**FLUOROHISTOCHEMISTRY AND ELECTRON MICROSCOPY
OF THE CHROMAFFIN CELLS IN THE ADRENAL GLAND
OF THE CLAWED TOAD, *XENOPUS LAEVIS****

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Received for publication November 1, 1982 and in revised form April 23, 1983

Chromaffin cells in the interrenal gland of clawed toad (*Xenopus laevis*) were studied by a combination of fluorescence histochemistry and electron microscopy. Microspectrofluorometrical analyses indicated that there were adrenaline(A)-containing and noradrenaline(NA)-containing chromaffin cells, both of which were scattered within the interrenal tissue. In addition, it was found that there existed a third type of chromaffin cells which emitted a primary catecholamine fluorescence. This cell type could be identified electron microscopically as small granule chromaffin (SGC) cells, based on the size of secretory granules (100 nm in diameter) which were considerably smaller than those that of NA (200 nm) or of A (250 nm) cells.

It is now well-established that chromaffin cells contain noradrenaline (NA) or adrenaline (A) in the adrenal glands of many species (1, 2, 5, 6, 9, 10, 13, 17, 18, 20). Recently, ultrastructural studies have proved the existence of small granule chromaffin (SGC) cells which can be distinguished from the NA or A cells in the adrenal medulla of mammals, birds and reptiles (5, 6, 9, 18, 20). However the SGC cells of the anuran adrenal gland have been reported only in *Rana catesbeiana* (8).

The present study aimed to identify the NA, A and SGC cells in the adrenal gland of the clawed toad (*Xenopus laevis*) as the lowest anuran, by a combination of fluorescence histochemistry and electron microscopy.

MATERIALS AND METHODS

Seventeen adult clawed toads (*Xenopus laevis*) of both sexes were used. After decapitation, the adrenal glands buried in the connective tissue ventral to the kidney were removed and quickly dissected into small pieces. For light microscopic examination, the tissue blocks were placed in Bouin-Holland's fixative, dehydrated and embedded in Paraplast. Sections of adrenal glands were stained with Mayer's hematoxylin and eosin or with lead hematoxylin. Chromaffin reaction was also

* This work was supported by Grants-in-Aids for Scientific Research from the Ministry of Education, Science and Culture, Japan (Nos. 344016 and 57770049).

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performed with a technique of Santer *et al.* (15).

For fluorescence microscopy, small blocks of fresh adrenal glands were processed according to Falck *et al.* (4). Structures of fluorescent cells in tissue sections were observed under a fluorescence microscope (Nikon) equipped with a combination of an exciting filter (IF 395–425), a dichroic mirror (DM 445), and a barrier filter (Schoett GG 455). Excitation and emission spectra of these fluorescent cells were recorded with a microspectrofluorophotometer (Nikon SPM-RFL system) (11).

In order to correlate findings obtained by fluorescence microscopy and by electron microscopy, tissue blocks were fixed overnight with 4% paraformaldehyde and 0.5% glutaraldehyde, and embedded in Araldite. Ultra-thin sections (about 0.2 μm thick) of thus fixed specimens, when mounted on reference grid meshes, could be examined by fluorescence microscopy as well as by electron microscopy (7).

For electron microscopy, tissue blocks were fixed with 2.5% glutaraldehyde for 1 hr and post-fixed with 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4) for an additional 1 hr. These tissues were dehydrated with graded ethanol and embedded in Epon-812. Semi-thin sections were stained with Richardson's methylene blue-azure II (14), or with Singh's ammoniacal silver solution (16) (argentaffin reaction). Adjacent ultra-thin sections were stained with uranyl acetate and lead citrate and observed under an electron microscope (Hitachi H-500).

RESULTS

Chromaffin cells were stained positively with lead hematoxylin and had an affinity for chromic salts (chromaffin reaction). The cells were seen in small groups (five to ten cells within each group) intermingled with adrenocortical cells which themselves were not stained with the above methods.

A fluorescence histochemical observation of the adrenal gland revealed that there were two types of chromaffin cells (Fig. 1); one emitted a strong green-yellow fluorescence (Fig. 2A, emission maximum: 480 nm) and the other a weak blue-green fluorescence (Fig. 2B, emission maximum: 470 nm). Excitation spectra of the green-yellow cells showed a major peak at 410–415 nm and a minor peak at 320–325 nm. The hydrochloric acid vapor treatment for several seconds caused a downward shift of the major peak to 370–380 nm and an augmentation of the minor peak. Additional hydrochloric acid treatment for several min made

FIG. 1. A fluorescence micrograph of weakly blue-green fluorescent (A) and intensely green-yellow fluorescent (NA) cells. $\times 700$

FIG. 2A,B. Excitation and emission spectra of green-yellow fluorescent cells (A) and of bluegreen fluorescent cells (B). cont.: before treatment with HCl vapor; 3 sec.: specimens treated for 3 seconds with HCl vapor; 3 min.: specimens treated for 3 min with HCl vapor.

FIGS. 3A,B. A A. fluorescent micrograph of a semi-thin section of specimen fixed with 4% paraformaldehyde and 0.5% glutaraldehyde. NA and SGC cells show fluorescence. B. An electron micrograph of the same section as Fig. 3A. NA and SGC cells are distinguished. A, $\times 1,400$ B, $\times 8,400$

the major peak almost disappear (Fig. 2A). On the other hand, excitation spectra of the blue-green fluorescent cells also showed a major peak at 410–415 nm and a minor peak at 320–325 nm. After the hydrochloric acid vapor treatment for only several sec, the major peak rapidly disappeared while the minor peak was augmented (Fig. 2B).

Since it was possible to observe the two types of fluorescent cells even in ultra-thin sections of resin embedded tissue, such a fluorescent cell could precisely be corresponded with granule-containing cells under the electron microscope. Thus it was confirmed that the green-yellow fluorescent cells were identical with large granule-containing cells, while the blue-green cells were the same as the small granule-containing cells (Figs. 3A, B).

In glutaraldehyde-osmium fixed specimens, semi-thin sections had been examined by light microscopy and the results were compared with the electron microscopic findings in adjacent ultra-thin sections. Cells stained dark blue with

FIGS. 4A–C. A. A semi-thin section stained with Richardson's solution. B. The same section as Fig. 4A stained with Singh's silver method. C. An electron micrograph showing NA, A and SGC cells using subsequent thin section of Fig. 4A or 4B. A, B, $\times 1,800$ C, $\times 7,800$

FIGS. 5A–C. Nerve terminals (nt) contacting with A (A), NA (B) and SGC (C) cells. These terminals (presumably cholinergic) contain many clear synaptic vesicles and large granular vesicles. $\times 25,000$

FIGS. 6A, B. A. A cholinergic nerve terminal (nt 1) and a second type of nerve terminal (nt 2). The second type contains small cored vesicles and large granular vesicles. B. A synapse contacting with NA cell of the second type of nerve terminal (nt 2). A, B, $\times 29,000$

Richardson's solution and black with the ammoniacal silver corresponded to large (200 nm in diameter) electron dense granule-containing (LDC) cells. Likewise, light blue and non-argentaffin cells corresponded to large (250 nm in diameter) moderately dense granule-containing (LMC) cells (Figs. 4A, B, C). In addition to these two types of cells, a third type of chromaffin cells was recognized. These

cells were stained blue or brown in their peripheral cytoplasm, and contained electron dense granules which were smaller in size (100 nm in diameter) and fewer in number than those of the other two type cells (Figs. 4A, B, C). Such small granule chromaffin (SGC) cells often showed small polygonal features and possessed spherical nuclei with distinct nucleoli. Cell organelles of the SGC cells (Golgi complex, mitochondria and rough endoplasmic reticulum) were much more developed than those of the other two types of chromaffin cells. Occasionally, these SGC cells extended thin cytoplasmic processes in which many secretory granules were packed (Fig. 4C).

The majority of nerve fibers terminating with the chromaffin cells, showing synapse-like contacts with LDC or LMC or SGC cells, contained a large number of clear synaptic vesicles and a few large granular vesicles (Figs. 5A, B, C). Another type of nerve terminal was identified by the presence of many small cored vesicles and a few large granular vesicles. This type of terminal appeared to make synapse-like contact only with LDC cells (Figs. 6A, B).

DISCUSSION

In good agreement with previous studies (1, 2, 10, 13), the LDC cells and the LMC cells contained different types of catecholamines. Our microspectrofluorometrical study (12) indicates, most probably, that the strong green-yellow fluorescent cell contains NA which is stored in the large dense granules while the weak blue-green fluorescent cell contains A which is in the large moderately dense granules.

In addition to these chromaffin cells, the present study demonstrates that small granule chromaffin (SGC) cells occur in the adrenal gland of the clawed toad. The SGC cells are characterized by the presence of small granules, well-developed cytoplasmic organelles, and thin cytoplasmic processes. The most important finding of the present study is that these thin cytoplasmic processes, containing many small granules and microtubules, protrude into the intercellular space of other chromaffin cells. Moreover, using the same section, it was proven that the SGC cells contain primary catecholamine (probably NA or dopamine) similar to the case of *Rana catesbeiana* (8). Such a feature suggests that the SGC cells preserve neuronal characters and control other types of chromaffin cells by a paracrine mechanism.

With regard to the innervation of these three types of chromaffin cells, cholinergic inputs to the NA and A cells have been found by De Robertis (3) and Unsicker (19, 20). Our data supports the existence of cholinergic control not only on the NA and A cells but also on the SGC cells. Furthermore, another type of nerve terminal, probably noradrenergic, appears to innervate only the NA cells. The present report gives evidence for multiple controls at least on the NA cells. Recent immunohistochemical studies indicate that there are many peptidergic neurons in the peripheral nervous system. Further studies are necessary to elucidate the multiple neuronal control on these chromaffin cells.

ACKNOWLEDGMENTS

The authors are greatly indebted to Dr. H. Kimura in our department for valuable criticism.

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