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SHORT COMMUNICATION

CORRELATION OF IRIDESCENCE WITH CHANGES IN IRIDOPHORE PLATELET ULTRASTRUCTURE IN THE SQUID *LOLLIGUNCULA BREVIS*

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Cephalopods are renowned for their unique colour change system in which neurally controlled chromatophore organs in the dermis work in combination with subjacent iridescent cells (iridophores) to produce colours and patterns of a wide range (Messenger, 1979; Hanlon, 1982). Some iridophores in squids are physiologically active and reversibly change from non-iridescent to iridescent (Hanlon, 1982). We discovered examples of these active iridophores in the dorsal mantle of the squid *Lolliguncula brevis*. The state of iridescence in these dorsal mantle iridophores can be controlled *in vitro* in isolated dermal layers with the putative neuromodulator acetylcholine (ACh), as well as by means of other factors (Hanlon & Cooper, 1985). We compared the ultrastructure of non-iridescent and iridescent iridophores in these isolated dermal layers to determine the cytological basis for the change in optical properties. In this paper we report the correlation of structural changes within the iridophore platelets with the appearance of iridescence.

Schooling *L. brevis* maintained in laboratory tanks (Hanlon, Hixon & Hulet, 1983) occasionally show iridescent pink on the dorsal mantle, varying from bright in the dorsal mantle collar to a soft sheen in other dorsal regions. The iridophores responsible for this iridescence are in multiple layers in the collar, but in a single layer elsewhere. We excised dermal tissue containing the single iridophore layer for our study (Fig. 1A). The dermal layers were pinned out in Petri dishes filled partially with Sylgard® 184 elastomer (pre-blackened with osmium) and covered with freshly prepared artificial sea water (in mmol l^{-1}: NaCl, 500; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 12; unbuffered with a pH of approximately 6·5; prepared from refrigerated stock solutions). The iridophores were illuminated with 45° incident light (fibre optics), with the direction of the light beam from dorsal towards ventral in relation to tissue orientation. Care was taken not to vary the intensity, angle of incidence, or direction of incident light when comparisons of iridescence were undertaken. The tissue was illuminated only briefly during observations or photography. Experiments were carried out at maintenance tank temperatures of 20–21 °C. Experimental solutions (2–3 ml) were pipetted onto the drained iridophore layers, and results were observed

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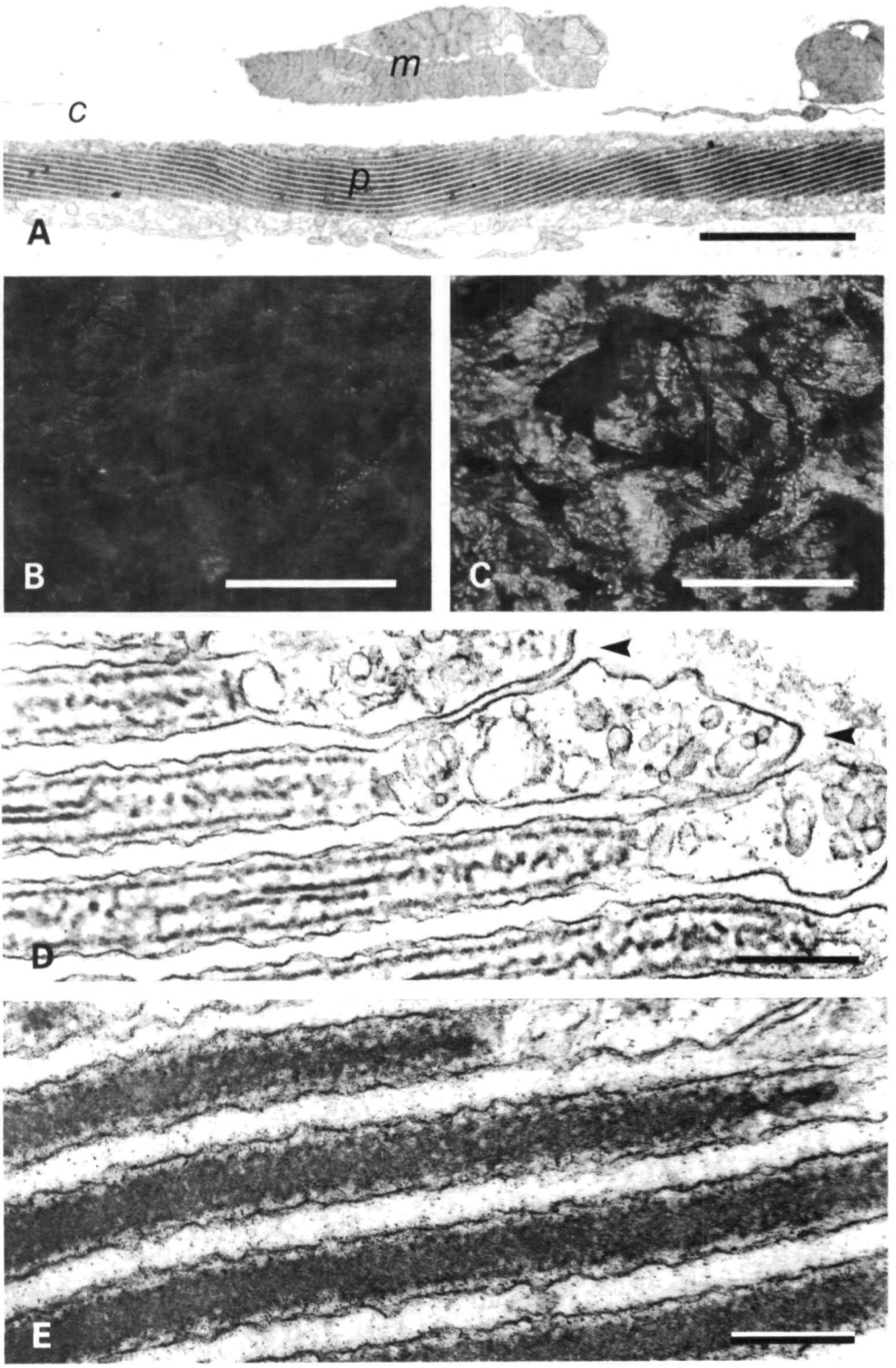
and photographed with a Leitz Orthoplan microscope equipped with a 25× long-working-distance lens.

Most iridophores in freshly excised skin appeared colourless, with non-iridescent, diffuse blue-to-violet iridophores scattered among the colourless cells (Fig. 1B). Sometimes small flecks of iridescent-appearing blue were observed on the non-iridescent blue cells, and some cells or parts of cells were brightly iridescent reds and other colours. This iridescence faded shortly after immersion of the excised tissue in artificial sea water. Application of ACh ($1{\cdot}11$–$2{\cdot}75\times10^{-7}$ mol l^{-1} in artificial sea water visible in the iridophores) resulted in the blue and colourless cells turning to metallic iridescent reds and golds, with some iridescent blues and greens (Fig. 1C); the overall macroscopic appearance was red to orange when viewed normal to the surface. Topical application of ACh to intact dorsal mantle skin also resulted in the iridophores becoming iridescent, with a pink appearance when viewed against the underlying whitish mantle muscle. The change to iridescence *in vitro* began within a few seconds and was usually complete within 3–5 min. The individual cells did not change colour all at once; patches of both non-iridescent blue and iridescent colours were visible simultaneously in a single cell. Washing with artificial sea water reversed the effect of ACh, and reapplication of ACh resulted in the reappearance of iridescence. This change to iridescence from non-iridescence is apparently mediated by Ca^{2+} since the Ca^{2+} ionophore A23187 (10^{-5} mol l^{-1}) in artificial sea water also caused the appearance of iridescence. Neither ACh nor the Ca^{2+} ionophore caused iridescence when applied in artificial sea water lacking Ca^{2+}. Equimolar atropine blocked ACh-induced iridescence, while tubocurarine did not, suggesting the involvement of muscarinic receptors in this system. The excised iridophore layers in our study contained connective tissue, muscle bundles, and some nerve fibres, as well as the iridophores (all chromatophore dermal layers were removed). Squid iridophores have not been shown to have direct muscle or nerve connections, but it is possible that the ACh acted primarily on one of these other tissue components, with a secondary effect on the iridophores. However, KCl, applied in a concentration (80 mmol l^{-1}) that caused chromatophore muscle contraction in intact skin, had no effect on the iridophores' response to ACh or on reversal to non-iridescence. Tetrodotoxin (10^{-7} mol l^{-1}) also had no effect. The iridophores themselves may be planar sides by membranes that often appear to be continuous with the plasma

Fig. 1. Comparison of non-iridescent and iridescent iridophores. (A) Electron micrograph of part of a non-iridescent dorsal mantle iridophore in an excised dermal layer. *p*, platelets; *m*, muscle; *c*, collagen. Scale bar, 5 μm. (B) Light micrograph of non-iridescent iridophores in excised tissue layer in artificial sea water, incident illumination at 45°. Scale bar, 200 μm. (C) The same tissue layer as in B. Treatment with $2{\cdot}75\times10^{-7}$ mol l^{-1} acetylcholine caused the iridophores to change from non-iridescent to iridescent. Scale bar, 200 μm. (D) Ultrastructural correlate to B. The platelets in this non-iridescent iridophore contain ribbon-like and flocculent material, as well as electron-lucent regions. Arrowheads indicate apparent connection of platelet membranes with plasma membrane. Scale bar, 200 nm. (E) Ultrastructural correlate to C. Compare the uniformly densely the target cells in this excised tissue. The iridophore platelets are bound on their granular appearance of these iridescent platelets with the fragmented ultrastructure of non-iridescent platelets in D. Scale bar, 200 nm.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membrane, making the space between the platelets extracellular (Fig. 1D, arrowheads). This cytological feature should facilitate putative neuromodulator or ionic access to the platelet regions of these cells. ACh is known to have a modulatory effect on the chromatophores of squids (Florey & Kriebel, 1969; Florey, Dubas & Hanlon, 1985), and ACh is present naturally in the dorsal mantle iridophore dermal layer of *L. brevis* at levels of approximately 1 nmol ACh mg^{-1} protein (32 $\mu g\, g^{-1}$ wet weight). Thus, it is possible that ACh is responsible for the appearance of iridescence in these iridophores in the intact animal as well as in our *in vitro* system.

Multilayer stacks of intracellular platelets are responsible for the structural colours of squid iridophores (e.g. Arnold, 1967; Mirow, 1972; Cloney & Brocco, 1983). Development of a fixation procedure that preserved the pre-fixation iridescent state was necessary to compare the ultrastructure of the platelets when they were non-iridescent and iridescent. Exposure of the iridophores to glutaraldehyde and/or paraformaldehyde fixatives resulted in all the dorsal mantle iridophores in excised layers becoming iridescent, regardless of prior treatment or iridescent state. However, iridophores fixed by quick total immersion in solutions containing 2% osmium tetroxide maintained their non-iridescent or iridescent state throughout fixation and after infiltration and polymerization of Spurr's resin (Spurr, 1969). Best cytological preservation was achieved by simultaneous fixation in 2% OsO_4, 2% paraformaldehyde, 2·5% glutaraldehyde, 3% NaCl, 0·1 mol l^{-1} phosphate buffer, pH 7·6, for 30 min in the dark in an ice bath (after Franke, Krien & Brown, 1969).

Transmission electron microscope (TEM) examination of fixed material revealed a striking difference in the iridophore platelet ultrastructure between non-iridescent and iridescent iridophores (Fig. 1D,E). Non-iridescent iridophores (Fig. 1D) had platelets containing electron-lucent and electron-dense regions, with an overall ribbon-like and flocculent appearance. The ribbon-like electron-dense material was 10–15 nm thick, while the flocculent material varied from 10 to 75 nm in dimension. Conversely, iridescent iridophores (Fig. 1E) had electron-dense platelets that were nearly uniform and densely granular, with no electron-lucent areas. Platelets of both iridescent and non-iridescent iridophores appeared to vary in orientation from parallel to the plane of the surface of the skin to tilted at an angle of up to 45°. The platelets were often 'wavy' rather than 'flat', and sometimes the platelets were folded within the cells. Non-iridescent platelets all had the fragmented ultrastructure, regardless of their prior treatment (i.e. artificial sea water alone, ACh treatment followed by reversal to non-iridescent, ACh or Ca^{2+} ionophore treatment in artificial sea water lacking Ca^{2+}). All iridescent platelets had uniform ultrastructure, whether the iridescence was induced by ACh, Ca^{2+} ionophore or aldehyde exposure. When ACh was applied for only 1–2 min prior to fixation, the ultrastructure of the individual platelets appeared to be in transition from fragmented to uniform. A few tissue samples contained both non-iridescent and iridescent iridophores and had a mixture of the two platelet ultrastructural types, as would be predicted. This may be one of those rare instances where ultrastructure is altered by the intracellular action of a transmitter substance.

The ultrastructural appearance of non-iridescent, colourless cells suggests that their platelets transmit rather than reflect light; the diffuse blues are presumably from Rayleigh-type scattering, with particles much smaller than the wavelength of blue light scattering blue and violet light more strongly than light of longer wavelengths (Jenkins & White, 1957). Apparently the electron-dense, uniform ultrastructure of iridescent platelets is responsible for the observed iridescence in these cells. Iridescent squid iridophore platelets reportedly act as thin-film interference reflectors (Denton & Land, 1971; Land, 1972) or as diffraction gratings (Brocco & Cloney 1980; Hanlon, Cooper & Cloney, 1983) to produce structural colours. Interpretation of the physical basis of the iridescence of these dorsal mantle squid iridophores is complicated by the large variation in platelet orientation within the intact cells, and will require further study.

The nature of the activity of physiologically active iridophores in animals is not fully explained. One must distinguish between the different mechanisms involved in varying iridescent colours of cells *versus* changing the cells from non-iridescent to iridescent. Most suggestions concerning mechanisms that vary iridescent colours cite alterations in array, tilt or spacing of cellular crystals in vertebrates (Rohrlich, 1974; Lythgoe & Shand, 1982) or of reflecting platelets in cephalopods (Kawaguti & Ohgishi, 1962; Young & Arnold, 1982). The evidence from our squid study indicates that the basic difference between iridophores that are in a non-iridescent state and those that are iridescent is the ultrastructural change *within* the platelets. This change in ultrastructure probably reflects changes in refractive indices within the platelets, with non-uniform discontinuous regions (Fig. 1D) coalescing into material of uniform refractive index (Fig. 1E); the result is a change in optical properties. To our knowledge this is the first report of this mechanism of colour production.

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