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Neuroendocrine Diffuse System of the Respiratory Tract of *Rana temporaria:* An Immunocytochemical Study

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The neuroendocrine cell population of the respiratory system of *Rana temporaria* has been studied by means of immunocytochemical methods at the light-microscopic level. Isolated or clustered endocrine cells have been found in the epithelium of the buccal cavity, glottis, larynx, and lung. Nine different types of endocrine isolated cell types can be distinguished according to their immunoreactivity to several regulatory peptides [calcitonin, substance P, bombesin, peptide histidine isoleucine (PHI), cholecystokinin (CCK), and endothelin 1] and neuroendocrine markers (7B2, chromogranin, and serotonin). Neuroepithelial bodies are innervated clusters of cells simultaneously immunoreactive for serotonin and 7B2. Nerves and/or neurons have been detected in different regions of the respiratory system using antibodies against protein gene product 9.5, serotonin, calcitonin gene-related peptide (CGRP), substance P, PHI, helodermin, and CCK.

The respiratory tract of amphibians has been the object of several studies that were focused mainly on its peculiar morphology (Goniakowska-Witalinska, 1978, 1980a) and on the functional relationships between the several respiratory regions (Czopek, 1965; Stark-Vancs *et al.,* 1984; Maina, 1989). In the adults of most amphibian species, the oxygen exchange takes place not only at the pulmonary respiratory barrier, but also through the skin and the mucosa of the buccal cavity, which are greatly vascularized (Czopek, 1965). In anurans, apart from the skin, the respiratory organs include the buccal cavity, the airways or "conducting portion" (glottis and larynx), and a pair of saccular septated lungs. The few physiological studies published on the control of the respiratory function of amphibians have been concentrated mostly on the autonomic innervation of the saccular lungs and the effects of neurotransmitter antagonists on the vascular or septal smooth muscle (Campbell, 1971a, b; Campbell and Duxson, 1978; Campbell *et al.,* 1978; Holmgren and Campbell, 1978; Stark-Vancs *et al.,* 1984). Studies on the mammalian respiratory tract have shown that, apart from the classical neurotransmitters, active amines and peptides released either from mucosal endocrine cells of the diffuse endocrine system or from sensory or autonomic nerve fibers exert a very important role in the control of pulmonary physiology (Barnes, 1989; Springall *et al.,* 1991). Very little is known about the cells that produce and release regulatory factors in the respiratory tract of amphibians. There are some descriptions of the endocrine cells present in the epithelium of the lung of several amphibian species by means of classical histological and/or ultrastructural (Wasano and Yamamoto, 1978; Rogers and Haller, 1978, 1980; Goniakowska-Witalinska, 1980a, 1981, 1982; Matsumura, 1985; Scheuermann *et al.,* 1989; Goniakowska-Witalinska and Cutz, 1990; Goniakowska-Witalinska *et al.,* 1990, 1992; Adriaensen *et al.,* 1994) and immunocytochemical (Cutz *et al.,* 1986; Scheuermann *et al.,* 1989; Goniakoska-Witalinska *et al.,* 1990, 1992; Saldise *et al.,* 1992; Adriaensen *et al.,* 1994; Kusakabe *et al.,* 1995) techniques. The literature concerning the peptides present in the amphibian pulmonary innervation is still

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less abundant (Cutz *et al.*, 1986; Scheuermann *et al.*, 1989; Saldise *et al.*, 1992; Adriaensen *et al.*, 1994). None of the above-mentioned articles have presented data on the distribution of the different types of endocrine cells and nerve fibers throughout the several regions that compose the respiratory tract. In the present paper we aimed for a complete immunocytochemical characterization of the elements of the diffuse endocrine system and the peptidergic innervation of the respiratory tract of the frog *Rana temporaria*. We have also studied the distribution of these cells throughout the buccal cavity, glottis, larynx, and saccular lungs of this species.

MATERIAL AND METHODS

Thirty adult specimens of frog (*R. temporaria*) were euthanized by decapitation and their lungs with the larynx, glottis, and part of the buccal cavity were used.

Paraffin-Embedded Tissue

The material was fixed in Bouin's fluid for 24 hr. The pieces were dehydrated and embedded in paraffin, and 3-μm-thick sections were cut. Sections were stained with hematoxylin–eosin, Masson trichome, and periodic acid–Schiff (PAS) techniques for the histological studies and with the Grimelius silver nitrate technique (Grimelius, 1968) for the study of the argyrophilia of endocrine cells. An immunocytochemical demonstration of the endocrine cells was performed by use of the avidin–biotin complexes (ABC) technique (Hsu *et al.*, 1981).

Endogenous peroxidase was blocked by exposure to 0.3% hydrogen peroxide in methanol for 30 min, and nonspecific immunoreaction was blocked with normal swine serum for 30 min at room temperature. Sections were then incubated with one of the primary antisera listed in Table 1 at optimal working dilution. This primary incubation was performed overnight at 4° in a moist chamber. After thorough rinsing with Tris buffer saline (TBS) (0.05 *M;* pH 7.36; 0.5 *M* NaCl), secondary antiserum (biotinylated swine anti-rabbit immunoglobulins, 1:200, Dakopatts, Glostrup, Denmark, code E353) was applied for 30 min at room temperature. Sections were washed with TBS, and then avidin–biotin peroxidase complex solution (ABComplex, 1:100, Dakopatts, code K355) was applied for 30 min. Immunoreacted sections were washed in TBS (0.05 *M;* pH 7.36), and the peroxidase reaction was developed in a solution of 3,3′diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, code D-5637) 0.03% w/v in TBS and H_2O_2 (0.006%). Some sections were lightly counterstained with Harris's hematoxylin. Primary and secondary antibodies and the ABC complexes were diluted in TBS.

Resin-Embedded Tissue

Small pieces (1 mm^3) of lung tissue from 10 frog specimens were fixed in Zamboni's liquid (Stefanini *et al.*, 1967) for 24 hr at 4°, dehydrated with ethanol, washed with propylene oxide, and embedded in Epon 812 (Luft, 1961). Semithin (1-μm-thick) sections were cut and transferred onto glass slides, and the plastic was entirely removed with sodium methoxide (Mayor *et al.*, 1961) or with aged saturated sodium hydroxide in ethanol (Lane and Europa, 1965). Some deplasticized sections were stained with borated methylene blue, and others were used for the ABC technique.

For immunocytochemistry, the sections were attached to the glass by heating at 60° overnight. The Epon was removed according to the Lane and Europa (1965) method. The sections were rinsed in absolute ethanol, then hydrated in graded ethanol, and washed twice in TBS. Then the ABC technique was applied according to the described method, increasing the incubation times in the primary antiserum to 42–48 hr and in the secondary antisera and ABC complex to 1 hr.

Controls

Immunocytochemical controls included: (1) use of normal rabbit serum, diluted 1:20, instead of the primary antiserum; (2) positive control with mammalian tissues (gastrointestinal, respiratory, and neurohormonal systems) known to be immunoreactive for each antiserum; and (3) absorption tests. The specificity of the antisera that gave immunoreactivity was examined. Prior to immunostaining each antiserum was absorbed by incubation overnight at 4° with 1–20 nmol of its respective antigen per milliliter of optimally diluted antiserum. Absorption tests were carried out on reverse-face sections (Osamura *et al.*, 1980). The antigens used are listed in Table 2. All of the absorption tests carried out confirmed the specificity of the immunocytochemical results.

RESULTS

The main components of the respiratory system of *R. temporaria* are shown in Fig. 1A. The respiratory system of *R. temporaria* consists of the buccal cavity, communicating with the larynx through the glottis, and two saccular lungs. The wall of the buccal cavity is lined by a columnar pseudostratified epithelium and contains the salivary glands (Fig. 1B). The epithelium rests on a thick layer of dense connective tissue

TABLE 1
ANTISERA USED IN THIS STUDY

Antisera raised to	Source (code)	Dilution
7B2	RPMS[a] (2012)	1:10000
Chromogranin	RPMS (1285)	1:500
NSE (neuron-specific enolase)	RPMS (1735)	1:500
PGP 9.5 (protein gene product 9.5)	RPMS (1648)	1:1000
Serotonin	Incstar[b] (20080)	1:30000
Serotonin	RPMS (645)	1:4000
Histamine	Peninsula[c] (61069)	1:1000
CGRP (calcitonin gene-related peptide)	RPMS (1204)	1:4000
Calcitonin	RPMS (272)	1:1000
Substance P	RPMS (1651)	1:500
Bombesin	RPMS (627)	1:2000
GRP (gastrin-releasing peptide)	RPMS (1750)	1:500
VIP (vasoactive intestinal peptide)	Incstar (20077)	1:500
VIP	RPMS (128)	1:500
VIP	RPMS (925)	1:500
VIP	RPMS (652)	1:500
PHI (peptide histidine–isoleucine)	RPMS (889)	1:3000
PHI	RPMS (938)	1:500
Helodermin	RPMS (1417)	1:1000
CCK (cholecystokinin)	Incstar (20078)	1:500
CCK	RPMS (1686)	1:1000
Somatostatin	Incstar (20067)	1:500
Somatostatin	RPMS (1460)	1:500
Somatostatin	RPMS (1082)	1:500
Leu-Enk (Leu–enkephalin)	RPMS (774)	1:500
Leu-Enk	RPMS (1634)	1:500
Met-Enk (Met–enkephalin)	RPMS (1657)	1:500
Met-Enk	Incstar (20065)	1:500
Big endothelin	RPMS (2086)	1:1000
ET1 (endothelin-1)	RPMS (2020)	1:500
ET1	RPMS (2092)	1:1000
ET2 (endothelin-2)	RPMS (2015)	1:1000
ET3 (endothelin-3)	RPMS (2008)	1:1000

Note. All the antisera were raised in rabbits.
[a] Royal Postgraduate Medical School, Hammersmith Hospital, London, UK.
[b] Incstar Corp., Stillwater, MN, USA.
[c] Peninsula Labs, Merseyside, UK.

and an outer layer of smooth and striated muscle cells. The buccal cavity communicates with the lumen of the larynx through the glottis, which is covered by a cylindrical and ciliated bistratified epithelium (Fig. 1C). The larynx, supported by the cartilagi arytenoidea and cricoidea, connects directly with the lungs.

As in other anuran species, the lungs of *R. temporaria* are simple, sac-like structures. The interior of each lung is divided by septa into "alveolar sacs" (Fig. 2A). The apical portions of septa, oriented toward the center of the lung, are widened by the presence of smooth-muscle bundles and blood vessels that are oriented parallel to the lung wall (Fig. 2B). The inner core of the septum wall is made up of connective tissue and also has single or grouped smooth-muscle cells. Both sides of this central region of the septa are occupied by a capillary network covered by the respiratory epithelium composed

TABLE 2
ANTIGENS

Antigens	Source (code)	Dilution[a]
7B2	RPMS[b] (2012)	0.1
Serotonin	Sigma[c] (H5755)	10
CGRP	Sigma (C4904)	1
Tirocalcitonin	Sigma (T3535)	10
Substance P	Sigma (S6883)	20
Bombesin	Sigma (B5508)	1
PHI	Sigma (P5048)	20
Neurotensin	Peninsula[d] (7351)	10
CCK	Sigma (C2901)	10
WMDF NH_2	Sigma (T6515)	10
ET1	Peninsula (6901)	20

[a] Nanomoles of its respective antigen per milliliter of optimally diluted antiserum.
[b] Royal Postgraduate Medical School, Hammersmith Hospital, London, UK.
[c] Sigma Chemical Co., St. Louis, MO, USA.
[d] Peninsula Labs, Merseyside, UK.

of flattened pneumocytes. The apical expansion of the septa is also covered by respiratory epithelium interspersed with areas of pseudostratified ciliated epithelium with mucous and endocrine cells (Fig. 3). In the saccular lung the endocrine cells are found exclusively in the latter epithelium.

By means of silver staining and immunocytochemical techniques, endocrine cells have been found in the epithelium of all regions of the respiratory system, from the buccal cavity to the lungs. These endocrine cells appeared generally as isolated single cells but in the lung they also formed clusters of 5–20 cells called neuroepithelial bodies (NEBs).

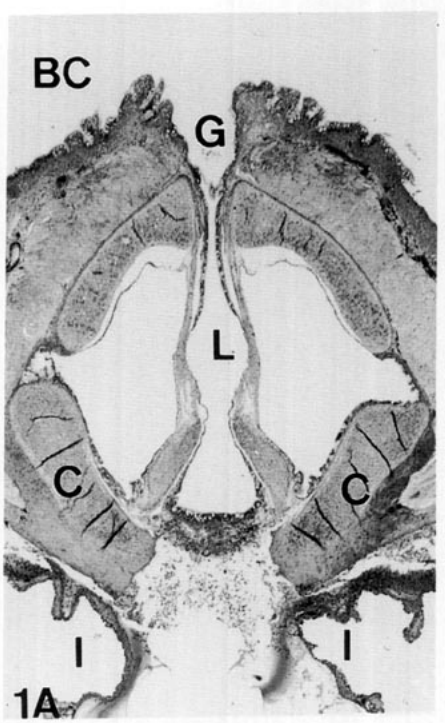
The Grimelius silver nitrate technique revealed some isolated argyrophilic endocrine cells and nerve fibers (Figs. 3 and 4) throughout the respiratory system. In contrast, numerous cells, apparently belonging to different cell types, can be observed by immunocytochemical techniques using several antibodies directed to mammalian regulatory factors. The results of the immunocytochemical tests are summarized in Table 3.

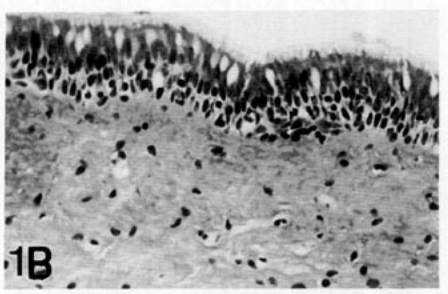
In the epithelium of the buccal cavity a relatively large number of cells immunoreactive for serotonin (Fig. 5) and bombesin (Fig. 6) antisera were observed. Peptide histidine isoleucine (PHI) (Fig. 7)- and cholecystokinin (CCK) (Figs. 8 and 9)-immunoreactive cells were also present in smaller numbers within this epithelium. In most cases the endocrine cells were in close contact with the epithelial basal lamina; some of them appeared round or oval-shaped (Figs. 5, 6, and 8), whereas others were large with the apex reaching the lumen (Fig. 9) or showed basal cytoplasmic processes (Fig. 8). Some nerve fibers and nerve-cell bodies could be demonstrated by immunostaining with an antibody against the mammalian neuroendocrine general marker protein gene product 9.5 (PGP 9.5) (Fig. 10). Numerous sub- or intraepithelial calcitonin gene-related peptide (CGRP)- and PHI-immunoreactive nerves (Figs. 11 and 12) with prominent varicosities were also observed in this region. Some nerve trunks positive for CGRP and serotonin were located in the submucosa (Fig. 13). A few nerves with CCK-like immunoreactivity were also identified in the vessels of the buccal cavity (Fig. 14).

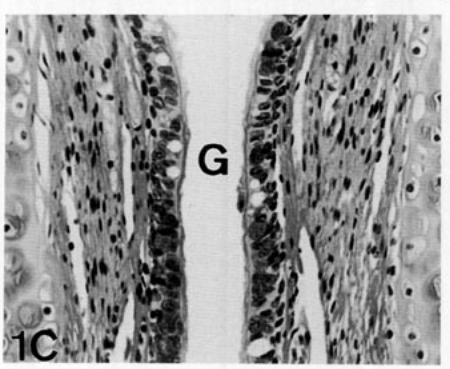
In the bistratified ciliomocous epithelium of the glottis, 7B2- and ET1-immunoreactive cells were observed (Figs. 15 and 16). Some of the endocrine cells of the glottis epithelium were also immunoreactive for chromogranin (Fig. 17). No immunoreactive nerve fiber was observed in this region with the antibodies used in the present study.

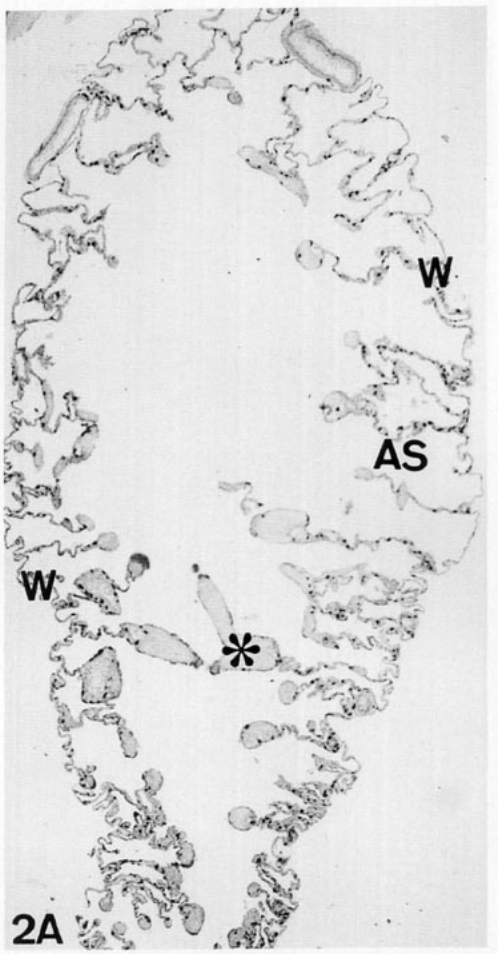
In the larynx, chromogranin-, substance P-, bombesin-, and ET1-immunoreactive cells were observed (Figs. 18–21). The neural elements found in the larynx consisted mainly of numerous CGRP-immunoreactive subepithelial nerve fibers (Fig. 22). A few other nerve fibers immunoreactive for substance P were also detected (Fig. 23).

In the saccular lungs, endocrine cells occur either individually or in groups forming NEBs, which are located only in the most apical portions of the septa. NEBs are made up of 2–20 cylindrical cells arranged in oval groups, generally only one cell layer thick. NEBs are usually covered by a thin layer of flat epithelial









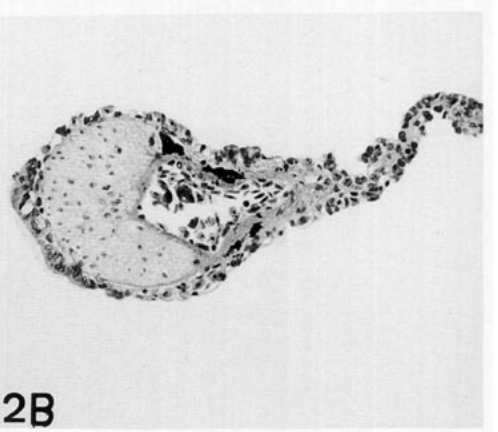


FIG. 1. (A) Low-power photomicrography of the main elements of the respiratory system of *Rana temporaria.* BC, buccal cavity; G, glottis; L, larynx; C, cartilagi; I, lungs. (B) Detail of the epithelium of the buccal cavity. (C) Detail of the glottis (G). Hematoxylin–eosin (×15; ×150; ×150). FIG. 2. (A) Longitudinal section of the lung of *Rana temporaria.* The connective septa arising from the external wall (W) of the lung are widened by smooth-muscle bundles (asterisk) at their apical part. AS, alveolar sacs. PAS (×15). (B) Detail of one of the septa of the saccular lung. Hematoxylin–eosin (×125).

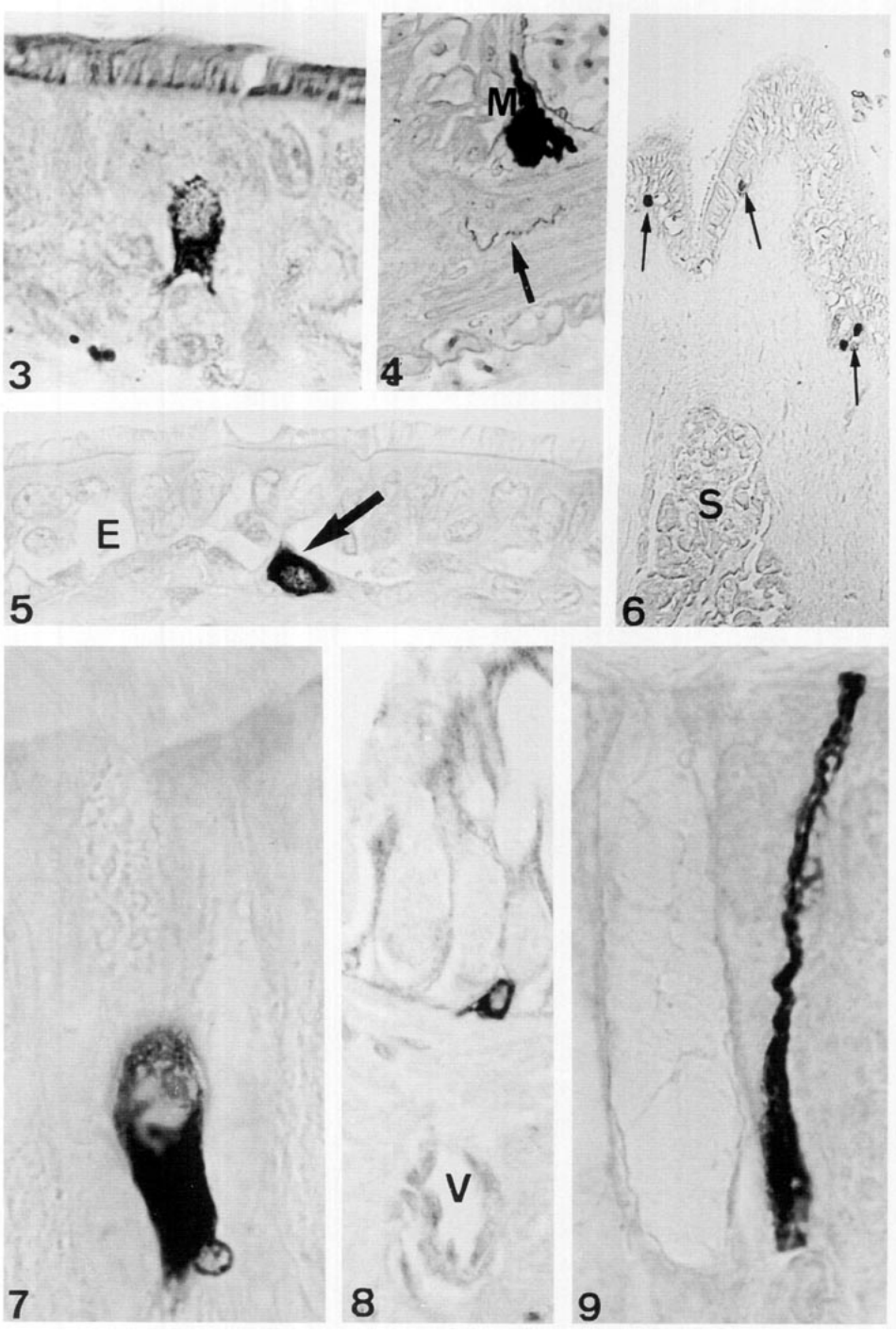


FIG. 3. Argyrophilic endocrine cell in the pseudostratified ciliated epithelium of the apical portion of a lung septum. Grimelius stain (×1500). FIG. 4. Argyrophilic nerve (arrow) in the central region of a septum. M, melanin. Grimelius stain (×400). FIG. 5. Serotonin-immunoreactive cell (arrow) in the epithelium of the buccal cavity (E). ABC technique (×400). FIG. 6. Several bombesin-immunoreactive cells (arrows) in the epithelium of the buccal cavity. S, salivary glands. ABC technique (×150). FIGS. 7–9. PHI (Fig. 7)- and CCK (Figs. 8 and 9)-immunoreactive cells in the epithelium of the buccal cavity, one of them showing an apical cytoplasmic process (Fig. 9). V, blood vessel. ABC technique (Fig. 7, ×1500; Fig. 8, ×650; Fig. 9, ×1500).

TABLE 3
FREQUENCY AND DISTRIBUTION OF THE IMMUNOREACTIVITY IN THE LUNG OF *Rana temporaria*

Antisera	Bucal cavity	Glottis	Larynx	Lung
7B2		Cells*		NEBs***
Chromogranin		Cells*	Cells*	Cells**
PGP 9.5	Nerves*			Nerves*
	Neurons*			
Serotonin	Cells***			Cells***
	Nerves*			NEBs***
				Nerves*
CGRP	Nerves**		Nerves**	Nerves***
Calcitonin				Cells*
Substance P			Cells*	
			Nerves**	
Bombesin	Cells***		Cells*	Cells**
PHI	Cells**			Cells**
	Nerves***			Nerves***
Helodermin				Nerves*
CCK	Cells***			Cells**
	Nerves*			
ET1		Cells*	Cells*	Cells*

Note. The frequency of immunoreactivity was classified according to the following scale: ***numerous; **moderate number; *few.

cells. In the lung, immunostaining with antibody against serotonin showed a positive reaction both in single cells and in NEBs (Figs. 24–26). Serotonin-immunoreactive single cells can be either of the close (Fig. 24) or of the open (Fig. 25) type, depending on the presence of direct cytoplasmic connection with the luminal space. 7B2 immunostaining was observed only in NEBs (Fig. 27), while the several types of single cells were immunoreactive for chromogranin (Fig. 28), calcitonin (Fig. 29), bombesin (Fig. 30), PHI (Figs. 31 and 32), CCK (Fig. 33), and ET1 (Fig. 34). The lungs of *R. temporaria* also contained numerous CGRP- and PHI-immunoreactive nerves (Figs. 35–37) CGRP-immunoreactive nerve fibers were located mainly in close contact with the pseudostratified ciliated or respiratory epithelium, whereas PHI-immunoreactive fibers were found mainly in relation to the muscle cells of the septa. PGP 9.5-immunoreactive fibers were found mainly near the mesothelium. Nerve-cell bodies of the lung were negative for this marker (Fig. 38). Serotonin-immunoreactive nerve trunks were also detected (Fig. 39). Immunostaining with antibody against helodermin showed a positive reaction in subepithelial nerve fibers (Fig. 40).

Absorption tests (Fig. 35) confirmed the specificity of the antisera, the immunoreactivity of each antiserum being abolished by the addition of the corresponding antigen. Antisera against CCK gave a negative result when absorbed both with CCK and with the tetrapeptide WMDF amide.

No immunoreactivity was found after the application of antisera against histamine, GRP, VIP, somatostatin, Leu-Enk, Met-Enk, big endothelin, ET2, and ET3.

DISCUSSION

The present paper presents a detailed description of the diffuse neuroendocrine system of the respiratory tract of the anuran *R. temporaria* by means of classical histological techniques and immunocytochemistry with antibodies directed against mammalian general neuroendocrine markers and active factors. According to the immunocytochemical results a complex and diverse population of different endocrine cell

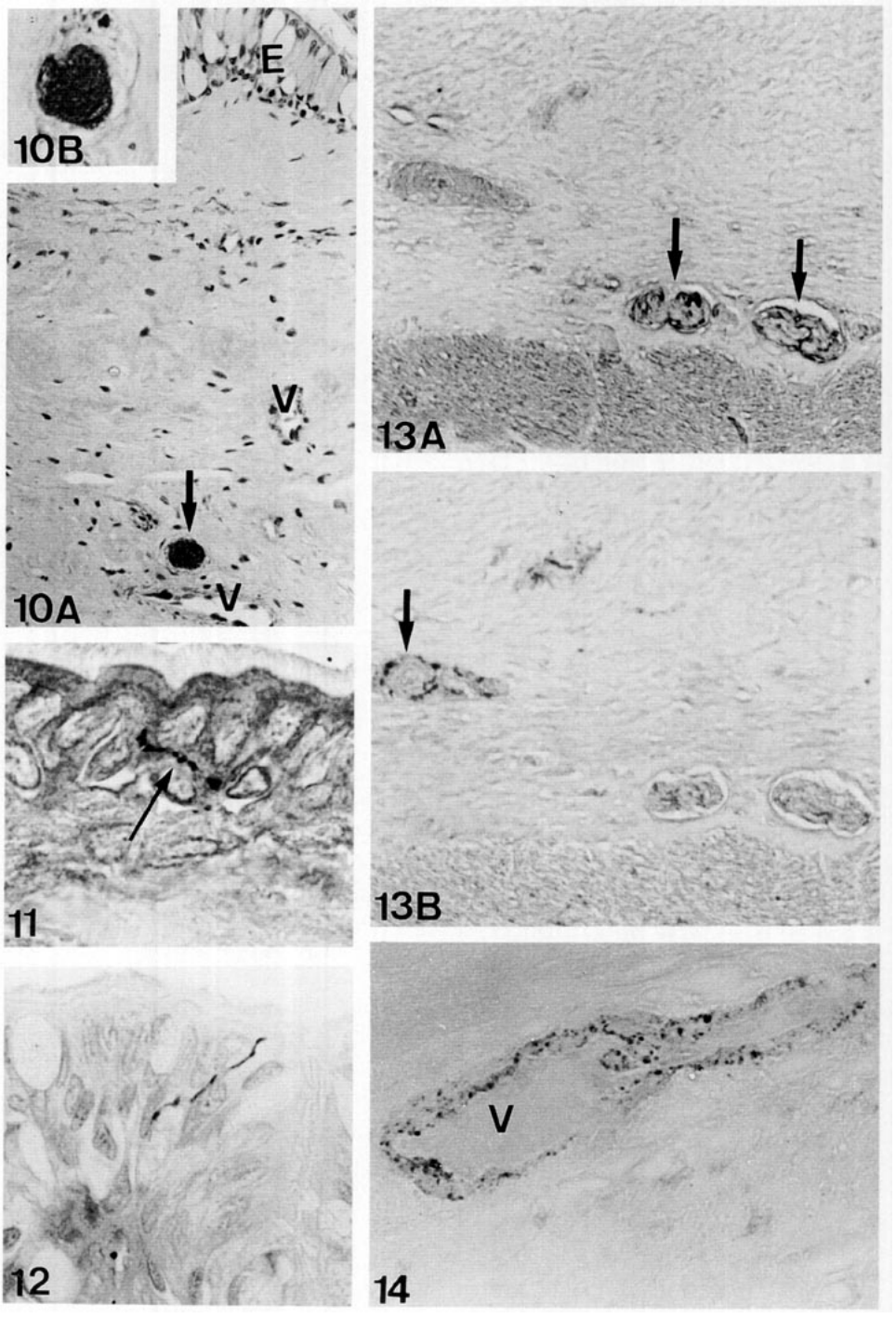
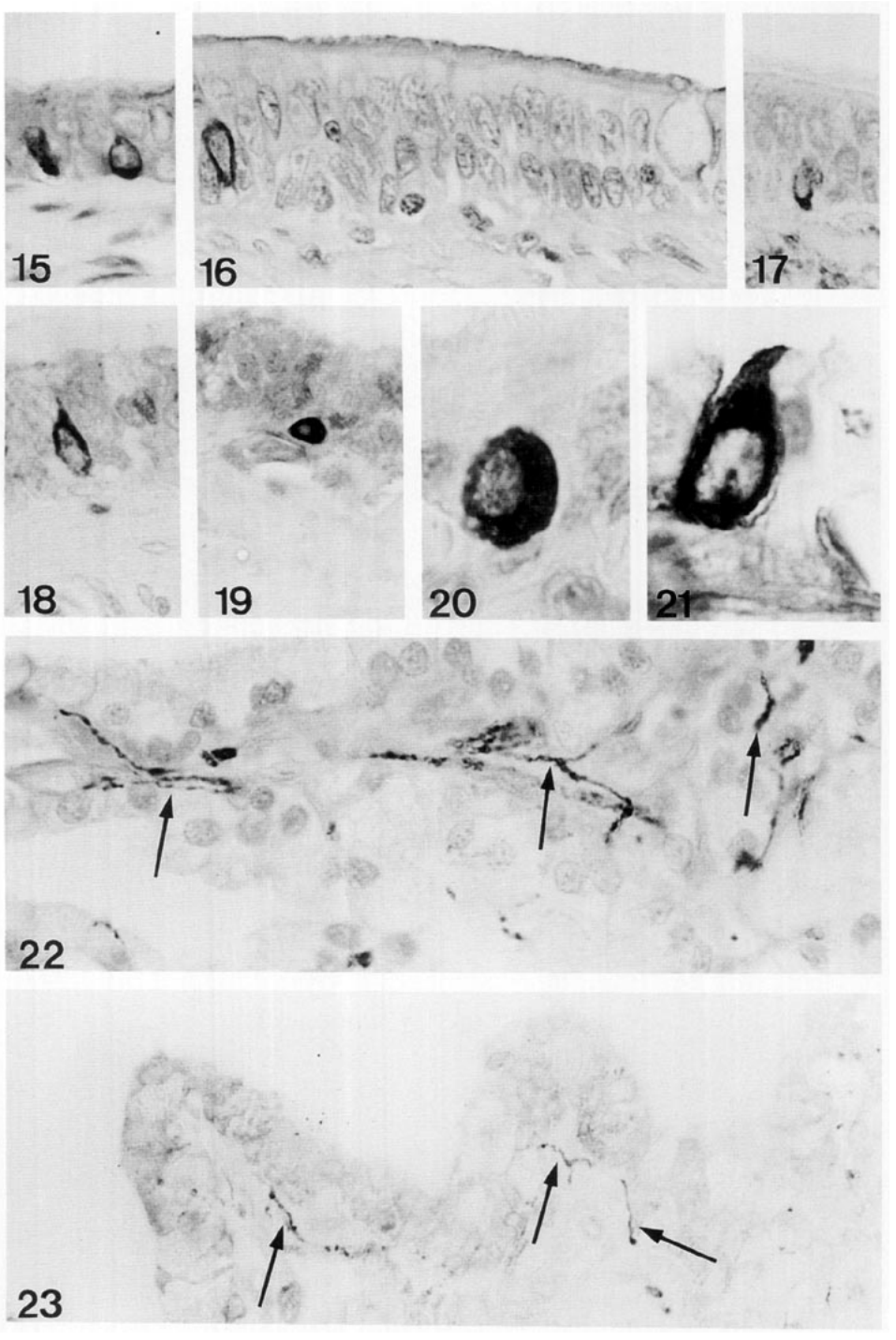
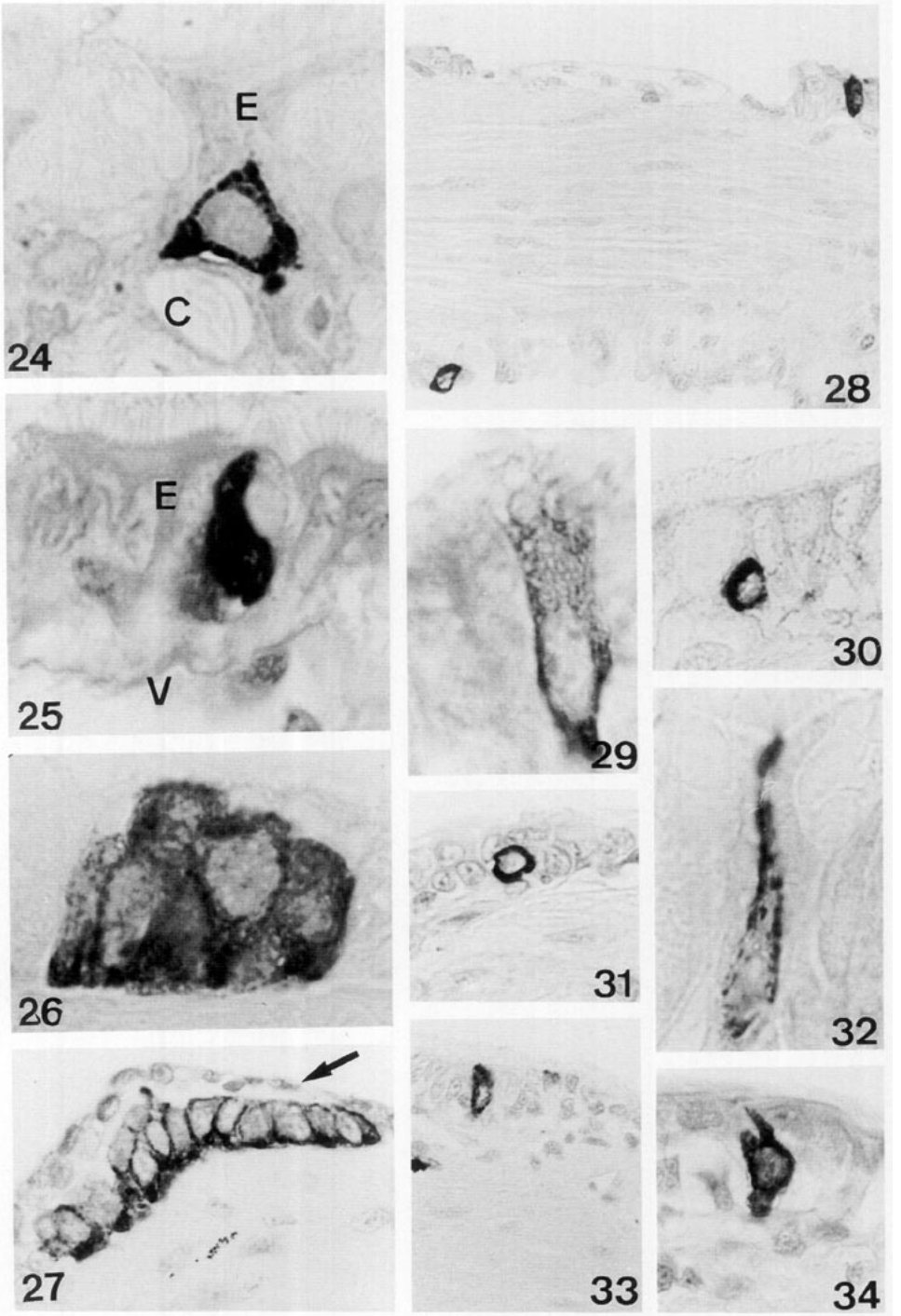


FIG. 10. (A) Microganglion located within the neural plexus of the buccal cavity in which one neuron positive for the mammalian neuroendocrine marker PGP 9.5 (arrow) is seen. (B) Detail of a PGP 9.5-immunoreactive neuron of the buccal cavity. E, epithelium; V, blood vessels. ABC technique (A, ×150; B, ×400). FIGS. 11 AND 12. CGRP (arrow, Fig. 11)- and PHI (Fig. 12)-immunoreactive intraepithelial nerves in the buccal cavity. ABC technique (×650). FIG. 13. Serial adjacent sections of the buccal cavity wall immunostained with anti-serotonin and anti-CGRP. The particular nerve fibers positive for each antisera within the nerve trunks obliquely sectioned are different (arrows). ABC technique (×200). FIG. 14. CCK-immunoreactive nerve fibers innervating a blood vessel (V) in the buccal cavity. ABC technique (×650).



FIGS. 15–17. 7B2 (Fig. 15)-, endothelin (Fig. 16)-, and chromagranin (Fig. 17)-immunoreactive cells in the bistratified ciliomucous epithelium of the glottis. ABC technique (×650). FIGS. 18–21. Chromogranin (Fig. 18)-, substance P (Fig. 19)-, bombesin (Fig. 20)-, and endothelin (Fig. 21)-immunoreactive cells in the epithelium of the larynx. ABC technique (Figs. 18 and 19, ×650; Figs. 20 and 21, ×1500). FIGS. 22 AND 23. CGRP (Fig. 22)- and substance P (Fig. 23)-immunoreactive subepithelial nerve fibers (arrows) in the larynx. ABC technique (×650).



FIGS. 24 AND 25. Serotonin-immunoreactive cell in the ciliomucous epithelium (E) of the lung (Fig. 24). Figure 24 corresponds to a semithin section of plastic-embedded material. A similar serotonin-immunoreactive cell shows an apical cytoplasmic process toward the lung lumen (Fig. 25). They are located near capillaries (C) or other blood vessels (V). ABC technique (×1500). FIGS. 26 AND 27. Serotonin (Fig. 26)- and 7B2 (Fig. 27)-immunoreactive neuroepithelial bodies (NEBs) in the septa of the lung. The NEBs are covered by flat nonendocrine epithelial cells (arrow). ABC technique (×650). FIGS. 28–34. Chromogranin-, calcitonin (Fig. 29)-, bombesin (Fig. 30)-, PHI (Figs. 31 and 32)-, CCK (Fig. 33)-, and endothelin (Fig. 34)-immunoreactive isolated cells in the epithelium of the septa of the lung. ABC technique (Figs. 28, 30, and 33, ×400; Figs. 29, 32, and 34, ×1500; Fig. 31, ×650).

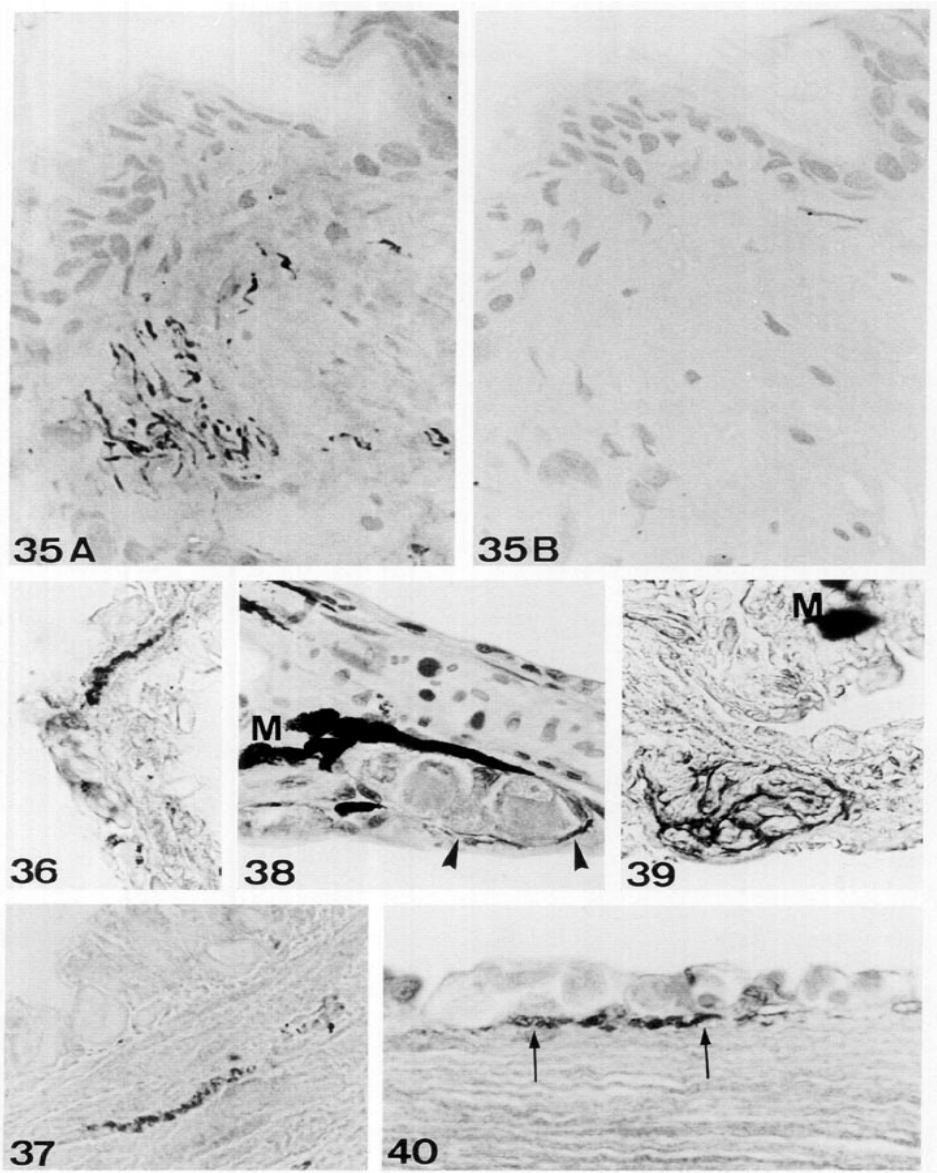


FIG. 35. (A) CGRP-immunoreactive nerve fibers in the connective layer of the apical portions of the septa. (B) Absorption control. Semithin sections of plastic-embedded material. ABC technique (×650). FIGS. 36 AND 37. CGRP (Fig. 36)- and PHI (Fig. 37)-immunoreactive nerve in the central region of the septum of the lung. ABC technique (×650). FIG. 38. Positive PGP 9.5-immunoreactive nerve fibers (arrowheads) and two negative nerve-cell bodies in the lung. M, melanin. ABC technique (×400). FIG. 39. Serotonin-immunoreactive nerve trunk in the wall of the lung. M, melanin. ABC technique (×400). FIG. 40. Helodermin-immunoreactive subepithelial nerve fibers (arrows) in the septa of the lung. The fibers run under the respiratory epithelium composed of pneumocytes and a capillary network. ABC technique (×650).

types and nerve fibers is present in the four regions of the respiratory tract of *Rana* studied. Differences in the tissue distribution of neuropeptide-containing fibers have already been described in mammals and are related to their role in the regulation of several functions such as bronchial and vascular tone or exocrine gland secretion (Martling, 1987; Martling *et al.*, 1990). The differences in the distribution of endocrine cells and nerve fibers shown in the studied regions of frog respiratory tract could be related to the diverse functional role of the oral mucosa, the airways, and the lung.

The present paper is the first report of endocrine cells in the epithelium of the oral mucosa, the glottis, and the larynx of amphibians. The literature available on the elements of the diffuse endocrine cells of the respiratory system of amphibia is still scarce and is related mainly to the isolated cells or the NEBs, which can be found in the saccular lungs of anura (Wasano and Yamamoto, 1978; Rogers and Haller, 1978, 1980; Goniakowska-Witalinska, 1980a, 1981; Goniakowska-Witalinska and Cutz, 1990; Goniakowska-Witalinska *et al.*, 1990; Saldise *et al.*, 1992) or urodela (Goniakowska-Witalinska, 1982; Matsumura, 1985; Scheuermann *et al.*, 1989; Goniakowska-Witalinska *et al.*, 1992; Adriaensen *et al.*, 1994). Recently, a study of the presence of sensory neuropeptides in the pharynx of a frog has been reported (Kusakabe *et al.*, 1995). In our study, isolated endocrine cells immunoreactive to several antibodies against mammalian regulatory factors were found for the first time throughout the whole respiratory tract, including the buccal cavity, glottis, larynx, and lung. NEBs could be found only within the respiratory epithelium of the saccular lung, at the apical regions of the lung septa. This apical localization of the NEBs is also common in the other amphibian species studied. In mammals, only isolated endocrine cells, but not NEBs, are found in the epithelium of the trachea and main extrapulmonary bronchi (Sorokin and Hoyt, 1989). Mammalian NEBs are present only in the intrapulmonary airways, mainly at their branching points (Hoyt *et al.*, 1982).

Although we have carried out a careful control to ensure specificity of the reactions, a methodological limitation is linked to our immunocytochemical study due to the use of antibodies directed to molecules isolated from mammals. At present several regulatory peptides that are structurally and functionally related to equivalent mammalian peptides have been extracted, isolated, and characterized from amphibians. The great similarity of the amino acid sequences among amphibian and mammalian related peptides (Pollock *et al.*, 1988; Johnsen and Rehfeld, 1992) and the cross-reactivity of polyclonal antibodies directed against these peptides give support to the immunocytochemical approach that we have used. In any case, our study shows that, according to the immunocytochemical reactivity, a complex system of different endocrine cell types is present in the respiratory tract of *Rana.*

Several active amines and peptides have been shown to be present in the mammalian lung endocrine cells and NEBs. Among them, serotonin, calcitonin, CGRP, and bombesin (gastrin-releasing peptide) are the most commonly reported (Springall *et al.*, 1991). Considerable variation has been found between mammalian species in terms of the presence and/or abundance of these factors within the endocrine cells (Polak *et al.*, 1993). Other peptides found in mammalian airway endocrine cells are Leu–enkephalins, somatostatin, CCK, endothelin, helodermin, etc. (Polak *et al.*, 1993; Adriaensen and Scheuermann, 1993; Cardell *et al.*, 1993).

Serotonin was the first active product that was demonstrated to be present in lung endocrine cells (Lauweryns *et al.*, 1973). The presence of serotonin in amphibian lung endocrine cells was previously shown in other amphibian species (Rogers and Haller, 1978; Wasano and Yamamoto, 1978; Goniakowska-Witalinska, 1980b; Cutz *et al.*, 1986; Scheuermann *et al.*, 1989; Goniakowska-Witalinska and Cutz, 1990; Saldise *et al.*, 1992; Adriaensen *et al.*, 1994). The function of the serotonin produced by these cells is still a matter of debate (Hoyt *et al.*, 1993; Gosney, 1993). In the case of *R. temporaria,* serotonin is produced not only by isolated and grouped cells of the lung, but also by some of the endocrine cells of the buccal cavity. It is

also present in nerve fibers of both the buccal cavity and the saccular lung. The presence of serotonin within nerve fibers was not reported in previous studies carried out in amphibians (Cutz *et al.*, 1986). In mammals, some of the motoneurons that innervate the upper respiratory muscles are in close proximity to serotonin-immunoreactive fibers (Holtman, 1988; Jiang *et al.*, 1991). It has been postulated that serotonin and substance P may have a general role in influencing respiratory motor outflow in mammals (Holtman, 1988).

It is well established that lung endocrine cells of mammals store and release calcitonin and/or CGRP. In the thyroid parafollicular cells, CGRP has been shown to be a product of alternative splicing of the calcitonin messenger. The immunocytochemical studies carried out previously in the lung of amphibians have reported negative (Cutz *et al.*, 1986) and positive (Adriaensen *et al.*, 1994; Gomi *et al.*, 1994) results with antibodies against calcitonin. In *R. temporaria,* the calcitonin-immunoreactive cells are found exclusively as isolated cells within the respiratory epithelium of the saccular lung. As is the case in other amphibian species studied, no immunoreactivity was found in endocrine cells when using antibodies against mammalian CGRP that labeled nerve fibers in the same sections. Interestingly, cells and NEBs immunoreactive for CGRP and calcitonin have been reported in the lung of several species of reptiles (Ravazzola *et al.*, 1981; Van den Steen *et al.*, 1994; Beorlegui *et al.*, 1994a, b), with partial colocalization of calcitonin and CGRP in the same cells (Beorlegui *et al.*, 1994b). In the lung of birds, CGRP-immunoreactive endocrine cells have also been reported (López *et al.*, 1993). This is the first report of CGRP-immunoreactive nerve fibers in the whole respiratory tract (buccal cavity, larynx, and lung) of amphibians. Very recently Kusakabe *et al.* (1995) showed its presence in nerve fibers of the lung and the pharynx. In the mammalian lung, CGRP is present in sensory intra- and subepithelial nerves and is related to the process of neurogenic inflammation with vasodilation, extravasation, and edema of the lung (Martling, 1987; Kowalski *et al.*, 1989). In the present study, CGRP-immunoreactive nerve fibers have been found mainly in relation to or within the epithelium of the buccal cavity, the larynx, and the lungs. The intra- and subepithelial location of these nerve profiles supports the hypothesis that CGRP-immunoreactive nerves in amphibia also have a sensory role, at least in the regions studied. The lack of CGRP-immunoreactive fibers in the glottis is difficult to interpret and could probably be due to the fact that this region has very specific functions that are not directly related to gas exchange but to phonation and inspiration of air. The other peptide related to sensory innervation in the mammalian lung is substance P (Lundberg *et al.*, 1984). In *R. temporaria* substance P-immunoreactive fibers have been found only in the larynx, while the lungs of other amphibian species studied contain numerous substance P-immunoreactive nerve fibers, located mainly in the submucosa of the ciliated epithelium (Cutz *et al.*, 1986). The interspecific variation concerning the presence or absence of particular peptides that has already been stressed in the case of mammals (Polak *et al.*, 1993) seems also to be applicable at inferior phylogenetic levels.

Bombesin is a 14-amino-acid peptide that was isolated from the skin of the amphibian *Bombina* sp. (Anastasi *et al.*, 1971). Bombesin was the first peptide that was shown to be present in the mammalian lung endocrine cells (Wharton *et al.*, 1978). The human counterpart of amphibian bombesin is gastrin-releasing peptide (GRP), a 27-amino-acid peptide that has high homology with bombesin at its C-terminus (McDonald *et al.*, 1979). In amphibia, bombesin-immunoreactive cells had been reported so far only in the lung of three species of urodela, *Triturus alpestris* (Cutz *et al.*, 1986), *Cynops pyrrhogaster* (Adriaensen *et al.*, 1994), and *Hynobius nebulosus tokyoensis* (Gomi *et al.*, 1994), and in one species of anura, *Bufo bufo* (Saldise *et al.*, 1992). In several other species of anura studied, bombesin or GRP-like immunoreactive cells apparently were absent (Cutz *et al.*, 1986). In *R. temporaria,* we have demonstrated the presence of bombesin-immunoreactive endocrine cells not only in the lung, but also in the epithelium of the larynx and the oral

cavity. The bombesin-like cells that we have found in the airways are of the closed type, i.e., they do not reach the lumen by apical cytoplasmic processes. This is also the case for the bombesin-immunoreactive cells that have been found in the gut of *Rana* (Díaz de Rada *et al.*, 1987). Several reports support a possible involvement of the bombesin released by lung endocrine cells in the proliferation and differentiation of the neighboring cells (Sunday *et al.*, 1993; Speirs *et al.*, 1993).

In our study we have found a number of cells immunoreactive for CCK that are present in the buccal cavity and in the lungs, but not in the conducting airways. A few single cells with CCK-like immunoreactivity were previously reported in the lung of *Hyla arborea,* but not in other amphibian species studied (Cutz *et al.*, 1986). The synthesis and release of CCK by hamster lung endocrine cells have recently been reported by Wang and Cutz (1993). It has been suggested that CCK could be involved in a variety of functions, including the regulation of lung development and growth (Wang and Cutz, 1993). Endothelin-like immunoreactive cells were found in the glottis, larynx, and lung of *R. temporaria.* A subpopulation of the endocrine cells of the lung of rats is also immunoreactive against endothelin (Seldeslagh and Lauweryns, 1993).

Whereas there are several reports of VIP-immunoreactive neurons in the gut of anurans (Davies and Campbell, 1994; Osborne and Gibbins, 1988; Adriaensen *et al.*, 1994), neither we nor other groups have found VIP-like immunoreactivity in the intrinsic neurons of the lung of the frog. In mammals, VIP is considered to be one of two possible neurotransmitters for the nonadrenergic noncholinergic relaxatory innervation, together with the recently discovered gas nitric oxide (NO). In the intrinsic neurons of the gut of amphibians, VIP and the enzyme responsible for NO synthesis, NO-synthase (NOS), are colocalized. Most (if not all) of the intrinsic neurons of the lung of *R. temporaria* are immunoreactive for NOS (Bodegas *et al.*, 1994), but none of them seem to store VIP. NOS-like immunoreactivity has also been reported in cell bodies and nerves of the lung of the urodele *C. pyrrhogaster* (Adriaensen *et al.*, 1994). In this species VIP colocalized with NOS only in some nerve fibers but not in cell bodies. These findings support the hypothesis that in the respiratory tract of amphibians NO is the main NANC neurotransmitter.

This study provides clear evidence that the respiratory tract of *R. temporaria* contains chemically distinct populations of endocrine cells and nerve fibers, similar to the situation found in the airways of mammals. The different distributions of these cell populations throughout the respiratory tract suggest that the neuroendocrine system in the frog is highly specialized and that it has distinct roles in the several regions that compose the amphibian respiratory tract.

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