflected wavelengths (Foster, 1933; Denton and Land, 1971; Morrison et al., 1991). The most likely explanation, that the squid iridophores were protected by connective tissue and that the solutions had no access to the iridophores, was disputed after the same experiments were repeated on isolated squid iridophore cells and, clearly, the access problem in these preparations was minimal. The failure of the iridophores to respond to changes in osmolarity may also be because the iridophore spaces do not contain cytoplasm, a possibility that has already been proposed by Cooper et al. (1990).

In contrast to the results of the study of Cooper et al. (1990) and Hanlon et al. (1990), the results of the present study show that the iridophores of all stripes are active, that is, all respond to certain drugs with changes in the spectrum of the light they reflect. These changes can also be observed in living squid.

The properties of isolated iridophore cells

Lucifer Yellow

A striking result of the Lucifer Yellow injections was that the dye diffused into the iridosomes. It has so far been assumed that the iridosomes, which contain the plates and spaces (2–10 per iridosome), are separate units within an iridophore cell (Mirow, 1972; Cloney and Brocco, 1983). The Lucifer Yellow injections presented here show that the iridosomes are interconnected and that during iridophore activity they most probably function as one unit. Furthermore the dye diffused uniformly into the iridophore cell. It therefore seems certain that the iridosomes are not electrically coupled, but that they are one unit.

Cytoplasmic Ca^{2+} measurements

The resting Ca^{2+} concentrations calculated during this study are within the physiological range, which reflects the healthy conditions of the cells. Similar concentrations have been obtained for isolated squid synaptosomes from the optic lobe (Benech et al., 2000) and isolated squid chromatophore muscles (Lima et al., 2003). It can therefore be concluded that the process of enzymatic isolation and Fura-2 loading did not impair the healthy condition of the iridophore cells.

Cytoplasmic Ca^{2+} was found to increase transiently in response to ACh, carbachol and muscarine, but not to nicotine. This study suggests that ACh acts locally, on a post-synaptic level, *via* muscarinic acetylcholine receptor. This is an interesting finding, as electron microscopy has revealed no nerve terminals in the vicinity of iridophores (Cooper et al., 1990) and electrical stimulation of fresh tissue had no effect on reflectivity (see also Cooper and Hanlon, 1986; Cooper et al., 1990). How ACh is released remains to be established. Hanlon et al. (1990) suggest that it may act as a hormone. Certainly, ACh is abundant in the brain and optic lobes of several cephalopod species (Tansey, 1979).

Addition of KCl resulted in an increase in cytoplasmic Ca^{2+}, which shows clearly that isolated iridophores are excitable

cells that respond to changes in the electrical properties of their membrane, revealing the existence of voltage-activated channels. It is, however, interesting to note that KCl had no effect on whole skin preparations of iridophores, in contrast to some tropical fish, where increasing the external KCl concentration changed reflectivity (Oshima et al., 1985a; Fujii et al., 1989; Nagaishi and Oshima, 1989; Goda and Fujii, 1998). The lacking KCl response in whole skin preparations may be due to an accessibility problem, as other tissue surrounds the iridophores. Also, the K^+ concentrations inside the iridophore cells (as well as outside) when in intact tissue are not known. Therefore, it is impossible to predict the magnitude of the induced depolarisation (by increasing the external K^+ concentration to 50 mmol l^{-1}), as the potassium equilibrium was not determined.

The response to caffeine and the Ca^{2+} response in the absence of external Ca^{2+} show the existence of Ca^{2+} stores inside the cells. The experiments described here strongly suggest the existence of ryanodine receptors on the Ca^{2+} stores in iridophore cells, as caffeine has been used as a specific agonist of ryanodine receptors (Shmigol et al., 1995; Verkhratsky and Shmigol, 1996; Koizumi et al., 1999; Huang 1998). Hanlon et al. (1990) suggested the existence of Ca^{2+} stores, although no exact localisation could be inferred from their study. The results presented here, on both fresh tissue and isolated cells, suggest that Ca^{2+} is released from the stores independently of extracellular Ca^{2+}. This is an interesting finding, as all muscular systems of invertebrates need external Ca^{2+} for contraction, a feature shared with vertebrate cardiac muscles (Fabiato, 1983; Inoue et al., 1994). In isolated iridophores both KCl and muscarine evoked vigorous Ca^{2+} transients, suggesting that calcium-induced-calcium-release may not be the only mechanism for triggering Ca^{2+} release. Further research is required to investigate this point.

It is unclear to date what the role of Ca^{2+} is during iridophore activity. Ca^{2+} is a trigger for many physiological phenomena. For example, fluctuations in intracellular Ca^{2+} concentration can change the structure of microtubules (Weisenberg, 1972; Zhang et al., 1992; Barton and Goldstein, 1996; Jones et al., 1980). Ca^{2+} is also required for the movement of flagella and cilia (Holwill and McGregor, 1975; Walter and Satir, 1979). Both microtubules and microfilaments have been observed in fish and lizard iridophores (Harris and Hunt, 1973; Rohrlich, 1974; Nagaishi and Oshima, 1992; Oshima and Fujii, 1987). Neither microfilaments nor microtubules have been detected in squid iridophores, but the possibility that they provide the mechanism of iridophore colour change in squid should be further investigated.

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