Published in final edited form as:

Comp Biochem Physiol B Biochem Mol Biol. 2007 April ; 146(4): 477–481.

Substance P and acetylcholine are co-localized in the pathway mediating mucociliary activity in *Rana pipiens*

C. J. Hernández¹, T. Ortiz¹, C. Rosa K. Foster¹, M Tyagi¹, N. Lugo², R. Albrecht³, and S. Chinapen⁴

¹ Department of Biology, University of Puerto Rico at Humacao, Humacao, Puerto Rico, 00791

² Institute of Neurobiology, University of Puerto Rico, San Juan, Puerto Rico

³ Veterinary Department, University of Wisconsin, Madison, Wisconsin, USA

⁴ Department of Physiology/Pathology, San Juan Bautista School of Medicine, Caguas, Puerto Rico 00726

Abstract

Mucociliary activity is an important clearance mechanism in the respiratory system of air breathing vertebrates. Substance P (SP) and acetylcholine play a key role in the stimulation of the mucociliary transport in the frog palate. In this study, retrograde neuronal tracing was combined with immunocytochemistry for SP and choline acetyl transferase (ChAT) in the trigeminal ganglion and for neurokinin-1 receptor (NK1R) in the palate of *Rana pipiens*. The cells of origin of the palatine nerve were identified in the trigeminal ganglion using the retrograde tracer Fluorogold (FG). Optimal labeling of FG cells in the trigeminal ganglion was obtained at 96 h of exposure. Immunofluorescent shows that SP and acetylcholine are co-localized in 92% of the cells labeled with FG in the trigeminal ganglion. NK1 receptors were found in the membrane of epithelial and goblet cells of the palate. Ultrastructural study of the palate showed axonal-like endings with vesicles in connection with epithelial and goblet cells. These results further support the concerted action of both neurotransmitters in the regulation of mucociliary activity in the frog palate.

Keywords

Amphibian; Confocal Microscopy; Fluorogold; Immunofluorescent; mucociliary activity; SP NK1 receptors

1. Introduction

The primary task of the mucociliary system is to propel a mucus blanket containing various materials and particles (bacteria and cellular debris) over the epithelial tissues to the pharynx where they are swallowed or expectorated (Sade et al., 1970; Sleight 1982; Sleight et al., 1988; Zagoory et al., 2002). Highly cooperative beating of cilia (Gheber and Priel 1989, 1994) at high frequencies enables the mucociliary system to carry relatively large objects, at remarkable velocities.

¹Corresponding author. Tel.: +787-850-9455, fax: + 787-859-9459, E-mail address: c_hernandez@webmail.uprh.edu (C.J.Hernández).

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The frog palate has been used by several investigators to study the regulation of mucociliary activity and transport. It is composed of stratified mucociliary epithelium and underlying connective tissue that is vascularized and highly innervated (Chu et al. 1995). Intra-epithelial nerve endings were observed terminating on secretory basal cells, ciliated cells and goblet cells in outgrowths of palate epithelium placed in tissue culture (Chu and Kennedy, 1994).

Stimulation of mucociliary systems is regulated via acetylcholine (Aiello et al., 1991, Zagoory et al., 2001), SP (Hernández & Aiello, 1992), Lindberg et al. 1986a, 1986b), nitric oxide (Alberty et al., 2004) and purinergic compounds (Briman et al., 2000a, Zagoory et al., 2002). The action of acetylcholine and part of the effect of SP in the mucociliary system in the frog palate were blocked by atropine. In vivo studies by Hernández and Aiello (1992) showed that electrical stimulation of the palatine nerve and intracardiac injection of acetylcholine or SP increased mucociliary clearance of the palate and this effect was partially blocked by atropine or spantide (inhibitor of SP) respectively and completely by the two administered concurrently. Furthermore, Aiello et al., 1991 showed that acetylcholine and SP increased and stabilized ciliary beat frequency.

This evidence suggests that SP appears to act directly and indirectly through the release of acetylcholine (Aiello et al., 1991, Hernández et al., 1992). The modulatory action of SP and acetylcholine may be related in their internal molecular pathways. Both transmitters have been shown to mobilize intracellular Ca^{+2} stores via phospholipase C (PLC) and consequently increase mucociliary activity (Bennet et al., 2005 and Zagoory et al., 2002).

Immunocytochemical evidence obtained by Hernández et al., (2003) supports the pharmacological role of SP in the regulation of mucociliary activity in *Rana pipiens*. SP containing fibers with varicosities were found in the connective tissue layer of the palate and also adjacent to blood vessels. SP-labeling was also observed in small to medium cells of the trigeminal ganglion. However, it still needs to be established whether the SP-labeled cell bodies are the cells of origin of the palatine nerve. It would also be pertinent to see whether the SP-labeled nerve terminals/fibers synapse on SP, NK-1 receptors, localized on the membrane of epithelial and goblet cells of the palate. In addition, even though pharmacological studies demonstrated that acetylcholine is a stimulator of mucociliary activity, its localization in this pathway in *Rana pipiens* has not been demonstrated.

This study was undertaken to: (1) establish whether the SP-labeled cell bodies are the cells of origin of the palatine nerve, (2) if acetylcholine, identified by the presence of choline acetyl transferase (ChAT), the enzyme that catalyzes the synthesis of acetylcholine, is co-localized with SP in the cell of origin of the palatine nerve, (3) if the nerve endings are in close relation with epithelial and goblet cells of the palate and (4) if NK1 receptors are present in the cell membrane of epithelial and goblets cells.

2. Materials and Methods

Optimal survival time for the retrograde tracer Fluoro-Gold (FG) in trigeminal ganglion

Twenty two northern leopard frogs (*Rana pipiens*) obtained from Charles D. Sullivan Co., Inc. (Nashville, TN, USA) were used in this experiment. Each animal was anesthetized by immersion in 2% tricaine and placed on its back on a dissecting tray, thereby exposing the palate. Two percent (2%) of Fluoro Gold (Fluorochrome Inc., Denver, CO, USA) soaked gel foam (10 μ L) was ipsilaterally inserted beneath the palatine nerve in each animal. The nerve was transected, and the proximal end was placed in contact with the Fluoro Gold soaked gelfoam. After a survival time of 24, 48, 72 and 96 h the animals were re-anesthetized and perfused transcardially with 20 mL of phosphate buffer saline pH 7.4 (PBS) followed by 10

mL of 4% paraformaldehyde in PBS. The trigeminal ganglion was dissected, post-fixed for 4 h at 4°C and cryoprotected. The tissue was frozen and 20 µm sections were cut, mounted serially and visualized with an epifluorescence microscope (Nikon Eclipse 80i) with UV excitation (Ex 330–380 nm).

Triple-labeling methodology for SP, ChAT and the retrograde tracer FG

Ten (10) animals were prepared with FG using the optimal survival time (96 h). FG-labeled trigeminal ganglion sections were mounted on super frost/plus slide and processed for the immunofluorescence detection of SP and ChAT. Sections were first incubated in blocking serum for 1 h at room temperature (0.5% normal goat serum (NGS), 3% Triton X-100, 0.001% thimerosal in PBS), rinsed and incubated in 1:100 goat anti-ChAT serum (Chemicon International, Temecula, CA, USA) in PBS with 0.5 % NGS, and 3% Triton X-100 overnight at 4° C, rinsed and incubated for 1 h at RT in 1:200 FITC conjugated donkey anti-goat serum (Jackson ImmunoResearch, West Grove PA, USA). After rinsing, sections were incubated in 1:100 rabbit anti-SP serum (ImmunoStar, Hudson, WI, USA) in PBS with 0.5 % NGS, and 3% Triton X-100 at RT for 1hr, then rinsed and incubated in 1:200 rhodamine conjugated goat anti-rabbit serum (Jackson ImmunoResearch) for 1h, rinsed and cover slipped. The overlapping epifluorescent images of FG, SP and ChAT were analyzed using the image analyses program Micro Computer Imaging Device (MCID), Imaging Research Inc., Canada and confocal microscope (Zeiss) for the double labeled cells.

NK1 receptor localization

The immunofluorescent method performed by Nakaya et al., 1994 and Zhang et al., (2006) was followed for this experiment. Briefly, four (4) frogs were anesthetized and perfused with 20 mL of 5mM PBS, pH 7.4 followed by 10 mL of 4% paraformaldehyde in 0.1M sodium phosphate buffer pH 7.4. The palates were dissected, post-fixed overnight in the same fixative and then cryoprotected in 30% sucrose. Sagittal sections of 20 µm were cut on a cryostat and processed for detection of the anti-substance P receptor (NK-1) (Chemicon International, Temecula, CA, USA). All steps were carried out on a shaker at RT. Sections were mounted on Superfrost/Plus microscope slides, rinsed in PBS (3 times for 5 min) and incubated in the blocking agent as mentioned above. After rinsing, the sections were incubated overnight in rabbit anti NK-1 (1:1000) diluted with 0.3% Triton X-100, 0.5% normal serum in PBS. Then the sections were rinsed (3 times for 15 min) and incubated for 1 h at RT in a 1:200 dilution of RITC conjugated goat anti-rabbit (Chemicon International) in the same diluent. After rinsing the sections were coverslipped for viewing with fluorescent and confocal microscopy.

Identification of nerve ending in the palate using TEM

The animals were anesthetized and perfused with 2% glutaraldehyde in 0.1M PBS. The dissected palate was post-fixed for 4 h in the same fixative solution. Dissected segments of palate were dehydrated in a series of ethanol (50, 70 and 95%), for 5 min each, in anhydrous, 100% ethanol for 3 times @ 5 min each, and then in propylene oxide 3 times for 5 min each. The sections were infiltrated with a mixture of 1:1(v:v) un-accelerated epon/araldite (resin without DMP-30) and propylene oxide, for 3 h at RT using continuous gentle rotation. The second infiltration was in 100% of un-accelerated resin for 12 to 16 h at RT using continuous gentle rotation. The segments were then embedded in 100% of the epon/araldite resin and polymerized in a 52°C oven for 48 h. Thin sections (50–60 nm) were cut with a diamond knife on an -RMC-MT 7000- ultramicrotome and mounted on nickel mesh grids and counterstained with uranyl acetate and lead citrate. Grids were examined in a transmission electron microscope (PHILIP 201).

3. Results

SP and acetylcholine are co-localized in the cells of origin of the palatine nerve

Optimal labeling of the cells of origin of the palatine nerve was identified in the trigeminal ganglion after 96 hrs of exposure to FG (Fig 1). Using the immunofluorescent technique combined with FG labeling we found that substance P and acetylcholine are co-localized in the cells of origin of the palatine nerve in the trigeminal ganglion (Figs. 1, 2,3,4). The overlapping immunofluorescent images of FG, SP and ChAT were analyzed using Micro Computer Imaging Device (MCID), Imaging Research Inc., Canada (Fig. 4). In fig. 4, ninety two percent (92%) of the cells of origin of the palatine nerve (FG) were double labeled for SP and ChAT. All of the FG-labeled cells were labeled for SP and 96% with ChAT. Statistic analysis for three (3) replicates shows that 68% ($s = 4.82$) of FG cells were double labeled with SP and ChAT, 78% ($s = 1.32$) with FG and SP and 75% ($s = 3.94$) with FG and ChAT. Confocal Microscopy confirms the co-localization of SP and ChAT (Fig. 5). No immunoreactivity was observed when the 2 primary antibodies were omitted during the processing of the tissue which was viewed with the same filters as the experimental sections. This experiment was replicated five times, obtaining similar results.

Substance P NK1 receptors are localized in the palate

Substance P NK1 receptors were found in the membrane of epithelial and goblets cell in the palate, which is innervated by the palatine nerve (Fig. 6). No immunoreactivity was observed when the primary antiserum was omitted from the processing of the tissue which was viewed with the same filters as the experimental sections

Ultrastructural study

Axonal-like endings containing small and large spherical synaptic vesicles in apparent synapses with epithelial and goblet cells were observed in the palate (Fig. 7).

4. Discussion

Using the retrograde tracer fluorogold, it was confirmed that the cells of origin of the palatine nerve are localized in the trigeminal ganglion and 92% of these cells are colocalized with SP and acetylcholine. The colocalization of these neurotransmitters was confirmed with statistic analysis for three (3) replicates. SP receptors (NK1) were also localized in the membranes of epithelial and goblet cell of the palate.

Pharmacological studies showed that SP and acetylcholine increases mucociliary activity (Hernández and Aiello, 1992; Aiello et al., 1991; Chu et al., 1995). In the present work we showed the anatomical co-localization of SP and acetylcholine in the pathway mediating mucociliary activity and the presence of NK1 receptors in the palate. Combining these results strongly indicate the concerted action of both neurotransmitters in the regulation of mucociliary activity in the frog palate. The modulatory action of these two transmitter systems may be related to their internal molecular pathways. Both transmitters have been shown to mobilize intracellular Ca^{2+} stores via phospholipase C (PLC).

The neurokinin-1 receptor (NK1R) is a member of the G protein-coupled receptor (GPCR) family of membrane proteins. The NK1R typically couples to **Gq** family of G proteins. Thus, stimulation of the receptors lead to activation of phospholipase C and mobilization of intracellular Ca^{2+} (Bennet et al., 2005). It is well established that Ca^{2+} is an important mediator of mucociliary activation, as demonstrated by Verdugo, 1980 and Zagoory et al., 2002.

Similarly, stimulation of muscarinic receptors by acetylcholine leads to activation of phospholipase C (PLC) and mobilization of Ca^{2+} from the intracellular stores, which results in formation of calcium-calmodulin complex (Ca-CaM) and the activation of guanylyl cyclase (GC) and adenylyl cyclase (AC). The increased levels of GMP and AMP activate the corresponding kinases; cGMP-dependent protein kinase (PKG) and protein kinase A (PKA). The concerted action of PKG, PKA and elevated Ca^{2+} results in stimulation of the ciliary beating as demonstrated by Zagoory et al. (2002).

Clearly it is well established by different researchers that the intracellular molecular mechanism used by SP and Acetylcholine are similar and substantiate their role in the regulation of mucociliary activity in the palate of *Rana pipiens*. This is explained by the synergistic effect of SP and acetylcholine in the regulation of mucociliary activity and the co-localization of these two neurotransmitters in the cell of origin of the palatine nerve.

Pharmacological study in the palate of *Rana pipiens* suggests that SP appears to act directly and indirectly through the release of acetylcholine (Aiello et al., 1991, Hernández et al., 1992). The direct action of SP in the regulation of mucociliary activity is supported by the localization of NK1 receptors in the cell membranes of the epithelial and goblets cells in the palate. Ultrastructure study show that axonal like endings are in apparent synapses with epithelial and goblet cells of the palate. These axonal like endings contain small and large spherical synaptic vesicles which are characteristic of peptide (SP) and acetylcholine (Peter et al., 1991). We expect that future immuno-electronmicroscopy studies will corroborate this assumption.

The indirect regulation could be explained by the synapses of SP on cholinergic neurons in the sphenopalatine ganglion as was described by Lindberg et al., 1986a, 1986b). Another possibility is that SP regulates the release of acetylcholine through the afferent fibers from the trigeminal ganglion to the medulla. Similar findings between SP and acetylcholine had been observed in other neurological pathways. Morphological studies show that SP-containing axonal terminals make synapses on cholinergic neurons in the striatum and basal forebrain regions (Bolam et al., 1986). Physiological and pharmacological data indicate that neurokinin receptor antagonists enhance the release of acetylcholine in the hippocampus (Kart et al., 2004).

To conclude, in this work we show that the cell of origin of the palatine nerve contains SP and acetylcholine. SP receptors (NK1) were localized in the membranes of epithelial and goblet cells of the palate. Our findings support the view that both neurotransmitters are involved in the regulation of mucociliary activity in the frog palate. Further studies will be on molecular characterization and expression of NK1 receptor in this system. This will involve the use of laser microscopy and real time RT-PCR.

Acknowledgements

This research was supported by a Grant of National Institute of Health (P 20 RR-0164740) and the Puerto Rico-Alliance for Minority Participation program.

The authors wish to thank Dr. Ervin Reyes and Ioannis M. Torres for their support on this project and students Nydiaris Hernández, Joel Lopez, Mario Ortiz, Marina Martinez, Jesus Gil and Isalis Sanchez.

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Fig 1.
Cells of the trigeminal ganglion with optimal labeling of FG at 96 h

Fig 2.
Cells of the same section in Fig. 1 labeled with SP

Fig 3.
Cells of the same section in Fig. 1 labeled with Chat

Fig 4. Overlapping images of Figs. 1–3 using Image Analyses Program. Note that the triple-labeled cells appear cream in color and the double-labeled cells are orange.

Fig 5.
Confocal microscope image showing the double-labeled cells of SP and ChAT (orange color).

Fig 6.
Substance P NK1 receptors in the membrane of epithelial and goblets cell in the palate

Fig 7. Axonal-like endings (with vesicles) in apparent synapses with epithelial and goblet cells in the palate.