Acta Biologica Hungarica 55 (1–4), pp. 221–232 (2004)

FUNCTIONAL MORPHOLOGY OF THE SALIVARY GLAND OF THE SNAIL, *HELIX POMATIA:* A HISTOCHEMICAL AND IMMUNOCYTOCHEMICAL STUDY*

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(Received: August 31, 2003; accepted: December 1, 2003)

Functional morphology of *Helix pomatia* salivary gland cells was studied at light microscopic level by using different histochemical methods. Three cell types could be demonstrated in the salivary gland: mucocytes, granular and vacuolated cells. The distribution and the number of the different cell types were different in active and inactive snails. In active feeding animals, dilatated interlobular salivary ducts were observed, which were never present in inactive ones. In active animals an additional cell type, the cystic cell could also be observed. Periodic acid Schiff staining revealed both mucuos and serous elements in the salivary gland. Furthermore, hematoxyline-eosin staining indicated the occurrence of a cell layer with high mitotic activity in the acini. Applying immunohistochemical methods with monoclonal mouse anti-human Ki-67 clone, B56 and polyclonal rabbit anti-human Ki-67 antibodies, we also were able to demonstrate the occurrence of dividing cells in the salivary gland. Analysis of 1–2 µm semi-thin Araldite sections stained with toluidine-blue showed that the saliva can be released, in addition to possible exocytosis, by the lysis of cystic cells. Using an apoptosis kit, we could also establish that this process was due to rather an apoptotic than a necrotic mechanism. In the salivary gland of active snails, where an intensive salivation takes place, significantly more apoptotic cells occurred, if compared to that of inactive animals. It is suggested that programmed cell death may also be involved in the saliva release.

Keywords: Salivary gland – cystic cell – dividing cell – apoptosis – snail

INTRODUCTION

In gastropod molluscs, the salivary glands (SG) consist of numerous acini and the saliva secreted is forwarded from the acini into the interlobular ducts that converge on the paired musculous primary salivary ducts (SD), which finally open into the buccal cavity. In SD, the saliva is driven by the peristaltic contractions of the muscle fibres of the duct towards the buccal mass [12]. The rich innervation and blood supply of the SG, including the SD, suggest a complex mechanism of central regulation and modulation of the process of salivation [2, 8]. The non-synaptic innervation by different peptidergic (FMRFa, MIP) elements of the SD muscle cells and the gland cells was demonstrated by electron microscopic immunocytochemistry [5, 6].

* Presented at the 10th ISIN Symposium on Invertebrate Neurobiology, July 5–9, 2003, Tihany, Hungary.

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0236-5383/$ 20.00

The secretory product is a complex substance consisting of mucous fluid and enzymes. It was suggested that different gland cell types are responsible for the production of the different components of the saliva. Early histological and histochemical studies revealed a remarkably wide range of cell types in the SG of pulmonates. In the SG of *Helix pomatia,* for example, Pacaut and Vigier [15] distinguished five cell types, while Krijgsman [9] described as many as seventeen different cell types. Recent morphological findings have led to similar conclusions: Walker [19] described ten, Beltz and Gelperin [2], Moreno et al. [14] and Serrano et al. [17] four, Moya et al. [14] six cell types in six helicid species. It was proposed that the different cell types represent only the morphological modifications of a single gland cell type [15]. Such a series of cellular modification may reflect the production of different constituents of the saliva, and was called a '*secretion cycle'* [3]. Luchtel et al. [12] have concluded, however, that such a functional relationship between the different cell types is not convincing and the existence of them is highly questionable. Consequently, the earlier hypothesis of secretion cycle was not accepted by these authors.

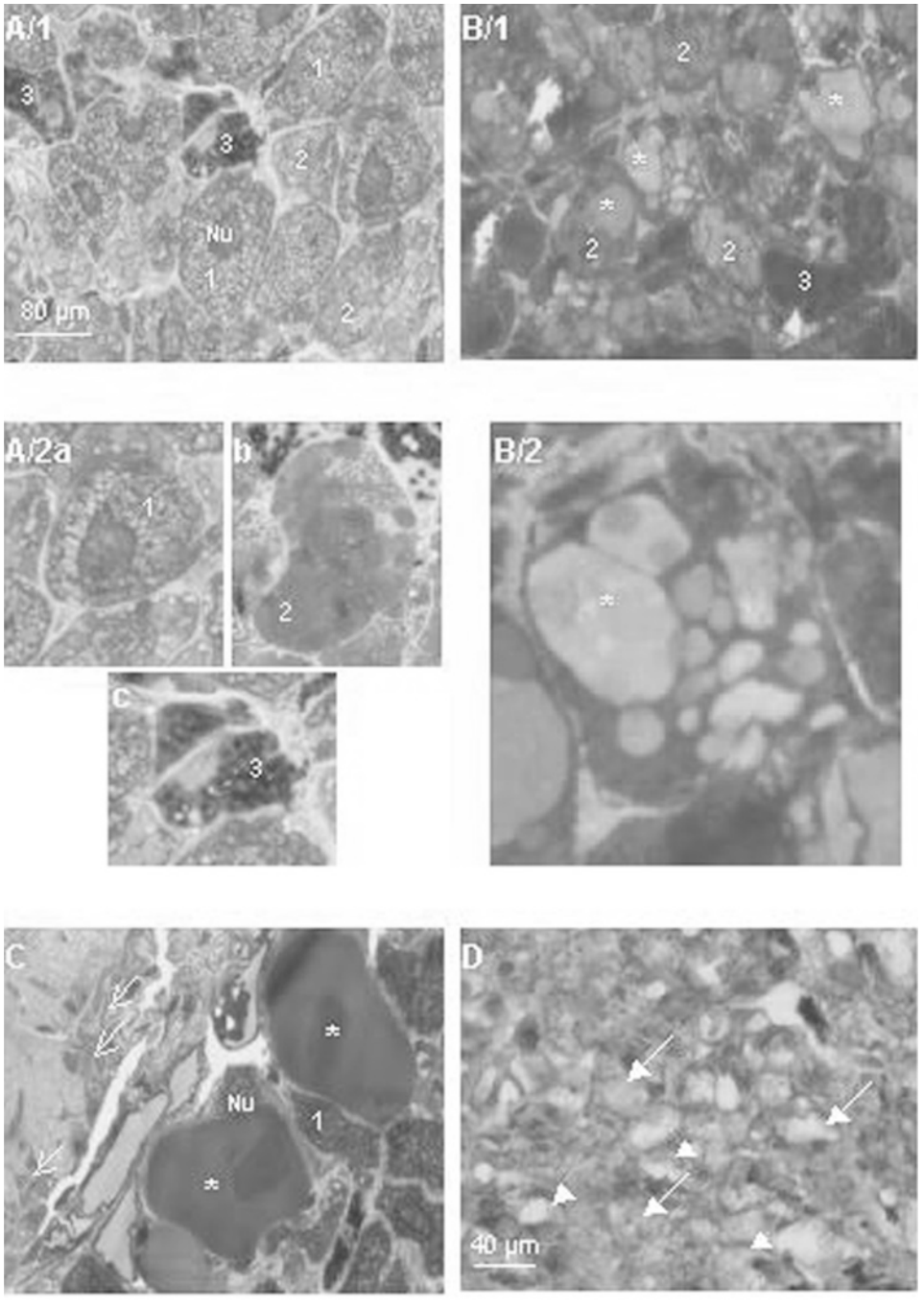
Based on the above findings, the morphological basis of the mechanism of saliva production was examined in the present study in the snail, *Helix pomatia,* in order (i) to identify the different cell types occurring in the salivary gland, and (ii) to get insight into the possible release mechanism of the saliva at cellular level.

MATERIALS AND METHODS

Animals

Adult specimens of the pulmonate snail, *Helix pomatia,* were used for the experiments. Animals were kept under dry conditions (inactive state) in an aquarium at a temperature of 20–24 °C. Animals were activated by placing them under wet conditions and supplying them with food (active state). Active state of the SG was also elicited by the electrical stimulation of the salivary nerve of inactive animals. For the stimulation 10 mV and 250 ms long impulse at 1 Hz for 5 min were applied by bipolar electrodes placed onto the salivary nerve.

Fig. 1. Cell types in the SG of *Helix pomatia.* A/1: Mucocytes (1), vacuolated cells (2) and granular cells (3), identified in a semithin section stained with toluidine-blue. Nu = nucleus. Scale bar: 80 μm. A/2: Higher magnification micrographs of SG cell types. A/2a: Mucocytes filled with vesicles containing fine granular material. A dense area at their periphery often characterizes these vesicles. A/2b: Vacuolated cells containing large, slightly stained droplets. A/2c: Granules in granular cells display a dark staining. B/1, B/2: In vacuolated cells (2) the droplets are of large size and show a tendency to fuse forming large saliva droplets (star). C: In cystic cells (star), the whole intracellular space is filled with a large, single saliva droplet. Arrows indicate muscle cells. Mucocytes marked with 1. D: Mucous (arrow) and serous cells (arrowheads) are distributed evenly the salivary gland as revealed by PAS-technique. Scale bar: 40 μm


A/1
1
3
3
2
Nu
1
2
80 µm
B/1
2
*
*
*
2
2
3
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1
b
2
B/2
*
c
3
C
*
Nu
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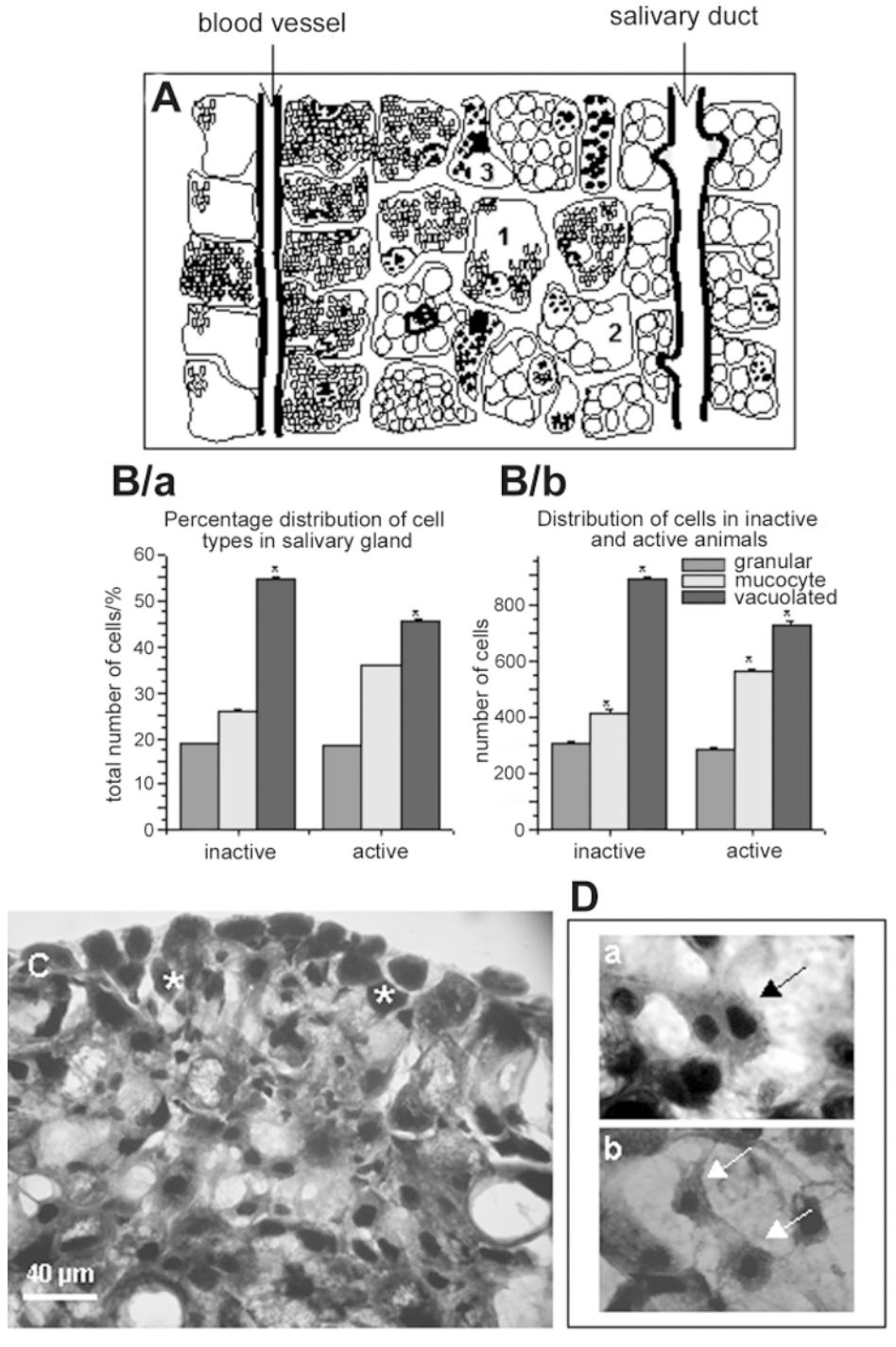
D
40 µm


blood vessel
salivary duct
A
1
2
3
B/a
Percentage distribution of cell types in salivary gland
total number of cells/%
60
50
40
30
20
10
0
inactive
active
B/b
Distribution of cells in inactive and active animals
granular
mucocyte
vacuolated
number of cells
800
600
400
200
0
inactive
active
C
*
*
40 µm
D
a
b

Histochemistry

For general histology, SG were dissected and fixed overnight at 4 °C in 0.1 M phosphate buffer (PB, pH = 7.4) containing 1% paraformaldehyde and 2.5% glutaraldehyde. After fixation, samples were washed in PB and cut into small (approx. 1 mm × ×3 mm) pieces, postfixed with 0.5% OsO_4 diluted in Na-cacodylate buffer (pH = 7.2) for 1 hour at 4 °C, dehydrated in graded ethanol and propylene oxide, and then embedded in Araldite. Semithin (1–2 μm) sections cut on an LKB Nova ultramicrotome were stained with 1% toluidine-blue.

In order to distinguish the mucous and serous cells in the SG, and to demonstrate cell proliferation, histochemical methods were applied. Pieces of SG were fixed in 4% paraformaldehyde (PFA) diluted in PB for 4 hour at 4 °C, then cut into 15 μm thick serial sections with a cryostat, dried onto glass slides, dehydrated in graded ethanol and xylene. Series of sections were partly treated for periodic acid Schiff (PAS) staining for 10 min, partly used for hematoxyline-eosin (HE) staining. Following both toluidine-blue and histochemical stainings the sections were analyzed by a Zeiss Axioplan compound microscope.

Immunocytochemistry

SGs were dissected and pinned out in a Sylgard-coated dish containing 4% PFA diluted in 0.1% M phosphate buffer saline (PBS, pH = 7.4). Following fixation overnight at 4 °C, the samples were cut into smaller pieces and washed several times in PBS. Ten μm serial cryostat sections were placed on chrome-alum gelatine coated slides and processed for two- (fluorescence) or three-step (peroxidase-anti-peroxidase, PAP) immunohistochemistry. Immunofluorescence labeling was performed as follows: (i) 5% normal goat serum (NGS) in PBS containing 0.1% bovine serum albumin (BSA), 0.1% Triton X-100 and 0.01% Na-azide (PBS-TX-BSA-azide);

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Fig. 2. Distribution of SG cells in inactive and active snails. A: Schematic drawing of the distribution of cell types in the salivary gland. The number of mucocytes (1) is higher near the blood vessel, whereas the number of vacuolated cells (2) is higher in the vicinity of the salivary duct. B/a: Percentage distribution of cell types (normalized to the total number of cells observed in approx. 1.1 mm^2 area) in the salivary gland. B/b: Relationship between the different cell types (granular cells, mucocytes and vacuolated cells) in inactive and active animals. The distribution of cell types is similar to that shown in B/a. In both cases, the statistical difference is significant between vacuolated cells and granular cells. The number of vacuolated cells in active animals is lower than in inactive animals. Contrary, the number of granular cells in active animals is higher than in inactive animals. In both graphs the statistical difference (marked with star) was at 0.05. C: HE stained cryostat sections showing the inhomogeneous distribution of gland cells in the SG. Intensive staining is seen close to the outer surface of the acini (stars). D: Higher magnification details from HE stained sections revealing the presence of dividing cells in the SG. a) Two nuclei of a dividing cell (arrow). b) Two new cells separating from each other (arrows)

(ii) monoclonal mouse anti-human Ki-67 clone, B56 antiserum (Histopathology Ltd., Hungary) diluted to 1 : 100, or polyclonal rabbit anti-human Ki-67 antiserum (Dako) diluted to 1 : 50, overnight at 4 °C; (iii) fluoresceine isothiocyanate (FITC) tagged rabbit anti-mouse IgG or swine anti-rabbit IgG (Dako) diluted to 1 : 30, 1.5 hour at room temperature. FITC labeled sections were mounted in a 1 : 1 mixture of glycerine-PBS. PAP immunohistochemistry was performed as follows: (i) 5% NGS in PBS containing PBS-TX-BSA-azide; (ii) monoclonal mouse anti-human Ki-67 clone, B56 antiserum diluted to 1 : 100; (iii) peroxidase conjugated rabbit anti-mouse (Dako) diluted to 1 : 50, 2 hour at room temperature; (iv) PAP diluted to 1 : 200 overnight at 4 °C; (v) PAP labeled sections were developed in 0.05M TRIS-HCl (pH = 7.6) containing 0.05% 3,3'-diaminobenzidine (Sigma) and 0.01% H_2O_2. All antisera were diluted in PBS-TX-BSA-azide, and each incubation were followed by several washing in PBS. Sections were dehydrated in graded ethanol, cleared in xylene, mounted in Canada-balsam. Sections were analyzed in a Zeiss Axioplan compound microscope equipped with the appropriate filters for FITC-labeling or in normal light for PAP-labeling.

Apoptosis detection

An Apoptosis Detection Kit, Annexin V-CY3 (Sigma) was used to demonstrate the presence of apoptotic cells. Experiments were made on both tissue pieces and dissociated cells. Dissociated cells were centrifuged (with 2000 G, 3 min.), washed twice with PBS, and suspended approximately at a concentration of 0.5×10^5 cells/ml. Thereafter the cell suspension and tissue pieces were washed three times with aliquots of 50 µl of 1× Binding Buffer. 50 µl Double Label Staining Solution was added to 50 µl cell suspension and to tissue pieces of the SG (To prepare 2 ml of Double Label Staining Solution, 20 µl Annexin V-CY3 and 20 µl 6-CFDA were added to 200 µl 10× Binding Buffer and 1.76 ml de-ionised water). Preparations were incubated for 10 min at room temperature and washed twice with aliquots of 50 µl of 1× Binding Buffer. Labelled tissues and cells were placed on gelatine coated slides, and observed in a Zeiss Axioplan microscope equipped with appropriate filters.

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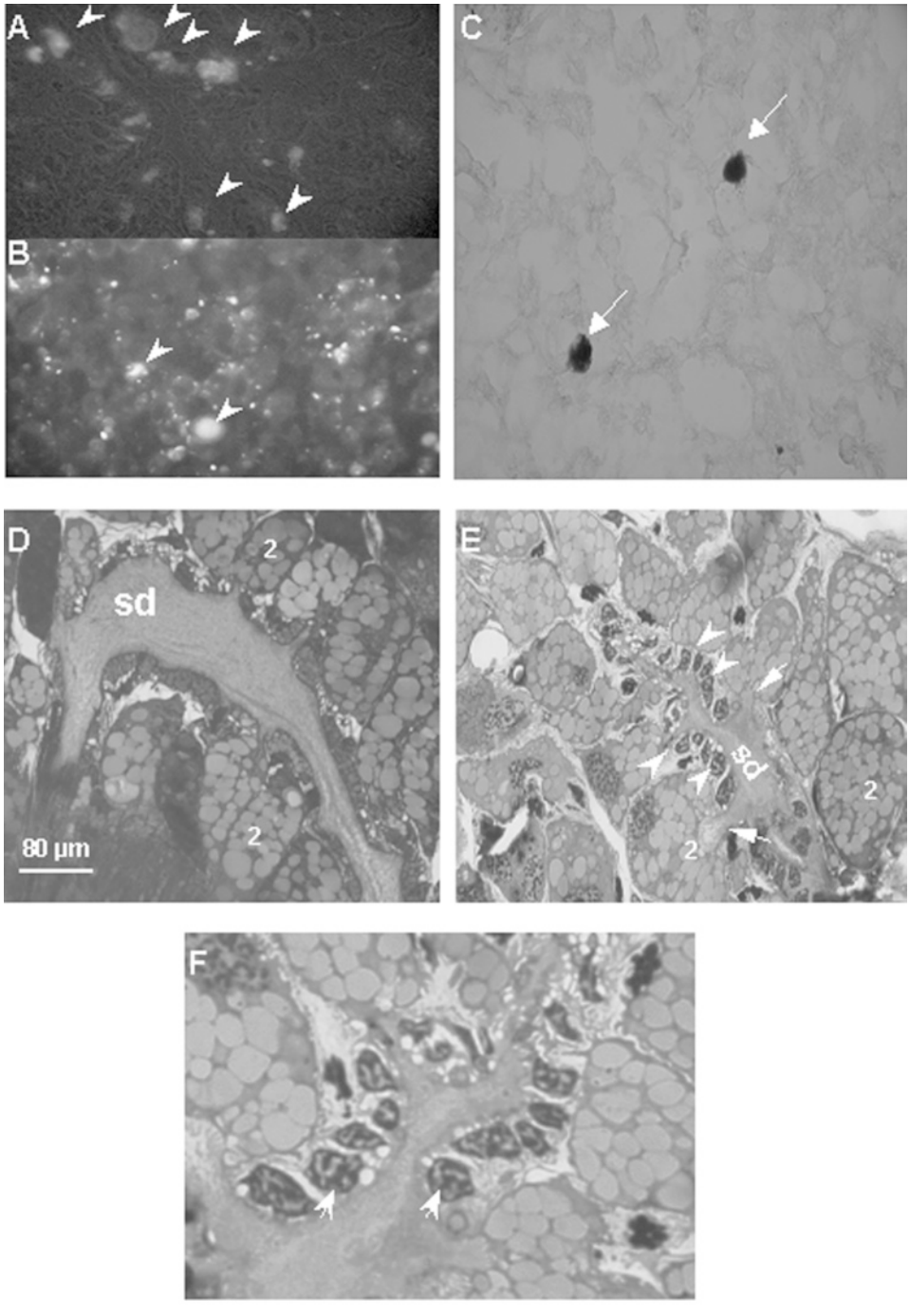
Fig. 3. A, B: Early proliferation of gland cells in the acini demonstrated by monoclonal mouse anti-human Ki-67 clone, B56 antibody (A) and polyclonal rabbit anti-human Ki-67 antibody (B). Proliferating cells are indicated by arrowheads. FITC-immunfluorescence technique. C: Dividing gland cells (arrows) demonstrated by PAP-technique. D, E: Interlobular salivary ducts in the SG of active animals. Vacuolated cells (2) are concentrated near the duct. Fine processes of the salivary duct are deeply invaginated (arrows) into the vacuolated cell. Cell debris (nuclei) is indicated by arrowheads. F: Higher magnification micrograph showing cell debris (nuclei, arrows; in E, arrowheads) along a duct in the SD


A
B
C
D
sd
2
2
80 µm
E
sd
2
2
F

RESULTS

General cell morphology

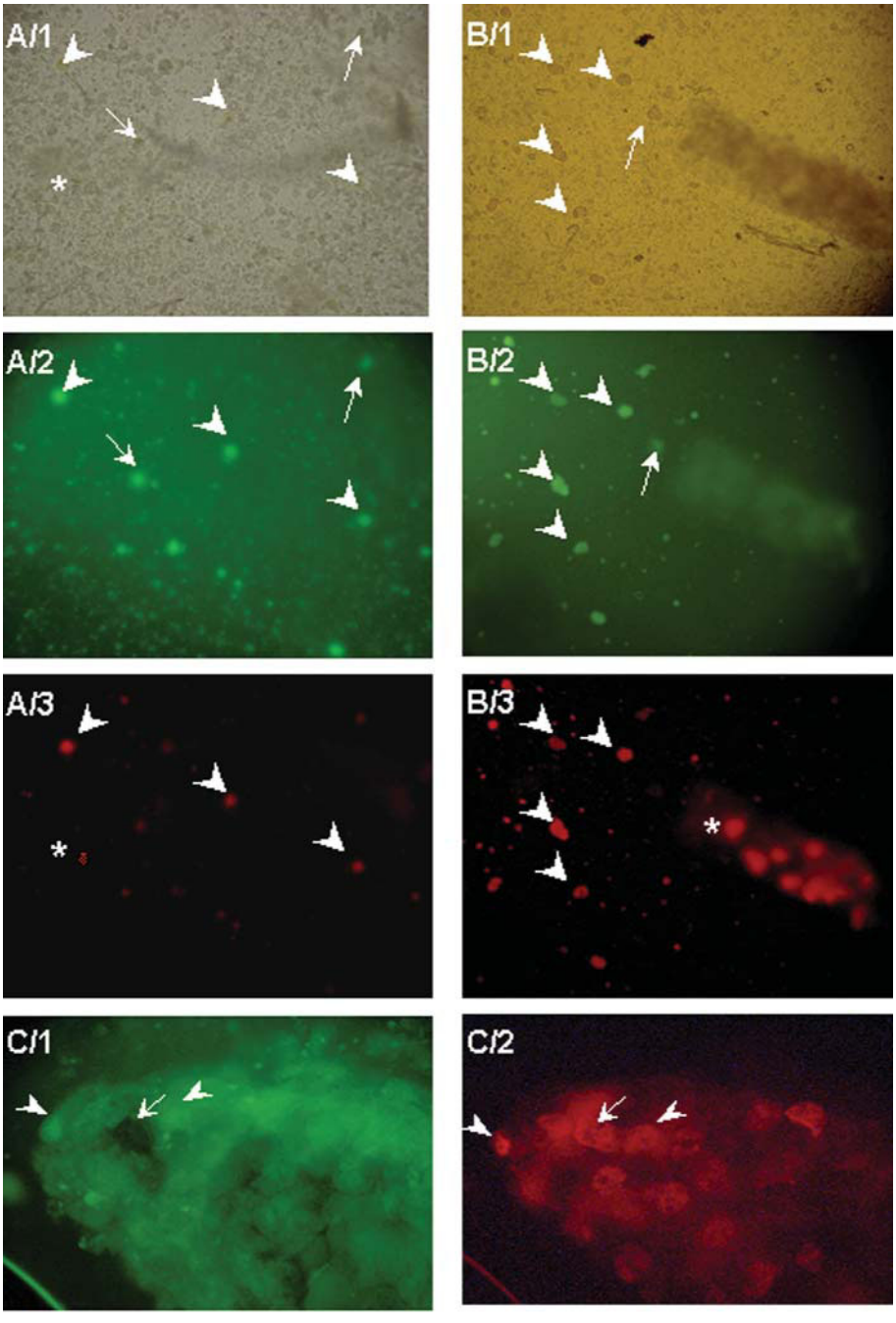
In the SG of *Helix pomatia,* three gland cell types could be identified: (i) mucocytes (Figs 1A1, A/2a), (ii) vacuolated cells (Figs 1A1, A/2b) and (iii) granular cells (Figs 1A1, A/2c). These cell types correspond to the cell types I, II and III described in *Limax* [2] and in *Aplysia* [11]. In toluidine-blue stained semithin sections, vacuolated cells and mucocytes were the most abundant, meanwhile the number of granular cells was less and they were evenly distributed. Mucocytes contained densely packed electron translucent secretory vesicles of approx. 4 μm in diameter. A more intensely stained area at their periphery often characterized these vesicles. Granular cells contained dark granules, whereas in vacuolated cells large size (approx. 8 μm) vesicles were seen stained in light blue and showing a tendency to fuse (Figs 1B/1, B/2). As a consequence, large saliva droplets could frequently be found within the cells up to a stage when the whole intracellular space was filled with a single large saliva droplet (Fig. 1C). The latter stage of the vacuolated cell was described as an additional cell type, the cystic cell, and was observed in the SG of all pulmonate gastropods studied [1, 3, 17].

Histochemistry and immunocytochemistry

Different cells could be distinguished based on their histochemical staining properties. In cryostat sections stained with PAS technique, both blue and red coloured cells were observed, indicating the presence of serous and mucous cell types in *Helix* SG. It seems that mucocytes and granular cells were red coloured and vacuolated cells were stained in blue (Fig. 1D).

The different cell types were not homogeneously distributed in the acini. Vacuolated cells tended to cluster near the SDs, meanwhile mucocytes were numerous nearby the blood vessels (Fig. 2A). Differences could also be observed in sections obtained from the SG of inactive and active animals, respectively. Figure 2B shows that there is no significant difference in the number of granular cells, but the

Fig. 4. Double staining of dissociated gland cells with 6-CFDA and Annexin V-Cy3. Cells were dissociated from inactive (A column) and active (B column) snails. A/1; B/1: Cell suspension seen at normal light illumination. A/2; B/2: Only living cells (arrows) are labeled with 6-CFDA (green). A/3; B/3: Apoptotic cells (arrowheads) stained both with Annexin V-Cy3 (red) and 6-CFDA (green, A/2; B/2). Necrotic cells (star) are labeled only with Annexin V-Cy3 (red, A/3; B/3). C: Double staining of the SG tissue (whole-mount) with 6-CFDA and Annexin V-Cy3. C/1: Living cells are stained with 6-CFDA (green arrowheads). C/2: Note that apoptotic cells are labeled both with Annexin V-Cy3 (red, arrowheads) and with 6-CFDA (see C/1, green, arrowheads), mean-while necrotic cells are stained with Annexin V-Cy3 (red, arrow), but not by 6-CFDA (C/1, arrow)


A/1
B/1
A/2
B/2
A/3
B/3
C/1
C/2

number of mucocytes is higher and the number of vacuolated cells is lower in active animals, as compared to those obtained from inactive snails.

The uneven distribution of the different cell types in the acini and in the SG of inactive and active animals suggested a continuous renewal of the cells in the SG. Following HE staining, an outer layer of gland cells could also be observed (Fig. 2C). Further analysis of HE stained sections also revealed the presence of gland cells being in an active mitotic process or dividing (Fig. 2D). Early proliferation of gland cells in the acini was also demonstrated using immunocytochemical methods. Both monoclonal (mouse anti-human Ki-67) and polyclonal (rabbit anti-human Ki-67) antibodies were applied to follow the proliferation of SG cells in inactive and active snails, labeling a large nuclear protein (345, 395 kDa) preferentially expressed during active phases of the cell cycle but being absent in resting cells. Both antibodies gave a positive result, resulting in immunolabeling of numerous nuclei of dividing cells (Figs 3A, B, C). Both immunfluorescence and PAP-technique gave the same results.

In active animals, dilatated intralobular salivary ducts were observed (Fig. 3D), which were never present in inactive snails. Along the duct, cell debris was found, which might be a result of gland cell degeneration and disintegration (Figs 3E, F). In several sections, the duct was seen to protrude into the vacuolated cell, possibly indicating the exocytotic release sites of the saliva. For the release of the saliva there are two possibilities: it could be released from the gland cells by exocytosis, or by the lysis of the active cells through the process of apoptosis or necrosis. In the present study, we have investigated in these latter possibilities and applied an apoptosis detection technique for the demonstration (Apoptosis Detection Kit, Annexin V-CY3). Similar results were obtained applying the apoptosis detection technique on cells dissociated from inactive (A column) as well as from active (B column) animals (Fig. 4). Living gland cells were labeled only with 6-CFDA (green, Figs 4A/2, B/2, C/1), mean while necrotic cells were marked only with AnnCy3 (red, Figs 4A/3, B/3, C/2). Cells in the early stage of apoptosis were labeled with both AnnCy3 and 6-CFDA (Figs 4A/3, B/3, C/2). The number of apoptotic cells was increased in active animals, compared to that of the inactive ones.

DISCUSSION

Histological, histochemical and immunocytochemical studies were performed on the SG of the pulmonate gastropod, *Helix pomatia,* in order to study the morphological basis of salivation. Three different cell types could be distinguished in SG: granular cells, vacuolated cells and mucocytes. Due to their abundance, vacuolated cells and mucocytes seem to be the main cellular components of the salivary gland in both inactive and active snails. In general, the histology and cellular organization of the SG of different pulmonates was found to be strikingly similar [17], and our present observations are also in accordance with the previous findings.

Several authors referred to a cystic cell type in the SG of helicid snails, which is characterized by a single, large secretory vacuole surrounded by a thin peripheral

layer of the cytoplasm [1, 3, 4, 16, 17]. A similar cell type, however, was not observed in *Aplysia depilans* [11]. Quattrini [16] was on the opinion that the large central vacuole of the cystic cell type is a result of the fusion of smaller vesicles and considered the cystic cell, to represent the last stage of the secretory cycle. Serrano et al. [17] has also observed cystic cells, but was on the opinion that the assumption about the secretory cycle was wrong, since the morphology of cystic cells remained always homogeneous, without showing transitional stages between them selves and the gland cells. In these studies, however, a distinction between active (feeding) and inactive snails was not made. According to our observations, cystic cells occurred regularly in active animals and were never present in inactive ones. This could be an explanation why in early studies the number of cystic cells was found to be small. Furthermore, we have regularly observed that vesicles tend to fuse in vacuolated cells in active animals leading possibly to the appearance of a cystic cell. Therefore, we suggest that cystic cells represent indeed the last stage of vacuolated cells, rather than an additional, fourth cell type of the *Helix* SG.

According to our data obtained by histochemical (PAS) technique, different cells identified in the SG may contain different saliva component. Neutral and acid mucins (stained red) could lubricate the food, while the proteins (stained blue) could constitute the enzymatic supply in the early phase of digestion [13]. No attempt has yet been made to correlate morphologically different cell types with the histochemical data, although Boer et al. [3] have concluded that on the basis of toluidine blue staining no close relationship could be established between the different cells types based on the nature of their secretion products. According to their study, the different cell types are not evenly distributed in SG. Hence, it is unlikely that the cell types described as distinct ones, would be only the morphological variations of one or two functional cell types, thereby contributing to the secretion cycle [3].

The different gland cell types are not evenly distributed in the *Helix* SG and they show a tendency to cluster. There are more mucocytes close to the blood vessels, whereas more vacuolated cells can be found close to the SDs. Furthermore, proliferating cells, demonstrated by HE staining and immunolabeling, could be observed on the periphery of acini, while cell remnants (toluidine blue) close to intralobular ducts. This latter observation suggests that there is a continuous ‘renewal cycle’ of secretory cells in the SG. Therefore, it is proposed that the release of the saliva from cystic cell can not exclusively be a result of exocytosis. It is possible that parallel with exocytosis another physiological way of the saliva release exists: the disruption of the cystic cells (holocrine release versus apocrine or merocrine release). In order to support the latter suggestion we have determined, using an apoptosis detection kit, whether this process would correspond to necrosis or programmed cell death [7, 18]. It was found that in active snails, where intensive salivation takes place, more apoptotic cell could be detected compared to that of inactive animals, indicating that part of the cells was eliminated. This finding is also supported by the another observation of this study. Namely, the number of vacuolated cells is lower in active than in inactive snails. Thus, the differences in the number of the different cell types in inactive and active snails seem to reflect the physiological state of the gland cells. It is con-

cluded that apoptosis does occur and can be a physiological way of the saliva release from secretory cells in *Helix pomatia* SG. This is the first report describing apoptosis in non-neuronal cells of molluscs. In *Aplysia* sensory neurones both apoptotic and necrotic cell death were described as a result of hydrogen-peroxide induced cytotoxicity [10].

ACKNOWLEDGEMENTS

This work was supported by OTKA grants No. T043216 to T.K. and No. 34106 to K.E.

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