Serous gland polymorphism in the skin of Phyllomedusa hypochondrialis azurea (Anura, Hylidae): response by different gland

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ORIGINAL ARTICLE

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Serous gland polymorphism in the skin of *Phyllomedusa hypochondrialis azurea* (Anura, Hylidae): response by different gland types to norepinephrine stimulation

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Abstract Stimulation by norepinephrine in physiological concentration was used on the dorsal skin of the Argentine tree-frog *Phyllomedusa hypochondrialis azurea* to trigger contraction of myoepithelial cells encircling the serous glands and provoke secretory release. This hylid species possesses two kinds of serous cutaneous glands, producing secretory granules or vesicles (type Ia and Ib serous units, respectively), along with serous-derived glands which synthesise lipids and store them in complex aggregates (type II units). Structural and ultrastructural observations on myoepithelia, secretory units and gland products collected in saline after discharge, revealed consistent but different responses in the three types investigated. Type Ia glands reacted intensely to treatment, with both contractile and secretory responses, type Ib glands were only mildly affected in their myoepithelia and glands of type II were not affected at all. According to data available in the literature, these findings suggest that: (a) the dense (type Ia) granules are expelled as a phasic response through bulk (holocrine) discharge, (b) the secretory (type Ib) vesicles are released as a tonic response through a merocrine mechanism and (c) lipid (type II) aggregates are exuded as a secretory component of a complex behavioural response which tends to reduce transcutaneous water loss. Furthermore, these findings indicate that the use of pharmacological modulation of myoepithelial activity allows selective collection of skin products in species characterised by serous gland polymorphism.

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Introduction

First accounts on serous gland polymorphism in anuran skin (i.e. occurrence of three or more types of cutaneous glands with different secretory products) were reported in species of the Old World genus *Bombina* and later confirmed in South American toads and frogs belonging to several families (Table 1).

The secretory cycle in serous glands is a prolonged activity (both biosynthetic and maturational; see Delfino 1991) which radically modifies secretory product morphology. Therefore, several criteria have been given so that true gland polymorphism can be distinguished from any morphological variability in secretory granules due to maturational processes. The former trait derives by divergent evolution of secretory units during ontogenesis (Delfino 1977; Delfino et al. 1998a), and the latter is due to modification of the secretory granules during the gland cycle (Delfino et al. 1999).

Since different serous glands in the same species may also exhibit peculiar features in their myoepithelia (Bani 1976; Delfino et al. 1999), it seemed appropriate to test whether functional myoepithelial cell differences are associated with serous secretory unit polymorphism. Accordingly, we carried out a morphofunctional investigation in order to ascertain the contribution of the muscular sheath to the differential characterisation of these glands. We chose to study *Phyllomedusa hypochondrialis azurea* for this purpose, on account of the large degree of gland polymorphism in this species (see Table 1), testing the contractile activity of the myoepithelia using norepinephrine stimulation. We decided to continue this pharmacological approach following previous investigations with catecholamines (Dockray and Hopkins 1975; Holmes et al. 1977; Holmes and Balls 1978; Delfino et al. 1982), which, in turn, had been prompted by consistent transmission electron microscope reports on the or-

Table 1 Anuran species characterised by serous cutaneous gland polymorphism

Species (current name)	Former name/s in references	Family	Region	Author	Method/s	Polymorphism degree
Bombina bombina (Linnaeus, 1761)	*Bombinator igneus* (Laurenti, 1768)	Bombinidae	Europe	Bertossi (1937)	Light microscopy, histochemistry	Two gland types
Bombina orientalis (Boulenger, 1890)		Bombinidae	Asia	Delfino et al. (1990)	Transmission electron microscopy	Two gland types
Bombina pachypus (Bonaparte, 1839)	*Bombinator pachypus* Bonaparte, 1839; *Bombina variegata pachypus* (Bonaparte, 1839)	Bombinidae	Europe	Vialli (1934) Delfino (1976)	Light microscopy, histochemistry Transmission electron microscopy	Two gland types
Bufo granulosus major Müller and Hellmich, 1936		Bufonidae	South America	Delfino et al. (1999)	Transmission electron microscopy	Two gland types
Melanophryniscus cupreuscapularis Cèspedez and Alvarez, 1999	*Melanophryniscus stelznerii* (Weyenbergh, 1875–1876)	Bufonidae	South America	Delfino et al. (1998b)	Transmission electron microscopy	Two gland types
Phyllomedusa hypochondrialis azurea Cope, 1862		Hylidae	South America	Delfino et al. (1998c)	Transmission electron microscopy	Three gland types, including a lipid-producing, derived type
Phyllomedusa sauvagei Boulenger, 1882		Hylidae	South America	Delfino et al. (1998c)	Transmission electron microscopy	Three gland types, including a lipid-producing, derived type

thosympathetic control on anuran serous glands (Whitear 1974; Sjöberg and Flock 1976). To evaluate the effectiveness of norepinephrine treatment, we observed both myoepithelial and secretory compartments, paying attention to changes in the arrangement of the contractile and biosynthesis apparatuses, respectively. Subsequently, in order to complement tissue observations, the secretory products obtained from stimulation were collected in saline and processed for ultrastructural analysis.

Materials and methods

Specimen collection and pharmacological treatment

Adult specimens of *Phyllomedusa hypochondrialis azurea* Cope, 1862 were collected near Corrientes Capital (Argentina), and tissue samples underwent preliminary treatments in the Departamento de Biología, Universidad del Nordeste. Skin strips of small surface areas (4–8 mm^2) were removed from the backs of the control (three) and experimental (two) animals. The former were anaesthetised and killed with 0.2% chlorobutanol, and the latter were kept at 4°C before killing by decapitation. This procedure avoided any interference from chlorobutanol treatment during pharmacological tests, while minimising stress and pain in frogs. For experimental purposes, the skin strips were immersed in amphibian Ringer, containing 3×10^{-4} M norepinephrine (Delfino et al. 1982) and followed under the stereomicroscope for 10 min, until secretory release ceased.

Preparation for transmission electron microscopy (TEM)

After secretory discharge, the tissue specimens were prepared for TEM examination by the routine methods described below. In addition, the secretory granules were collected with a Pasteur pipette and suspended in Eppendorf test tubes containing 1 ml amphibian Ringer solution, then subjected to the procedures used for preparing isolated cells and cell organelles for TEM. Granules were pelleted at 10,000 *g* for 5 min by centrifugation, and then processed like the skin strips (Dockray and Hopkins 1975). Experimental and control skin specimens, as well as pellets, were treated for prefixation (3 h, 4°C) with a glutaraldehyde–paraformaldehyde mixture, according to Karnovsky (1965), and washed in 0.1 M, pH 7.0, cacodylate buffer (the same used in prefixation and subsequent steps). This material was sent in 2–4 ml of the solution (with the addition of a drop of prefixative mixture) to the Dipartimento di Biologia Animale e Genetica (Università di Firenze, Italy). The skin specimens and secretory product pellets were again rinsed (the tissue strips also reduced in size) and postfixed (1 h, 30 min) in 1% OsO_4, dissolved in the buffer. After rinsing, the samples were dehydrated in graded ethanol, soaked in propylene oxide and infiltrated with Epon 812. After polymerisation, the Epon blocks were cut with a NOVA LKB ultramicrotome into 0.5- to 1.5-µm semithin sections and ultrathin sections with gold yellow-silver grey interference colour. Semithin sections were stained with buffered toluidine blue and used for preliminary light microscope (LM) observations. Ultrathin sections were collected on 300-mesh, uncoated copper grids, and electron-dense stained with saturated, uranyl acetate hydroalcoholic solution, followed by 2 mg/ml lead citrate alkaline solution. TEM observations were performed (at 80 kV) with a Siemens 101 electron microscope. To exclude any non-specific patterns of gland stimulation during observations, we avoided analysing sample areas involved in preparative handling.

Results

Control specimens

Light microscopy

Three gland types, characterised by syncytial secretory units, can be identified on the basis of the peculiar fea-

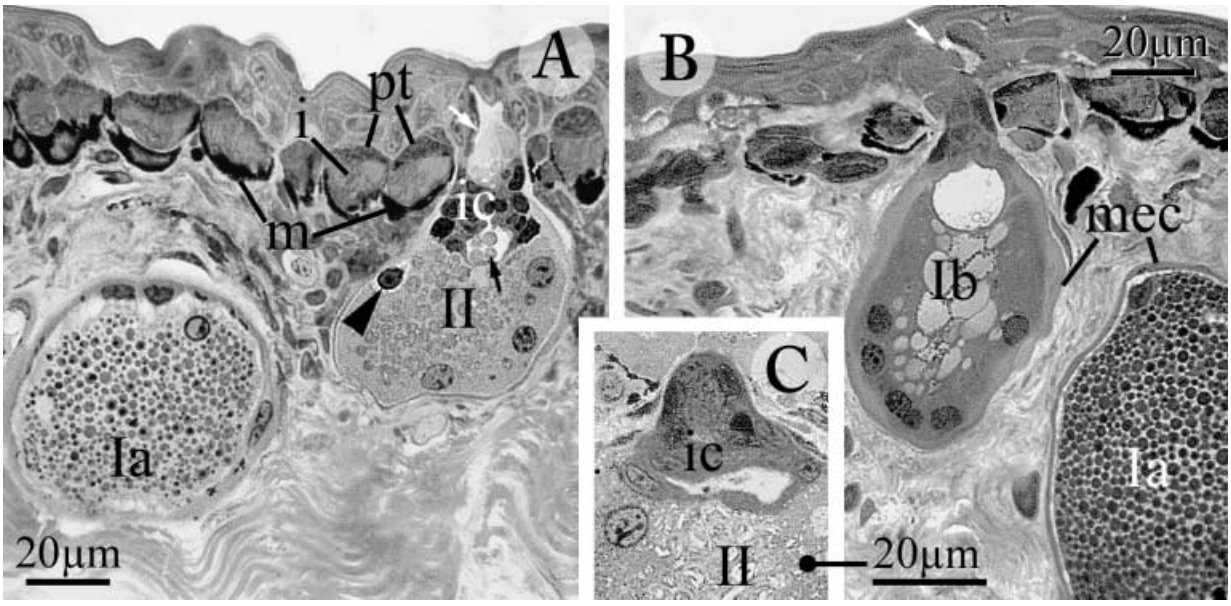


Fig. 1A–C Control specimens: structural aspects of serous glands. **A** Closely contiguous type *Ia* and *II* glands can be recognised by their peculiar contents, consisting of dense particles and heterogeneous bodies (lipid deposits), respectively. Notice multicellular chromatic units beneath epidermis, including melanophores (*m*), iridophores (*i*) and pterinophores (*pt*); also notice type II secretory product in the gland lumen (*black arrow*) beneath the intercalary tract (*ic*); *arrowhead* points to a migrating cell. **B** Differential features are obvious between type *Ia* and *Ib* glands; the latter produce secretory vesicles containing a moderately electron opaque product. Notice peripheral myoepithelial cells (*mec*). In both **A** and **B**, *white arrows* point to the horny wall of the gland duct. **C** Typical aspect of neck region in a type *II* glands: laminar cells pertaining to the intercalary tract (*ic*) form a screen between gland lumen and secretory syncytium

tures of their products. Type Ia glands contain spheroidal dense granules, rather homogeneous in size (about 3–5 nm in diameter) and somewhat heterogeneous in substructure (Fig. 1A, B). Type Ib units have larger vesicles (up to 10 nm in diameter) holding a translucent material and closely adhering to each other (Fig. 1B). Finally, type II glands contain discrete secretory aggregates of varying sizes (Fig. 1A) which, at higher power magnification, display a complex substructure (Fig. 1C). The muscle sheath (myoepithelium), intercalary tract (neck) and horny-lined duct (Fig. 1A–C) complete the gland structure, following the common architecture of amphibian cutaneous glands. The interstice between myoepithelium and secretory unit is usually slender, but is sometimes enlarged to accommodate migrating cells (Fig. 1A). As a peculiar feature in type II glands, laminar cells descend from the neck towards the secretory syncytium and form a cytoplasmic screen around an exiguous lumen (Fig. 1C). However, this cell layer does not hinder gland product release, since discrete secretory particles may be detected inside the lumen (Fig. 1A).

Transmission electron microscopy

Ultrastructural investigation confirms the common architecture of type I (a and b) and type II glands. The syncytial secretory units are functionally engaged in intracytoplasmic product storage, as confirmed by an extremely reduced organelle machinery segregated towards the gland periphery (Fig. 2A). As stated in the previous paragraph, the features of the secretory product are unique to each gland type. Type Ia granules are extremely electron dense, but provided with a somewhat spongy-like substructure with a lighter portion inside (Fig. 2A). Type Ib vesicles contain a faint to finely grained product, with occasional dense particles adhering to their limiting membranes (Fig. 2B). Crowding of the secretory product in the central cytoplasm causes the vesicles to become polyhedral (Fig. 2B); those contiguous to the exiguous gland lumen tend to adhere to the syncytium plasmalemma with their limiting membranes and release their fine material into this cavity through relatively wide openings (Fig. 2B), which do not affect cytoplasm integrity. The features of type II gland product are unusual among anurans. The product consists of subspherical aggregates of translucent to moderately opaque rods (Fig. 2C, D), which have been histochemically characterised as lipid accumulations (Blaylock et al. 1976). The myoepithelial cells ensheathing the different secretory units share common subcellular features, including dense bodies scattered among the contractile filaments (Fig. 2A, E, *inset* in B). However, the contractile sheaths of type II glands appear to be rather discontinuous (Fig. 2C), thus increasing gland–stroma exchange. Detailed analysis of the cell screen separating the type II syncytium from the exiguous lumen is highly informative about how the secretion is released: it occurs through local openings, resulting from the detachment of contiguous cells, and also involves the syncytium cytoplasm (Fig. 2E).

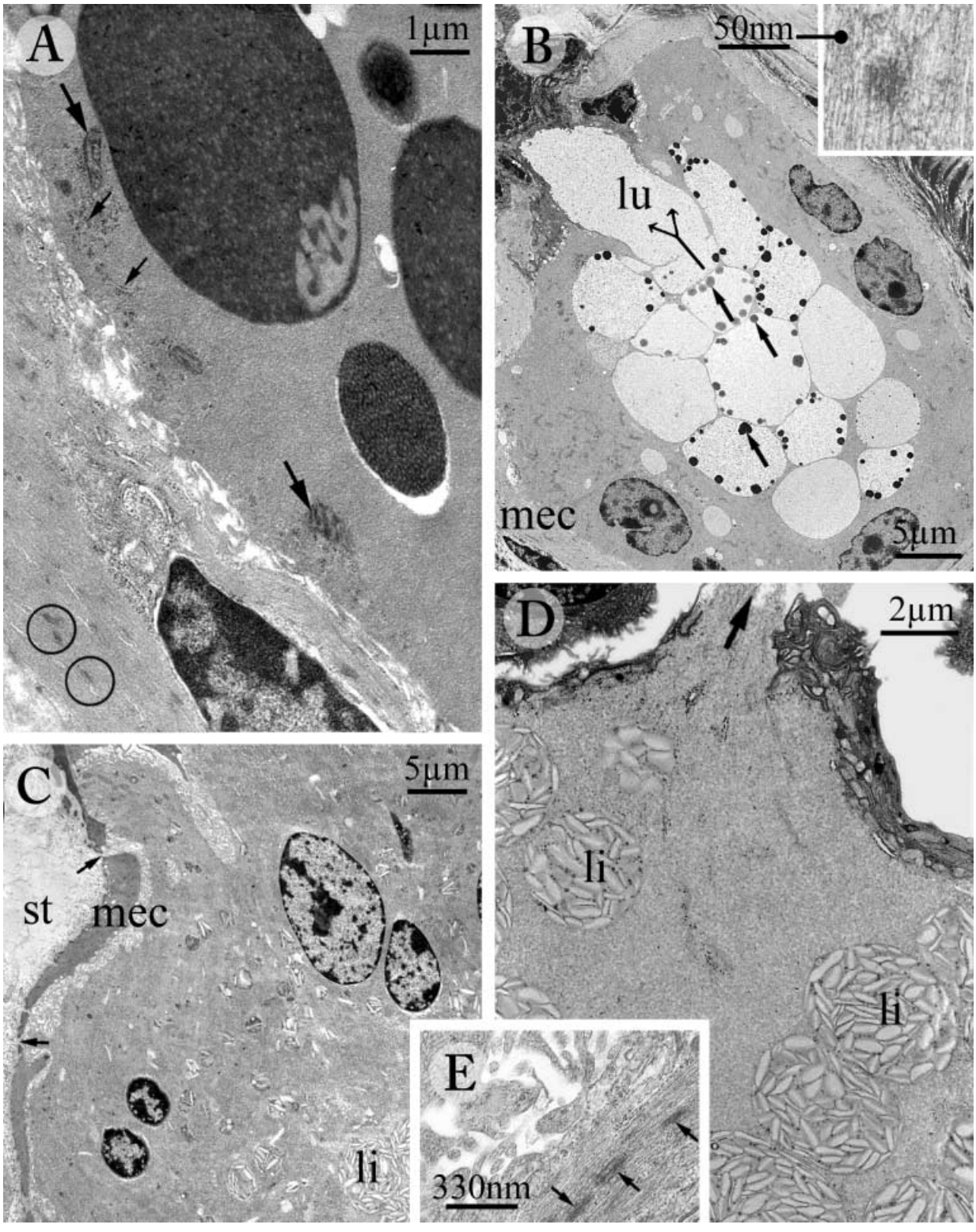


Fig. 2A–E Control specimens: ultrastructural aspects of serous glands. **A** Type Ia gland: peripheral portion of the secretory syncytium and myoepithelium. Serous granules are electron dense, but contain a single lighter zone. Scanty mitochondria (*large arrows*) and short rough endoplasmic reticulum cisterns (*small arrows*) occur in the peripheral, syncytial cytoplasm, whereas dense bodies (*encircled*) can be detected between contractile filaments. **B** Low power micrograph showing usual features of type Ib gland. The secretory syncytium contains large vesicles with peripheral dense particles (*arrows*) in a finely grained background. Notice release patterns (*forked arrow*) into the exiguous gland lumen (*lu*), which contains finely grained product and therefore resembles an elongated secretory vesicle. *Inset* shows dense body in the contractile apparatus of myoepithelial cell. *mec* Myoepithelial cell. **C** Type II glands display the usual arrangement of serous glands: syncytial nuclei are arranged in a single row in the peripheral cytoplasm, which contains scanty secretory product; lipid deposits (*li*) occupy the central zone of the syncytium; *arrows* point to gaps between myoepithelial cells (*mec*). *st* Stroma. **D** Hypothetical path of secretory release, through openings between flat cells encircling the lumen; lipid deposits (*li*) follow the cytoplasm towards this cavity (*arrow*). **E** Dense bodies (*arrows*) in type II gland myoepithelial cells

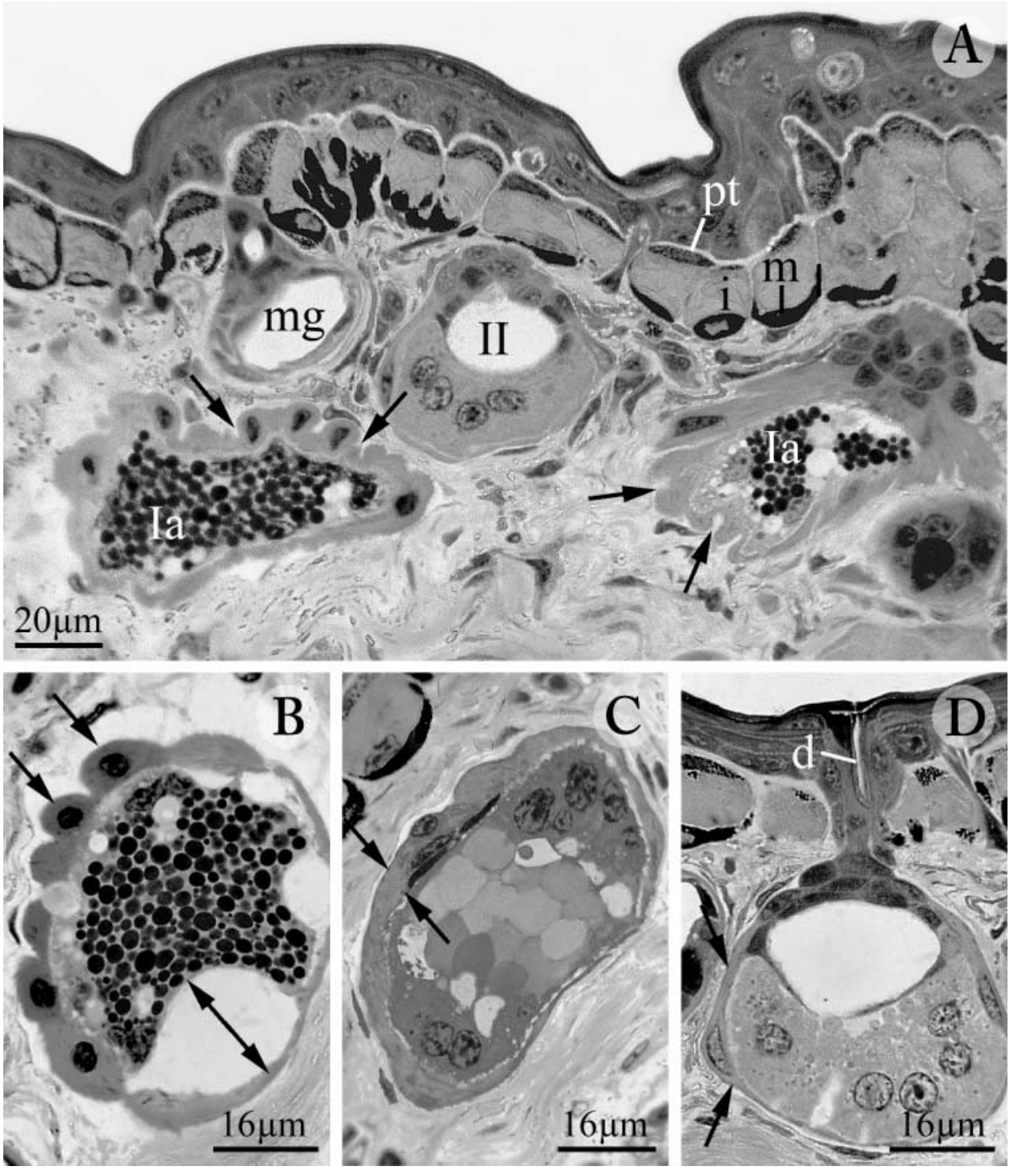
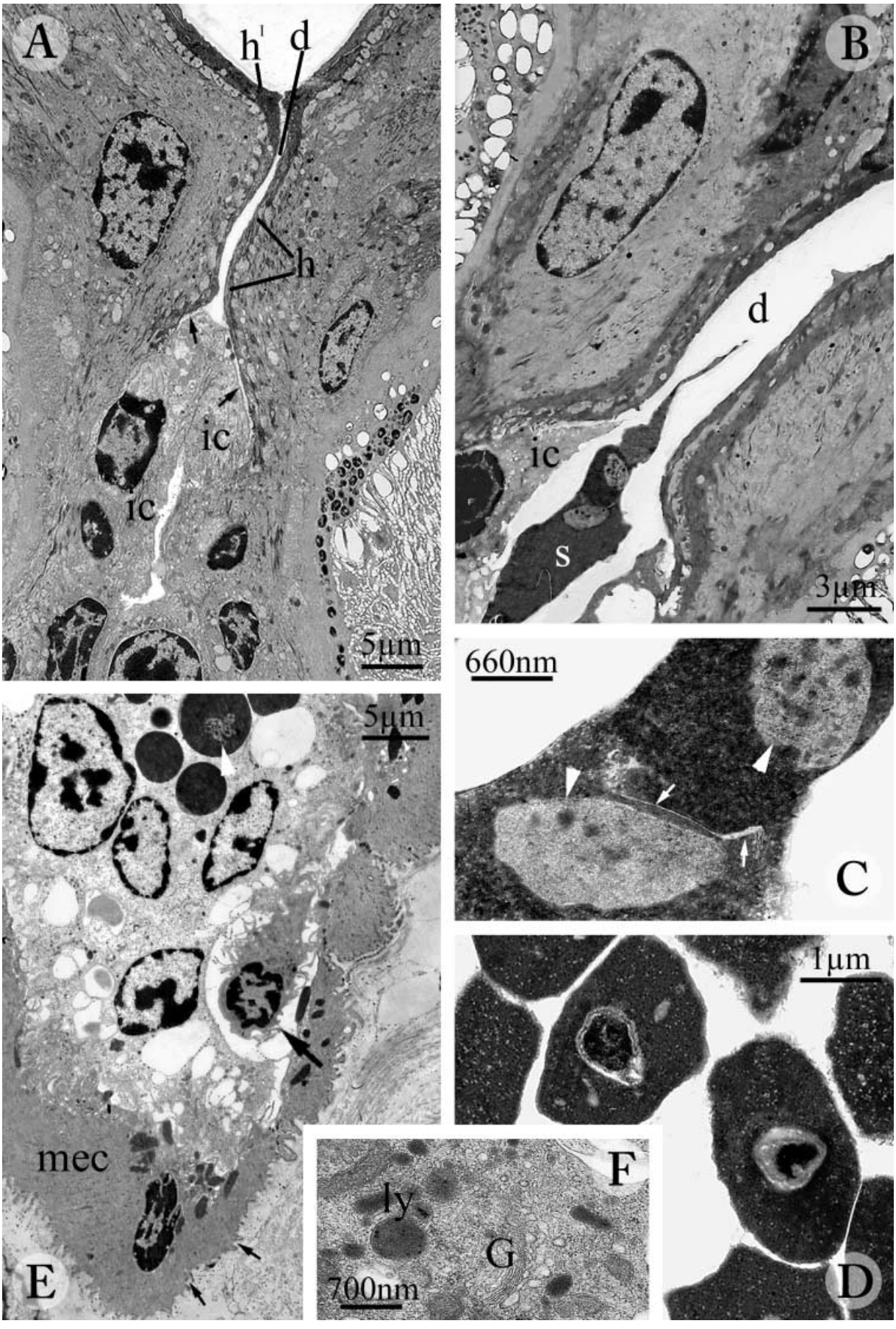


Fig. 3A–D Treated specimens: structural aspects of serous glands after exposure to norepinephrine. **A** Comparison between type *Ia* and *II* glands reveals obvious contractile responses only in myoepithelial cells pertaining to the former: notice woven-like arrangement of contractile cells (*arrows*) and compressed secretory units. Also notice mucous gland (*mg*) and cells of the chromatic units (*m*, *i* and *pt*). **B** Beside dome-like myoepithelial cells (*parallel arrows*), type Ia glands exhibit altered relationships between secretory and contractile compartments: notice unusually wide interstices (*double-headed arrow*). **C** Type Ib glands only exhibit somewhat thickened myoepithelia (*facing arrows*). **D** This longitudinal section shows that type II glands are unaffected by pharmacological stimulation; notice flat myoepithelial cell (*arrows*) and slender duct lumen (*d*) through epidermis

Treated specimens

Light microscopy

Figure 3A–D illustrates the effects induced by norepinephrine stimulation on experimental skin strips. In type Ia glands, contraction patterns consistently characterise the myoepithelial layers, which exhibit woven-like profiles in section, resulting from bulges (usually holding nuclei) alternating with thinner portions (Fig. 3A, B). Myoepithelial cell contractile activity causes a striking change in the arrangement of the secretory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units, so that they appear rather compressed (Fig. 3A) or considerably detached from the myoepithelium (Fig. 3B). Type Ib myoepithelium is only moderately thickened, whereas the main organisation of the secretory syncytium is retained (Fig. 3C). Patterns observed in type II glands are even more similar to those described in control specimen, including flat myoepithelial cells, normal arrangement of secretory syncytium and an empty lumen (Fig. 3A, D).

Transmission electron microscopy

Norepinephrine-induced contraction of myoepithelial cells affects all the component parts of type Ia glands. Cells of the intercalary tract were pushed towards the skin surface, occupying the transitional space between neck and duct (Fig. 4A). This intermediate zone represents the lower end of the horny duct wall, continuous with the external epidermis layer. Remnants of the secretory product, which had been discharged into the saline of the experimental medium, can still be recognised in this space as an amorphous, electron-opaque material but also with less dense portions (Fig. 4B). Higher power magnification reveals that this material is actually discontinuous in structure, due to occurrence of interposed boundaries (Fig. 4C). Therefore, the material trapped between the neck and duct corresponds to discrete structures, compressed together during poison discharge. This means that they are secretory granules, as confirmed by comparison with the secretory product collected in saline (Fig. 4D). The pelleted material consists of granules, very similar in substructure to the secretory bodies contained in the neck duct lumen, including the occurrence of single, less dense parts. Similar features (Fig. 4C, D) have already been described in the control specimens (Fig. 2A), and can also be seen in the granules remaining in the secretory units after discharge (Fig. 4E). These secretory units undergo a dramatic change following myoepithelial cell contraction. The regular arrangement of syncytial nuclei, typical of control glands, is notably altered so that they become randomly distributed in a rather translucent cytoplasm (Fig. 4E). The myoepithelial cells show a thick contractile apparatus and deep indentations on the stromal side (Fig. 4E), whereas their boundary with the secretory syncytium exhibits local enlargement of the interstice. These dilated spaces may hold individual cells, characterised by slender cytoplasmic processes (Fig. 4E). As confirmed by their cytoplasm content, including Golgi stacks and lysosomes (Fig. 4F), these cells are migrating macrophages.

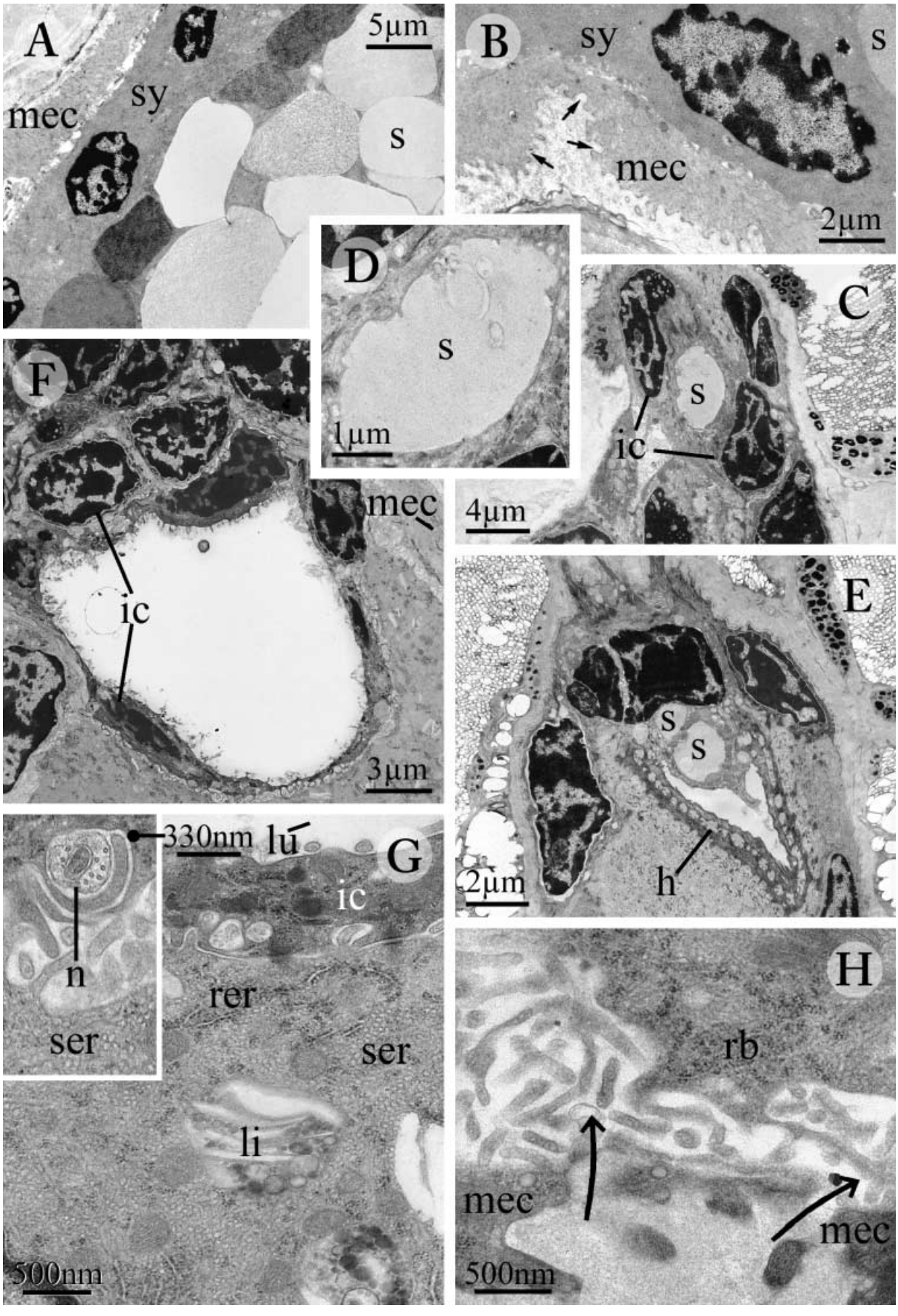
As already detected under LM, type Ib glands in treated specimens maintain the usual features of non-reactive units, namely large amounts of secretory product and nuclei arranged in a single, peripheral row (Fig. 5A). Only indentations on the stromal myoepithelial cell surface seem to indicate a slight response to the catecholamines from the contractile sheath (Fig. 5B). Cells in the intercalary tract also retain their common orientation, enclosing a proper cavity (Fig. 5C). This intercalary lumen holds a faint material (Fig. 5D), resembling the product contained in the vesicles of the secretory unit (Fig. 5A), and conveys it towards the horny lined duct lumen (Fig. 5E).

Norepinephrine treatment did not act on type II glands, which as a rule show a continuous layer of flat cells encircling the small lumen, devoid of any secretory product (Fig. 5F). No pattern of secretory release is obvious at the boundary between the flat intercalary cells and syncytial cytoplasm (Fig. 5G); this zone is characterised by a network of slender cytoplasmic processes from opposite facing cells and thin axons (Fig. 5G, *inset*). The secretory unit cytoplasm facing the flat cell screen holds lipid aggregates (Fig. 5G) and biosynthesis organelles, namely rough endoplasmic reticulum cisterns (Fig. 5G) and smooth endoplasmic reticulum tubules (Fig. 5G and *inset*). The flat myoepithelial cells form a discontinuous layer, still allowing exchange between stromal environment and peripheral cytoplasm, rich in free ribosomes (Fig. 5H).

◀ **Fig. 4A–F** Treated specimens: type Ia glands and type Ia serous product. **A** Myoepithelium contraction causes marked rearrangement of the structure of the intercalary tract, where the tips of contractile cells converge. Notice intercalary cells (*ic*) pushed (*arrows*) towards the duct lumen (*d*). *h* Horny wall of duct lumen, h^1 horny epidermal layer. **B** Secretory product in the transitional zone between neck and lumen. *ic* Intercalary cell, *d* duct lumen, *s* secretory product. **C** Enlargement of the previous image, revealing boundary interface (*arrows*) between two parts characterised by lighter zones (*arrowheads*). **D** Secretory product collected after discharge; compare with **C**, **E** and Fig. 2A. **E** Peripheral portion of a gland, showing contracted myoepithelial cells (*mec*) and rearranged orientation of nuclei in the peripheral syncytium. *Arrowhead* points to the light part of a granule, *large arrow* to a migrating cell, *small arrows* mark indentations on external myoepithelial cell (*mec*) surface. **F** Detail of the cytoplasm in the above migrating cell, showing Golgi stack (*G*) and lysosomes (*ly*)

Discussion

Norepinephrine stimulation of serous glands in *P. hypochondrialis* disclosed obvious differences in release responses, consistent with the secretory type considered. Type Ia glands gave intense responses, type Ib glands released only scanty amounts of product, slightly more than the controls, whereas lipid-producing (type II) units were totally unaffected by the treatment. These findings, resulting from observations on different gland compartments (secretory and contractile), agree with the results of the analysis of the secretory products collected in saline, which consisted of type Ia granules. We should, however, stress that type Ib product consists of faint material, hardly detectable in pellets. The above data fit the results obtained from *Bombina pachypus* (see Delfino 1980; Delfino et al. 1982): in the yellow-bellied toad, pharmacological stimulation was more effective on glands producing dense granules than glands producing vesicular-like material.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Comparing experimental findings with patterns observed in the serous glands of control specimens, it appears that secretory discharge from type Ia units occurs as a phasic process whereas type Ib glands apparently display tonic activity of release, slightly influenced by norepinephrine stimulation. Furthermore, patterns detected in type Ia glands after secretory release are consistent with a "bulk discharge mechanism", namely a peculiar holocrine process resulting from intense myoepithelial cell compression, and involving emission of large amounts of secretory product along with portions of cytoplasm (Delfino 1980; Delfino et al. 1996, 2001; Melis et al. 2000). The occurrence of migrating cells in type Ia glands after secretory discharge stresses the degenerative aspects of such a mechanism. Macrophages are ordinary cell components found in the periglandular stromal environment, extending their patrolling range to the gland interstices; nevertheless, these migrating cells are remarkably mobilised after secretory discharge to remove cytoplasmic debris and prepare gland restoration (Faraggiana 1938, 1939; Delfino 1980). On the other hand, secretory release from type Ib glands, which is also detectable in control specimens, appears to be merocrine in nature, since it does not involve any cytoplasmic loss.

The different release mechanisms observed in the two gland types can be analysed according to the hypothesis proposed by Daly et al. (1987), which considers that anuran skin poisons have two roles, namely regulative and antipredatory. Secretory release from Ib glands seems to fit a regulative role since it is a continuous process and involves small amounts of product. Active molecules may diffuse into epidermal interstices from the duct lumen, passing through its wall, which is discontinuous in structure (Delfino 1991). Therefore these molecules can perform their activity in the cutaneous and subdermal environments, as suggested by Zasloff (1987) in *Xenopus laevis* (Daudin, 1802) for poison fractions with broad-spectrum antimicrobial activity. On the other hand, bulk discharge from type Ia glands seems to fit a defensive strategy against large predators, since it allows considerable amounts of noxious substances to be released.

Despite the different effects of stimulation, myoepithelia in both type Ia and Ib glands of *P. hypochondrialis* react to norepinephrine, mimicking a reflex response which pertains to the orthosympathetic division of the autonomic nerve system. Results of pharmacological stimulation also demonstrate that lipid release from type II glands is not connected with epinephrine stimulation, although it may occur in control specimens, possibly as a response to handling. This paradoxical report suggests that lipid release is not a mere reflex evoked by orthosympathetic stimulation, but it is inserted in a stereotyped "wiping" behaviour, which serves to spread lipids on the body surface, counteracting dehydration (Blaylock et al. 1976). Wiping behaviour is a graduated activity and implies that frogs can evaluate the degree of disruption of the surface skin layers during handling (Blaylock et al. 1976). It appears, therefore, that the adrenergic mechanism we reproduced in vitro is not adequate to elicit the secretory response included in this complex strategy of skin homeostasis.

As suggested by pharmacological investigation, the adrenergic responses of anuran poison glands may be finely regulated by endogenous factors such as agonists and ions (Holmes et al. 1977; Holmes and Balls 1978; Delfino et al. 1982). A pharmacological approach was not the main aim of this investigation, nonetheless our results stress that different serous glands in the same species possess neural-contractile apparatuses with specific properties, although myoepithelia share common ultrastructural features. Improved pharmacological approaches would therefore furnish researchers with suitable methods for obtaining pure serous secretions from skins with several gland types. Routine techniques of poison collection, which usually involve mechanical compression of living specimens (Meyer and Linde 1971) are not specific since the material spread over the body surface includes both poison products and mucus. As an alternative method, ether stimulation triggers systemic reactions, and requires preliminary manoeuvres of the animals to deplete the urinary bladder and avoid contamination by urine (Kiss and Michl 1962). On the contrary, norepinephrine stimulation of skin strips allows the collection of products whose origin from serous glands may be ascertained, provided the method is complemented by microscope observation (Barberio et al. 1987).

◀ **Fig. 5** Treated specimens: type Ib (**A–E**) and II glands (**F–H**). **A**, **B** Peripheral zones of a gland, showing normal arrangement of secretory (*sy*) and contractile compartments (*mec*). *s* Secretory product, *arrows* in **B** point to indentations of myoepithelial cell. **C** Oblique section of neck region, showing intercalary cells (*ic*) and intercalary lumen, containing faint secretory product (*s*). **D** Detail of the secretory product (*s*) in **C**. **E** Transitional zone between intercalary tract and duct; the former contains secretory product (*s*), the latter is characterised by a horny wall (*h*). **F** Periluminal zone, just beneath the intercalary tract: notice flat intercalary cells (*ic*) forming a continuous screen separating the empty gland lumen from the syncytium. *mec* Myoepithelial cell. **G** Notice biosynthesis organelles (smooth and rough endoplasmic reticulum complements, *ser* and *rer*, respectively) in the syncytial cytoplasm contacting flat intercalary cells (*ic*) around the gland lumen (*lu*). *Inset* shows axonal ending (*n*) in the interstice between intercalary cells and secretory syncytium, containing smooth endoplasmic reticulum (*ser*). *li* Lipid deposit. **H** Boundary between myoepithelium (*mec*) and secretory syncytium: the contractile sheath is discontinuous (*bowed arrows*), allowing stromal-glandular flow. *rb* Free ribosomes

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