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Neuroepithelial Endocrine Cells in the Lung of *Rana ridibunda*: An Immunohistochemical Study

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Abstract: In this study, neuroepithelial endocrine cells were for the first time identified in the lung of *Rana ridibunda*. The presence of these cells was investigated using endocrine markers by the immunohistochemical method at the light microscopic level. Immunoreactive - cells containing serotonin, calcitonin, met-enkephalin and leu-enkephalin were observed alone or in clusters within the respiratory epithelium. In general, clustered - cells containing serotonin were detected among the ciliated cells and goblet cells of the pseudostratified epithelium on apical dilation of the primary septa where the inhaled - air is first met. Among pneumocytes, single and clustered neuroepithelial endocrine cells display calcitonin, met-enkephalin and leu-enkephalin immunoreactivity in the rest of the epithelium. All immunoreactive - cells have different functions in different regions of the lung in *Rana ridibunda* by the paracrine/endocrine pathway, taking into consideration their morphological properties, localization in the epithelium and distribution in the lung.

Key Words: pulmonary neuroendocrine cells, lung, immunohistochemistry, serotonin, calcitonin, enkephalins, *Rana ridibunda*.

Rana ridibunda'nın Akciğerindeki Nöroepiteliyal Endokrin Hücreler: İmmünohistokimyasal Bir Çalışma

Özet: Bu çalışma ile nöroepiteliyal endokrin hücreler *Rana ridibunda* akciğerinde ilk kez çalışıldı. Bu hücrelerin varlıkları endokrin işaretleyiciler kullanılarak immünohistokimyasal metodla ışık mikroskobu seviyesinde araştırıldı. Serotonin-, kalsitonin-, met-enkefalin- ve leu-enkefalin-immünoreaktif hücreler tek ya da yığınlar halinde solunum epiteli içinde gözlendi. Serotonin içeren yığınlar halindeki hücreler, çoğunlukla solunum havasının ilk karşılandığı yerler olan birincil kıvrımların genişlemiş apikal bölgelerindeki psödostratifiye epitelde, silli ve goblet hücrelerinin aralarında belirlendiler. Pnömositler arasındaki tek ve yığınlaşmış nöroepiteliyal endokrin hücreler, epitelin geri kalan kısmında kalsitonin, met-enkefalin ve leu-enkefalin immünoreaktivitesi gösterdiler. Tüm immünoreaktif hücrelerin akciğerdeki dağılımları, epiteldeki yerleşim yerleri ve morfolojik özellikleri dikkate alındığında, onların *Rana ridibunda* akciğerinin birkaç bölgesinde parakrin/endokrin yol ile farklı görevlere sahip olabilecekleri sonucuna varılmıştır.

Anahtar Sözcükler: solunum nöroendokrin hücreleri, akciğer, immünohistokimya, serotonin, kalsitonin, enkefalinler, *Rana ridibunda*.

Introduction

Lungs contain neuroepithelial endocrine cells (pulmonary neuroendocrine cells, (PNECs)) together with pneumocytes, ciliated cells, goblet cells and basal cells in the inner surface epithelium. Investigations into PNECs in recent years have provided data about the morphological, histochemical and immunohistochemical characteristics of these cells. So far, the presence of a number of active peptides and hormones in PNECs has been detected by means of immunocytochemistry and fluorescence studies (1-3).

Since PNECs are characterized by considerable diversity among different animal groups with regards to their location, morphology and even the contents of their secretory material, they are of considerable interest to researchers. PNECs have considerably diverse characteristics as mentioned above (4), and there is wide speculation about the functions of these neuroendocrine cells in the lung. Therefore, there is a need to investigate the distribution of these cells in the lung, their location in the epithelium, the contents of their secretory materials and their relationship with other types of cells. Studies of

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PNECs are mostly carried out on the lungs of mammals, but are limited to the lower vertebrates (5-7). However, significant comments on the functions of PNECs and their mechanisms of action, the characteristics of PNECs in many species, including lower vertebrates, should be based on taking the evolutionary development of species into consideration.

The aims of this study were to demonstrate the distribution of PNECs containing serotonin, calcitonin, met-enkephalin and leu-enkephalin in the lung of *Rana ridibunda*, and to explain their morphological characteristics, location and distribution in the epithelium. In addition, this study was performed to understand their interactions with other epithelial cells and connective tissue structures.

Materials and Methods

In this study, 10 adult males and females of *Rana ridibunda* (Amphibia-Anura), weighing from 37 g to 93.7 g, were used. The frogs were dissected after being collected from their natural habitat. After the spinal cord had been destroyed, the lungs of the animals were fixed in Bouin's fluid for 24 h. The pieces of lung were dehydrated and embedded in paraffin. An indirect immunohistochemical method using a streptavidin-biotin complex (StrepABC) technique was applied to paraffin sections of 4 µm thickness. The deparaffinized sections were treated with 30% hydrogen peroxide in methanol for 30 min. Non-specific immunoreaction at room temperature was blocked for 20 min with normal - goat serum, included in the Histostain-plus Bulk kit (Zymed 85-9043). Sections in a humidity chamber were incubated with primary rabbit antisera against serotonin (1:100; Zymed, 18-0077), calcitonin (1:100; Zymed, 18-0012), met-enkephalin (1:3500; Chemicon, AB1974) and leu-enkephalin (1:3500; Chemicon, AB1975) for 1 h at room temperature. The sections were further processed by using a Histostain-plus Bulk kit. Briefly, biotinylated-secondary antibodies were coupled to streptavidin-biotinylated horseradish peroxidase. The peroxidase reaction was developed by DAB-plus kit (Zymed, 00-2020) and AEC substrate. During each incubation step the sections were washed in phosphate-buffered saline (PBS, 0.01 M; pH: 7.4). Primary antisera were diluted in PBS containing 0.1% normal - bovine serum.

Immunoreactivity was controlled in three ways: (a) replacement of the primary antiserum by non-immune serum or PBS; (b) use of isotype rabbit antiserum instead of primary antiserum (Zymed, 08-6199) and (c) positive tissue control with rat thyroid, lung and frog brain.

Results

General Morphological Findings

We identified the three-fold (primary, secondary and tertiary) septa that protruded deeply from the inner surface of the lung walls into the lumen. The primary septa with dilated apical ends were the longest and thickest. All of the septa were interconnected. The lungs were divided by these septa into large and small air sacs. The respiratory epithelium covering the inner surface of the lung was in the form of a pseudostratified epithelium (PSE) on the dilated apical end of the primary septa, whereas it was in the form of a single-layer epithelium in the remaining parts of the lung. The respiratory epithelium exhibited local variations in terms of the distribution of different cell types. The PSE contained ciliated cells, goblet cells, basal cells and pulmonary neuroendocrine cells. On the other hand, the single-layer respiratory epithelium contained pneumocytes, goblet cells and pulmonary neuroendocrine cells.

Immunohistotochemical Findings

PNECs containing serotonin, calcitonin, met-enkephalin and leu-enkephalin were distinguished by coloured cytoplasm surrounding the immunonegative nucleus in the respiratory epithelium. They appeared to occur alone and in clusters, called the neuroepithelial endocrine cells (NECs) and neuroepithelial bodies (NEBs), respectively.

Anti-serotonin-immunoreactive cells

Serotonin-immunoreactive cells were numerous around the central lumen and the upper half of the lungs. They were observed within both the pseudostratified epithelium and also within the single-layer epithelium region close to the PSE. However, it was found that these immunoreactive cells were mostly situated within the PSE on the dilated apical regions of the primary septa that protruded toward the center of the lung lumen (Figure 1). NECs and NEBs containing serotonin in PSE were generally embedded on the basal part of the epithelium or near its base. Nevertheless, serotonin-immunoreactive

cells extended in different several levels inside PSE (Figure 1), appearing to reach the lung lumen (Figure 2). NECs and NEBs containing serotonin were observed among the ciliated cells, goblet cells and pneumocytes (Figures 1-4). It was generally observed that the number of cells in NEBs (with 2-6 cells) within the single-layer epithelium was lower than those within the PSE (with 2-17 cells). They were arranged either side by side or one on top of the other in the epithelium (Figures 3 and 4).

Serotonin-immunoreactive cells possessed various shapes such as cylindrical, spherical, pear-shaped or pyramidal. Their nuclei were found to be in accordance with the shape of the cells. In addition, they were fairly large and sometimes invaginated. Many dark-brown secretory granules were found in serotonin-immunoreactive cells. These sometimes appeared scattered throughout the cytoplasm and sometimes concentrated in the basal region of the cell (Figure 5). Generally, it was detected that serotonin-immunoreactive cells were associated with a capillary (Figure 5).

Anti-calcitonin-immunoreactive cells

Calcitonin-immunoreactive cells in the respiratory epithelium were less abundant than serotonin-immunoreactive cells. These cells were found either as NECs or NEBs with 2-6 cells among pneumocytes in a single-layer epithelium (Figure 6). However, NECs containing calcitonin were also observed among the ciliated and goblet cells in the PSE (Figure 7). They possessed fewer secretory granules than the serotonin-immunoreactive cells and were generally scattered in the cytoplasm. Calcitonin-immunoreactive cells and their nuclei were sphere-shaped. The nuclei occupied a wide area in the cell volume.

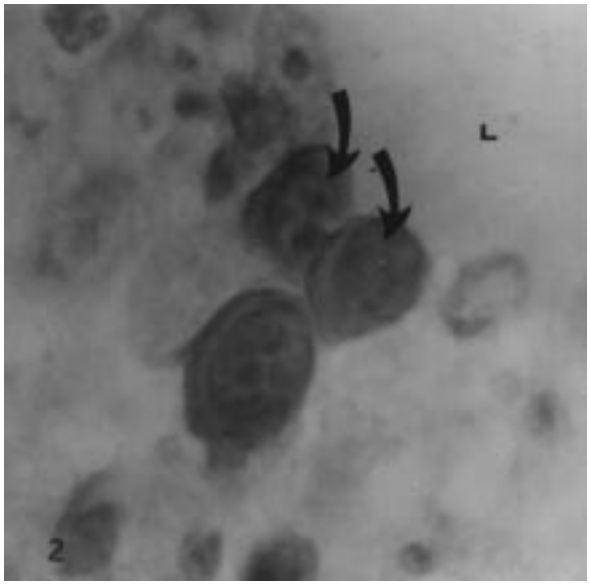


Figure 2. Arrows show serotonin-immunoreactive cells in relation to the lumen (L), x 1850.

Anti-met-enkephalin- and anti-leu-enkephalin-immunoreactive cells

NECs and NEBs (with 2-3 cells) containing met-enkephalin were found among pneumocytes in the single-layer epithelium (Figures 8a,b). Leu-enkephalin-immunoreactive cells were mostly observed alone among pneumocytes within the single-layer epithelium (Figure 9). Both of these immunoreactive cells were oval-shaped and their nuclei were also oval or spherical in shape. Their secretory granules were seen throughout the cytoplasm.

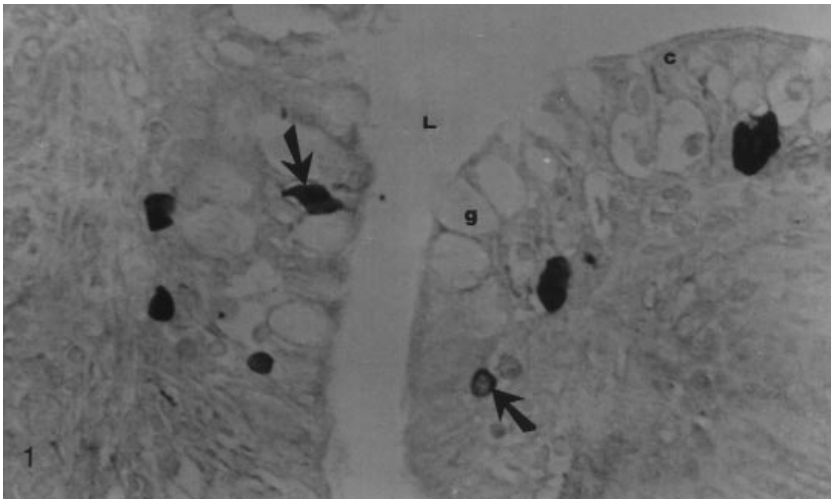


Figure 1. Serotonin-immunoreactive cells in the apical region of the primary septa: goblet cells (g), ciliated cells (c), lumen (L), x 680.

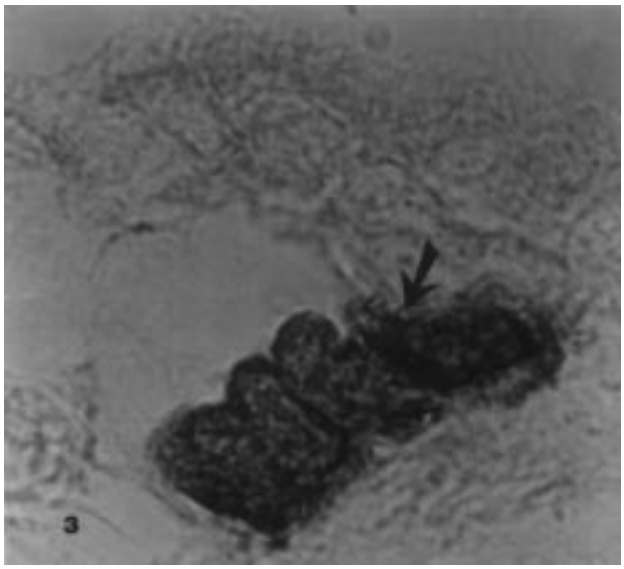


Figure 3. Arrow points to serotonin-immunoreactive cells in the basal region of the PSE, x 1850.

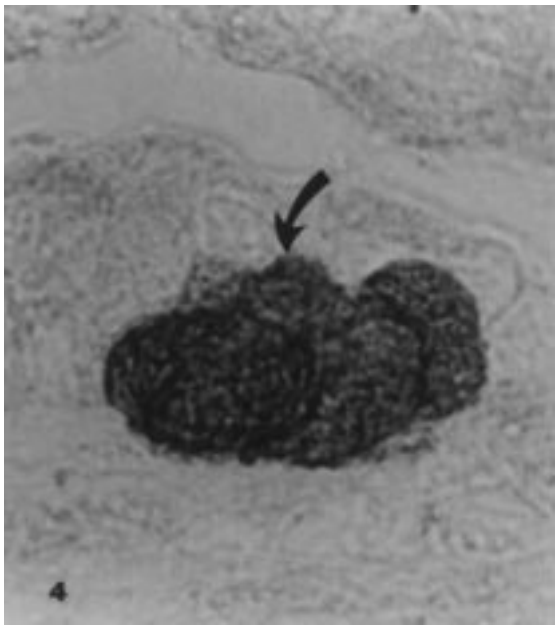


Figure 4. Serotonin-immunoreactive cells among pneumocytes (arrow), x 1850.

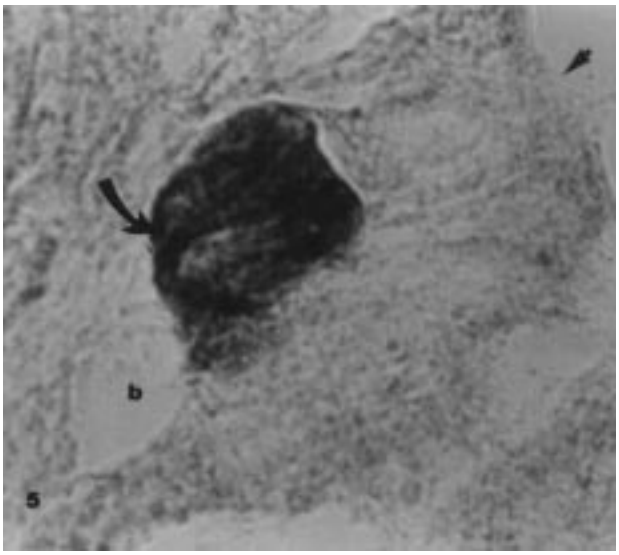


Figure 5. Serotonin-immunoreactive cells in basal region of the PSE; secretory granules (arrow); blood capillaries (b); cilia (arrowhead), x 1850.

Discussion

It is known that many vertebrates, from air-breathing fish to mammals, have PNECs in their lungs (8-11). They were found both alone and in clusters as neuroepithelial bodies. During the last 20 years, investigations have focused on the identification of their structural and immunocytochemical characteristics, and on the immunomodulator function of PNECs (1,3). The morphology and distribution of PNECs in the lungs of vertebrates are characterized by diversity. PNECs have different properties among species with regard to their distribution in the respiratory epithelium. In mammals, NECs are scattered in the epithelium lining upper airways, whereas, in contrast to NECs, NEBs are found almost exclusively in the intrapulmonary airways (1). In the present study, the numbers of NEBs in *R. ridibunda* lungs were higher than those of NECs. Similar findings were obtained from amphibian samples, such as *Rana temporaria* (7), *Bufo marinus* (9) and *Bufo bufo* (12). Other vertebrates such as reptiles and amphibians apart from mammals and birds have a structurally simple lung containing unbranched conducting airways (13). Therefore, their PNECs are localized differently in the ciliated epithelium of lung septa in the reptilian and amphibian lung (9,10,12). NECs and especially NEBs of *R. ridibunda* were mostly observed within the PSE in the dilated apical region of the primary septa near the central lung lumen where the inhaled-air is first met, just like in *R. temporaria* (7), *B. marinus* (9) and *Bufo viridis* (12). In addition, PNECs of *R. ridibunda* were also found in the remaining epithelial areas other than the PSE like the PNECs of *Bombina orientalis* (14).

The PNECs connecting to the lumen are called "open-type" while others that seem not to reach the lumen are

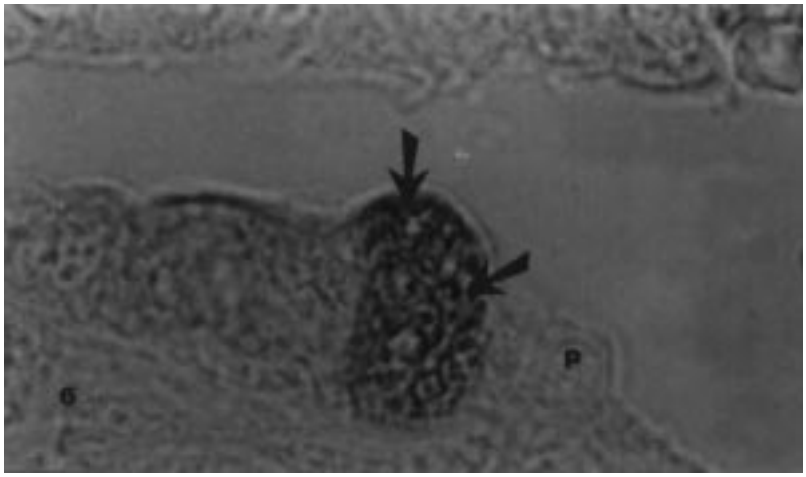


Figure 6. Arrows indicate calcitonin-immunoreactive cells among pneumocytes (p), x 1850.

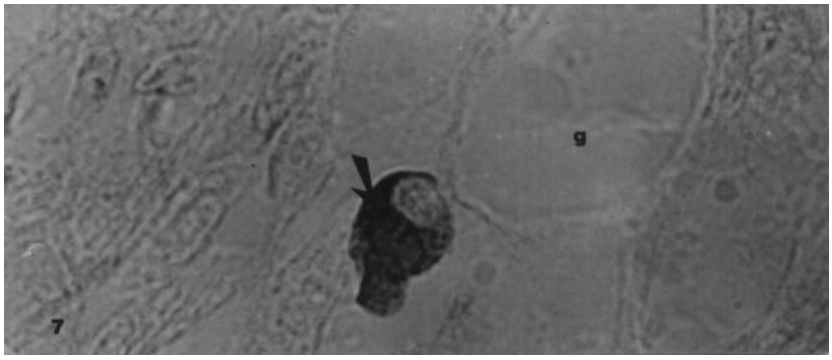
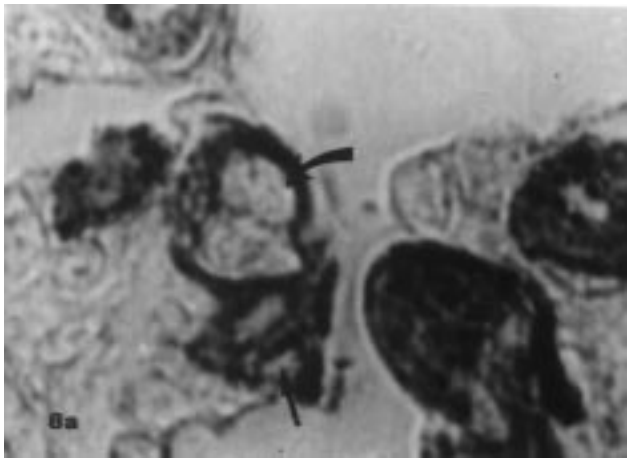
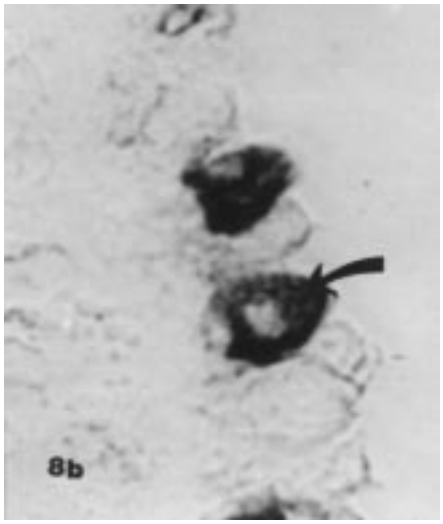


Figure 7. Arrow shows calcitonin-immunoreactive cells in PSE, (g) goblet cell, x 1850.





Figures 8. a,b. Thick arrows indicate met-enkephalin-immunoreactive cells; fine arrows show erythrocytes, for Figure 8a x 1150, for Figure 8b x 960.

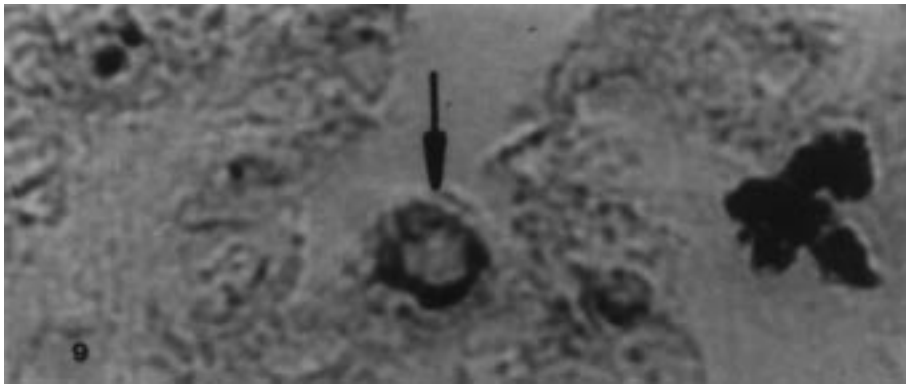


Figure 9. Arrow shows leu-enkephalin- immunoreactive cell in single-layer epithelium, x 1150.

called "closed-type" endocrine cells (15). It was observed that many NEBs located in the mammals, respiratory epithelia were covered by a cell with many microvilli, appearing on their apical surface and not reaching the lumen (4). *Podarcis hispanica* is a reptile, with NEBs of the open-type cell (10). Amphibians have generally closed-type PNECs surrounded by a thick layer of other epithelial cells. Open-type PNECs were found in only two amphibian species; *Ambystoma tigrinum* and *B. marinus* (9,15). Given their location in the epithelium, some PNECs in *R. ridibunda* are covered by ciliated cells, goblet cells and pneumocytes, and are thus mostly closed-type endocrine cells. Nevertheless, it was observed that NEBs containing serotonin of the open-type are connected to the lumen, as in *A. tigrinum* and *B. marinus*. Researchers (9,15) have stated that open-type PNECs containing serotonin perform a chemoreceptor function in the lung. In the light of their distribution in the lung and localization in the epithelium, it is suggested that open-type PNECs containing serotonin in the lung of *R. ridibunda* also possess as a chemoreceptor function.

Serotonin is one of the first biogenic amines investigated in PNECs. The existence of serotonin has been detected in NECs of *T. alpestris* (5) and *Ambystoma mexicanum* (16) and in both NECs and NEBs of *Hynobious nebulosus tokyoensis* (6), *R. temporaria* (7), *B. orientalis* (14) and *A. tigrinum* (15). In many of the examples mentioned above, serotonin-immunoreactive cells have been found at the base or close to the base of the PSE. There are many smooth muscle cells in the connective tissue, which are found just under the PSE. In our study, serotonin-immunoreactive cells were particularly observed at the base of the PSE on the dilated apical part of the primary septa. In addition, it was established that some of these cells at the base of the epithelium rest against the wall of the blood capillaries. The physiological role of serotonin in the lung is not yet known. It is suggested, however, that serotonin may have a vasoconstructive effect, and it may also affect lung circulation by stimulating the contraction of the smooth muscles of blood vessels (5). In addition, it may modulate mucous secretion, the action of cilia and the contraction of smooth muscle cells in the connective tissue (10). Considering that the serotonin-immunoreactive cells of our subjects were mostly located among the ciliated cells and the goblet cells, and near the blood capillaries and smooth muscle cells, it may be concluded that the functions of the neuroendocrine cells containing serotonin in our subjects were similar to those in the animals mentioned above.

Using immunocytochemical studies, it was established that PNECs in the lungs of some amphibian species, such as *Bombina variegata* and *Hyla arborea*, do not contain calcitonin (5), while calcitonin does exist in some examples, such as *A. mexicanum* and *H. nebulosus tokyoensis* (6,16). In the epithelium of *R. temporaria*, exterior to the ciliated epithelial region, calcitonin-immunoreactive NECs have been found (7). However, calcitonin was observed both in NECs and particularly in NEBs in the single-layer epithelium of *R. ridibunda*. In addition, one NEC was located exclusively at the base of the ciliated epithelium in *R. ridibunda*. Gomi et al. (6) observed calcitonin-immunoreactive NEBs and NECs located in the ciliated epithelium in *H. nebulosus tokyoensis*. It was concluded that calcitonin-immunoreactive cells play an important chemothrophic role (3). However, all these suggest that more research is needed to understand the function of calcitonin in the lung.

Met-enkephalin and leu-enkephalin-like immunoreactivity were noted in PNECs of *A. tigrinum* (15). NECs containing-leu-enkephalin were found in *B. variegata* lungs (12). On the other hand, neither met-enkephalin- nor leu-enkephalin-immunoreactive cells were observed in the lung of *R. temporaria* (7). Both of these immunoreactive cells were found in the lungs of *R. ridibunda*. Cutz et al. (5) indicated that the PNECs consisting of both met-enkephalin and leu-enkephalin controlled the metabolism and secretory activity of the other neighbouring epithelial cells in the lung. In the light of these findings, it may be considered that the PNECs containing met-enkephalin and leu-enkephalin possessed the same functions in the lung of *R. ridibunda*.

So far, the exact role of PNECs in the lung has not been established. When the distribution, morphological and immunohistochemical characteristics of PNECs are investigated, it appears that these neuroendocrine cells of *R. ridibunda* are similar to the PNECs of other amphibian and vertebrate species with regard to the characteristics mentioned above. Several researchers (17-19) have stated that PNECs play an important role in lung biology by the paracrine/endocrine pathway. Experimental data on PNECs physiology in the lungs of amphibians has still not been applied. The morphological characteristics and the localization of open-type NEBs containing serotonin on the apical ends of primary septa meeting with inhaled-air in *R. ridibunda* showed that these neuroendocrine cells may play a role as chemoreceptors. According to our findings, it may be considered that the PNECs of *R. ridibunda* possess the same functions such as chemoreceptors, and paracrine/endocrine functions for lung biology and physiology. However, more experimental data verifying their physiological properties as well as their ultrastructural characteristics are required to define the biological functions of these cells.

Acknowledgements

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