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Chemical skin defence in the Eastern fire-bellied toad *Bombina orientalis*: an ultrastructural approach to the mechanism of poison gland rehabilitation after discharge

Sara Quagliata[1], Cecilia Malentacchi[2], Filippo Giachi[1], Giovanni Delfino[1]*

[1]*Dipartimento di Biologia evoluzionistica dell'Università, via Romana 17 I-50125 Firenze, Italy. *Corresponding author.* E-mail: giovanni.delfino@unifi.it
[2]*Dipartimento di Fisiopatologia clinica dell'Università, viale Pieraccini 6 I-50139 Firenze, Italy*

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Abstract. Type I serous glands in the skin of the Eastern yellow-bellied toad *Bombina orientalis* released their product massively after 10^{-3} M nor-adrenalin (NA) stimulation, mimicking orthosympathetic control on poison emission in chemical skin defence. Features of cutaneous glands involved in this bulk discharge were observed under light and electron microscopes. Furthermore, restoration of depleted glands was followed after 1, 2 and 3 weeks, and compared with serous biosynthesis during larval gland development. Bulk discharge was caused by contraction of myoepithelial cells (mec_s) encircling the secretory units. Mec compression dramatically affected the secretory unit, but parts of this syncytial cytoplasm were saved from degeneration and cooperated in gland renewal with stem cells from the gland neck. These adenoblasts underwent proliferation and secretory cytodifferentiation, until merging with the syncytium. Cytoplasm that had resumed secretory activity showed the features typical of larval gland development: the endoplasmic reticulum (rer) cisterns were aligned in close parallel arrangement and Golgi stacks released minute type I granules. Secretory rehabilitation led to increasing amounts of granule content. In the meantime, rough cisterns decreased in number and assumed the less ordered pattern described in control specimens. Data collected in the present study revealed that chemical skin defence in anurans is a multi-factorial mechanism involving specific activities: mechanical from mec_s, biosynthetic from secretory syncytium and proliferative from intercalated stem cells.

Keywords. Serous glands, anuran skin, secretory release, ultrastructure.

INTRODUCTION

Pioneering light microscope (LM) studies on the serous (poison or granulous) cutaneous glands in yellow bellied toads of the genus *Bombina* (formerly *Bombinator*) provided

evidence on the features of their serous product and the syncytial structure of their secretory units (Faraggiana, 1937, 1939; Bertossi, 1937). The secretory product consists either of small (average diameter 2 μm) or large (10 μm) granules contained in the secretory syncytium of type I or, respectively, II glands. These glands perform a functional cycles consistent with a defence role (Faraggiana, 1939; Delfino, 1978, 1980): smooth muscle cells (myoepithelial cells or mec_s) ensheathing the secretory unit contract, causing granules to be discharged towards the skin surface through the intraepidermal duct. Since secretory discharge involves both granules and syncytial cytoplasm, it is referred to as holocriny (Faraggiana, 1938b). Serous gland holocriny requires a turnover mechanism based on proliferation and secretory differentiation of the stem cell pool in the "neck" (Faraggiana, 1939), a regenerative region intercalated between the secretory unit and duct. Investigations into several frog and toad species revealed that this renewal mechanism is a shared functional trait within the anuran order (Faraggiana, 1938a, 1939).

LM and transmission electron microscope (TEM) investigations subsequently confirmed these results, while providing ultrastructural details of the cytodifferentiation processes of intercalated tract cells (Delfino, 1978, 1980). Furthermore, TEM analysis revealed that the manufacture of serous product does not actually involve any degenerative processes –the damage observed merely derives from not-adequate fixation methods and/or compression by mec_s. Therefore, the concept of "bulk discharge" has been introduced to describe the main functional trait of these glands: secretory release from anuran poison glands is a massive process affecting secretory granules as well as the syncytium storing them (Delfino et al., 1996). Bulk discharge has been investigated in several hylid species, with observations extending to the serous product collected after release; this material contained nuclei and complements of the rough endoplasmic reticulum (rer), along with integral secretory granules (Melis et al., 2000; Delfino et al., 2002, 2006; Nosi et al., 2002), thus mec compression, whilst indeed damaging the syncytial cytoplasm, left the secretory product intact.

Whereas the peculiar features of secretory discharge from anuran cutaneous serous glands is widely confirmed in current literature, further investigation is required to clarify their restoration mechanism. Indeed, along with stem cells, secretory rehabilitation may also involve residual portions of the pre-existing syncytium (Neuwirth et al., 1979; Delfino, 1980). To evaluate how the secretory syncytium and stem cells contribute to serous gland restoration, we carried out an experimental study on *Bombina orientalis*, following the early, intermediate and ultimate steps of the renewing process. Our research, which also involves a comparison between adult glands during restoration and larval glands during development, aims to complete previous studies on chemical skin defence in this species (Delfino et al., 1990; Sanna et al., 1993).

MATERIALS AND METHODS

Specimens and pharmacological treatment

We procured adult specimens (8) and tadpoles (12 specimens, ontogenetic range 34-43, according to Gosner, 1960) of the eastern red-bellied toad *Bombina orientalis* (Boulenger, 1890) from authorized dealers and acclimatised them for three weeks to laboratory condi-

tions in the Dipartimento di Biologia evoluzionistica (Università di Firenze, Italy). During this period adults were fed on meal worms (larval *Tenebrio molitor*) and kept in 20 °C water under a natural light cycle. Larval specimens were fed on boiled spinach. Before pharmacological treatment and/or sacrifice, adult and larval specimens were kept at 4 °C to reduce response to external stimulation. This procedure, which avoided use of anaesthetics in pharmacological tests, minimised stress and pain in the animals during manipulation. To observe control glands, and glands immediately after stimulation, we removed twelve skin strips of small surface area (25-36 mm^2) from the backs of two toads (killed with 0.2% chlorobutanol) and pooled them together. Half of the pooled samples were immersed in amphibian Ringer containing 10^{-3} M nor-adrenalin (NA), and their responses checked under a stereomicroscope (SLM, Delfino, 1980), until secretory discharge ceased (usually, within 15-20 min at room temperature). The control skin samples (six) from the same pool were immersed in the Ringer solution, observed with the SLM for 20 min, and processed in the same manner as the experimental and larval strips for microscopic observation (see below). Skin strips collected from larval specimens were 16 mm^2 wide. To follow gland regeneration, we injected 10^{-3} M NA (1 ml) into the dorsal lymph sacs of 6 animals and photographed their responses to the pharmacological treatment with a Coolpix 4500 digital camera. After treatment, we kept the toads under the same acclimatization conditions described above for one, two and three weeks. Two specimens were sacrificed at the end of each interval, and 25-36 mm^2 skin strips (4 each toad) removed from dorsal areas 5 mm away from the injection site.

Preparation for microscopic analysis

Skin strips from experimental (0, 1, 2, 3 weeks) and control specimens as well as tadpoles, were treated for prefixation (3 h, 4 °C) with a glutaraldehyde-paraformaldehyde mixture in 0.1 M, pH 7.0 cacodylate (Karnovsky, 1965), and then washed in this buffer solution. The skin samples were then reduced into 4 mm^2 surface area strips, and post-fixed (1.5 h) in OsO_4 (1% in cacodylate). After rinsing in the same buffer, the samples were dehydrated in graded ethanol, soaked in propylene oxide and infiltrated in Epon 812. After 72 h polymerisation, Epon blocks were cut with a NOVA LKB ultramicrotome into 1-1.5 μm semithin sections and yellow, interference colour ultrathin sections. Semithin sections were stained with buffered (1% borax) toluidine blue, and LM informative pictures were collected with the Coolpix camera adapted to a Leitz DIALUX LM. Ultrathin sections, collected on 300 mesh copper grids, were electron-dense stained with a saturated, hydroalcoholic solution of uranyl acetate, followed by a lead citrate alkaline solution (2 mg/ml). TEM observations were performed at 80 kV with a Philips M300 transmission electron microscope (Laboratorio di Botanica Generale, Università di Firenze).

RESULTS

Macroscopic (SLM) observations

After NA injection, the toads assumed a resting posture or only moved slowly, but in no case displayed the typical *Unkenreflex*. Within five minutes, a foamy product was dis-

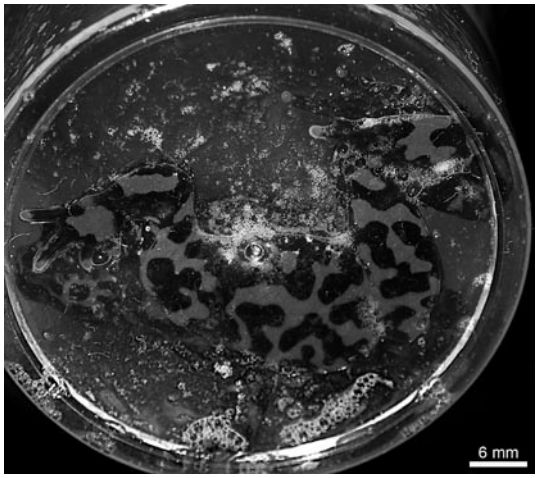

Fig. 1. (SLM) Secretory product released after NA injection.

charged onto the skin surface and was later detected on the floor of the pot in which toads had placed. The serous product had obviously condensed on this substrate and formed yellowish bloblets (Fig. 1). Secretory discharge ceased within twenty minutes.

Microscopic analysis

Since consistent findings revealed that nor-adrenalin stimulation only affected serous type I glands, type II and mucous glands are described only for general comparison.

The serous cutaneous glands in control animals exhibited the usual LM features, including the spheroid (Fig. 2A) or ellipsoidal shape of the syncytial secretory unit (Fig. 2B) with a peripheral row of nuclei and thin sheath of myoepithelial cells. Mucous glands consisted of discrete, secretory cells in various phases of activity, as suggested by the varying height and density of their cytoplasms (Fig. 2A). Type I and II serous secretory units contained remarkable amounts of granules that occupied the syncytial compartment almost entirely, whereas the lumen was extremely reduced or totally missing (Fig. 2A, B). Ultrastructural analysis of type I serous units revealed scanty and slender rer cisterns and small Golgi stacks at the boundary between myoepithelium and secretory syncytium, along with rod-shaped mitochondria (Fig. 2C). Myoepithelial cells were spindle-like in shape and contained a myofilament apparatus with homogeneous density (Fig. 2D). Thin nerve endings appeared in the interstice between contractile and secretory compartments: they held dense-cored synaptic vesicles and established neuro-contractile contacts with myoepithelial cells (Fig. 2D). This association between axonal endings and myoepithelial cells represents the neuro-contractile apparatus responsible for regulating secretory discharge from serous glands in anuran skin defence responses.

Type I serous glands in skin specimens fixed immediately after pharmacological treatment showed the typical histological features of myoepithelium contraction and secretory

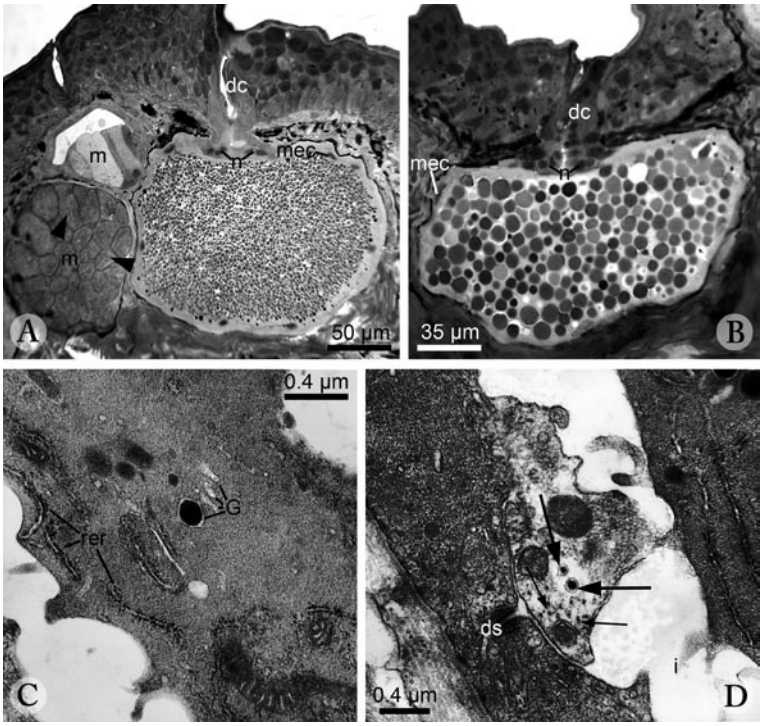

Fig. 2. Control specimen (LM: A, B and TEM: C, D): A) Contiguous type I and mucous glands, characterised by small secretory granules in the syncityum and variable staining intensity of mucocytes, respecitvely. The polygonal profiles (arrowheads) are mucous cells in tangential section. B) Type II gland with large granules of varying densities. C) Biosynthesis apparatus and mitochondria in Type I secretory syncytium. D) Same as above: nerve ending in the interstice between myoepithelium and secretory syncytium. Notice synaptic vesicles and neurotubules (large and thin arrows, respectively). dc = duct, ds = desmosome, G = Golgi stack, i = interstice, m = mucous gland, mec = myoepithelial cell, n = gland neck, rer = rough endoplasmic reticulum.

release. These morphological traits included thickened myoepithelial sheaths (Fig. 3A, B), while secretory discharge patterns ranged from fully depleted glands (Fig. 3A) to others still containing secretory products (Fig. 3B). Totally discharged glands resembled empty ice-pouches in shape, with remarkably thickened mec_s around an extremely reduced cavity (Fig. 3A). This hollow is not a lumen but a residue of the compartment that in control glands once held the secretory unit cytoplasm. Indeed, it contained sparse remnants of the syncytium, including degenerating nuclei. Glands with residual granules still somewhat resembled the control glands, although the syncytium nuclei were crowded centrally in the secretory unit (Fig. 3B). Compared with type I glands, type II and mucous glands were not affected by pharmacological treatments and closely resembled their control counterparts (Fig. 3C, D). TEM observation of type I glands disclosed in detail ultrastructural features brought about by pharmacological stimulation. In smooth muscle cells the peripheral cytoplasm bulged locally towards the secretory compartment (Fig. 4A) and

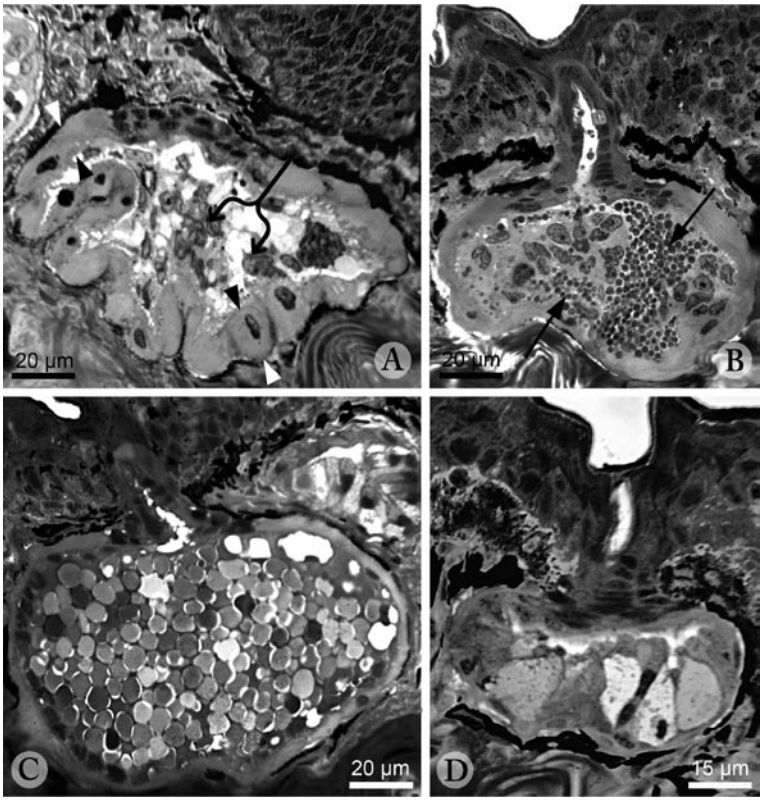

Fig. 3. Treated specimens immediately after NA injection (LM). A) and B) Type I glands which underwent full and partial depletion, respectively. Notice thickened mec_s (opposite arrowheads) and cytoplasm waste (forked arrow) in A, residual granules (arrows) and central nuclei in B. C) and D) Type II gland and mucous gland, respectively, resembling control specimens (compare with 2A and B). Notice in D variable secretory features in mucocytes.

there were comb-like profiles on the, opposite, dermal side (Fig. 4B). The bulges had a transparent sarcoplasm (Fig. 4A) since myofilaments were concentrated in the inner cell regions, forming thick bands (Fig. 4A, B). In glands that were not fully depleted, remants of syncytium contained closed membrane profiles, which were never observed in control specimens. The cytoplasm at the boundary with the contractile cells held small vesicular structures (diameter range: 0.2-1 µm) with a light inner compartment (Fig. 4A), whereas in the central regions of the residual syncytium larger vesicles (2-6 µm) were prominent, with a denser content that resembled the cytoplasm background (Fig. 4C).

In the first week after treatment, type I glands were engaged in early phases of renewal activity, that involved both stem cells in the gland neck (intercalated tract) and residual portions of the secretory syncytium. As revealed by LM analysis, undifferentiated cells in the intercalated tract underwent mitotic processes and migrated toward the secretory compartment (Fig. 5A). Under the TEM, their light background cytoplasm, clearly distinguishable from the denser cytoplasma of the contiguous syncytium (Fig. 5B), was rich in free ribos-

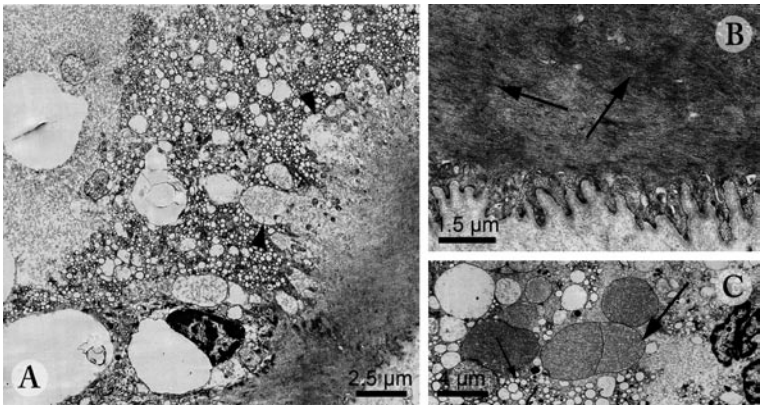

Fig. 4. Same as above (TEM). Ultrastructural patterns of type I secretory syncytia and myoepithelia. A) Mec cytoplasm form electron transparent bulges towards the secretory syncytium (arrowheads), where two distinctive regions are obvious: an outer one with minute vesicles, and an inner one with a moderately dense background. B) Comb-like mec profile on the dermal side, and thick bands of myofilaments (arrows). C) Detail of the boundary zones between the two syncytium regions, notice wide and minute vesicular profiles (large and small arrows, respectively).

omes and contained peculiar mitochondria that were ring-shaped in section and possessed tubular christae (Fig. 5C). Before undergoing secretory cytodifferentiation and merging into the syncytium, the adenoblasts derived from stem cell proliferation, could still be recognised on account of their high nucleo-plasmatic ratio and structureless cytoplasm (Fig. 5D). The still thick myoepithelium encircled the residual secretory syncytium, that had resumed an obvious functional polarization, as suggested by the peripheral row of nuclei in semithin sections (Fig. 6A). In glands undergoing renewal, the peripheral cytoplasm of the secretory unit was denser and contained minute opaque granules, whereas the inner cytoplasm was lighter with typical type I granules (Fig. 6A, B). Although relative amounts of inner granules and peripheral cytoplasm might vary, depending on the level of the section and/or phase of gland restoration, the centripetal, functional polarization of the secretory unit was a consistent feature. The biosynthesis machinery included flat cisterns of rough endoplasmic reticulum (rer), arranged in a closely parallel pattern and interspersed among secretory granules (Fig. 6C). Minute stacks of Golgi saccules (dictyosomes) were also observed, with flat saccules of the *trans* face involved in condensing tiny secretory granules (Fig. 6D). Mitochondria were abundant in this phase, and were often ring shaped in section (Fig. 6E), closely resembling those described in proliferating neck cells.

After two weeks, an increase of type I product throughout the secretory syncytium was detected under the LM, and suggested that serous gland rehabilitation had reached intermediate steps. The degree of granule accumulation varied considerably: they were relatively dispersed when newly synthesized (Fig. 7A) or occupied the entire syncytium when new and pre-existing products coexisted (Fig. 7B). Single centrioles in the syncytial cytoplasm were sometimes observed under the TEM (Fig. 7C), as possible remnants of the mitotic processes described in the first week. The close parallel orientation of rough cisterns was virtually lost (Fig. 7D), whereas granulogenesis still proceeded in Golgi areas

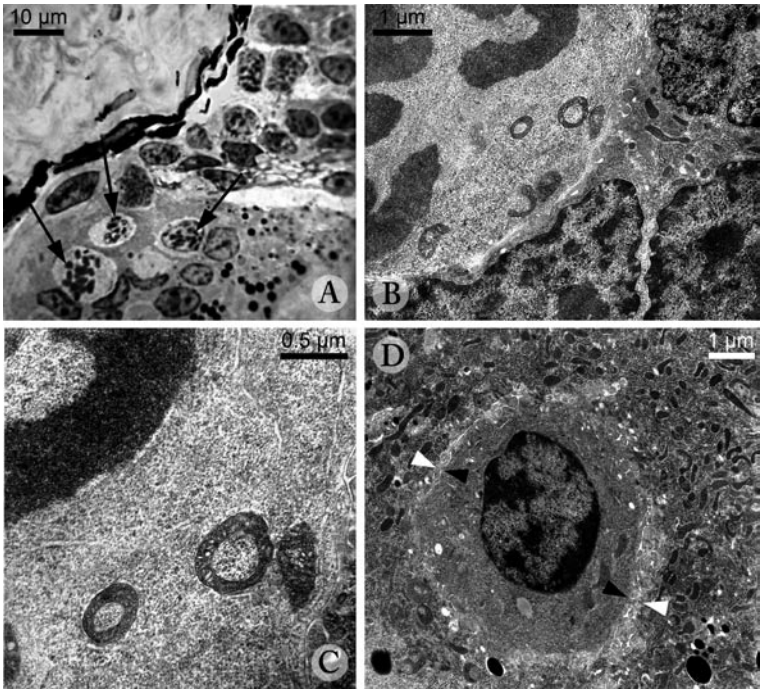

Fig. 5. Early steps of gland renewal a week after NA injection (LM: A and TEM: B, C, D). A) Mitotic processes (arrows) involving cells from neck region. B) Detail of the previous gland, compare the secretory syncytium (right) with cell involved in mitosis (left). C) Peculiar mitochondria in this cell, compare with 6E. D) Cell originated by mitotic process (opposite arrowheads) within the syncytium.

(Fig. 7E). When glands in early and intermediate steps of renewal activity were compared with larval glands in pre-metamorphic stages), rer and Golgi apparatuses exhibited similar features (Fig. 7F, G) despite the different conditions that drove the processes, experimental *vs.* ontogenetic. As a slight difference, rer profiles in developing glands included small rough membrane vesicles and moniliform complements (Fig. 7F).

As observed under LM, secretory product accumulation proceeded in the final stage of gland renewal, at the third week after discharge, which involved both fully depleted glands (Fig. 8A) and others that underwent partial depletion (Fig. 8B). As a rule, newly formed granules were larger than in the early steps of functional rehabilitation, possibly due to reciprocal, serial confluences (Fig. 8A, B). In both cases, the myoepithelia were relaxed, although some glands still maintained an irregular shape (Fig. 8A). Ultrastructural changes in the biosynthesis apparatus led these glands to closely resemble control glands in ultrastructural features of their secretory units. There were fewer rer cisterns which were located at the boundary between secretory unit and myoepithelium. Most rough profiles were found in the perinuclear cytoplasm, approximately parallel to the secretory-contractile interface (Fig. 8C, D). The Golgi apparatus maintained its supra-nuclear location and was involved in the usual condensation activity (Fig. 8C), producing granules that underwent serial merging processes (Fig. 8E).

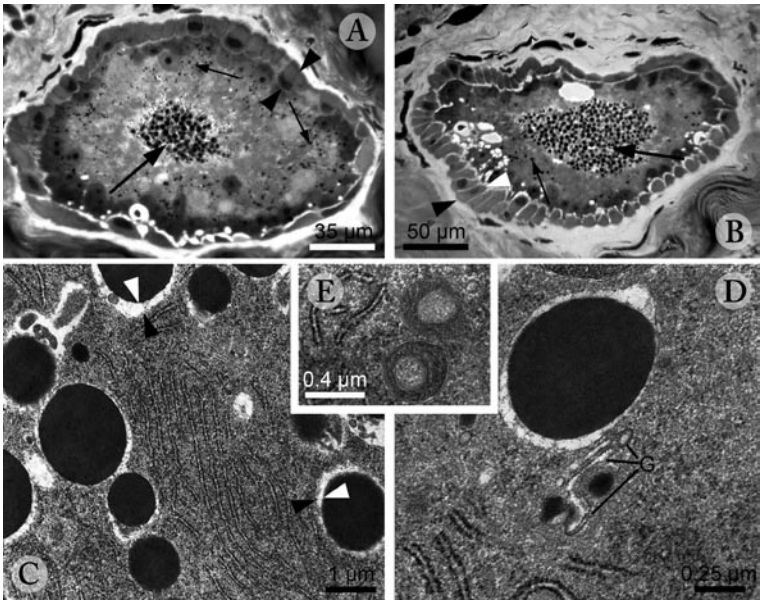

Fig. 6. Same as above (LM: A, B, TEM: C, D, E). A) and B) Renewing glands with different amounts of pre-existing secretory material (large arrows), along with newly synthesized granules (small arrows). Notice thickened myoepithelial cells (opposite arrowheads). C) Rer cisterns arranged in parallel; opposite arrowheads point to perigranular compartment enlargements. D) Granulogenesis patterns in Golgian area. E) Peculiar mitochondria in restored secretory syncytium: compare with 5C. G = Golgi stack.

DISCUSSION

Chemical skin defence in extant anurans is a complex survival device, based on biosynthesis, storage and release of noxious (toxic/repellent) molecules, and dependent on an efficient turnover activity. Serous glands provide all these functions on account of their complex morpho-functional architecture, including the syncytial secretory unit (poison production and accumulation), neuro-muscular apparatus (secretory discharge) and intercalated tract (regenerative activity). Our results confirm previous studies on the release mechanism (Holmes et al., 1977; Holmes and Balls, 1978; Delfino et al., 1982): poison discharge from anuran skin glands is an adrenergic mechanism, i.e., mediated by the orthosympathetic nervous system. This is also suggested by the occurrence of dense-cored synaptic vesicles in effectory nerve endings (Dockray and Hopkins, 1975; Delfino, 1979; Delfino et al., 1990; Delfino, 1991; Delfino et al., 1992, 1995a, b, 1998a, b, 1999a, b; Melis et al., 2000; Arifulova et al., 2007), complemented by hystochemical data (Sjoberg and Flock, 1976), and therefore it pertains to the repertory of fight or flight responses. Studies on the yellow bellied toads of the genus *Bombina* suggest that this mechanism is highly discriminatory since it only involves type I glands, whereas electric stimulation is effective on both serous gland types (Faraggiana, 1939; Delfino, 1978, 1980). Pharmacological reproduction of the orthosympathetic mechanism in living *Bombina* specimens allowed selective stimulation of type

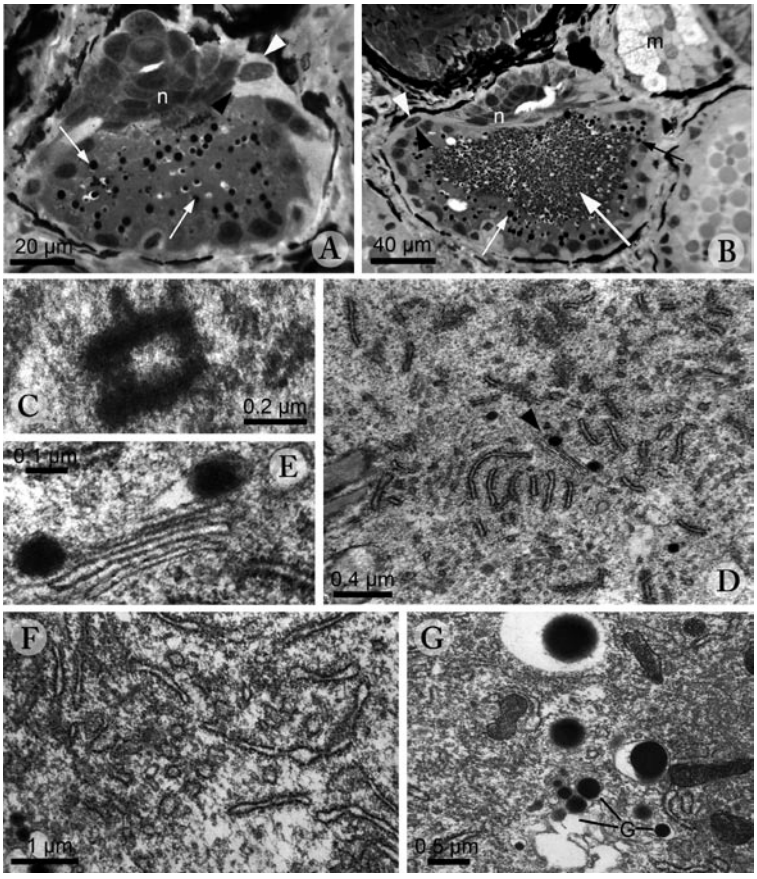

Fig. 7. Intermediate steps of gland renewal two weeks after NA injection (LM: A , B and TEM: C, D, E) and development of larval glands (stages 36-38, TEM: F, G). A), B) These type I glands have been sectioned along the larger longitudinal diameter as demonstrated by occurrence of gland neck in both. The gland on the left was completely depleted: it holds newly formed secretory products (arrows) throughout the syncytial cytoplasm. In the gland on the right, old and new granules co-exist (large and small arrows respectively). Notice thick myoepithelia in type I glands (opposite arrowheads), and glands of the mucous and type II, serous type (right part of B) resembling control specimens. C) Centriole in the secretory syncytium, possibly residual from mitotic processes. D) These rer cisterns display a less ordered arrangement compared with 6C; arrowhead points to an area enlarged in E. E) Detail of D, showing a Golgi stack. F, G) During ontogenesis, rer and Golgi apparatus patterns exhibit features resembling those of gland renewal. G = Golgi stack, m = mucous gland, n = neck.

I serous glands (Delfino, 1980; Delfino et al., 1982), and collection of adequate amounts of poison to assay its biological properties (Barberio et al., 1987; Mastromei et al., 1991; Balboni et al., 1992; Sanna et al., 1993). The NA treatments could be repeated after some weeks on account of the regenerative processes we have described in this study.

Secretory unit rehabilitation requires coordinated activities in the intercalated tract and residual syncytium. Stem cells proliferate and merge together in the common cyto-

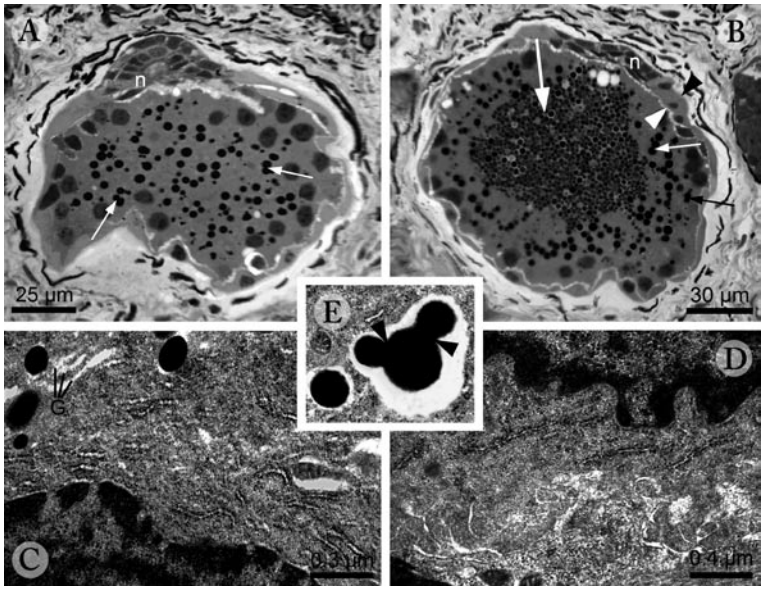

Fig. 8. Treated specimen three weeks after NA injection (LM: A, B and TEM: C, D, E). A) and B) Notice increased amounts of secretory granules In these glands that correspond to glands in 7A and B. Serous products include newly formed granules involved in reciprocal merging processes (small arrows) and pre-existing granules (large arrow). Myoepithelial cells (opposite arrowheads) appear to be relaxed. C) Rer cisterns are scanty and reduced to the periphery of the syncytium; notice a Golgi stack. D) Rer profiles beneath the nuclear level. E) Merging between newly formed granules (arrowheads). G = Golgi stack, n = neck.

plasm, as confirmed by the occurrence of similar mitochondria with exclusive features in both discrete cells and the syncytium. Three-dimensionally, these organelles are tube- or cup-shaped, a morphological adaptation which increases the surface area involved in exchange between the cytoplasm and inner compartments. The proliferation-differentiation-merging sequence we observed repeats the same phases described during ontogenesis, when adenoblasts from the presumptive gland neck contribute to the gradual increase in size of the secretory unit (Delfino et al., 1988, 1993, 1994, 2001a; Terreni et al., 2003). Since this sequence has also been described in single neck cells under resting conditions (Delfino et al., 1990, 1992), it appears to be a constitutive process of anuran serous glands.

Early steps in functional rehabilitation involve enhanced biosynthesis processes as suggested by numerous rer cisterns with the typical parallel arrangement already described in type I serous gland buds of *Bombina pachypus* (Delfino, 1977a, b). Three weeks after stimulation, the rer machinery had virtually resumed the traits described in control glands (Delfino et al., 1990), with scanty, peripheral cisterns.

Confirming previous studies (Delfino, 1980; Melis et al., 2000; Delfino et al., 2006), stimulated mec_s resemble smooth muscle fibres undergoing contraction (Wagenwoort and Dingemans, 1985; Kargacin and Fay, 1987). Their compression caused substantial damage to the syncytium, similar to the effects of manual squeezing (Toledo et al., 1992). Despite these degenerative changes, which also involved loss of the plasmalemma encir-

cling the exiguous lumen, the residual secretory syncytium resumed its activity. It should be recalled that secretory granules are provided with limiting membranes, as emphasised by the distinctive halos encircling them (Delfino et al., 2001b). Furthermore, these perigranular compartments are constantly involved in reciprocal merging processes (Delfino, 1991), resulting in a somewhat continuous, membrane bounded compartment extending throughout the syncytial cytoplasm. When mec_s force secretory granules towards the duct lumen, a continuation of the external environment, part of this inner membrane system is maintained, and guarantees isolation of the cytoplasm compartment. The occurrence of peculiar vesicular profiles in residual syncytia suggests that membrane patches may fuse together, resembling an unusual, short-cut secretory process (Neuwirth et al., 1979). On the other hand, membrane fusion may contribute to restoring the plasmalemma, when it occurs at the boundary with the external environment.

The ultrastructural evidence collected in this experimental study on *B. orientalis* serous glands stresses their functional traits, coherent with chemical skin defence against predators. Although mec contraction seems to be an all-or-nothing event, poison discharge from a relatively wide skin area is modulated by several factors, both exogenous and endogenous (Holmes et al., 1977; Holmes and Balls, 1978; Delfino et al., 1982) that may vary depending on the actual defence demands. This means that the extent of gland depletion, as well as the number of glands involved, may be lower under natural than experimental conditions, so that a relatively short time-lapse would be required to rehabilitate the defence mechanism.

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