An Immunohistochemical Study of Chromaffin Cells and Nerve Fibers in the Adrenal Gland of the Bullfrog, *Rana catesbeiana*

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Summary. Histochemical and immunohistochemical studies of the adrenal gland of bullfrogs gave the following results.

1. The occurrence of two types of cells, intensely and weakly chromaffin cells, was demonstrated by a dichromate-chromate fixation. The former cells corresponded to strongly argentaffin cells in Masson-Hamperl's method and were regarded as noradrenaline-containing cells. The latter cells corresponded to weakly argentaffin cells and were identified as adrenaline-containing cells.

2. Enkephalin- and serotonin-like immunoreactivities were found in the adrenal chromaffin cells. The combination of immunohistochemistry and fluorescence histochemistry indicated three types of cells in the adrenal gland: cells with adrenaline and serotonin; those with noradrenaline and serotonin; and those with adrenaline, enkephalin and serotonin.

3. Varicose nerve fibers with substance P-, calcitonin gene-related peptide (CGRP)-, peptide HI(PHI)- and gastrin releasing peptide (GRP)-like immunoreactivities were found on solitary or small clustered chromaffin cells. The CGRP-fibers were also located among chromaffin cells forming large clusters. Neuropeptide Y(NPY)-immunoreactive fibers, as well as the other four fibers, were seen around the small arteries and arterioles supplying the adrenal gland.

4. Double immunostaining for substance P and CGRP demonstrated the occurrence of nerve fibers with three kinds of immunoreactivities in the adrenal gland: fibers with both substance P- and CGRP-like immunoreactivities; those with substance P-like immunoreactivity without CGRP-like immunoreactivity; and those with CGRP-like immunoreactivity without substance P-like immunoreactivity. Double staining for substance P and CGRP also showed the presence of dorsal root ganglionic neurons which contained the three above-mentioned immunoreactivities. Thus, these neurons are suggested to supply the adrenal gland.

5. In the paravertebral sympathetic ganglia, most small neurons were immunopositive for NPY. These neurons were presumed to supply the small arteries and arterioles in the adrenal gland.

The adrenal chromaffin cells have long been regarded as merely involved in secreting amines, especially adrenaline and noradrenaline. Recent immunohistochemical studies, however, have shown that the cells synthesize, store and release a variety of bioactive peptides such as enkephalins (Schultzeberg et al., 1978; Linnoila et al., 1980; Pelto-Huikko et al., 1982; Livett et al., 1982; Kobayashi et al., 1983a), substance P (Kuramoto et al., 1985b), neuropeptide Y (NPY) (Varndell et al., 1984; Kuramoto et
al., 1986; Lundberg et al., 1986; review; Sundler et al., 1986), vasoactive intestinal polypeptide (VIP) (Bryant et al., 1976; Holzwarth 1984; Kondo et al., 1986), somatostatin (Lundberg et al., 1979), neurotensin (Lundberg et al., 1982a) and calcitonin gene-related peptide (CGRP) (Rosenfeld et al., 1983; Kuramoto et al., 1987). In addition, most of these peptides have been revealed to be contained not only in the chromaffin cells, but also in the nerve fibers supplying them.

All of the above mentioned investigations have been made in mammals. As far as we are aware, no studies on the occurrence of bioactive peptides are available concerning submammalian adrenal glands, except for two reports in amphibians. Lebouleguer et al. (1983) have revealed that, in the adrenal gland of Rana ridibunda, most of the chromaffin cells showed VIP-like immunoreactivity, although methionin (Met)- and leucine (Leu)-enkephalin-like immunoreactivities were detected in about 40% of them. Kondo and Yui (1984) have also confirmed the presence of Met-enkephalin-like immunoreactivity in half of the total chromaffin cells in the adrenal gland of Rana catesbeiana. These two works, however, gave little immunohistochemical information regarding the neuronal components of the adrenal gland.

In the present study, we demonstrate that enkephalin- and serotonin-like immunoreactivities are present in the chromaffin cells of the bullfrog adrenal gland. Furthermore, we are first to report that the intra-adrenal nerve fibers exhibit immunoreactivities for five kinds of peptides.

**MATERIALS AND METHODS**

*Tissue preparation*
Bullfrogs (*Rana catesbeiana*, 400-600 g) of both sexes were used in this study. Under anesthesia, the animals were perfused first with a 0.68% saline solution through the heart, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). The adrenal glands, the paravertebral sympathetic ganglia (6th-10th) and the dorsal root ganglia (6th-10th) of both sides were dissected out and immersed in the same fixative for 2-4 hr. After rinsing in 0.1 M phosphate buffer for 1 hr, the tissues were soaked for 2-4 days in a 0.1 M phosphate buffer containing 30% sucrose. They were frozen in liquid nitrogen, cut in a cryostat either at 15-20 µm or serially at 4 µm and mounted on poly-L-lysine-coated glass slides.

*Fluorescence for catecholamines*
It has been established that fixation with 4% paraformaldehyde is sufficient for demonstrating noradrenaline fluorescence in the adrenal chromaffin cells of mammals (Falck and Torp, 1961; Eränkö, 1967) and frogs (Kataoka and Yamamoto, 1980; Kondo and Yui, 1984). Cryostat sections of the adrenal gland were covered with a buffered glycerin, then viewed and photographed with a Leitz fluorescence microscope. Filters of Set D were used to detect noradrenaline fluorescence. After observation, the specimens were immunostained.

*Immunohistochemistry*
Cryostat sections of tissues were incubated for 12-18 hr at room temperature with the antisera shown in Table 1. The sites of antigen-antibody reaction were detected with the peroxidase-antiperoxidase (PAP) procedure according to Sternberger (1979). The relations between different peptides and between peptides and amines in
chromaffin cells and nerves were examined by the double immunofluorescence staining method put forth by ERICHSEN et al. (1982). After being photographed for noradrenaline fluorescence, sections of the adrenal gland were treated with a mixture of rabbit anti-methionin (Met)-enkephalin antiserum and rat monoclonal anti-serotonin antiserum (YC5/45) for 3 hr at room temperature. In addition, sections of the three tissues under study were incubated with a mixture of rabbit anti-calcitonin gene-related peptide (CGRP) antiserum (RPN1842) and rat monoclonal anti-substance P antiserum (NC1/34HL) under similar conditions. These sections were then incubated for 45 min at room temperature with a mixture of FITC-labeled goat anti-rabbit IgG (Biomedical Technologies Inc., Cambridge, USA) and rhodamine-labeled goat anti-rabbit IgG (KPL, Maryland, USA). Some sections of the sympathetic ganglia were incubated with a mixture of guinea pig anti-CGRP antiserum (GP-1-5) and rabbit anti-luteinizing hormone releasing hormone (LHRH) antiserum, followed by incubation with a mixture of FITC-labeled goat anti-guinea pig IgG (KPL, Maryland, USA) and rhodamine-labeled goat anti-rabbit IgG (KPL) under the same condition as described above. The double immunostained sections were mounted in a buffered glycerin and viewed using the Leitz fluorescence microscope equipped with filter sets L2.1 and N2.1 for FITC and rhodamine fluorescence, respectively. The details of the antisera used in this study are shown in Table 1.

To check the specificity of the immunostainings, certain sections were incubated with antisera pretreated with corresponding synthetic peptides (20–50 μg/ml of the diluted antisera) for 24 hr at 4°C. In addition, anti-Met-enkephalin antiserum treated with synthetic leucine (Leu)-enkephalin and anti-Leu-enkephalin antiserum with synthetic Met-enkephalin were used in the absorption tests.

**Histochemical procedures**
The adrenal glands of freshly sacrificed bullfrogs were dissected out and fixed with a dichromate-chromate mixture solution (HILLARP and HÖKFELT, 1955) for observation of chromaffin reaction. Following post-fixation with 1% formalin, they were embed-

### Table 1. Details of antisera used for immunohistochemistry

| Antisera    | Code     | Host          | Dilution  | Source                        |
|-------------|----------|---------------|-----------|-------------------------------|
| CGRP        | RPN1842  | Rabbit        | 1:2,000   | 1:200                         |
| CGRP        | G-1-5    | Guinea pig    |           | Amersham International plc, UK |
| GRP         | R-6902   | Rabbit        | 1:3,000   | Dr. K. YAMAGUCHI              |
| PHI         | R-8201   | Rabbit        | 1:2,000   | Dr. N. YANAIHARA             |
| VIP         | R-502    | Rabbit        | 1:1,600   | Dr. N. YANAIHARA             |
| Substance P | NC1/34HL | Rat (monoclonal) | 1:100   | Sera-Lab, UK                 |
| Substance P | R-2404   | Rabbit        | 1:2,000   | Amersham International plc, UK |
| NPY         | RPN1702  | Rabbit        | 1:1,600   | UCB-Bioproducts, Belgium      |
| Leu-ENK     |          | Rabbit        | 1:1,600   | UCB-Bioproducts, Belgium      |
| Met-ENK     |          | Rabbit        | 1:1,600   | UC Biologics                  |
| Met-ENK.Arg-Arg-Leu | R-0171 | Rabbit        | 1:3,000   | Dr. N. YANAIHARA             |
| Serotonin   | YC5/45   | Rat (monoclonal) | 1:400 | Sera-Lab, UK                 |
| LHRH        |          | Rabbit        |           | Immuno Nuclear Corp., USA     |
ded in paraffin. Sections were cut and mounted on glass slides coated with gelatin. In addition, some of the tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. To examine the argentaffin reaction, Masson-Hamperl's method modified by SINGH (1964) was applied to the paraffin sections. Cryostat sections were made and stained for cholinesterase by Karnovsky's method (KARNOVSKY, 1964) to examine cholinergic elements within the adrenal gland.

RESULTS

Histochemical features of the bullfrog adrenal gland

A distinct chromaffin reaction was found in a number of cells in the adrenal gland by fixation with a dichromate-chromate solution. These chromaffin cells were generally present singly or in clusters of variable sizes (Fig. 1a). They were polygonal in shape, and many of them possessed long cytoplasmic processes. They were intermingled with summer cells showing a spherical or oval appearance and cortical lipid cells, both of which were components occupying large parts of the adrenal gland. The chromaffin cells in the adrenal gland were divided into two types on the intensity of the reaction: one type of cell was tinged dark brown and the other type cell, light brown after the dichromate-chromate fixation (Fig. 1a). The former cells tended to form large clusters, while the latter were mostly distributed solitarily. Neither the cortical lipid cells nor the summer cells showed the chromaffin reaction.

The argentaffin reaction by the Masson-Hamperl's method was positive in all of the chromaffin cells. The argentaffin cells were clearly separated into two types: one type appearing dark brown, the other faintly brown (Fig. 1b). These strongly and weakly argentaffin cells corresponded in distribution and frequency to the intensely and weakly chromaffin cells, respectively. The cytoplasm of the summer cells was stained slightly brown. No cortical lipid cells showed the argentaffin reaction.

On observation with the fluorescence microscope, noradrenaline fluorescence was found in many chromaffin cells; the cells fluoresced at variable intensities of bright green among the cells. The noradrenaline fluorescent cells often formed large clusters (Fig. 2c). Their distribution and frequency were much the same as those of the cells showing the stronger chromaffin or argentaffin reaction. In addition, most of the summer cells were recognized as showing a blue-white fluorescence (Fig. 2c). The cortical lipid cells exhibited no fluorescence.

The Karnovsky's method showed no nerve fibers positive for the cholinesterase reaction in the adrenal gland, whereas the chromaffin cells were mostly stained dark brown by the method. Neither summer cells nor cortical lipid cells showed the cholinesterase reaction.

Peptide- or amine-containing chromaffin cells in the adrenal gland

After incubation with various antisera, Met-enkephalin-, Leu-enkephalin- and serotonin-like immunoreactivities were observed in the chromaffin cells of the adrenal gland. On comparing serial sections, Met-enkephalin-immunoreactive cells completely corresponded to Leu-enkephalin-immunoreactive cells. The enkephalin-cells (Fig. 2a) occupied about 50% of the total chromaffin cells, while serotonin-cells (Fig. 2b) counted for 70-80% of them. Most of the enkephalin-cells were found solitarily or in
occasional small clusters. The serotonin-cells also were only occasionally solitary in
distribution, usually forming large clusters. The intensity of the immunoreaction for
serotonin varied from cell to cell, whereas that for enkephalin was uniform in most of
the positive cells.

Comparison of the distribution of enkephalin-like immunoreactivity, serotonin-
like immunoreactivity and noradrenaline fluorescence showed that enkephalin-like

Fig. 1. a and b. Micrographs of the bullfrog adrenal gland stained by dichromate-chromate
fixation (a) and Masson-Hamperl's method (b). a. Many noradrenaline cells reacting
in dark brown (arrows) are seen in clusters. Some adrenaline cells tinged light
brown (arrowheads) are also found in the clusters. b. Numerous noradrenaline cells
colored dark brown (arrows) are seen in a large cluster. A few adrenaline cells
tinged weak brown (arrowheads) are present in the cluster. Note summer cells
stained slightly brown (★). a, b: ×510
Fig. 2. a-c. Micrographs of the same section of the adrenal gland which was double-immunostained with the antiserum against Met-enkephalin (FITC labeling) (a) and with the antiserum against serotonin (rhodamine labeling) (b) and shown by noradrenaline fluorescence (c). A few cells (1) are non-fluorescent for noradrenaline and positive for both Met-enkephalin and serotonin. A few cells (2) are non-fluorescent for noradrenaline, negative for serotonin and positive for Met-enkephalin. Some cells (3) emit bright green fluorescence and are positive for serotonin and negative for Met-enkephalin. Note that summer cells (*) show blue-white fluorescence in c. a-c: ×580
Fig. 3. a-f. Sections of the bullfrog adrenal gland immunostained with anti-substance P (a, f), anti-CGRP (b), anti-PHI (c), anti-GRP (d) and anti-NPY (e) antisera. a. Substance P-fibers located on chromaffin cells and around an arteriole (arrow). b. Many CGRP-fibers are seen among chromaffin cells in a large cluster. c. Many PHI-fibers are present in a nerve bundle (arrows) and on chromaffin cells. d. GRP-fibers are seen on chromaffin cells, but absent from a chromaffin cell cluster (C). e. NPY-fibers run along an arteriole. No NPY-fibers are seen on chromaffin cells. f. Substance P-fibers surround the sinusoidal capillaries. a-f: $\times 260$
immunoreactivity was present in the non-noradrenaline fluorescent cells, and not in noradrenaline fluorescent cells; the serotonin-like immunoreactivity was found in most of the noradrenaline fluorescent cells and a part of non-noradrenaline fluorescent cells. Thus, both immunoreactivities were detected in a part of non-noradrenaline fluorescent cells (Fig. 2a-c). Neither summer nor cortical lipid cells displayed immunoreactivities for enkephalins and serotonin. No immunoreactivity to the other antisera which are shown in Table 1 was recognized in the chromaffin cells of the bullfrog adrenal gland.

**Immunoreactive nerve fibers in the adrenal gland**

Substance P-, CGRP-, GRP-, PHI- and NPY-immunoreactive fibers were found numerous in the bullfrog adrenal gland.

Fibers containing substance P-, CGRP-, PHI- and GRP-like immunoreactivities were varicose in profile and distributed on solitary or small-clustered chromaffin cells in the adrenal gland (Fig. 3a-d). The CGRP-fibers could be also found among those chromaffin cells forming large clusters (Fig. 3b), though here the substance P-, PHI- and GRP-fibers were scarce.

NPY-like immunoreactivity was not found in any of the fibers distributed on the chromaffin cells of the gland. This immunoreactivity could be found only in fibers supplying the small arteries and arterioles (Fig. 3e).

Numerous peptidergic type-fibers were found along the small arteries and arterioles in the adrenal gland. Among these fibers, NPY-immunoreactive fibers were most numerous in the periarterial portions. CGRP-, substance P- and GRP-immunoreactive fibers occurred with the same frequency, but they were fewer than the NPY-fibers. PHI-immunoreactive fibers were least in number in the portions. Substance P- (Fig. 3f) and GRP-immunoreactive fibers also surrounded the capillary sinusoids in the gland. The former fibers occurred more frequently than the latter. There was little relationship between the capillaries and CGRP-, PHI- and NPY-immunoreactive fibers. In the adrenal gland were larger and smaller nerve bundles, in which peptidergic type-fibers were numerous. No ganglion cells in the adrenal gland were immunopositive to all antisera shown in Table 1.

Double immunofluorescence staining for substance P and CGRP revealed that the substance P-fibers were more abundant than the CGRP-fibers. A number of the CGRP-fibers located on solitary or small-grouped chromaffin cells were immunopositive for substance P, whereas the CGRP-fibers innervating a part of the chromaffin cells in large clusters proved immunonegative (Fig. 4a, b). On the other hand, many of the substance P-fibers distributed on the chromaffin cells exhibited CGRP-like immunoreactivity, though some did not show it (Fig. 4a, b). In the areas around the small arteries and arterioles, the substance P-fibers corresponded to at least most of the CGRP-fibers (Fig. 4c, d).

**The paravertebral sympathetic ganglia**

Principal neurons of a large (40–65 µm) and a small type (20–35 µm), as well as some small intensely fluorescent (SIF) cells, compose the paravertebral sympathetic ganglia of the bullfrog. Preganglionic nerve fibers with CGRP-like immunoreactivity are located on most of the large neurons and a few of the small neurons, coiling around their axon hillock region (Fig. 5a). Details of immunohistochemical findings of the
CGRP-preganglionic fibers in the sympathetic ganglia in the bullfrog have been published elsewhere (KURAMOTO and FUJITA, 1986). Substance P-preganglionic fibers were also in close contact with the axon hillock portion of a few principal neurons in the sympathetic ganglia (Fig. 5b). Among larger and smaller nerve bundles, there was an abundance of varicose substance P-immunoreactive fibers (Fig. 5b). Although PHI-, GRP- and CGRP-immunoreactive fibers with varicosities were also found in the bundles, they were less numerous than the substance P-fibers. NPY-like immunoreactivity was localized in most of the small neuronal somata and in nerve fibers around blood vessels (Fig. 5c). In the sympathetic ganglia of bullfrogs, SIF cells were

Fig. 4. a-d. Immunofluorescent micrographs of the same section in the adrenal gland double-immunostained with the antiserum against CGRP (FITC labeling) (a, c) and with the antiserum against substance P (rhodamine labeling) (b, d). a and b. Terminals of CGRP-fibers (arrowheads) on many chromaffin cells in a cluster are negative for substance P. A few substance P-fibers (long arrows) are negative for CGRP, whereas a few nerve terminals show both immunoreactivities (short arrows). ×350. c and d. CGRP-fibers (arrowheads) located on many chromaffin cells and around an arteriole are positive for substance P. A part of CGRP-fibers show no substance P-like immunoreactivity (arrows). ×220
Fig. 5. a-d. Sections of the bullfrog sympathetic ganglion immunostained with anti-CGRP (a, d), anti-substance P (b) and anti-NPY (c) antisera. a. Numerous neurons are innervated by preganglionic fibers containing CGRP-like immunoreactivity. ×160. b. Substance P-fibers (arrows) are seen in nerve bundles. A neuron (*) is innervated by a substance P-fiber. ×210. c. A number of small neurons and perivascular fibers (arrowheads) show NPY-like immunoreactivity, whereas large neurons and SIF cells are negative for NPY. S SIF cells. ×160. d. Many CGRP-nerve terminals (arrowheads) are seen among SIF cells forming a cluster, whereas a part of SIF cells (*) are not innervated by the fibers. ×330

Fig. 6. a-d. Immunofluorescent micrographs of the same section from the sympathetic ganglion double-immunostained with the antiserum to CGRP (FITC labeling) (a) and with the antiserum to substance P (rhodamine labeling) (b), and with the antiserum to CGRP (FITC labeling) (c) and with the antiserum to LHRH (rhodamine labeling) (d). a and b. Preganglionic nerve terminals showing both CGRP- and substance P-like immunoreactivities are located on two neurons (*), whereas CGRP-fibers innervating other neurons do not exhibit any substance P-like immunoreactivity. A few of varicose substance P-fibers (arrows) are positive for CGRP, whereas many of them show no CGRP-like immunoreactivity. ×220. c and d. A few neurons (L) are innervated by nerve fibers showing LHRH-like immunoreactivity but no CGRP-like immunoreactivity, whereas CGRP-immunoreactive terminals on some neurons (C) are negative for LHRH. ×350
present singly or in variously sized clusters. CGRP-nerve terminals were also in contact with a part of the SIF cells forming clusters (Fig. 5d).

Double immunofluorescence staining for substance P and CGRP revealed that only a few of CGRP-preganglionic fibers innervating the postganglionic neurons exhibited substance P-like immunoreactivity (Fig. 6a, b). In the nerve bundles, some of the varicose substance P-fibers were immunopositive for CGRP, though many of them were immunonegative for it (Fig. 6a, b). On the other hand, LHRH-like immuno-
Fig. 7. a and b. Immunofluorescent micrographs of the same section from the dorsal root ganglion double-immunostained with the anti-substance P (rhodamine labeling) (a) and anti-CGRP (FITC labeling) (b) antisera. Many of the small and medium neurons showing substance P-like immunoreactivity are positive for CGRP, but some of them (arrows) do not exhibit CGRP-like immunoreactivity. Several large neurons (*) show weak CGRP-immunoreaction, whereas they are negative for substance P. V blood vessel. ×290
reactivity was detected in the preganglionic fibers innervating a large number of the small neurons in the sympathetic ganglia (Fig. 6d). The double immunostaining for LHRH and CGRP showed that the respective immunoreactive fibers were located on different neurons (Fig. 6c, d).

Other antisera used (Table 1) showed no immunoreactivities within the sympathetic ganglia.

The dorsal root ganglia

Neurons containing substance P- and CGRP-like immunoreactivities were found in the posterior dorsal root ganglia. The substance P-immunoreactive neurons surpassed the CGRP-neurons in number. Intense or weak immunoreaction for substance P was found in many small (20–30 \( \mu \text{m} \)) and medium (40–50 \( \mu \text{m} \)) neurons, but not in large (80–100 \( \mu \text{m} \)) ones (Fig. 7a). On the other hand, CGRP-like immunoreactivity was recognized in a number of the small and medium neurons and a few of the large neurons. The small and medium neurons showed varyingly intense CGRP-immunoreaction distributed rather uniformly in the cell, whereas the large neurons generally showed a weaker immunoreaction for this peptide (Fig. 7b).

Double immunostaining for substance P and CGRP showed that neurons exhibiting three kinds of immunoreactivities were present in the ganglia (Fig. 7a, b): a number of the small and medium neurons contained both substance P- and CGRP-like immunoreactivities; some of them with substance P-like immunoreactivity but without CGRP-like immunoreactivity; and a few of the large neurons with CGRP-like immunoreactivity but without substance P-like immunoreactivity.

Specificity controls

As a result of the absorption tests, individual immunoreactivity was completely inhibited by the antiserum preabsorbed with the respective synthetic peptide. On the other hand, Met-enkephalin-like immunoreactivity was not affected by the Leu-enkephalin-pretreated anti-Met-enkephalin antiserum, but Leu-enkephalin-like immunoreactivity was completely eliminated by the anti-Leu-enkephalin antiserum pretreated with synthetic Met-enkephalin.

DISCUSSION

Histochemical findings

Histochemical aspects of the present study enabled us to confirm the occurrence of two types of chromaffin cells in the bullfrog adrenal gland: cells stained dark brown and cells stained light brown. In Masson-Hamperl's argentaffin reaction, the former were tinged dark brown and the latter, faintly brown. In mammals, it has been established that the dark brown cells are noradrenaline-containing cells and the light brown ones, adrenaline-containing elements (review: COUPLAND, 1965b). This relation between coloration in chromaffin reaction and the kinds of amines has been clarified through in vitro experiments on the chromaffin reaction of each of the known amines (COUPLAND, 1954; HILLARP and HÖRFELT, 1955) and, more recently, on the immunocytochemical detection of phenylethanolamine-N-methyltransferase (PNMT) which occurs in the
adrenaline cell (bovine, rat; NAGATSU et al., 1974, 1979a). The relation between the kinds of amines and the colors in the chromaffin reaction should hold true also for amphibian adrenal cells, and in the bullfrog the dark brown chromaffin cells are rightly judged to be noradrenaline cells and the light brown cells, adrenaline cells. As for an immunohistochemical study of PNMT, there are two papers by NAGATSU et al. (1979a, b) in the bullfrog, but the authors regrettably do not mention the relation between the coloration of the cells and positivity for the enzyme.

In their study of *Rana nigromaculata*, NAKAI and IWASHITA (1976) demonstrated two types of chromaffin cells on the basis of the electron microscopic structures of the granules. This study by NAKAI and IWASHITA (1976), as well as other investigations on the fine structure of chromaffin granules, have suggested that, from the level of amphibia to that of mammals, adrenaline cells and noradrenaline cells can be consistently identified with the same morphological criteria (noradrenaline cells with intensely dense and variably shaped granules, adrenaline cells with less dense and mostly round granules).

The summer cells, though weakly positive in argentaffin reaction, were negative in chromaffin reaction which is known to be much less sensitive for monoamines than modern techniques such as the formaldehyde-induced fluorescence (FIF) method. In the FIF reaction, the summer cells give off a bluish fluorescence, clearly indicating the occurrence of an amine or amines. As the bluish fluorescence of the summer cells is different in color from the bright green one of the noradrenaline cells, it seems possible that the kinds of the amines contained in the cells may also be different. This suggestion may be supported by the immunohistochemical finding that the summer cells of bullfrogs are negative for catecholamine-synthesizing enzymes, such as tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) and PNMT (NAGATSU et al., 1979a, b). With regard to the amine content of the summer cells, KAWAMURA (1986) demonstrated that the summer cells emitted a blue fluorescence when the adrenal gland of bullfrogs was treated with o-phthaldialdehyde, presuming that the cells contain a small amount of histamine within the granules. On the other hand, VOLK (1972) has suggested by several histochemical techniques that the summer cells may contain renin or a renin-like substance within their granules. However, immunohistochemical or biochemical studies should be performed to corroborate the presence of these substances in the summer cells.

According to the classical view, the adrenal medulla of mammals is innervated by cholinergic preganglionic nerves from the spinal cord (review: COUPLAND, 1965b). Staining for cholinesterase is useful when demonstrating the cholinergic system within the adrenal medulla (review: COUPLAND, 1965b). The presence of the cholinergic fibers is further supported by the ultrastructural features of nerve terminals, which are characterized by the occurrence of two different populations of vesicles: an abundance of small clear vesicles with a mean diameter of 37 nm, and a few large cored vesicles with a mean diameter of 70 nm (COUPLAND, 1965a). In the adrenal gland of birds and reptiles, UNSICKER (1973, 1976) has observed that the same types of vesicles within the nerve endings on the cholinergic fibers are present in the adrenal gland of these animals. However, little information is available regarding the occurrence of cholinergic fibers in the adrenal gland of amphibians.

Although our attempt of cholinesterase staining by Karnovsky's method failed, for unknown reasons, to find positive fibers in the adrenal gland, small clear vesicles morphologically corresponding to the cholinergic vesicles in mammals, birds and reptiles can nevertheless be found within the nerve endings on the adrenal chromaffin
cells of the bullfrog by ultrastructural observations (WEIGHT and WEITSEN, 1977). Therefore, it is suggested that the chromaffin cells in the bullfrog adrenal gland are innervated by cholinergic fibers.

**Enkephalin- and serotonin-containing chromaffin cells**

The present results indicate that there are populations of the chromaffin cells containing enkephalin in the bullfrog adrenal gland. The occurrence and frequency of the enkephalin-containing cells demonstrated in the present study are in agreement with the results indicated by KONDO and YUI (1984) in the same species. LEBOULENGER et al. (1983) have shown that enkephalin-like immunoreactivity was found in about 40% of adrenal chromaffin cells of the frog, *Rana ridibunda*, and that most of the chromaffin cells were immunopositive for vasoactive intestinal polypeptide (VIP). Our finding that the enkephalin-containing cells occupied about 50% of the total chromaffin cells is similar to their finding, although we failed to detect any VIP-like immunoreactivity in the chromaffin cells. This discrepancy may be due to species differences, although one should also consider that the anti-VIP antiserum used in the present study may be different in characterization from that applied by LEBOULENGER et al. (1983). CUTZ et al. (1986) have provided evidence that the regulatory peptides in endocrine cells in the lung of amphibians vary from species to species. It thus seems also possible that the difference between species in the amphibian class may occur in the bioactive peptides of the adrenal chromaffin cells.

As compared with the peptides contained in mammalian adrenal chromaffin cells, the kinds of peptides in the cells of the bullfrog are far fewer than those in mammals. In the adrenal chromaffin cells of rats, enkephalins (SCHULTZBERG et al., 1978; KOBAYASHI et al., 1983a; KONDO et al., 1984), NPY (VARNDELL et al., 1984; KURAMOTO et al., 1986; LUNDBERG et al., 1986), substance P (KURAMOTO et al., 1985b), VIP (BRYANT et al., 1976; KONDO et al., 1986) and CGRP (ROSENFELD et al., 1983; KURAMOTO et al., 1987) have been shown to occur, while the present finding in the bullfrog reveals the presence of only enkephalins. In domestic fowls, Met-enkephalin-, Leu-enkephalin- and NPY-like immunoreactivities are found in the chromaffin cells of the adrenal gland (KURAMOTO, unpublished data). It seems, therefore, that fewer kinds of peptides occur in the adrenal chromaffin cells of submammalian vertebrates than of mammals. This may be partly accounted for by the possibility that submammalian peptides, if present, may be different in molecular structure, so that they may not be detectable with the antisera against mammalian peptides.

Through the combination of immunohistochemistry and fluorescence histochemistry, enkephalin-immunoreactive cells were found to be adrenaline-containing cells. This was further supported with the immunohistochemical finding by electron microscopy that the enkephalin-immunoreaction was confined to the secretory granules of adrenaline-type cells (KONDO and YUI, 1984). With regard to the co-existence of enkephalins and adrenaline or noradrenaline in the chromaffin cells of mammals, there are definite species differences. An enkephalin-like peptide is contained in both adrenaline- and noradrenaline-containing cells in humans, dogs, cats and rats (SCHULTZBERG et al., 1978; KOBAYASHI et al., 1983a; LINNOILA et al., 1980; review: KONDO, 1985), while the peptide is restricted to the adrenaline-containing cells in bovines and hamsters (LIVETT et al., 1982; PEHLT-BUIKKO et al., 1982). However, significance of such a difference in the distribution pattern of enkephalin in the chromaffin cells is unclear.

The results of the absorption tests using Leu-enkephalin give evidence that it is
Met-enkephalin that occurs in the chromaffin cells of the bullfrog adrenal gland. It seems doubtful whether Leu-enkephalin is contained in the chromaffin cells, since the antiserum to Leu-enkephalin showed a complete cross-reactivity with synthetic Met-enkephalin. It has been established in the adrenal chromaffin cells of bovines and humans that several kinds of enkephalins—including Met-enkephalin—are derived from a precursor, preproenkephalin A (GUBLER et al., 1981; COMB et al., 1982; NODA et al., 1982). The antiserum to Met-enkephalin-Arg-Gly-Leu, one of the derivatives from preproenkephalin A, is useful in demonstrating the presence of the precursor (KOBAYASHI et al., 1983a; KONDO et al., 1984). Since no immunoreactivity to the antiserum is, however, observed in the chromaffin cells of the bullfrog adrenal gland, we were unable to determine whether or not the precursor exists in the chromaffin cells. It seems possible that the processing of the prepropeptides for enkephalins in bullfrogs may vary from that in mammals or that the Met-enkephalin in bullfrogs is derived from another precursor altogether.

The present study also demonstrated the occurrence of serotonin in the chromaffin cells of the bullfrog adrenal gland. Similarly, an immunohistochemical study has demonstrated that serotonin-like immunoreactivity was detected in adrenaline-containing cells of the rat adrenal medulla (VERHOFSTAD and JONSSON, 1983). In addition, experiments in vivo and in vitro in mammals have given evidence for serotonin being stored within the chromaffin granules (CARLSSON et al., 1963; SLOTKIN and KERSHNER, 1971; VERHOFSTAD and JONSSON, 1983). VERHOFSTAD and JONSSON (1983) have suggested that the preferential storage of serotonin in adrenaline-containing cells may be due to the affinity of serotonin to adrenaline-granules. This affinity, however, is refuted by our data in the bullfrog adrenal gland indicating that serotonin is contained in both adrenaline and noradrenaline cells.

**Peptidergic fibers innervating the adrenal chromaffin cells**

The present study demonstrated nerve fibers containing immunoreactivities for substance P, CGRP, GRP, PHI and NPY in the adrenal gland of bullfrogs. This is the first report on the occurrence of a peptidergic nerve system in the adrenal gland of submammalian vertebrates.

The peptidergic nerves have been known in mammalian adrenal gland. Many reports are available especially in the rat adrenal gland, where nerve fibers containing immunoreactivities for enkephalins (SCHULTZBERG et al., 1978; KOBAYASHI et al., 1983b; KONDO et al., 1984; PELTO-HUIKKO et al., 1985), substance P (KURAMOTO et al., 1985b), VIP (HÖKFELT et al., 1981; HOLZWARTh, 1984; KONDO et al., 1986), NPY (VARNDELL et al., 1984; KURAMOTO et al., 1986; LUNDBERG et al., 1986) have been identified. The peptide-containing fibers in the adrenal gland of bullfrogs are comparable to those of rats in the number of kinds of peptides. However, neither the enkephalin- nor VIP-fibers seen in rats are found in bullfrogs, whereas substance P-, CGRP- and NPY-fibers are common to both species. In the case of rats, enkephalin-like immunoreactivity is found in both chromaffin cells and nerve fibers in the adrenal gland, whereas in bullfrogs it is detected in chromaffin cells only. The same situation as in the bullfrog can be encountered in the adrenal medulla of human (LINNOILA et al., 1980). Thus, it seems likely that although the occurrence of enkephalin-like immunoreactivity prevails in the adrenal chromaffin cells of many species from frogs to the human, it varies from species to species in the adrenal nerve fibers. It is unclear whether the species difference in the occurrence of enkephalin-like immunoreactivity in the adrenal
fibers is due to a different processing of enkephalin-precursors or to the presence or absence of the precursors themselves.

The present study demonstrated that numerous substance P-fibers, as well as CGRP-fibers, are distributed in the adrenal gland of bullfrogs. In addition, double immunostaining for substance P and CGRP showed certain nerve fibers containing three combinations of both immunoreactivities: fibers with both immunoreactivities; those with substance P-like immunoreactivity and without CGRP-like immunoreactivity; and those with CGRP-like immunoreactivity and without substance P-like immunoreactivity. CGRP- and substance P-fibers have been also recognized in the adrenal medulla of rats as reported elsewhere (KURAMOTO et al., 1985b, 1987). Previous immunohistochemical studies have indicated the presence of CGRP-immunoreactive neuronal somata in the rat and cat dorsal root ganglia, where many of the CGRP-neurons are simultaneously immunopositive for substance P (GIBSON et al., 1984; LEE et al., 1986). Therefore, the reasonable suggestion that CGRP- and substance P-fibers in the adrenal gland originate from the dorsal root ganglia has been raised (KURAMOTO et al., 1985b, 1987). Considering the present results in which CGRP- and substance P-immunoreactive neurons were observed within the dorsal root ganglia of bullfrogs and that no ganglion cells with their immunoreactivities were found in the adrenal gland, it may be assumed that the CGRP- and substance P-fibers in the adrenal gland are derived from the dorsal root ganglia also in the bullfrog. The presumed origin could be further supported by the finding that the double staining for substance P and CGRP demonstrated the occurrence of dorsal root ganglion cells showing the above-mentioned three combinations of immunoreactivities.

Possible functions of the neuropeptides contained in the fibers supplying bullfrog adrenal gland will be now discussed.

Substance P has been shown to inhibit the release of catecholamines from isolated chromaffin cells of bovines and the guinea pig (MIZOBE et al., 1979; ROLE et al., 1981; LIVETT et al., 1983). Whether a similar effect of this peptide occurs in the adrenal chromaffin cells of bullfrog must be determined. The effects of CGRP upon the adrenal medulla are not known, either in mammals or in submammalian animals. As substance P and CGRP are colocalized in neurons, their possible synergic actions or interactions also pose an interesting problem for future studies.

That PHI represents a part of the molecule of mammalian prepro-VIP (ITO et al., 1983) is supported by immunohistochemical findings that VIP and PHI coexist in mammalian neurons (YANAIHARA et al., 1983; LUNDBERG et al., 1984). However, our previous studies have demonstrated that PHI-immunoreactive neurons are much more numerous than VIP-neurons in invertebrates such as the cockroach and Apópsta, suggesting that PHI is not necessarily included in the prepropeptide of VIP and that PHI may exert more important regulatory functions than VIP in those invertebrates (KURAMOTO et al., 1985a, YUI et al., 1985). In the present study, PHI-like immunoreactivity was detected in the adrenal fibers and in the sympathetic ganglia of the bullfrog, whereas no VIP-like immunoreactivity was recognized in them. Here also, PHI seems to occur independently from VIP. In contrast, however, VIP-like immunoreactivity can be recognized in the neurons of the intestine of the bullfrog in coexistence with PHI-like immunoreactivity (KURAMOTO, unpublished data). The reason for this discrepancy in the occurrence of PHI and VIP in the adrenal nerves must be explored.

GRP-immunoreactive fibers have been abundantly demonstrated in this study of the adrenal gland of the bullfrog, while corresponding fibers have not been shown in
mammals. There is a report (Brown et al., 1979) on the pharmacological effect of bombesin, amphibian peptide related to GRP, upon mammalian adrenal chromaffin cells. They demonstrated that bombesin stimulates the release of adrenaline from the chromaffin cells in the rat. It remains to be examined whether a corresponding effect can be found in the amphibian adrenal gland.

**Peptidergic fibers supplying the adrenal blood vessels**

In the adrenal gland of mammals, VIP-and NPY-immunoreactive fibers have been reported to be located around the blood vessels (Hökfelt et al., 1981; Varneddell et al., 1984; Kondo et al., 1986; Kuramoto et al., 1986; review: Sundler et al., 1986), whereas in amphibians no information is available regarding peptidergic fibers in the adrenal vessels.

Abundant NPY-immunoreactive fibers have been recognized around blood vessels in various organs of mammals (review: Sundler et al., 1986). The NPY-neurons have been further found in the superior cervical, celiac and pelvic ganglia, presumably representing the origins of the perivascular NPY-fibers (Uddman et al., 1984; Lundberg et al., 1985; Mattiasson et al., 1985). In the rat adrenal gland, a number of NPY-fibers surround the medullary arteries, penetrating the cortex into the medulla (Kuramoto et al., 1986). Among submammalian vertebrates, NPY-like immunoreactivity has been demonstrated in the spinal cord of lampreys (Van Dongen et al., 1985), but no reports are available concerning the occurrence of NPY-neurons in amphibians. The present study was first to demonstrate the presence of abundant NPY-fibers in the bullfrog adrenal gland. They were not correlated to the chromaffin cells or any parenchymal cells, but exclusively surrounded the small arteries and arterioles.

The finding that ganglion cells with NPY-like immunoreactivity were absent from the adrenal gland suggests that the adrenal NPY-fibers are of extrinsic origin. Actually, most of small neurons located within the posterior sympathetic ganglia were immunopositive for NPY. Therefore, it is reasonable to presume that the adrenal NPY-fibers originate from the small neurons in the sympathetic ganglia. This assumption may be supported by the fact that the posterior splanchnic fibers arising from the sympathetic trunk lower than the 6th ganglion innervate the adrenal gland in frogs (Pick, 1970). The principal neurons within the bullfrog sympathetic ganglia are adrenergic, as indicated by immunohistochemistry using the antisera specific for catecholamine-synthesizing enzymes (Kondo et al., 1982). Thus, the adrenal NPY-fibers of bullfrogs are considered to be adrenergic.

NPY has been known to be a potent vasoconstrictor in mammals (Lundberg and Tatemoto, 1982; Lundberg et al., 1982b; review: Sundler et al., 1986). Furthermore, pharmacological experiments have revealed that the vasoconstrictive effects of adrenaline and noradrenaline are greatly enhanced by NPY (Ekblad et al., 1984). It seems reasonable to also suggest that in the bullfrog NPY might cause the constriction of smooth muscles of the adrenal arteries and arterioles to regulate the blood circulation within the adrenal gland.

Substance P and CGRP have been shown to have a potent vasodilation effect in mammals (Eklund et al., 1977; Brain et al., 1985). Likewise, PHI has similar, though weaker, action upon the blood vessels of mammals (Lundberg and Hökfelt, 1983; Lundberg et al., 1984). In the bullfrog adrenal gland, numerous substance P-fibers, most of which corresponded to CGRP-fibers, were found around small arteries and
most of which corresponded to CGRP-fibers, were found around small arteries and arterioles; here, though, PHI-fibers were scarce. Therefore, the dilation action to the adrenal arteries and arterioles of bullfrogs, if present at all, seems to depend preferentially upon substance P/CGRP-fibers rather than upon PHI-fibers. In order to verify this, comparative data regarding the potency of the vasodilation effect of substance P and CGRP and PHI upon various vessels is needed. Furthermore, the effect of GRP upon blood vessels is unknown. However, since bombesin has been shown to induce hypertension in some mammals (ERSPAMER et al., 1972), the adrenal periarterial GRP-fibers may possibly be involved in vasoconstriction.

On the other hand, substance P- and GRP-fibers were also seen around the sinusoidal capillaries in the adrenal gland. Such a distribution may suggest the possibility that substance P- and GRP-like peptides are secreted from their fibers into the sinusoids. In addition, it is possible that the substance P-fibers may transmit some sensory information from the adrenal circumstances.

The sympathetic ganglia

Taking into consideration that NPY-like immunoreactivity can be observed in most of the small neurons in the sympathetic ganglia of bullfrogs, LHRH-preganglionic fibers are located on the NPY-neurons. Therefore, it is supposed that LHRH-like peptide from the preganglionic fibers regulates the release of NPY-like peptide from the terminals of the fibers, which are believed to be derived from the NPY-neurons within the sympathetic ganglia. The finding that CGRP-preganglionic fibers are located on numerous principal neurons, mainly large neurons, in the sympathetic ganglia of bullfrogs attracts our attention. An immunohistochemical finding by JAN et al. (1980) has shown that preganglionic fibers innervating most of the small neurons (C cells) contained LHRH-like peptide, corresponding to our findings. From the double immunostaining of our previous (KURAMOTO and FUJITA, 1986) and present studies, we were able to obtain the interesting result that respective fibers with LHRH- and CGRP-like immunoreactivities were localized on different neurons. Thus, it can be expected that distinct effects are exerted by LHRH and CGRP on the different neurons. However, JAN and JAN (1982) have demonstrated that late slow excitatory postsynaptic potential (EPSP) is generated by LHRH not only in C cells, but also in large neurons (B cells), assuming that the phenomenon is due to the diffusion of LHRH released from the preganglionic fibers on C cells, to B cells. It has yet to be established whether or not such a phenomenon is in relation with certain potent actions of CGRP. The influence of CGRP on the postganglionic neurons and the interaction of CGRP and LHRH remains to be clarified.

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