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Distribution and putative function of autonomic nerve fibres in the bill skin of the platypus (*Ornithorhynchus anatinus*)

Paul R. Manger[1], Janet R. Keast[2], John D. Pettigrew[1*] and Louise Troutt[1]

[1]*Vision, Touch and Hearing Research Centre, Department of Physiology and Pharmacology, and*
[2]*Department of Physiology and Pharmacology, University of Queensland, Brisbane, Queensland 4072, Australia*

The electroreceptors located in the bill skin of the platypus are modified secretory glands. The electroreceptive nerve terminals form bare endings in close proximity to the duct of these glands. In this study, we describe the autonomic innervation of the glands and a separate specialized autonomic innervation of the epidermal portion of the glandular duct. A range of immunohistochemical labels showed that the gland cells of the electroreceptors have a non-noradrenergic (putative parasympathetic) innervation. Phalloidin labelling revealed a 'sphincter' of epidermal luminal cells that labelled strongly for actin. These actin-dense keratinocytes were seen to have a noradrenergic (putative sympathetic) innervation. Fine-diameter sensory fibres containing substance P (presumably C-fibre thermoreceptors or polymodal nociceptors) were observed to terminate in the superficial epidermis surrounding the pore of the gland. When the bill of the platypus is dry these pores were closed. However, when room temperature water was washed over the bill, the pores opened. It is proposed that this autonomic and sensory innervation, along with the actin sphincter, mediates the opening and closing of the pores. By doing this, the platypus prevents the desiccation of the bare electrosensory nerve terminals when it is out of the water, and it may also be a way to regulate the impedance of the internal electrical circuit presented to the water at the pores.

Keywords: Monotremata; autonomic nerve reflex; platypus; electroreception; mechanoreception; trigeminal

1. INTRODUCTION

The bill of the platypus contains around 70 000 glands, both mucous and serous (Manger & Pettigrew 1996). Many of these glands are anatomically associated with arrays of sensory nerve fibres of trigeminal origin (Andres & von During 1988; Manger *et al*. 1995; Manger & Pettigrew 1996) that have been shown to be electroreceptive (Gregory *et al*. 1987, 1988). The mucous sensory glands are arranged in parasagittal stripes along the bill, and the serous sensory glands are distributed across the skin of the bill, with a maximum density at the rostrolateral poles (Andres & von During 1988). Ultrastructural studies of these electroreceptive nerve terminals show that they are bare nerve endings located at the basal epidermal portion of the gland duct, in contrast to the electroreceptive nerve terminals of fish, which are associated with a sensory cell (Andres & von Düring 1988; Manger *et al*. 1995). Therefore, the bare nerve terminal itself is used to detect any electrical stimuli. When the platypus is within the water, the pathway for conduction of the electrical stimuli has been proposed to be as follows: origin of the electrical stimulus, through the water, through the column of secretion from the gland associated with the electroreceptive nerve terminals, and onto the nerve terminals themselves (Manger *et al*. 1995).

However, the platypus does not spend all of its time in the water. It sleeps in a burrow that it digs into the bank of the streams it inhabits. Studies of both wild and captive platypus show that it spends at least 16 h of its day out of the water. As the electroreceptive nerve terminals are associated with the duct of the gland, it has been assumed that the secretion of the gland protects the electroreceptive nerve terminals from desiccation. However, the metabolic cost of constant secretion from all of the glands would be quite high. Electroreceptive fish obviously do not encounter problems with desiccation, although they do maintain constant secretion of ampullary canal fluid to protect the sensory cells from the osmotic effects that water may create. The metabolic cost associated with constant secretion of ampullary fluid is probably not as high as it may be for the platypus, as there are significantly fewer ampullary canals in most electroreceptive fishes compared with the glands of the platypus bill (Andres & von During 1988).

The pores of the glands in the platypus bill have been shown to be associated with an extraordinary elaboration of the superficial keratinocytes (Andres & von During 1988). These keratinous elaborations were proposed to have two functions: first, to hold the secretion of the gland in place to enhance discretion in detection of

*Author for correspondence (j.pettigrew@vthrc.uq.edu.au).

Table 1. *Primary and secondary antisera used for staining putative autonomic and sensory fibres*

(PGP 9.5, protien gene product 9.5; NPY, neuropeptide Y; VIP, vasoactive intestinal peptide; GAL, galanin; SP, substance P; CGRP, calcitonin gene-related peptide.)

substance	purpose	antibody source/dilution	secondary antibody/fluorophore
PGP 9.5	general neuronal marker	Ultraclone, 1:800	donkey anti-rabbit IgG/FITC
NPY	putative sympathetic axons	Auspep, 1:500	donkey anti-rabbit IgG/FITC
VIP	putative parasympathetic axons	INCstar, 1:2000	donkey anti-rabbit IgG/FITC
GAL	visceral motor and sensory axons	INCstar, 1:1000	donkey anti-rabbit IgG/FITC
SP	unmyelinated sensory nerves	Seralabs, 1:800	donkey anti-rat IgG/FITC
CGRP	unmyelinated sensory nerves	INCstar, 1:1000	donkey anti-rabbit IgG/Texas Red

electrical stimuli, and second, to hold secretion in place after the platypus has emerged from the water to prevent desiccation of the associated bare nerve terminals. A major flaw with these proposals is that the secretions are water soluble, and that even though the keratinous elaborations associated with the pores may indeed have some effect on movement of the secretion, any associated water that is running over the bill, such as when the platypus is swimming, will wash away the secretion.

Personal observations on anaesthetized platypus (from the study of Krubitzer *et al*. (1995)) and platypus that have been kept out of the water for a short period of time (from the study of Manger & Pettigrew (1995)) have shown that when out of the water, the glands of the platypus do not show any signs of secretion. However, when water is applied to the bill of an anaesthetized platypus, secretion begins almost immediately.

These observations led to the present study of the autonomic innervation of the glands and associated epidermis of the platypus bill. We report here the anatomical substrates for an autonomic reflex associated with the gland and the superficial array of keratinocytes that allows for the opening and closing of the pore. We show that there is an array of autonomic nerves associated with the gland, and that this array forms an association with a 'sphincter' of cells, near the opening of the duct, which label strongly for actin. Finally, experiments on two anaesthetized platypuses confirm that the superficial array of keratinocytes opens when stimulated with water, and then close when the bill is allowed to dry out. An analogous study has recently been reported for the pore canals of the sea urchin madreporite (Tamori *et al*. 1996).

2. MATERIALS AND METHODS

Two adult male platypuses were used in this study, and were a subset of the platypus used for other studies (Krubitzer *et al*. 1995; Manger & Pettigrew 1995, 1996; Manger *et al*. 1995). Lack of availability of platypuses did not allow us to confirm the present observations in more animals. Specimens of bill skin were obtained from the two platypuses following transcardial perfusion with 4% paraformaldehyde. Additional post-fixation treatment was carried out for some samples, as described below. From one of the platypuses, tissue was removed before fixation and immediately frozen for catecholamine analyses.

(a) *Immunohistochemistry and sucrose-phosphate-glyoxylic acid (SPG) staining of axons*

Fixed tissues were processed for immunohistochemical localization of the general nerve marker, protein gene product 9.5 (PGP), or for various neuropeptides, as described previously (Keast 1991, 1993). PGP was selected as it intensely stains both sensory and autonomic axons (Thompson *et al*. 1983) and, in the bill tissue of the platypus, has been found to mark electroreceptors and their associated innervation (Manger *et al*. 1995). Various other neuropeptide markers were selected for this study and are listed in table 1. An additional group of sections was stained with a mixture of substance P (SP) and calcitonin gene-related peptide (CGRP) antisera, to enable observation of axons containing both substances (a characteristic feature of visceral sensory axons in most mammals (Gibbins *et al*. 1987)).

An indication of which axons in the nerve supply are likely to be sympathetic, parasympathetic or sensory can be provided by the immunostaining for various peptide markers and catecholamines. Catecholamines (CAs) were considered to be indicative of sympathetic axons. In many other mammals, sympathetic vasoconstrictor axons can be distinguished from those having other functions by the presence in the former of neuropeptide Y (NPY) (Morris & Gibbins 1992). Parasympathetic axons are not known to contain CAs and are instead considered to be cholinergic; this could not be studied directly here owing to the lack of availability of antisera that stain peripheral cholinergic axons. Instead, vasoactive intestinal peptide (VIP) was taken as a marker of parasympathetic secretomotor nerves, as is the case in many mammalian secretory epithelia, such as the salivary glands (Morris & Gibbins 1992). Galanin (GAL) was included as another substance present in some visceral pathways (Hökfelt *et al*. 1993; Lindh *et al*. 1989), although there is no clear indication whether it is more typically found in sensory or motor types. Finally, SP and CGRP were selected as putative markers of unmyelinated sensory axons, as is the common case in other species (Gibbins *et al*. 1987).

Perfusion-fixed tissue samples were post-fixed in Zamboni's fixative (a mixture of picric acid and buffered formaldehyde), washed in phosphate buffered saline (PBS; 0.1 M, pH 7.0) and stored in PBS containing 30% sucrose until sectioning on a cryostat. Sections (15 µm) were dried onto subbed slides and incubated with droplets of diluted antiserum overnight in a humid chamber at room temperature. They were distributed consecutively

between groups of five slides such that adjacent sections were not stained for the same marker. A minimum of five sections per animal were stained for each substance. After washing off unbound antiserum, a further 1-h incubation was carried out with the appropriate secondary antiserum (1:50; Jackson, West Grove, PA, USA), as shown in table 1. Appropriate testing of secondary antisera was also carried out to ensure species specificity (e.g. primary antisera raised in rabbits were not recognized by secondary antisera raised against sheep or rat IgGs). All sections were finally coverslipped with buffered glycerol and viewed under a Leitz Laborlux microscope, using L3 or N2 filters for viewing flourescein isothiocyanate (FITC) or Texas Red, respectively. The PGP-labelled sections were visualized on a confocal scanning laser microscope. Collapsed Z-series images were taken of the PGP–FITC-labelled sections.

Visualization of CAs was done by a modification of the SPG (sucrose-phosphate-glyoxylic acid) method, as described previously (de la Torre 1980). Briefly, snap-frozen tissue was sectioned on a cryostat and sections (15 µm) then dipped briefly in substrate solution, heated at 95 °C, coverslipped with Depex and viewed under the fluorescence microscope (Leitz filter cube D). By using this method, CAs fluoresce green–blue and can be readily distinguished from serotonin, which emits yellow fluorescence; the method does not allow distinction between noradrenaline and dopamine. Ten sections from each animal were stained by the SPG method.

(b) *Phalloidin–FITC labelling of actin*

Specimens of bill tissue were taken and post-fixed overnight in 4% paraformaldehyde in 0.05 M phosphate buffer. Cryostat sections (20 µm) of the tissue were taken and washed in phosphate buffer. These sections were lysed in 100% acetone at −20 °C for 10 min. The sections were then incubated in a 2×10^{-6} M solution of phalloidin–FITC (Sigma) in phosphate buffer for 1 h at 37 °C. The sections were then stored at 4 °C overnight in this solution. After this overnight treatment the sections were washed in phosphate buffer and mounted in glycerol. The mounted sections were stored at 4 °C, away from light, until visualization under a confocal scanning laser microscope. Collapsed Z-series images were taken of the phalloidin–FITC-labelled sections.

(c) *Functional experiments*

Platypuses were anaesthetized with a 0.4 ml intramuscular injection of xylazine hydrochloride (100 mg ml^{-1}). The bill was placed under the ×100 objective of a dissecting microscope, the image of which was recorded onto videotape for later analysis. The bill was alternately irrigated with room temperature distilled water and allowed to dry. Any changes in the appearance of the superficial arrays of keratinocytes surrounding the pores of the glands associated with the electroreceptors was easily detectable when viewing the videotape.

3. RESULTS

(a) *Autonomic nerves*

Nerve fibres were stained by each of the antisera. The staining was assumed to represent specific immunoreactivity because: (i) all antisera have been thoroughly characterized previously, including absorption tests (Keast 1991), and are known not to react with the other substances included in this study; and (ii) each yields staining with a characteristic and unique distribution. Nevertheless, it is possible that the staining represents immunoreactivity towards peptides of slightly different structure than those against which the antisera were raised. For accuracy, it would be preferable to refer to each type of staining as (for example) substance P-like immunoreactivity. However, because of the range of substances studied, this would become cumbersome to the reader and we have chosen to aim for brevity by referring to each group of stained nerves as (for example) SP nerves.

Previous studies of the ultrastructure of the platypus electroreceptors have noted that there are many autonomic nerves surrounding the glands (Andres & von During 1988). This observation is confirmed by the present study. However, we observed that there were additional autonomic axons present, which were not restricted to innervation of the gland. Around each structure in the bill of the platypus, a plexus of autonomic axons (mucous gland electroreceptors, non-sensory mucous glands, sensory serous glands and push-rod mechanoreceptors), containing either VIP or NPY, was observed. A series of sections, orthogonal and parallel to the skin surface, were taken to examine the distribution and morphology of these autonomic axons. Autonomic axons were seen to arise from the same plexus as those that innervated the sensory portion of the glands. No attempt was made to reconstruct the tissue to allow individual fibres to be followed, or to allow the same individual glandular structure to be examined for each substance. Instead, our method of sampling, in which every fifth section was stained for a given substance, provided a better representation of overall tissue distribution of nerves of that type.

(b) *General features of nerve distribution*

Using PGP as a marker for sensory, motor and autonomic innervation, an extensive distribution of axons was seen in all tissues. In the dermal layer this included thick bundles of non-varicose (pre-terminal) axons, meandering among the glands and travelling close to the blood vessels. Thinner axon bundles and some apparently single, varicose (terminal) axons emanated from the large bundles and supplied the basolateral surfaces of the glandular epithelium. Many axon bundles travelled toward the epidermis and appeared to terminate near the various sensory receptors. These include both electrosensory and mechanosensory axons (Andres & von During 1988). The receptor terminals were also intensely stained, as described previously (Manger *et al*. 1995). Further innervation of the epithelial tissue was of two types. First, dense networks of finer axon bundles and single axons supplied the region near the superficial portion of the sensory receptors, surrounding the glandular ducts and the upper epidermal portion of the push-rod mechanoreceptors. Second, fine, varicose fibres were occasionally found traversing the epithelium near the duct and terminating very close to the surface of the epidermis.

Thus, for the glandular electrosensory structures in the bill skin of the platypus, there are at least four types of innervation. First, the electrosensory innervation, which

forms a large neural cuff of myelinated sensory fibres and terminals around the papillary region of the epidermal portion of the gland duct (Manger *et al*. 1995). This study deals with the three other areas of innervation: (i) innervation of the gland itself; (ii) innervation of the keratinocytes that surround the superficial epidermal portion of the gland duct; and (iii) the small varicose axons that terminate very close to the surface of the epidermis surrounding the pore of the gland duct.

For the push-rod mechanoreceptor, we have demonstrated five types of innervation. Three of these are mechanosensory, supplying: (i) paciniform corpuscles, which lie in the dermis below the rod; (ii) Merkel cells, which are invested in the deepest portion of the epidermal rod; and (iii) the vesicle chain receptors, which traverse the rod towards the surface of the epidermis (Andres & von During 1988; Manger & Pettigrew 1996). The present study deals with the additional two forms of innervation, namely the innervation of the keratinocytes that surround the superficial epidermal portion of the push rod, and the small varicose axons that terminate very close to the surface of the epidermis surrounding the protrusion of the rod from the surrounding epidermis.

(c) *Innervation of the glands*

Many axons travelled among the glandular acini. Various substances (CA, VIP and SP) were prevalent in the thicker axon bundles, the targets of which could be either dermal or epidermal structures (see above and figure 1*a*). CGRP, GAL and NPY axons were less common in these larger bundles (see figure 1*b*). Fine, varicose axons supplying the glands mainly contained VIP, although a few containing CA, NPY or GAL (figures 1*b* and 2*a*) were also seen. The most common nerve types associated with blood vessels were those containing SP/CGRP, CA or NPY.

(d) *Epidermal innervation*

No GAL, NPY or VIP axons were found in the epidermis. The most notable axon type present at this level contained CAs (see figure 2). These axons formed a dense network surrounding the keratinocytes that formed the upper portions of the duct of the glands, or the upper epidermal portion that surrounded the push rods (see figures 2*a*,*c*,*d*, and 3). Even more superficial to the axon network just described, rare varicose axons were seen to extend almost to the surface of the epidermis (see figure 4*a*,*b*). A few of these axons contained CAs, but most of these axons were seen to contain both SP and CGRP, or, less commonly, CGRP alone (see figure 4*c*,*d*).

(e) *Epidermal cells that label strongly for actin*

A series of sections, orthogonal to the skin surface, were taken and labelled for actin as described. Cells that labelled strongly for actin were easily distinguishable under the confocal microscope. A series of cells just below the level of the stratum corneum were seen to label intensely for actin. These cells were seen to form a ring, or 'sphincter', around the duct of a gland (see figures 5 and 6), or around the upper epidermal portion of the push-rod mechanoreceptor (see figure 5*b*). This sphincter of actin-dense cells had a depth of around 50 µm. These actin-dense cells were found to be at approximately the same depth as the termination of the autonomic axons

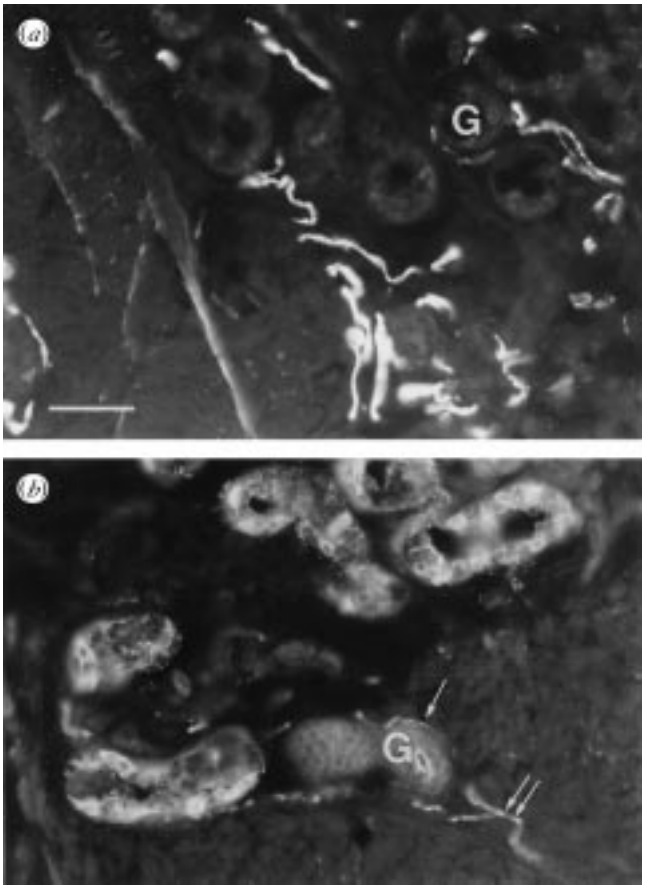

Figure 1. Neuropeptide-containing axons surrounding dermal glands. Micrographs are oriented such that the deeper dermis is at the bottom. (*a*) VIP-immunoreactive axons (which may be parasympathetic) are found in thick bundles travelling among the glandular acini and some finer bundles or single axons surround a few individual acini (example indicated by G). (*b*) GAL-immunoreactive axons are less commonly found among the glands, although a few fine fibres surround acini (example indicated by G and single arrow). Double arrows indicate a larger bundle of GAL axons more distant from the acinus. Note that some of the glandular epithelium is also labelled, possibly indicating an additional endocrine source of this substance. Scale bar, 50 µm.

that were found in the dermis. The phalloidin labelling of actin in this study was seen to intensely label the autonomic nerves associated with the actin sphincter (see figure 5*a*), whereas the large electrosensory and mechanosensory axons were unlabelled (see figure 6).

(f) *Effect of water on the superficial portion of the structures within the platypus bill*

The superficial keratinous elaboration surrounding the pore of each of the mucous gland electroreceptors is around 100 µm in diameter (Andres & von During 1988), and each shows a 'rose-like' morphology. These are the largest superficial structures on the surface of the bill skin and are very easy to visualize under a dissecting microscope. The other three structures have somewhat smaller superficial structures (mucous gland = 50 µm, sensory serous gland = 20 µm, push-rod mechanoreceptor = 30 µm), but are also distinguishable under a dissecting microscope.

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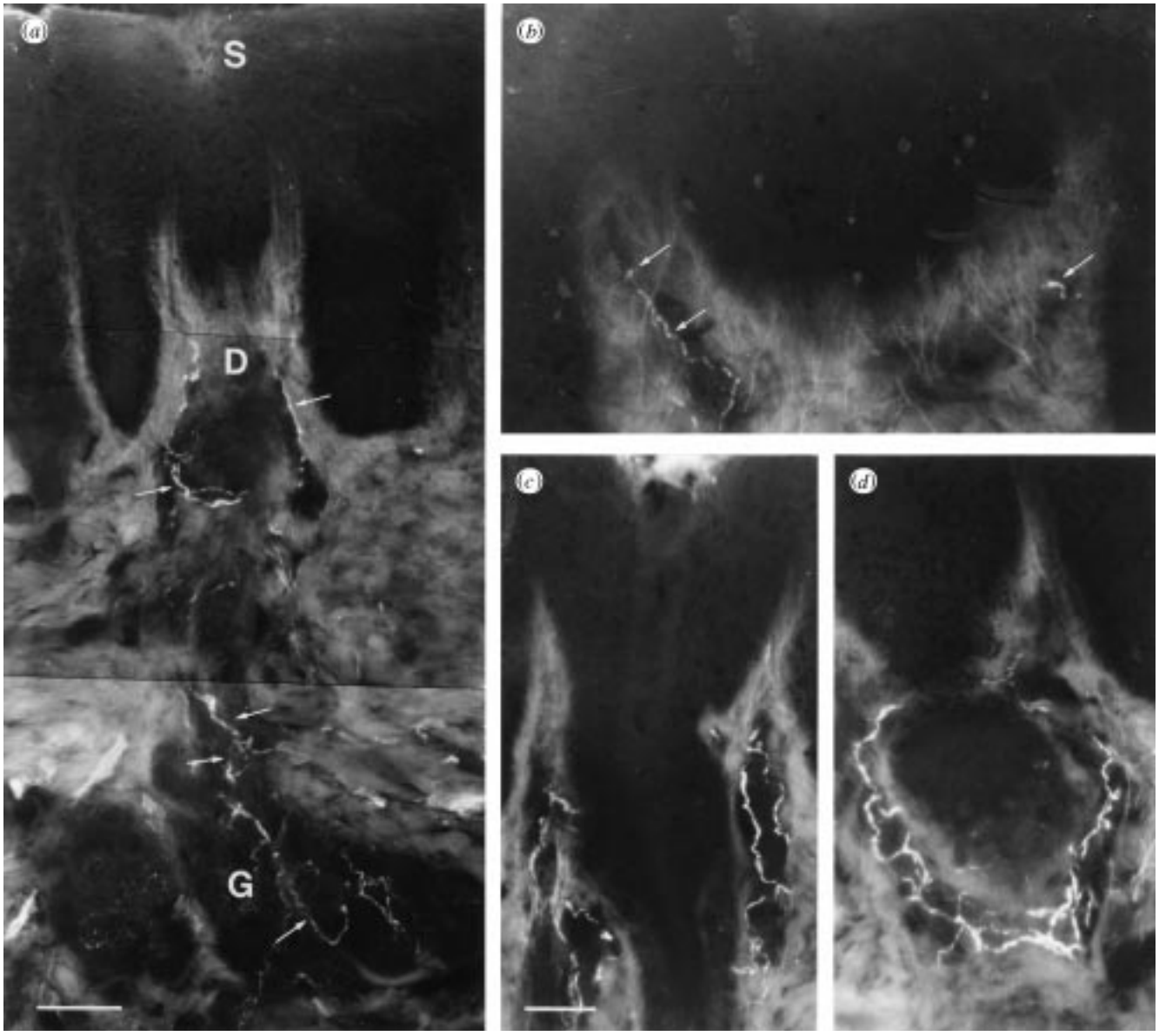

Figure 2. Catecholamine (CA)-containing axons supplying various tissues in the bill skin. These axons are probably sympathetic. All micrographs are oriented so that the surface epithelium is at the top. (*a*) This montage shows the dermal glands (G), duct of a mucous gland electroreceptor (D) and the surface of the epidermis (S). CA axons (examples indicated by arrows) travel among the dermal glands and progress toward the epidermal portion of the duct, where they form a plexus adjacent to the keratinocytes surrounding the gland duct; this region also labels strongly for actin (see figure 5). (*b*) CA axons (arrows) travelling close to the keratinocytes below the level of the stratum corneum, demonstrating the plexus that invests the actin-dense keratinocytes surrounding the gland duct. (*c*, *d*) CA axons travel past the region of the sensory nerve cuff and continue towards the epidermal portion of the gland duct, just below the level of the stratum corneum. As the axons pass the papillary region of the duct, they divide to form the plexus shown in (*b*). Scale bar in all figures, 50 μm.

After induction of anaesthesia, each platypus was placed in a dry wooden box, with dry cloth, allowing the bill to dry out. The dry bill was placed under the dissecting microscope and all four structures were visualized. The microscope was then focused on several (less than ten) of the structures and the bill was irrigated with water. As the experiment was being videotaped the changes in the appearance of the superficial structures could be timed.

It was found that when the bill was dry, the superficial elaborations of the structures in the platypus were in what can be described as a 'closed' position. In this position the rose-like structure of the superficial keratinocytes was not seen, and the elaborations of keratinocytes around the other structures of the bill were also less evident (see figure 7). However, 2–5 s after irrigation of the bill with water at room temperature, the keratinous elaborations were far more evident, appearing in the 'open' position (see figure 7). Simultaneously, the glands associated with these structures began to secrete either a mucous or serous secretion.

4. DISCUSSION

The immunohistochemical studies presented here have demonstrated that there is an extensive innervation of the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

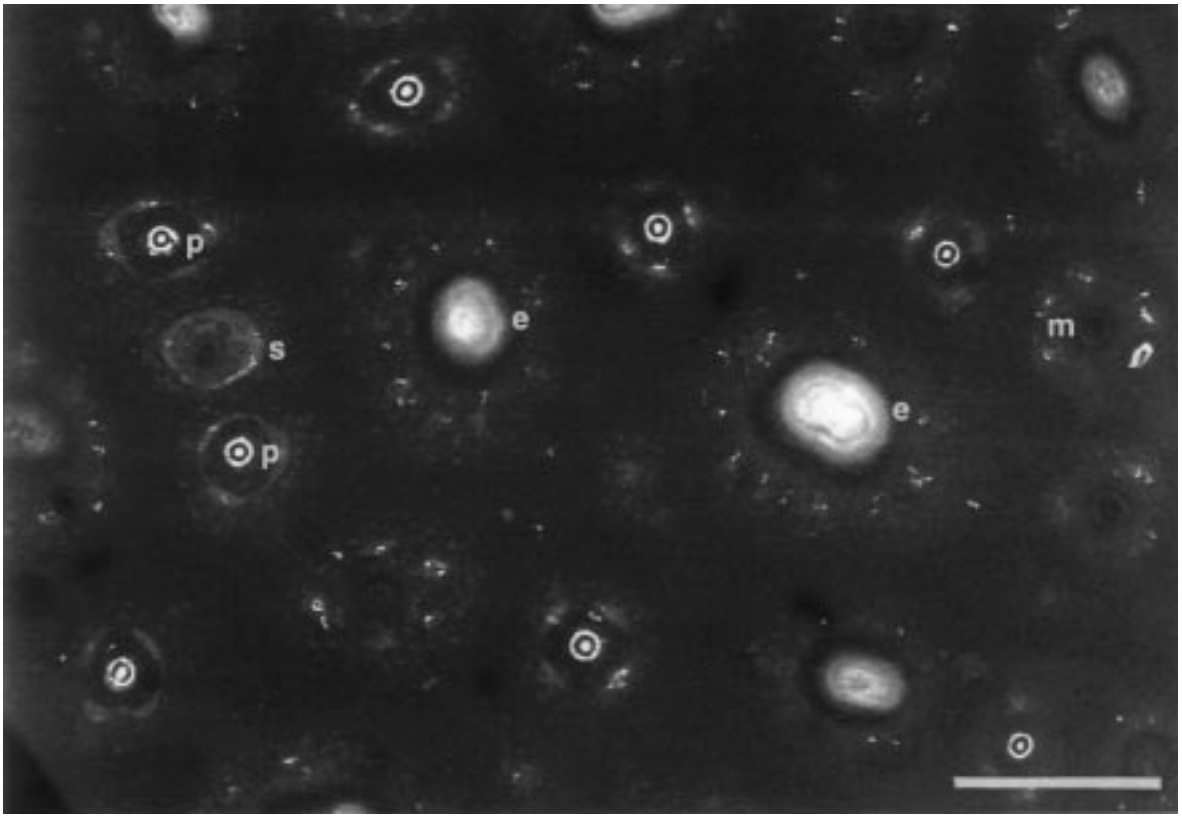

Figure 3. This scanning confocal micrograph shows the epidermis of the bill skin in a section taken parallel to the skin surface. All axons have been labelled with PGP. All four structures in the bill skin can be seen. The electrosensory mucous glands (e) are seen as brightly fluorescing circles. Each of these brightly labelled ducts is surrounded by 10–15 brightly labelled autonomic axons. The push-rod mechanoreceptors (p) are easily seen by the configuration of the mechanosensory vesicles chain, each of which appear as a bright dot, surrounded by a bright circle. Around each of these push rods, there are 3–4 brightly labelled autonomic axons. A sensory serous gland (s) is seen to the left of the micrograph between two push rods. Again, brightly labelled autonomic axons are seen to surround the duct, and to the upper right of the micrograph a non-sensory mucous gland (m), with associated autonomic axons, is seen. This micrograph demonstrates that all four structures have an associated autonomic innervation. Scale bar, 100 μm.

sensory and glandular structures in the bill of the platypus, much of which is likely to consist of autonomic axons. The primary targets of these axons are dermal blood vessels and glandular acini, as well as the epidermal walls surrounding the sensory structures, especially those regions that are seen to label strongly for actin. The presence of this autonomic supply has been suggested previously by the ultrastructural studies of Andres & von During (1988), but our work is the first to use histochemical markers to identify its likely sympathetic and parasympathetic components.

The peptide markers we have chosen in this study provide a good indication of the quite different target tissues of sympathetic and parasympathetic axons in the platypus bill skin. From our results it appears that the parasympathetic and sympathetic axons supply quite different targets. The parasympathetic axons, as marked by VIP immunostaining (and perhaps also GAL), exclusively supply the dermal glands. Sympathetic axons that contain CAs supply dermal blood vessels and the epidermal gland ducts in the region of the described actin sphincter. A much less prominent CA supply is provided to the dermal glands. It appears that the sympathetic vasoconstrictors may also contain NPY, but that the glandular sympathetic axons do not. This latter observation is similar to that provided in other mammals (Morris & Gibbins 1992).

However, it is not possible on the basis of chemistry alone to absolutely characterize the various axon types as sympathetic or parasympathetic. Such definition requires knowledge of the central origin of the preganglionic supply to the ganglion cells that provide the stained bill skin axons, information which was not determined in our experiments. Although it is highly likely that (for example) VIP is a marker of cholinergic parasympathetic axons, we must still exercise some caution with respect to categorization of the various types of peptide-containing fibres. Nevertheless, our studies clearly show quite extensive innervation of various glandular structures, and that the patterns of innervation differ widely between chemical groups. This provides good support for our proposal that sympathetic and parasympathetic axons subserve distinct functions in the platypus bill skin, as elsewhere.

This study has also demonstrated that there are some unmyelinated sensory axons that supply the bill skin of the platypus. In other mammals, SP and CGRP characteristically coexist in many small diameter, unmyelinated, sensory fibres (C fibres) (Gibbins *et al*. 1987). Such coexistence is seen in the platypus bill in axon bundles travelling adjacent to dermal blood vessels, the terminations of

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

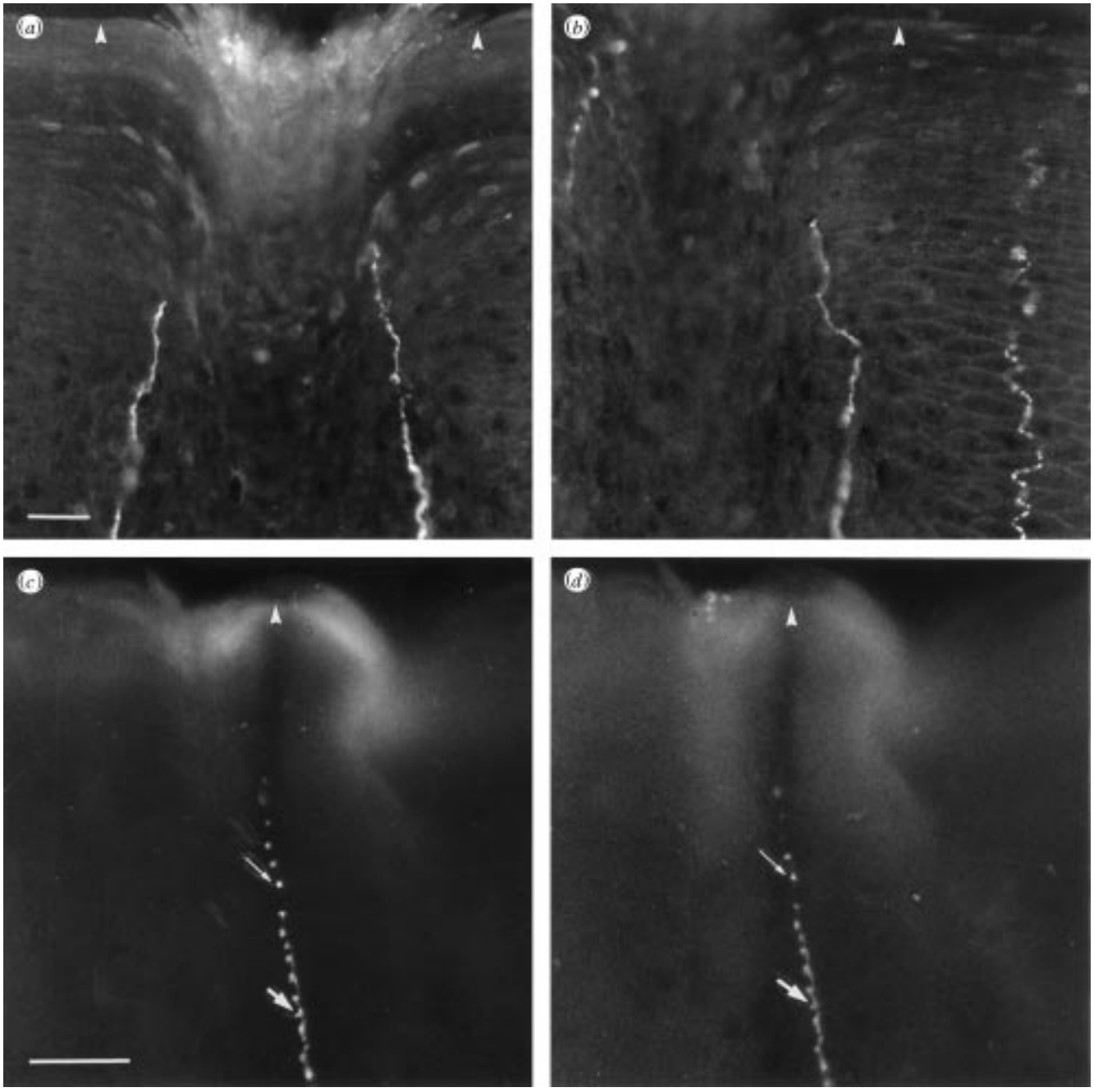

Figure 4. Putative sensory fibres in the epidermis. All micrographs are oriented so that the surface epithelium is at the top (and is indicated by arrowheads). (*a*, *b*) Fine axons containing PGP-immunoreactivity travelling close to the epidermal surface. In (*a*) these travel close to each side of a duct. (*c*, *d*) Varicose fibres double-stained for SP (*c*) and CGRP (*d*). Examples of varicosities containing both substances are indicated by matching pairs of arrows. Here these lie close to the epidermal surface and adjacent to a push-rod mechanoreceptor, the dome of which is easily seen (arrowheads). (*a*, *b*) Scale bar, 25 μm; (*c*, *d*) scale bar, 20 μm.

which are likely to be the fine varicose fibres found in the very superficial parts of the epidermis surrounding the electro- and mechanosensory receptors. The function of these sensory fibres is unknown, but in comparison with other tissues and species, may be either temperature receptors or nociceptors (see review by Holzer (1991)).

In summary, our study demonstrates that, apart from the major sensory function of the epidermal glands and the push rods, there are three other areas of innervation. First, there is parasympathetic innervation of the glands. Second, there is sympathetic innervation of an actin sphincter, and finally, there is sensory innervation of the epidermis surrounding the keratinous elaboration of the electroreceptors and mechanoreceptors found in the bill skin of the platypus (see figure 8). We envisage that this innervation provides the mechanism for the following proposed reflex pathway.

We postulate, first, that the sympathetic axons that innervate the actin-dense sphincter of keratinocytes release CAs to cause it to contract. In addition, we suggest that the C-type sensory fibres are activated by either the drying or the wetting of the bill (changes in

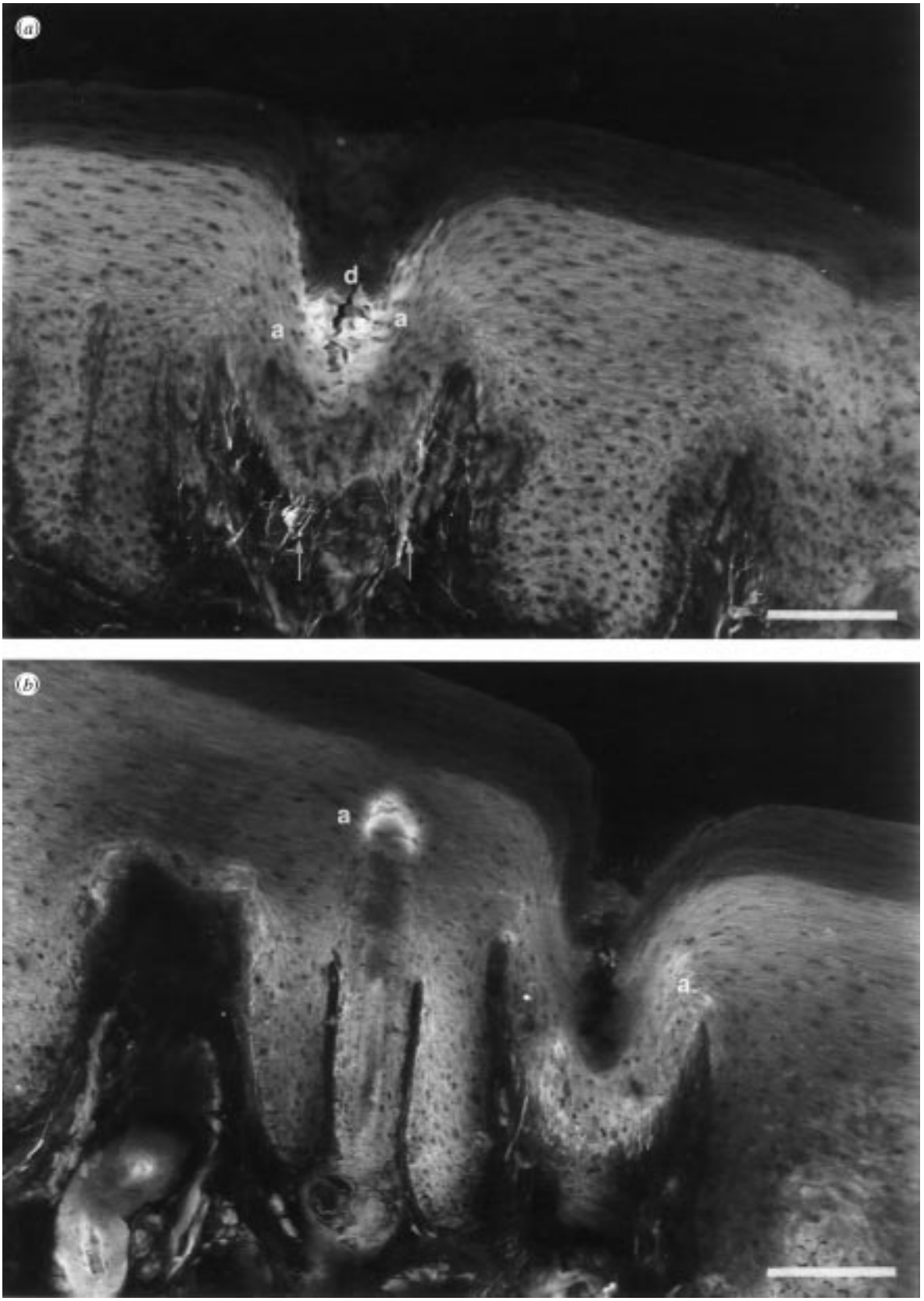

Figure 5. (*a*) Confocal scanning micrograph of a section through the upper portion of the duct of a mucous gland electroreceptor, labelled with phalloidin for actin. A ring or 'sphincter' of actin-dense cells (a) can be seen to surround the duct (d), just below the level of the stratum corneum. Autonomic fibres in the dermis can be seen to be extending towards these actin-dense keratinocytes. Scale bar, 100 µm. (*b*) Confocal scanning micrograph of a section through the skin of the platypus bill, labelled with phalloidin for actin, showing the full length of a push-rod mechanoreceptor beside the upper epidermal portion of the duct of a mucous gland electroreceptor. Around the upper portion of the push rod, a ring or 'sphincter' of heavily labelled keratinocytes (a) are seen. In this case the sphincter is only a few cells below the stratum corneum. Actin label can also be seen in the keratinocytes surrounding the duct of the mucous gland electroreceptor. Scale bar, 100 µm.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

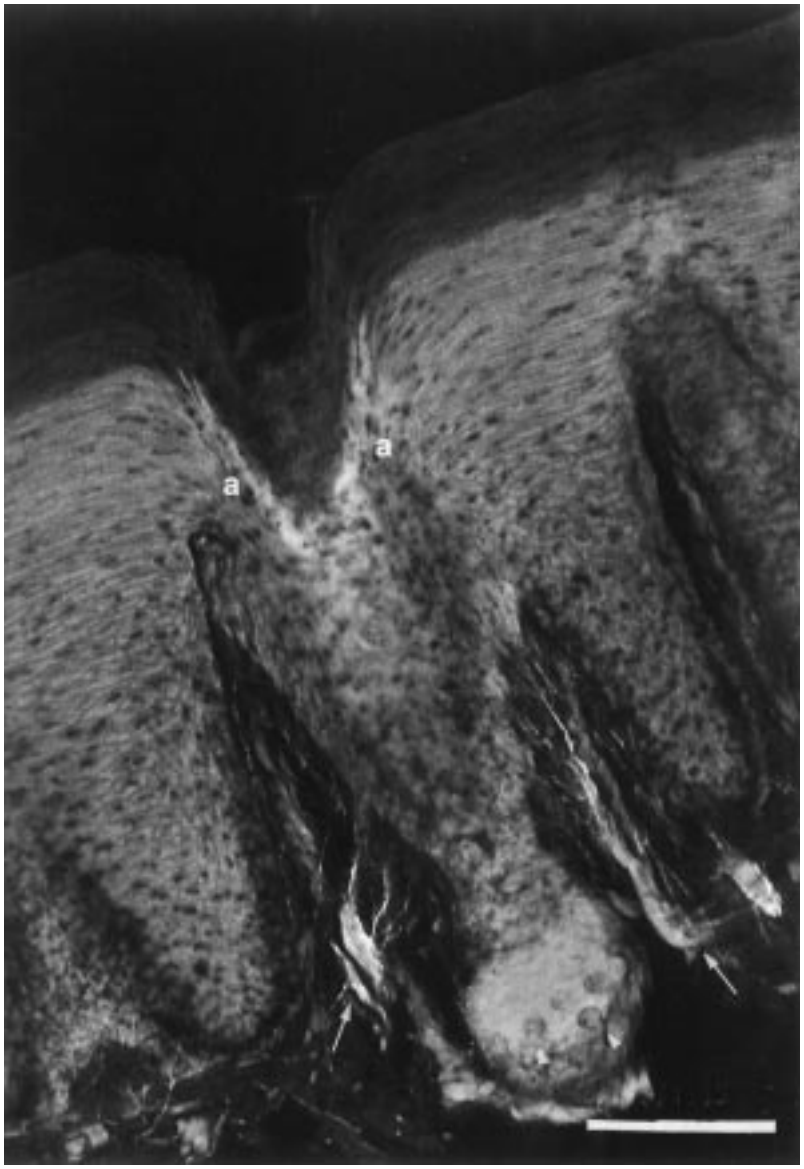

Figure 6. Confocal scanning micrograph of the epidermal portion of a mucous gland electroreceptor labelled for actin. The actin sphincter (a) can be seen around the duct, just below the stratum corneum. However, this micrograph clearly shows the preferential labelling of autonomic axons (long arrows) to sensory axons (short arrows). At the base of the papillary portion of the duct, the large bulbous portions of the electroreceptive nerve terminals are visible. In the dermal pegs to either side of the epidermal portion of the duct, brightly labelled autonomic axons are seen describing a course towards the actin sphincter. Scale bar, 100 μm.

temperature) to then initiate an autonomic reflex that enhances secretion while the pores are open, but to cause secretion to stop or slow when the pores are closed. Our experiments do not allow us to define which of the stimuli activate these sensory fibres, although we envisage that activation by wetting (i.e. cooling) is more likely. Furthermore, there are many possible mechanisms by which the appropriate changes in glandular activity could be initiated, and our experiments are unable to distinguish between these. One possible model is based on an assumption that the parasympathetic (VIP) axons stimulate secretion and, as mentioned above, sympathetic axons cause sphincter contraction. In this model, when the bill is dry, sympathetic axons would be tonically active (i.e. the sphincter is closed), and the parasympathetic axons would be silent (i.e. secretion is not stimulated). When the bill becomes wet, sensory fibres are activated and initiate a reflex such that the sympathetic pathway is switched off (i.e. sphincter is no longer contracted) and the parasympathetic pathway activated (i.e. glandular secretion is increased). Thus, when the bill is in the water, the pore will be open and the push rod will be allowed to move more freely.

The major functional finding presented in this paper is that the pores of the electroreceptor gland complexes in the platypus bill open and close according to whether or not they are immersed in water. This finding solves a major problem in understanding the mechanism of platypus electroreception, namely, how the electroreceptive nerve terminals are protected from desiccation when the platypus is out of the water. If the pores close when the platypus is out of the water, then a physical barrier between the dry external environment and the duct of the secretory gland is formed. Within the majority of the duct (except possibly for the superficial most portion) the secretion would continue to protect the nerve terminals from desiccation. A secondary implication of the pores closing as the platypus emerges from the water is to prevent dirt from clogging the pores. As the platypus sleeps in a burrow in the bank of the stream it inhabits, closing these pores to prevent clogging would prove useful. Third, this mechanism might provide a means for the platypus to regulate the impedance of the electroreceptive circuit that is presented to the aqueous environment. It is known that there must be a good match between the impedance of the electroreceptor

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

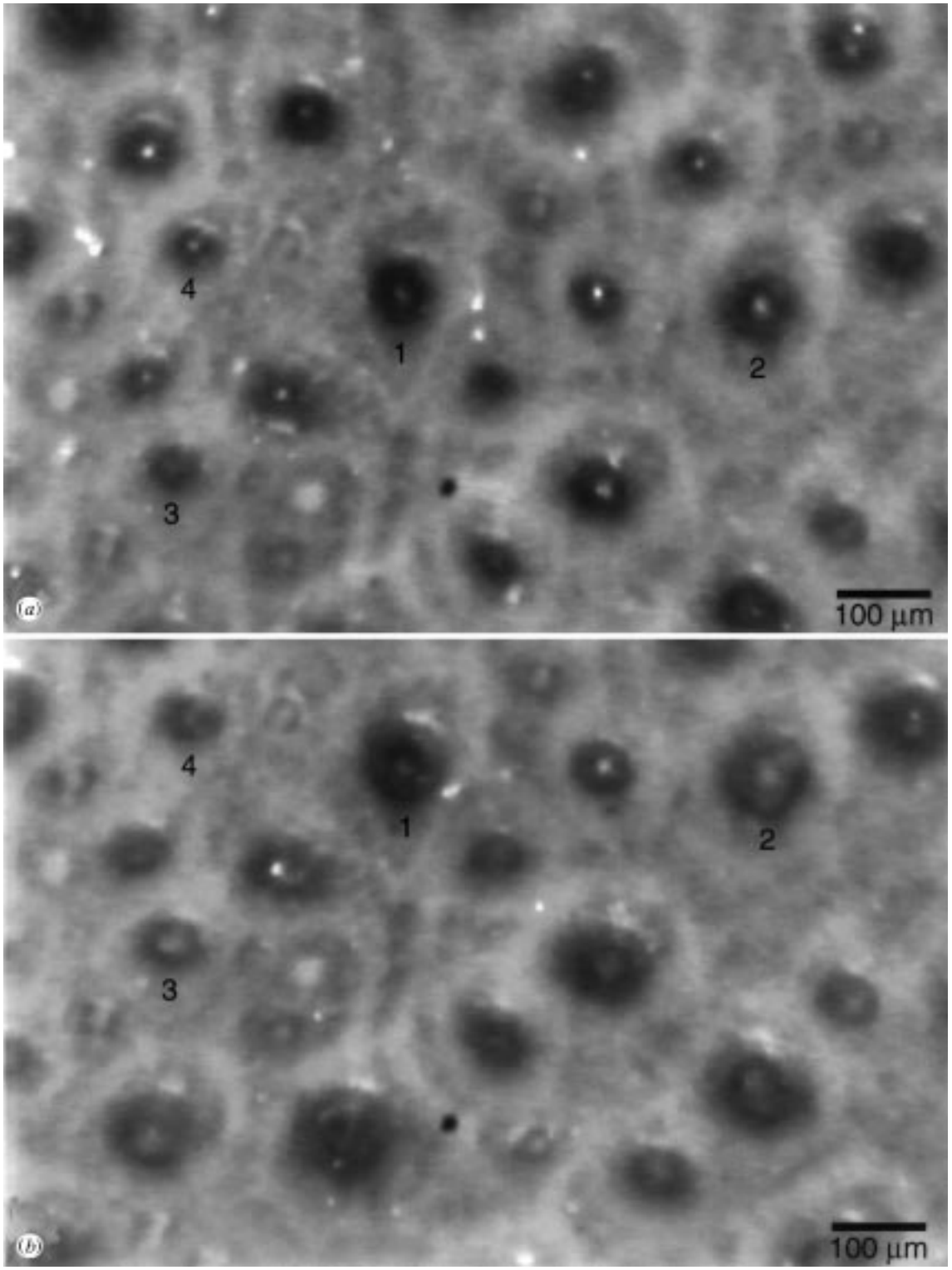

Figure 7. Two video images of the surface of the skin of the bill of a platypus. (*a*) Image taken immediately after wetting of the bill; (*b*) image taken *ca*. 3.5 s later. The labels '1' and '2' represent the pores of a mucous gland electroreceptor. The size of the pore has increased following wetting. More interestingly, however, is the ring of superficial keratinocytes that surround the pore, which are more obvious and which appear to extend above the surface of the skin. The label '3' indicates the tip of a push-rod mechanoreceptor, and it can be seen that after wetting the tip of the rod is more easily seen as it has become free of the surrounding epidermis. Perhaps least striking are the changes surrounding the pore of the serous sensory glands, '4', where a small change in size is the most obvious difference.

circuit and the water, as has been shown for three species of electric fish in the genus *Hypopomus*, which inhabit three different zones of water conductivity (C. Hopkins, personal communication). Although there is as yet no information available about impedance in either the electroreceptive circuit of the normal environment of the platypus, it seems likely that water conductivity may be an important factor limiting the range of signals

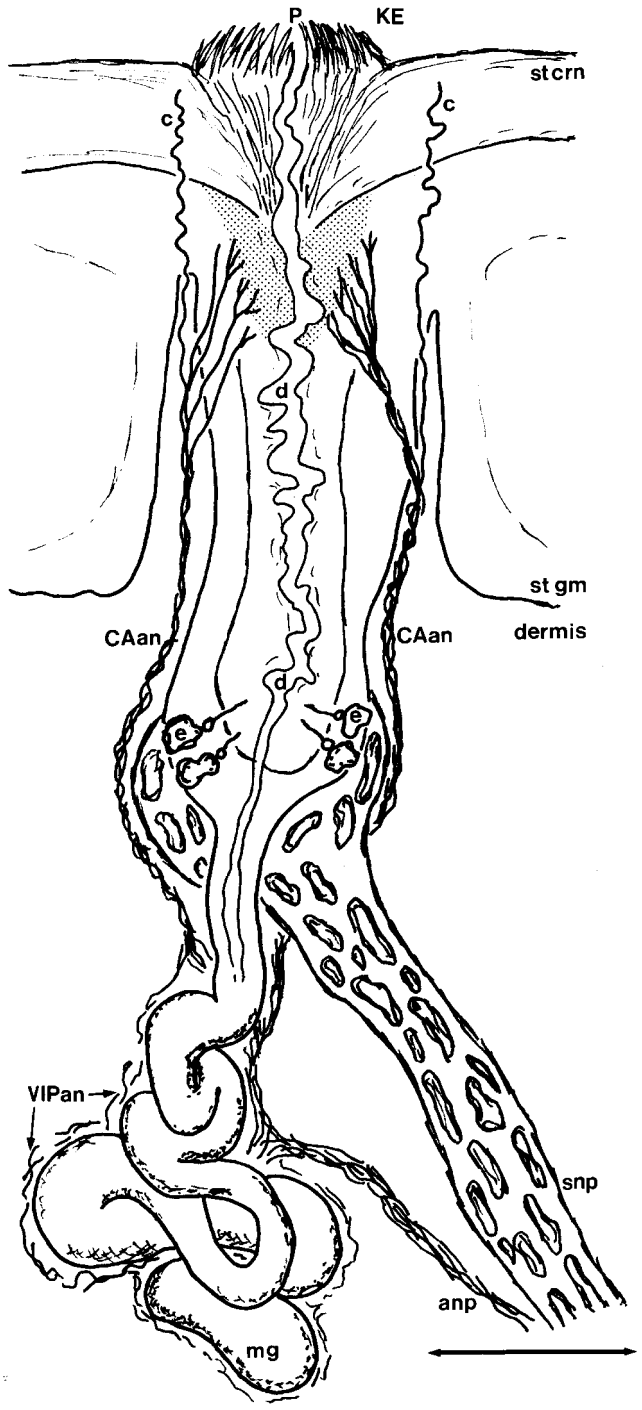

Figure 8. Summary diagram of the innervation, both sensory and autonomic, of a mucous gland electroreceptor in the bill skin of the platypus. Sensory nerves (snp) are seen to form a cuff around the epidermal papillary region of the gland duct and terminate (e) in the stratum germinativum (st gm) (Manger *et al*. 1995). The autonomic innervation (anp) is divided into two portions: VIP-containing axons (VIPan), which surround the gland (mg), and CA-containing axons (CAan), which innervate a sphincter of actin-dense cells (stippling) that surround the duct (d) at a level just below the stratum corneum (st crn). Small c fibres (c), assumed to be thermoreceptive, are seen to terminate in the stratum corneum surrounding the pore (p) of the gland, where an elaborate array of superficial keratinocytes (KE) are found. The autonomic innervation, thermoreceptors and actin sphincter form a reflex loop that allows the pore of the gland to close, and mucous secretion to cease, when the platypus is out of the (*opposite*) water, and the opposite when the platypus is in the water. This thereby provides two functions: protection of the electrosensory nerve terminals and lowering the metabolic cost of maintaining secretion in around 70 000 glands. Scale bar, 100 μm.

detected by the electroreceptive system. If the platypus could change its own circuit's impedance by altering the pore diameter and the constitution of the secretion, this would be a valuable attribute for impedance matching. This would increase the available habitat of the platypus as salinity varies significantly across their distribution. Finally, closing the pores and slowing, or stopping, the secretory action of the gland would provide significant savings in metabolic costs. Owing to the high density and number of glands on the bill of the platypus, with such significant functional importance to the animal, it is not surprising that such a specialized autonomic system has developed to protect the sensory terminals.

It is also significant that the sphincter of actin-containing cells and sympathetic innervation was seen to surround the push-rod mechanoreceptors. Anatomical studies have shown that the tip of the push rod is relatively free to move (Andres & von During 1984), thereby enhancing their tactile discrimination. When the platypus is in the water engaged in food search activities, enhanced sensitivity of the rods would be valuable. However, when in the burrow, such fine tactile discrimination is probably not necessary for the platypus. We envisage that contraction of the actin sphincter around the tip of the push rod would inhibit the freedom of movement of the push rod. It is noteworthy that if the tip of the platypus push rod were fixed, then its morphology would more closely resemble that of the push rods found in the echidna (Manger & Hughes 1992), European mole (Quilliam 1966) and the star-nosed mole (Catania 1995). In these three land-dwelling mammals, the tip of the push rod is fused with the surrounding epidermis. The temporary transformation of the platypus push rod by contraction of the actin sphincter to approximate the morphology of the push rods in other mammals suggests that these other mammals do not have the tactile discrimination provided to the platypus when it is in the water. However, the tactile discrimination of the platypus out of the water may be equivalent to these other animals.

The only previous report of a pore canal diameter being modified in a similar manner to that reported here for the platypus is that of Tamori *et al*. (1996). The report of Tamori *et al*. details functional changes in the pore canal size of the madreporite in a species of sea urchin. It is suggested that the changes in canal size controls the volume and pressure of fluid in the water-vascular system of this sea urchin. This situation is not analogous to the platypus, although the techniques employed by Tamori *et al*., especially the use of phalloidin to stain for actin filaments in the wall of the canal, are similar to those employed in the present study. The results of the present study, and that of Tamori *et al*. (1996), are suggestive of a similar format for the control of pore canal size across species. The specific point of biological interest is that two such evolutionarily distinct creatures should independently

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

solve the problem of control of pore size in such a similar manner.

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