The Functional Morphology of the Pulmonary Neuroendocrine Cells in the Lung of Larval and Adult *Rana ridibunda* (Amphibia-Anura)

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**Abstract**

The distribution and functional morphology of neuroendocrine cells (PNECs) were studied in the lung of tadpole and adult *Rana ridibunda*. The responses of PNECs to different experimental conditions were investigated in adult frog lungs. In tadpole lungs, bombesin- and somatostatin immunoreactive (IR) PNECs were found in the inner-lining epithelium. In adult frogs kept in cold water (+4 °C), mostly serotonin- and bombesin-IR cells had stored secretory material as well as serotonin-IR cells with released secretion, while somatostatin-IR cells with limited secretory material were scarce. In the dry aquarium, somatostatin-IR cells of adult frogs were at various secretory stages. In conclusion, the activities of PNECs varied by depending on the amount of pulmonary contribution of lungs into respiration.

**Key words:** NEBs; serotonin; bombesin; somatostatin; frog lung.  
**Abbreviations:** IR, immunoreactive; NEBs, neuroepithelial bodies; PNECs, pulmonary neuroendocrine cells.

**Introduction**

The airway epithelium of human and animal lungs contains highly specialized pulmonary neuroendocrine cells, distributed as solitary cells and as innervated clusters, neuroepithelial bodies (Scheuermann 1987; Sorokin and Hoyt 1987; Öztay 2006). The PNECs have been described by classical histological, ultrastructural and immunohistochemical methods in lungs of some urodelan and anuran amphibians. These cells are predominantly found on the ciliated epithelium along pulmonary vein in urodelan lungs and in the apical and lateral parts of the first and second order septa in anuran lungs. The PNECs are also seen on the third order septa covered with the respiratory epithelium (Goniakowska-Witalinska 1997).

The PNECs are capable of synthesizing and releasing serotonin and peptide hormones such as substance P, cholecystokinin, calcitonin, somatostatin, bombesin in amphibian lung as well as the other vertebrate lung (Polak et al. 1983; Bodegas et al. 1995; Öztay and Tabakoğlu-Oğuz 2003; Öztay 2006). Morphological evidences show that NEBs are located at or near airway bifurcation and strategically important areas for sensing changes in the airway gas concentration. Thus, they may have different important functions in the regulation of physiological processes in prenatal, early postnatal and adult life (Scheuermann 1987). The use of in vitro models of isolated NEBs combined with electrophysiological approaches and experimental physiological data have confirmed the direct evidence about oxygen-sensing mechanism of NEBs in response to hypoxic conditions (Cutz and Jackson 1999). Other functional considerations include the regulation of the differentiation, proliferation and growth
of the lung in addition to their chemoreceptor functions (Van Lommel 2001). More recently, ATP-storing NEBs innervated by purinergic P2X3 receptor expressing nerve terminals were classified as mechanosensors that are most likely able to accommodate various sensory modalities. They excited P2X3-IR vagal sensory nerve terminals, releasing ATP in rat lung (Brouns et al. 2003).

The specific functions of PNECs are still a subject of debate in spite of recent physiological and morphological results from modern histological methods. The PNECs are characterized by diverse distribution, structure and neurochemical content among vertebrates, and also species. In a point of phylogenetic view, amphibians are the first tetrapods that spend part of their time on land and constitute a suitable species for investigations of PNECs functions. Amphibians use one or a combination of different organs such as buccopharynx, skin, lungs and also gills in the larval stages during respiration. In many anurans, oxygen uptake takes places predominantly in the lungs, while removal of carbon dioxide is through the skin. Hibernating frogs ensure the decreased supply of oxygen through the skin (Withers 1992). The investigations indicate that the use of the amphibian lung and skin in the respiration could be altered under experimental conditions. This situation may provide an experimental model to assess the structural and functional alterations in PNECs that may play an active role during respiration as a result of these conditions (Öztay 2000; Öztay 2006).

The previous study documented the structural and functional characteristics of PNECs containing serotonin and bombesin in Rana ridibunda lung under normal and dry environmental conditions (Öztay 2006). The present paper describes the functional morphology and neurochemical coding of PNECs in the lung of tadpole and the adult frogs kept in cold water. In addition, the structural and functional characteristics of somatostatin-IR PNECs are examined in adult frog under dry environmental conditions.

**Material and Methods**

All experiments were conducted in strict adherence to the guidelines of Istanbul University for animal experimentation. Adult frogs, Rana ridibunda (Amphibia: Anura: Ranidae) of both sexes were collected near Büyük Çekmece Lake, Istanbul, Turkey. The species weighed between 30g to 101 g. The tadpoles were measured 2-3 cm in length. Tadpole development was staged according to Taylor and Kollros (1946). They were taken care of animals during experimental time in accordance with national laws and principles concerning animal care. The animals were divided into four groups, as follows: I- Tadpoles (n=7), II- Adult frogs (n=5, controls), III- Adult frogs kept in cold water at 4 °C for two weeks (n=7), and IV- Adult frogs kept in an aquarium without water at 20-24°C, 48-57 % humidity for two weeks (n=7). To maintain skin integrity, the skin of animals in group IV was moistened with water twice daily.

After decapitation and the spinal cord had been destroyed, the lungs of the animals were fixed in Bouin’s fluid for 24 h. 4 µm-thick paraffin histological sections of the tissue were obtained, stained with hematoxylin and eosin for microscopic observation. Strep ABC immunohistochemical methods were carried out in the lung sections using primary rabbit antiserum against serotonin (1:100; Zymed, 18-0077), somatostatin (1:100; Zymed, 18-0078) and bombesin (1:400; Novocastra, NCL-BOMp), as described before (Öztay 2006).

**Results**

**Tadpoles**

The tadpoles had generally lungs with smooth internal surface. A simple epithelium consisting of mostly spherical cells as well as few flattened and ciliated cylindrical cells covered the inner surface of the lung. Bombesin- and somatostatin-IR PNECs besides bombesin-IR nerves and/or neurons in the subepithelium were observed in the inner-lining epithelium (Figs. 1,2). There was no immunoreactivity for serotonin in the lung of tadpole.
Control Frogs

Somatostatin-, bombesin- and serotonin-IR cells were found at or near the base of the lung inner-epithelium. Somatostatin-IR cells constituted the smallest population in the lung dispersed as only solitary throughout the epithelium covering the inner surface of the lungs. The secretory material in somatostatin-IR cells was limited and scattered throughout the cytoplasm appeared as small granules (Fig. 3).

Frogs kept in cold water

The serotonin- and bombesin-IR cells were markedly reduced in size, especially the former that had either very short or no cytoplasmic extensions. The cytoplasm of both types of cells was mostly filled with large quantities of secretory material, as can be seen in Figs. 4 and 5, despite of several serotonin-IR NEBs released secretory material. Conversely, somatostatin-IR cells were less numerous and had limited amount of secretory material (Fig. 6). The sections stained with hematoxylin revealed that the nuclei of all IR cells show abundance of heterochromatic areas.

Frogs in the aquarium without water

The secretory material of the somatostatin-IR cells was spread over the entire cytoplasm in the form of a large number of small granules and a number of coarse granules, whereas the cells with a pale red-stained cytoplasm contained secretory material that was spread over the cytoplasm as small granules. The somatostatin-IR cells also displayed a multitude of small vacuoles in the cytoplasm (Fig. 7). In sections stained with hematoxylin, somatostatin-IR cells harbored nuclei with small heterochromatic areas.

Discussion

It has been reported that in fetal and neonatal mammalian lungs the PNECs are the first type of cell to mature and are present in higher numbers, which decreases with age. Experimental evidence showed that PNECs influence mammalian lung development and growth, following either paracrine or autocrine pathways (Cutz 1997; Van Lommel 2001). The NEB of developing lung in human and rabbits contains gastrin-releasing peptide/or bombesin, a growth promoter. The gastrin-releasing peptide receptor is colocalised with MIB-1, a nuclear marker of proliferating cells, in the epithelial cells but not in NEB cells (Wang et al. 1996). Contrary to mammals, the adult lungs of amphibians possess well-developed PNECs. However, ultrastructural observations revealed solitary PNECs in the ciliated epithelium of developing larval lung of Salamandra salamandra (Goniakowska-Witalinska 1982). The date indicated that PNECs can be distinguished and epithelial cells such as ciliated cells and goblet cells are started differentiation in stage III of larvae development (Goniakowska-Witalinska 1995). The present study demonstrated PNECs containing bombesin and somatostatin in the inner surface epithelium in larval lung of R. ridibunda, which are in stage IV of embryonic development. Particularly, a lot of bombesin content both in the PNECs and nerve fibres can indicate that the larval lung needs bombesin for lung growth and development, just as the fetal mammalian lung. Somatostatin immunoreactivity has not been reported until now in larval lung. Somatostatin negatively affects the activity of targets (Bentley, 1998). It was purposed that somatostatin in the PNECs of larval R. ridibunda lung had the similar function on the targets and could negatively regulate their activity. On the other hand, it is known that numerous serotonin-IR PNECs are present in the adult R. ridibunda lung (Öztay 2006). For this reason, it is very interesting to note the absence of serotonin in the PNECs of R. ridibunda tadpoles.
Fig 1. In the tadpole lungs, bombesin-IR was present in PNECs and nerve fibres (NF). L, lumen; n, nucleus of PNEC; arrow, secretory granules of PNEC, X 1600.

Fig 2. Somatostatin-IR PNECs were seen in the inner-epithelium of tadpole lungs. L, lumen; e, erythrocytes, thin arrow, secretory granules of PNEC; thick arrow, nucleus of PNEC, X 1600.

Fig 3. Arrows indicate somatostatin-IR PNEC in the lung of control frog, X 1600.

Fig 4. In frogs kept in cold water, serotonin-IR PNECs were characterised by stored secretory material. n, nucleus of serotonin-IR cell, X1600.

Fig 5. Arrow indicated bombesin-IR cells in frogs kept in cold water, X1600.

Fig 6. Somatostatin-IR cells (arrow) contained a limited amount of secretory material in frogs kept in cold water. n, nucleus of somatostatin-IR cell, X1600.

Fig 7. Some of the somatostatin-IR cells with small secretory granules contain many small vacuoles (arrow) in their cytoplasm in frogs kept in without water aquarium, X 1600.
In amphibian lungs, serotonin- and bombesin-IR cells were mostly seen at the base of ciliated epithelium on the apical ends of larger septa (Wasano and Yamamoto 1979; Cutz et al. 1986; Gomi et al. 1994; Gomi et al. 1990; Goniakowska-Witalinska et al. 1997; Öztay 2006). Particularly, the presence of serotonin-IR PNECs and their conservative localization in the amphibian and other vertebrate lungs reflects permanent biological functions in these regions. On the other hand, somatostatin-IR cells are rarely found and defined as solitary at the base of the ciliated epithelium in the lungs. In the human lung has been reported only in pathological conditions (Scheuermann et al. 1992). In addition, solitary somatostatin-IR PNECs is present in the ciliated epithelium of lungs from monkeys and lower vertebrates, such as Hynobius nebulosus tokyensis and Bombina variegata (Cutz et al. 1986; Gomi et al. 1994; Scheuermann et al. 1992). No somatostatin immunoreactivity has been observed in the lungs of Triturus alpestris, Rana lessonae, Rana temporaria, Bufo viridis, Bombina bombina and Hyla arborea (Cutz et al. 1986; Bodegas et al. 1995). The present study noted a different localization for somatostatin-IR cells in the lung: they are located in the respiratory epithelium besides the ciliated epithelium in the lungs of R. ridibunda. Additionally, the somatostatin-IR nerve fibers found in the lungs of R. ridibunda were not observed in the lungs of the other vertebrates.

Stewart et al. (2004) suggest the skin conductance for respiratory gases is sufficient for carbon dioxide excretion and is also sufficient for oxygen uptake if the partial of oxygen in the water is high enough at the low temperature in Ranid frogs. Another study showed that R. ridibunda rarely used, or did not use their lungs for respiration at low temperature (+4 °C), but the opposite was seen when the frog was maintained in a dry aquarium, depending upon the morphological observations on the epithelial cells and other structural components in the lungs (Öztay 2000). It is well known that content of serotonin in PNECs is released after hypoxic stimulus. However, in frogs kept in the cold water these cells had decreased cytoplasmic extensions, heterochromatic nuclei and stored secretory material despite of the presence of several serotonin-IR NEB-released secretory content. At the beginning of hibernation, frogs store a small amount of oxygen in their lungs, but survive from skin oxygen/ carbon dioxide exchange throughout hibernation, lasting 3-4 months. In the present study frogs were kept for two weeks in cold water. This period can be short to be constituted real hypoxia, because they can ensure the decreased supply of oxygen mostly through skin or rarely from stored air in the lungs. Moreover, their oxygen requirement is minimal due to depressed metabolic activity. The present suggestion can be supported by those obtained on NEBs of neonatal rabbits (Fu et al., 2002). They reported that hypoxia-induced secretion of serotonin from NEBs is a dose-dependent in the PO2 range 18-95 mmHg).

Bombesin promotes proliferation of epithelial and mesenchymal cells and also stimulates the differentiation of type II pneumocytes (Emanuel et al. 1999). That bombesin is effective on the contraction of smooth muscle cells through a paracrine/endocrine pathway during respiration, acting as a hormone or neurotransmitter in the lung of R. ridibunda is suggested (Öztay 2006). In frogs kept in the cold water of the present study bombesin-IR cells and nerve fibres were inactive structures with stored secretory material due to non-use of lung during the experimental time. These data indicated that bombesin–IR PNECs worked harmoniously with lung activities in the lung of R. ridibunda.

It is well known that somatostatin originating from the hypothalamus inhibits the release of growth hormone, while if originating in the intestines it inhibits the release of gastric secretion. If it originates from the pancreas inhibits the secretion of insulin and glucagons (Bentley 1998). It has been suggested that somatostatin in PNECs inhibits the secretion of regulatory peptides and contraction of smooth muscles in the bronchi (Cutz et al. 1986;
Scheuermann et al. 1992; Gomi et al. 1994; Goniakowska-Witalinska 1997). Based on these findings, it can be deduced that somatostatin found in the PNECs of R. ridibunda is an inhibitory peptide for the above-mentioned targets. In this case it may be a paracrine signaling pathway, because somatostatin-IR cells were scarcely localized close to capillaries. In frogs kept in the cold water, the limited observation of somatostatin-IR cells with limited secretory material suggest that their secretory material was released at the early stages of experiment, afterwards which decreased metabolic activity results in no more secretions at the lung level. By contrast, in frogs in the dry aquarium the somatostatin-IR cells were very active in synthesing and releasing secretory material. Furthermore, in this group, the presence of small vacuoles in the cytoplasm of many somatostatin-IR cells indicates that they are highly stimulated for release and synthesis of somatostatin. Similar observation was reported in neurosecretory cells of cerebral ganglia highly stimulated for release and synthesis of secretory material (Tabakoğlu-Oğuz 1975).

The present study described the presence of somatostatin in addition to the presence of serotonin, bombesin, calcitonin and enkephalins to be reported previously, in PNECs of adult R. ridibunda lung. Under the present experimental conditions, the PNECs of R. ridibunda lung exhibit diverse functional activities depending on the contribution of frog lung to respiration. Their structural characteristics exhibited by these cells under experimental conditions indicate the requirement for each secretory material. Furthermore, the study stated that bombesin- and somatostatin-IR PNECs in larval lung may play an active role during the embryonic development of lung.

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References
histology, ultrastructure and immunohistochemistry of the respiratory organs in non-mammalian vertebrates. Edited by L.M. Pastor, publications of Murcia University, Spain.


