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The aminergic and peptidergic modulation of salivary gland and molecular mechanism of saliva release in terrestrial snail (*Helix pomatia)*

PhD. thesis

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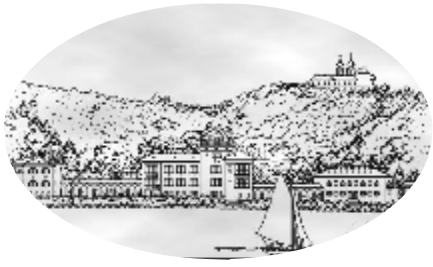

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INTRODUCTION

Saliva is responsible for the predigestion of food entering the mouth cavity through the mouth, its formation to mouthful, and for the promotion of swallowing. The salivary gland as a separate organ first appeared in bristle worms (Polychaeta), and then in all animal phyla in various developmental states. The composition of released saliva is determined by the predatory or herbivorous lifestyle. The salivary gland cells of some predator species are even specialized in poison production, such as the salivary glands of *Conus* snails which produce conotoxin, or the salivary glands of bloodsucker parasites which produce anticoagulant factors. Unlike in vertebrates, the secretory acini of the salivary glands of invertebrates consist of morphologically diverse cell types. Various cell types produce different components of the saliva, such as proteins, enzymes, mucus, poisons or anticoagulant factors. The function of the salivary cells is coordinated by the nervous system. In vertebrates, the salivary gland is under parasympathetic (cholinergic) and sympathetic (adrenergic) regulation. Cholinergic regulation is also present in the salivary gland of invertebrates for example in insects. However, several histochemical, electrophysiological and pharmacological examinations have shown that dopamine (DA) is the most important neurotransmitter in the nerve modulation of the insect salivary gland. Besides DA, other transmitters and/or modulators, such as serotonin (5-HT), proctolin and SG-SASP can also trigger saliva release in the salivary gland of insects. In the buccal ganglia of snails, DA-ergic neurons have been found, which regulate the food-in-take system and play a prominent role in the activation of the central pattern generator of feeding. However, the role of DA in the salivary gland has not yet been identified. Furthermore, the regulatory role of 5-HT and acetylcholine (Ach) in saliva secretion is not known either, in the salivary gland of snails.

The secretory product of the exocrine salivary gland is excreted into the mouth cavity through the salivary duct. Excretion from the salivary gland cells may take place by exocytosis and holocrine or apocrine mechanism. Holocrine secretion is known both in invertebrate and vertebrate organisms, such as, for example, in the sebaceous glands, the uropygial glands of birds, the axillary glands of fish, the harderian glands, and in the venom glands of fish, spiders and molluscs. In these glands, holocrine secretion results in continuous excretion, so mucus is released from stock when needed. During mucus release the acinar cells of the holocrine glands perish, transforming the whole cell into secretory product. According to some authors, holocrine secretion is a controlled mechanism, which could be similar to apoptosis or necrosis. The salivary gland cells of the snail species *Biomphalaria straminea* have been described to release mucus by holocrine secretion. However, the molecular mechanism of the saliva release by holocrine secretion is not yet known.

Molluscs represent the second largest animal group living on the Earth at present, only Arthropods have more species. The class of snails (Gastropoda) is the most populous one with in the phylum. Thanks to their few and easily to identifiable neurons, snails have become popular objects of neuronal research and of the cell-level examination of various physiological processes. We examined the salivary gland of the terrestrial snail (*Helix pomatia* L., Pulmonata, Gastropoda), the largest snail species in Hungary, using it as a relatively simple model to explore the cellular and molecular mechanisms of the salivation and to explore the regulatory and integrative role of the nervous system in this process. Chemical signalization and the mechanisms of modulation between the central nervous system and peripheral organs are similar to the processes in vertebrates in several cases, but they may be species specific as well.

AIMS

There are a number of frequently contradictory information on the morphology and neuronal regulation of the salivary gland of *Helix pomatia*. However, we do not have any information

about ion conductance of the acinar cells, both in resting and active states, about neurochemical modulation and electrical coupling of acini or the molecular mechanism of saliva release. Therefore, our aims were to study the aminergic and peptidergic modulation of the salivary gland of *Helix*, and the cellular mechanisms of saliva release, applying electrophysiological, immunohistochemical, biochemical and molecular biological methods.

Our aims were:

i) to identify the different gland cell types of the salivary gland of *Helix*;
ii) to describe the electrical properties of salivary gland cells, and to characterise their ion currents and ionic pump mechanisms;
iii) to examine the possibilities of cell to cell communication that ensure the synchronised activity of the gland, and to study appearance and distribution these molecules on the cell surface;
iv) to study the cellular mechanisms of saliva secretion, to follow up the destiny of cystic cells during the mechanism of saliva release;
v) to test the effect of transmitters and modulators on saliva secretion;
vi) to identify the peptide(s) that can effectively modulate saliva secretion;
vii) to observe the potential role of apoptosis in saliva release, and its responsiveness to transmitters and modulator molecules (DA, 5HT, ACh), and to a well known antiapoptotic peptide, PACAP.

MATERIALS AND METHODS

Experimental animals and preparation

Experiments were carried out on the salivary gland of adult active, inactive and hibernated specimens of *Helix pomatia*.

The relaxed animals were fixed in the preparation chamber, then the intestine with salivary gland and buccal mass was dissected. Thereafter, the salivary gland was detached from the intestine, and pinned out. In the case of electrical stimulation, the buccal ganglia, which contain the salivary gland innervating neurons, were also prepared together with the salivary gland. Approximately 2-3 mm^2 pieces of the anterior salivary gland were pinned out into the recording chamber and used for voltage clamp experiments. Both whole salivary glands and pieces of the gland were perfused at a rate of 1-2 ml/min with normal physiological saline (in mM; NaCl 80, KCl 4, $CaCl_2$ 10, $MgCl_2$ 5 and TRIS-HCl 10, dissolved in distilled water and adjusted with NaOH to pH=7.4)

Histochemistry

For light microscopic histochemical and electron microscopic experiments, the fixed (1% paraformaldehyde and 2.5% glutaraldehyde diluted in 0.1 M PBS) and dehydrated tissues were embedded into Araldite, then semi-thin and ultra-thin sections were prepared. The semi-thin sections were stained with 1% toluidine-blue. The ultra-thin sections were postfixed with 1% OsO_4 diluted in 0.1 M Na-cacodylate buffer then viewed in a JEOL 1200EX electron microscope.

In order to distinguish the mucous and serous cell types in the gland and to demonstrate cell proliferation, glands were treated for PAS-like and HE staining. The stained sections were analyzed in a Zeiss Axioplan microscope.

Immunocytochemistry

In our experiments indirect two-step methods (by fluorescent stains, or peroxidase conjugated IgG) or Sternberger's three-step peroxidase-anti-peroxidase (PAP) method (1979) was used. The samples were fixed in 4% paraformaldehyde, diluted in 0.1 M PBS, then incubated in 20%

sacharose solution, thereafter were embedded into Tissue-Tek and finally 12-16 µm thick cryostate sections were taken. The sections were incubated with primary and thereafter labeled with secondary antibodies.
The early apoptotic processes of salivary gland were visualized by Annexin V-CY3 and TACS-XL-DAB *in situ* apoptosis detection kit according to the manufacturer's instructions. MitoCapture apoptosis detection kit was used to detect changes in the mitochondrial membrane potential (MMP), associating with the early stages of apoptosis.

Western-blot analysis

Protein samples of salivary gland were prepared by homogenization in SDS-buffer. The homogenates were cleared by centrifugation then the supernatant was removed and diluted 1:1 in SDS sample buffer. After this the mixtures were denatured and samples were loaded and separated in SDS/PAGE (10% or 15% gels) and then electrophoretically transferred to PVDF membranes. The blots were blocked with dried skimmed milk then the proteins were incubated with the primary antibody and thereafter treated with HRP-conjugated secondary antibody. Blots were visualized in TRIS-HCl containing DAB and H_2O_2 solution. The average protein content of samples was 85 µg, determined with the Bradford method.

Control experiments in immunocytochemistry and WB analysis

Preabsorption control experiments were made for most antibodies with specific blocking peptides. No immunostaining could be observed after the control experiments. We did not have specific blocking peptides for innexin-2 and active-caspase-3 antibodies, so we controlled the antibody specificity in WB experiments, using homogenates of the salivary gland of *Locusta migratoria* and rat brain as a positive control. In the positive control experiments we could detect similar bands from snail samples and from the locust or rat. The antibodies reacted specifically in snail samples because in method control experiments we did not detect any reaction in blot. Actin was used as an internal control.

Electrophysiology

In case of nerve stimulation the square wave pulses were delivered by bipolar metal electrodes through the buccal commissure.
The transmitters (ACh, 5-HT, DA) were applied by a microperfusion system. The final concentration of the substances was not higher than 10^{-5}-10^{-6} M.
In voltage-clamp experiments the potassium currents were identified with specific blockers such as TEA and 4-AP. Inward calcium currents were blocked by adding $CdCl_2$ to the physiological solution. In Cl^- free solution Cl^- was substituted by acetate in equimolar amount. NPPB was used to block the Cl^- channels.
In order to measure the resting membrane potential of salivary gland cells and electrical changes upon nerve stimulation or transmitter application, and to record ion currents, an AxoClamp 2B amplifier was used. Data were filtered then digitalized by TL-1 or Digidata 1200 converter. Intracellular recording electrodes were pulled from borosilicate glass using a vertical puller. In the MP measurements 10-30 MΩ, while in voltage-clamp experiments 4-8 MΩ recording electrodes were used. In one-microelectrode voltage-clamp experiments the protocols were generated by pClamp 5.7.7. Data of electrophysiological traces were plotted and analyzed with Origin 7.5. software.

Induction and quantification of apoptosis in the salivary gland

In the salivary gland cells apoptosis was elicited chemically or by the electrical stimulation of the salivary nerve. The glands were incubated with DA and 5-HT at 10^{-4} or 10^{-8} M concentrations. In other cases, the salivary glands were preincubated with DA receptor antagonists (fluphenasine, eticlopride) or K-channel blockers (TEA, 4-AP) before DA treatment. After pharmacological treatments the samples were fixed in 4% PFA then 10 µm sections were made.

The number of TACS-XL positive cells was estimated by counting cells in three visual fields (1.8 mm^2 areas viewed at 400x magnification) per gland. The procedure was repeated on six to eight gland in each type of experiment. The number of specific labeled (TACS-positive) and non-labeled cells was counted on 1.8 mm^2 areas under a Zeiss Axioplan microscope. Data were plotted as means ± S.E.M. using Origin 7.5.

RIA

For identification of PACAP27 and PACAP38 and measurement of their content by RIA, the samples were prepared as previously described. C-terminal fragment of PACAP38 (PACAP24-38) and PACAP27 were iodinated and the reaction mixtures were separated on a reverse-phase HPLC column. The mono-iodinated products were applied as RIA tracers. Synthetic PACAP38 and PACAP27 were used as standards. During the assay procedures the 100 µl antisera, 100 µl RIA tracers and 100 µl standards or salivary gland extract samples were mixed and then the antibody-bound peptides were separated from the free ones by addition of 100 µl separation solution. Following centrifugation the tubes were gently decanted and the radioactivity of the precipitates was measured in a gamma counter. PACAP38 and PACAP27 concentrations of the salivary gland extract samples were read from the appropriate calibration curve.

Detection of cytochrome-c

In our experiments we used the salivary gland of inactive snails. One part of the glands served as control (n=6; 0.188g) and the second part (n=6; 0.197) was exposed to 10^{-4} M DA solution for 30 min. Thereafter the samples were homogenized in iso-osmotic sucrose solution. Differential centrifugation was carried out by modified according to Whittaker's method (1965) in order to separate the mitochondrial and cytosolic fractions. The pure cytosolic fraction contained the translocated cytochrome-c molecule. Release of the cytochrome-c from mitochondrial fraction was elicited by $CaCl_2$ and its absorbance (A) was measured continuously photometrically. The absorbance of cytochrome-c was measured in control and DA-treated samples at 540 nm by a spectrophotometer. The determined absorbance of cytochrome-c values was plotted using OriginPro 7.5. software.

RESULTS AND CONCLUSIONS

Four gland cell types could be identified in the salivary gland of *Helix* in toluidine-blue stained semi-thin sections: the mucocyte, the granular cell, the vacuolated cell and the cystic cell. The cystic cell is considered as the last stage of the secretory cycle, which is the maturation mechanism of vacuolated cells. During maturation, the vesicles in vacuolated cells fuse and form large saliva droplets inside the cystic cell. According to our observation, this cell type is frequent in the salivary gland of active animals, and has a role in saliva secretion. Early histological studies revealed a remarkably wide range of salivary gland cell types within one species. According to our opinion, the reason of it could be that seasonal changes and/or the active and inactive states the animals were disregarding. In the salivary gland the various cell types form acini, in which serous and mucous cell types were found to be interspersed, as revealed by PAS-like staining. Consequently, the released saliva is a mixture, containing digesting saliva full of enzymes and diluted saliva poor in proteins. Such mixed, so-called seromucous salivary glands are atypical in vertebrates.

A layer of dividing gland cells could be observed at the edges of the acini, following HE staining. The early proliferation of gland cells in the acini was demonstrated using Ki67 antibody, too. The number of dividing cells showed activity dependence, because cell proliferation was stronger in the salivary gland of active animals. According to literature data, saliva secretion from the cystic cell takes place by holocrine mechanism. This conlusion was supported by our data that cell debris was found along the intralobular duct, in toluidine-blue stained semi-thin sections of the

salivary gland of active animals. Similar to cell proliferation the amount of cell debris depended on the activity state. It was greater in active animals than in the salivary gland of inactive animals. We supported, that cell debris is the remnant of those gland cells, which show morphological and physiological changes typical for apoptosis. Cystic cells formed in the secretory cycle gave off their product through cell degeneration. The degeneration process of vacuolated and cystic cells was identified as apoptosis in the salivary gland of active animals, using Annexin V-Cy3 and TACS-XL *in situ* apoptosis detection kits.

The ultrastructural analysis of the salivary gland revealed various types of membrane contacts, including both close and loose membrane appositions intermingling between adjacent glandular cells. Unspecialized, loose cell contacts were characterized by wide extracellular space, the distance between the opposing gland cell membrane segments varying between 20-150 nm. Close membrane appositions were characterized by unspecialized membrane segments separated by a space of 10-20 nm. In other cases, longer parallel membrane segments displayed increased electron density, and were separated by wide intercellular space (~60 nm). These membrane contacts were identified as zonula adherens or desmosoma-like structures. Tight membrane contacts (2-4 nm), the classic communication channels in adjacent glandular cells, could also be identified occasionally along short membrane segments. These structures were identified as gap-junction-like contacts. The molecular structures that form gap-junction-like contacts, called innexins, could be detected with applied fluorescence immunohistochemical method. The identified gap-junction-like contacts were also supported by electrophysiological experiments. In electrophysiological recording gland cells revealed a good coupling coefficient from 7-10 cells distance (approx. 300 µm), so the electrical stimulus spreading from cell to cell was good. These cell contacts help synchronize gland secretion despite their poor innervations.

The resting MP value of salivary gland cells was in the range of -30 and -80 mV, the average value was -56.6 ± 9.8 mV (n=483) in normal physiological solution. This average value did not show any significant difference from the MP value of the exocrine salivary glands of vertebrates or insects. MP was determined principally by the distribution of K^+ across the membrane, but the electrogenic Na^+-pumps and Cl^- ions also have important role.

Following microelectrode penetration, salivary gland cells usually remained electrically silent, although in some cases spontaneous miniature potentials were detected. These miniature potentials occasionally were added up and we could record EPSPs, but overshooting action potential could be never recorded. Sometimes action potential-like potentials, so called junction potentials were recorded with amplitude values between 5 and 25 mV.

In order to study the ionic-currents of the salivary gland cells, single electrode voltage-clamp technique was used. Voltage-dependent inward and outward membrane currents could be recorded after the step depolarization of voltage-clamped gland cells. Four different voltage-clamp currents could be recorded from the salivary gland cells of the snail, as fellows: I_K, I_A, $I_{K(Ca)}$ and I_{Ca}. 62% of the total outward K-current consisted of TEA sensitive I_K components, while 27% was Ca^{2+}-dependent $I_{K(Ca)}$ K^+-current component. 4-AP sensitive I_A component was also recorded in a part of the gland cell population, making up 55% of the total current. The voltage-gated inward current probably carried by a T-type Ca-channel, and it could be blocked 100% by Cd^{2+}. These ion currents determined the electrical properties of gland cells.

Electrical stimulation of the salivary nerve, which originates from the buccal ganglia and innervates the salivary gland, elicited a depolarizing response of approx. 30 mV in the salivary gland cells. Similar depolarizing response could be elicited by ACh, 5-HT or DA microperfusion onto the cell surface. The concentration of transmitters was below 10^{-4}-10^{-5} M. The stimulation of the salivary nerve gave a strongest depolarizing effect. The MP change (depolarization) induced by nerve stimulation, ACh or monoamine treatment couldbe identified as secretory potential. Nerve endings, possible containing the transmitters that induce secretory potential, were formed among the gland cells. The varicosities were deeply embedded in the salivary gland cells and formed a close but unspecialized contact with gland cells, so we cannot speak about true synaptic

membrane appositions. We examined the effects of various transmitter molecules on the amount of mucus release. Secretory potential was induced most effectively by ACh, while the saliva secretion was regulated best by DA. The potent neuropeptides of the nervous system, such as MIP, FMRFa, CARP, etc., did not generate secretory potential, so these neuropeptides do not seem to function as transmitters.

In inactive animals TUNEL-positive (apoptotic) cells comprised a small proportion of the salivary gland, however, in gland of active snails the number of TUNEL-positive cells increased significantly. The vacuolated and cystic cells were identified as TUNEL-positive cells among gland cells. These results suggest that the vacuolated and cystic cells degenerated by apoptosis, and gave off their product during feeding. But what could be the apoptosis inducing signal? According to earlier biochemical measurements, DA concentration was significantly higher in the salivary gland of animals that are hungry or just about to feed, than in satiated animals. Our results suggest that the most potent transmitter in saliva secretion is DA, which is able to increase the number of apoptotic cells in the salivary gland of inactive animals. Similar results were obtained by nerve stimulation, during which DA was released from the nerve endings. DA acts through D2 receptors, because the selective blocking of these receptors with eticlopride blocked the effect of DA in eliciting apoptosis. TEA, the selective antagonist of I_K, prevented the effect of DA on inducing apoptosis, while the transient K-channel blocker 4-AP had no effect. These experimental results support that the effect of DA on eliciting apoptosis was exerted partly through D2 receptors and partly through the regulation of K^+ homeostasis.

The MP change (secretory potential) induced by DA was able to influence the mitochondrial transmembrane potential (MMP) through the decrease of intracellular K^+ concentration. The permeabilization of the outer mitochondrial membrane was formed by the pro-apoptotic stimuli. Furthermore, the WB analysis of salivary gland homogenate revealed the presence of a substantial endogenous level of inactive Bcl2 and active Bad proteins, which take part in the permeabilization of the mitochondrial membrane. The ratio of antiapoptotic (Bcl2) and proapoptotic (Bad) proteins determines the destiny of a cell by activating the intrinsic pathway of apoptosis. The ratio depended on the activity state of the animals and on the DA level. It was observed, that DA treatment increased the amount of active Bad molecules, supporting the role of DA in apoptosis. Through the permeabilized mitochondrial membrane, cytochrome-c, as the most importan apoptosis inducing factor was translocated to the cytosol. It was observed, that DA was able to induce the release the cytochrome-c from mitochondria in the gland of inactive animals. The release of cytochrome-c together with caspase-9 and APAF-1 molecules form the apoptosome, which activates caspase-3, the effector molecule of apoptosis, during the intrinsic apoptotic signal.

Active-caspase-3 molecules, the most important effector and leading molecule in apoptosis, were observed exclusively in the cytoplasm and membrane region of cystic cells by immonohistochemical and WB methods. DA significantly increased the number of cells containing active-caspase-3 both in inactive and active animals. However, eticlopride was able to block the caspase-3 activating effect of DA. The presence of caspase-8 was not found in signaltransduction pathways, so we concluded, confirmed that the classical extrinsic pathway of apoptosis is absent in the salivary gland of snails. However the extrinsic and intrinsic apoptotic pathways could be linked by an unknown mechanism. We hypothesized that another pathway and /or unknown molecule(s) connect the effect of DA with the intrinsic pathway of apoptosis. This was also supported by MßCD, which is a non-toxic oligosaccharide, during DA incubation. The effect of DA was blocked by MßCD, which is known to prevent the involvement of ceramide in the lipid raft formation of the membrane thereby blocking signal transduction pathway. Lipid depletion with MßCD in the salivary gland cell membrane significantly decreased the DA-induced cytohrome-c release into the cytosol. So we concluded that ceramid can be an important molecule in signal transduction pathway of the salivary gland.

Using anti-PACAP antibodies, immunopositive gland cells were detected in the salivary gland, while PACAP containing nerve elements were missing, as opposed to the exocrine glands of vertebrates. RIA measurements revealed the existence of both forms of PACAP in the gland tissue. According to our observation, the PACAP38 showed activity dependence in the salivary gland of *Helix*. Our biochemical measurements proved that PACAP is a physiological active molecule, because it increased the level of cAMP (cAMP assay kit, Biorad) by 50% in the salivary gland cells. We observed the antiapoptotic effect of PACAP in the salivary gland cell, since it significantly blocked the apoptosis inducing effect of DA and colchicine. The anti-apoptotic effect of PACAP was also detected on the number of active-caspase-3 molecules, since it DA could not increase the active-caspase-3 level in the gland cells, in the presence of PACAP.

SUMMARY

Our aim was to examine the function of salivary gland with special attention to molecular mechanism of saliva release, by electrophysiological, immunohistochemical, biochemical and molecular biological methods. The salivary gland of *Helix pomatia* is regulated, like all other organs, by the nervous system. The various gland cell types, whose electrical properties are very similar to the vertebrate exocrine salivary gland cells, form acini. In the acini, adjacent gland cells are electrically connected to each other. The good electrical coupling between gland cells has an important function in synchronizing the saliva release from gland cells in spite of their poor innervation. Neurotransmitters (DA, 5-HT, ACh) are released from deeply embedded nerve endings upon stimulation of the salivary nerve elicit depolarizing secretory potentials. The secretory potentials generate the saliva release through exocitosis or, in the case of vacuolated and cystic cells by holocrin secretion. The holocrin mechanism of the saliva secretion is manifest as apoptosis. The morphological and biochemical changes characteristic for apoptosis, such as the appearance of phosphatidyl-serine molecules in the outer cell membrane, the fragmentation of cytoplasm and nuclear elements, the degradation of proteins and the cell membrane, was detected in gland cells. Cell debris appearing along the intralobular salivary ducts is release by saliva. Apoptosis plays an important role in the saliva secretion of cystic cells and therefore it is considered to be a physiological process. Apoptosis is regulated by intrinsic and extrinsic signals. In the intrinsic way of apoptosis the mitochondrion-dependent pathway is activated and cytochrome-c is translocated from the mitochondrion to the cytoplasm. Our experiments revealed that the DA released from nerve endings, biding to the D2 receptors and stimulated cytochrome-c translocation from the mitochondria to the cytoplasm. This process is under the control of the pro- and antiapoptotic Bcl2 protein family. In addition, we observed that high K-efflux could also induce apoptosis in salivary gland cells. DA stimulated the TEA sensitive K-channels and increased K-efflux, which activated apoptosis. Low intracellular K^+ concentrations directly increase the amount of active-caspase-3, as well. We proved that the effect of DA in eliciting caspase-3, the effector molecule of apoptosis, was exerted partly through D2 receptors and partly through the regulation of K^+ homeostasis. The mechanism linking extrinsic and intrinsic apoptotic pathways is still unknown, as caspase-8 has not been found in the signal transduction pathway. DA-induced apoptotic ways are blocked effectively by PACAP, which is an endogenous peptide of *Helix* as well. The target molecule of PACAP is caspase-3.