LOCALIZATION OF CATECHOLAMINES IN THE EYES AND OTHER TISSUES OF APLYSIA

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Received for publication November 3, 1975, and in revised form July 1, 1976

A green fluorescence indicative of catecholamines (CA) was localized in the secondary cells (nonreceptor neurons), neuropile and optic nerve of the eye and other tissues in Aplysia by using the formaldehyde-induced fluorescence method for the demonstration of biogenic amines. The specificity of the induced fluorescence was confirmed by its absence in tissue not exposed to formaldehyde vapor, relatively rapid decay upon exposure to UV light and its chemical reduction by sodium borohydride. The fluorescence was greatly reduced in eyes treated with reserpine (depletes serotonin and catecholamines). Further confirmation that the green fluorescence in the eye was due to a CA and not to serotonin was obtained by showing that it was decreased or eliminated by a-methyl-para-tyrosine (an inhibitor of catecholamine synthesis), increased by incubation in dopamine and exhibited a peak emission (470 nm) characteristic of CA fluorescence. CA fluorescence was also observed in the neuropiles of the cerebral, pedal, pleural and parieto-visceral ganglia and in cells in the pedal ganglion, statocyst, mantle, anterior tentacles and siphon. The finding of CA in secondary neurons of the eye was unusual since CA-containing cells have not been observed in other gastropod eyes. The distribution of CA in Aplysia, in tissue other than the eye, is similar to that of other gastropod molluscs.

Dopamine is the most commonly occurring catecholamine in molluscs (29, 32). Recent biochemical studies have shown that dopamine, and the enzymes involved in its synthesis and catabolism, are present in the nervous system of the gastropod mollusc, Aplysia californica (5, 20, 21, 31). Dopamine was found to be the predominant catecholamine in the nervous system of Aplysia but was not found in single, identifiable cells of the central nervous system (21). Fluorescence histochemistry (9) has been used to localize serotonin and catecholamines in single cells of both vertebrates and invertebrates and has greatly aided the study of biogenic amine function in the nervous system (10, 32). In two previous studies of Aplysia, serotonin-containing nerve fibers in the heart (30) and serotonin-containing cells in the gill (27) were demonstrated with the formaldehyde-induced fluorescence histochemical method. Similar studies of catecholamine distribution in Aplysia have not been done.

In this study we used the formaldehyde-induced fluorescence histochemical method (9) to localize catecholamines in the eye and the central and peripheral nervous system of Aplysia. Previous studies have shown that the secondary cells (nonreceptor neurons) in the eye contribute to large spontaneous compound action potentials in the optic nerve (12). These synchronized nerve potentials exhibit a circadian rhythm of compound action potential frequency (11) and are suited for the sustained and massive release of a neurotransmitter or a neurohormone. Therefore, we were particularly interested in identifying a possible secretory substance in the secondary cells.

Our finding of catecholamines in secondary neurons of the eye was unusual since catecholamine-containing cells have not been observed in other gastropod eyes. We also found that the distribution of catecholamines in Aplysia, in tissue other than the eye, is similar to that reported for other gastropod molluscs (1, 7, 8, 16, 23, 28, 32) and in general, confirms previous biochemical studies of Aplysia (5, 21). The results of preliminary studies of the eye have been reported (18, 19).

MATERIALS AND METHODS

Aplysia californica, obtained from Pacific Bio-Marine (Venice, Calif.), were maintained in a contin-
uously aerated, artificial sea water tank at 15°C and were subjected to a light-dark cycle (LD, 12:12).

**Fluorescence histochemistry:** The tissue was rapidly dissected from each animal at about the same time of day, quenched in liquid Freon 12 cooled by liquid nitrogen and freeze dried for 48 to 72 hr at -50°C, 5 x 10^{-3} torr. Larger ganglia and peripheral tissue were cut into 2 to 3 mm² segments before freezing. Tissues were then exposed to paraformaldehyde vapors of 60% relative humidity for 1 hr at 60°C. Immediately after the vapor reaction, the tissues were embedded in paraplast and sectioned at 10μ. Sections were briefly deparaffinized in xylene and mounted in a low fluorescence mountant (Harleco or Entellan). A Zeiss microscope, equipped with incident illumination, a 450 nm dichroic mirror, a xenon XBO-150 lamp, a BG12 excitation filter and a Zeiss 47 or 50 barrier filter, were used to observe fluorescence. Photographs were taken with Tri-X or Plus-X (Kodak) film and developed in Diafine.

**Specificity tests:** Because the *Aplysia* eye contains structures that exhibit brown, yellow, orange and green autofluorescence, several specificity tests were done to differentiate between specific monoamine fluorescence and nonspecific autofluorescence. These tests included: (a) absence of fluorescence when exposed to the formaldehyde vapor, (b) fading of fluorescence when exposed to the UV light source, (c) chemical reduction of fluorescence in tissue sections by sodium borohydride (0.1% NaBH₄ in 90% propa- nol) (6), (d) absence or change in the fluorescence intensity following treatment of the tissue with reserpine, α-methyl-para-tyrosine (α-mpt) or dopamine. Reserpine depletes tissue monoamines while α-mpt specifically inhibits catecholamine synthesis from tyrosine. Dopamine has been shown to be accumulated by *Aplysia* nervous tissue (5). In this study isolated eyes or ganglia were placed in a culture medium (13) containing either 5 x 10^{-4} M reserpine (Sigma) or 10^{-3} M α-mpt (Sigma) for 20 hr or 10^{-3} M dopamine (Sigma) for 10-15 min. Effective dosage and time of application for each drug was determined by experimentation. Control tissue was treated similarly except that the drug was not present in the culture medium.

**Fluorescence microspectrophotometry:** The emission spectrum of fluorescence in the retinal cells, the optic nerve and the pedal ganglion was measured with an interference wedge inserted in a Photometer head (Zeiss MP-1) containing an EMI 9555 8B photomultiplier tube. The fluorescence intensity was read from a digital photometer (Pacific Photometric). The optical system consisted of a Zeiss II epi-illumination microscope with a BG12 excitation filter, an FL 450 dichroic insert, a short neofluar 40X phase objective and 440 nm barrier filter. A xenon XBO-150 lamp on a stabilized power supply was used as a light source. The excitation spot was slightly larger than the measuring aperture. The observed emission spectra were compared to previously reported spectra for biogenic amine fluorescence (CA; emission peak 475 nm and 5-hydroxy tryptamine; emission peak 525 nm) (2).

**RESULTS**

The morphology of the eye was described previously (12, 15) and is reviewed in Figure 1. The eyes are about 800 μm in diameter and are situated at the base of the posterior tentacles (rhinophores). The retina of the eye surrounds a central spherical lens and contains several thousand receptor cells, several hundred secondary cells, pigmented support cells and numerous nerve fibers. The retinal nerve fibers converge at the base of the eye and form a neuropile. The optic nerve emerges from the neuropile and enters the cerebral ganglion.

Differences in cellular location and shape were used to differentiate between receptor cells and secondary cells. Receptor cells and secondary cells are about 20 μm in diameter. The receptor cells have a distal and proximal process and a large round nucleus; secondary cells are ovoid cells with a single axon and a large ovoid nucleus. In addition, the receptor cells and the pigmented support cells surround the central lens while the secondary cells are located in the neuropile and along the periphery of the retina.

**Autofluorescence:** We found that the autofluorescence in all *Aplysia* tissue was relatively high and usually consisted of a cellular yellow and orange granular fluorescence, a diffuse yellow fluorescence particularly in receptor cells of the eye, and a dull green cellular and extracellular (connective tissue) fluorescence. In initial experiments the formaldehyde vapor reaction was performed at 80°C and both heat treated and untreated (no formaldehyde or heat) controls were used. The autofluorescence in heat treated tissue was generally more intense than in untreated tissue. Subsequently we performed the vapor reaction at 60°C and 70°C and found that optimal results, as determined by comparison in the microscope, were obtained at 60°C. Relative fluorescence intensities were not measured but the autofluorescence was less at 60°C and appeared similar to that in untreated tissue while the induced fluorescence was as intense as at 70°C or 80°C.

In addition, observation of the induced fluorescence was facilitated greatly by the use of an incident (epi-) illumination system. We found this system offered several advantages;
the major advantage was that light scatter from nearby autofluorescence only minimally interfered with the observation of induced fluorescence.

**Fluorescence histochemistry of the eye:**
The secondary cells and nerve fibers in the eye and optic nerve (Figs. 2 and 3) exhibited a green (500 nm barrier filter) or a blue-green (470 nm barrier filter) fluorescence when the eye was treated with formaldehyde vapor. The secondary cell and nerve fluorescence was more easily visualized near the base of the eye where there were fewer receptor and pigmented cells. The green fluorescence in the secondary cells and nerve fibers faded upon exposure to the UV light source but the yellow-orange fluorescence in the receptor cells did not. The green fluorescence was absent in eyes not treated with formaldehyde vapor although yellow fluorescence was present in the receptor cells (Fig. 4). When eyes were pretreated with reserpine to deplete biogenic amines (i.e., serotonin and catecholamines), the green fluorescence was not seen in the secondary cells or the neuropile (Fig. 5).

In addition, sodium borohydride, which reduces the biogenic amine fluorophore to a nonfluorescent form, reduced the secondary cell and nerve fluorescence but did not affect the receptor cell autofluorescence. These observations indicated that the green fluorescence represented a formaldehyde-induced fluorescence and that the yellow-orange fluorescence represented autofluorescent material. Furthermore, the green color of the induced fluorescence suggested that the secondary cells and nerve fibers in the eye and optic nerve contain a catecholamine and not serotonin which produces a yellow fluorescence.

The presence of a catecholamine was confirmed by experiments designed to change the catecholamine content of the tissue and thus the fluorescence intensity. When α-mpt was used to inhibit catecholamine synthesis, the green fluorescence was frequently reduced and occasionally eliminated in most of the secondary cells (Fig. 6) indicating that the concentration of catecholamine had decreased. The optic nerve fluorescence was decreased but not eliminated. Similarly, when dopamine, which is taken up by *Aplysia* nerve tissue (5), was used to increase the concentration of catecholamines, the intensity of the fluorescence in most of the secondary cells increased (Fig. 7).

The green fluorescence in the secondary cells and nerve fibers in control tissues that were cultured for 20 hr without the drugs was similar to freshly prepared tissue, suggesting the catecholamine concentration was not significantly changed in cultured tissue. Therefore, changes in fluorescence intensity were caused by the drugs.

The fluorescence in the eye was further characterized by measuring its emission spectrum. The emission spectrum of the secondary cells and nerve fibers in the eye and optic nerve was relatively narrow with a peak at about 470 nm, similar to that expected for catecholamine fluorescence (Fig. 8). Measurements of the receptor cell fluorescence showed a broad emission spectrum with a peak near 500 nm. That part of the receptor cell emission spectrum between 460 and 480 nm was nearly as intense as the induced fluorescence in the secondary cells suggesting a possible catecholamine fluorescence was present but was masked by the autofluorescence. However, no difference in the

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**Fig. 1.** Diagram of the *Aplysia* eye. The secondary cells are usually located near the base of the eye within the neuropile (cross-hatched area) and along the periphery of the eye. The characteristic shape and position of receptor cells (RC), pigment cells (PC) and secondary cells (SC), are shown in a section of the eye (upper right).

**Fig. 2.** Eye fluorescence. The peripherally situated secondary cells (SC) exhibit a green induced fluorescence. A yellow-orange autofluorescence is present in the receptor cell (RC) area. Lens (L). Dark pigment (P).

**Fig. 3.** Nerve fluorescence. Longitudinal section of the optic nerve (ON) showing the fluorescent fibers and the nonfluorescent nerve sheath (S).

**Fig. 4.** Receptor cell autofluorescence. In eyes not treated with formaldehyde, receptor cells (RC) exhibit a yellow-orange autofluorescence while the secondary cells and the neuropile (N) are not fluorescent. Nucleus (Nu). Pigment area (P).

**Fig. 5.** Reserpine treated eye. The secondary cell (outlined) and the surrounding neuropile are nonfluorescent. Secondary cell nuclei (SCN) are barely visible. Pigment (P).

**Fig. 6.** α-Methyl-para-tyrosine treated eye. Secondary cell (SC) fluorescence is decreased while autofluorescence in receptor cells (RC) is unchanged. Neurpule (N).

**Fig. 7.** Dopamine treated eye. Secondary cell (SC) fluorescence is increased. Receptor cells (RC). Dark pigment (P).
SECONDARY CELL spectra of receptor cells from formaldehyde-treated or untreated eyes was found. Since the eyes exhibit a circadian rhythm (11) that reflects the neuronal activity of the secondary cells (12, 14) one might expect a change in the contents of these cells during a 24-hr period. However, no qualitative difference in the fluorescence intensity or in the number of fluorescent cells was detected when some eyes were prepared at different times of the day.

Fluorescence histochemistry of other Aplysia tissue: The additional tissues investigated in this study included four ganglia in the central nervous system (pedal, pleural, cerebral and parieto-visceral), and several peripheral tissues (mantle, anterior tentacles and siphon).

In the pedal ganglion, which has been shown to contain a high concentration of dopamine (5, 21), a few small green fluorescent cells, 20-40 μm in diameter, were always found (Fig. 9) at the edge of the neuropile. These cells did not contain yellow granular autofluorescence as found in many of the larger cells. The peak emission of these cells and nerve fibers in the neuropile at 470 nm is characteristic of catecholamine fluorescence (Fig. 8). Measurements of the emission spectrum of yellow fluorescence in the giant serotonin-containing cells of the cerebral ganglion were made for comparison to the emission spectra of catecholamine-containing structures. However, reliable results were not obtained since these cells contain a large amount of yellow and orange autofluorescence and since the serotonin fluorescence decays rapidly.

The statocyst, which is located in the pedal ganglion, contained green fluorescent cells (Fig. 10). This fluorescence decayed upon exposure to UV light, was absent in untreated (no formaldehyde) and α-mpt treated pedal ganglia. Numer-

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**Fig. 9.** Pedal ganglion. Bright green fluorescent cell (arrow) and axon (A) adjacent to fluorescent neuropile (N).

**Fig. 10.** Statocyst. Green fluorescence in area of sensory cells (arrow), nonfluorescent sheath (S) surrounding pedal ganglion, and statoconia (St) within the statocyst are shown.

**Fig. 11.** Cross-section of the parieto-visceral ganglion. Green fluorescent fibers in central neuropile (arrows) are surrounded by nonfluorescent cells and fibers.

**Fig. 12.** Anterior tentacle. Network of fluorescent fibers (arrows) adjacent to external pigmented epithelium (PE).

**Fig. 13.** Mantle. Green fluorescent cell (arrow) with nonfluorescent nucleus adjacent to the pigmented epithelium (PE). Other fluorescent structures near the cell displayed an orange autofluorescence.

**Fig. 14.** Siphon. Cluster of green fluorescent cells (arrows) with nonfluorescent nuclei at the epithelial edge of the siphon. Muscle (M), contains yellow and orange autofluorescence.
ous green fluorescent fibers were also seen in the central areas of the neuropile in the cerebral, parieto-visceral and pleural ganglia but few green fluorescent cells were observed (Fig. 11).

Induced fluorescence was also seen in peripheral tissues. For example, adjacent to the pigmented epithelium of the mantle and the anterior tentacles, green fluorescent nerve fibers (Fig. 12), and small green cells, 20-30 m in diameter (Fig. 13) were observed. Some of these cells were near large (150-300 m in diameter) nonfluorescent cells. Green fluorescent fibers frequently appeared to end on or near the large nonfluorescent cells.

In the siphon several clusters of 10-20 green fluorescent cells, 20-40 m in diameter, were seen along the edge of the pigmented epithelium (Fig. 14). Occasionally, a group of these cells was found adjacent to a large nonfluorescent cell similar to those seen in the mantle and anterior tentacles. No fluorescent nerves were seen in the siphon, although they may have been obscured by the autofluorescent muscle fibers. The green fluorescence in the mantle, anterior tentacles and the siphon faded upon exposure to the UV light source and was reduced by sodium borohydride indicating it was due to the reaction between formaldehyde and a biogenic amine.

DISCUSSION

The secondary cells described in this paper are unique since they are the only catecholamine-containing neurons identified in a molluscan eye. Nerve fibers that contain a catecholamine have been observed in *Limax maximus* eyes (23) and in the optic nerve and tissue surrounding the retina of *Buccinum undutam* (8). The unusual finding of catecholamine-containing cells in the *Aplysia* eye may be related to their physiology. The secondary cells interact, probably via electrical junctions, to produce large, slowly conducted compound action potentials that can be recorded from the optic nerve (12). In addition, the eye exhibits a circadian rhythm of compound action potential frequency (11) that involves the interaction of the secondary cells (12, 14). A population of cells with similar physiologic properties has not been described in other molluscan eyes.

In contrast to the eyes, the distribution of catecholamines elsewhere in *Aplysia* is similar to that reported for other gastropod molluscs. In general, the results of this study and those of previous studies (1, 7, 8, 16, 23, 25, 28, 32) show that gastropod molluscs have: (a) numerous catecholamine-containing nerve fibers, but relatively few catecholamine-containing cells, in the ganglia of the central nervous system and (b) numerous catecholamine-containing cells and nerve fibers associated with peripheral structures and sensory structures such as the eyes, statocysts and anterior tentacles. A similar distribution of dopamine was found in biochemical studies of *Aplysia* (5, 21). In these studies high levels of dopamine were found in the neuropiles and some nerve trunks of ganglia in the central nervous system but dopamine was not found in single, identifiable cells isolated from the ganglia (21). The results of our study agree with the biochemical findings of dopamine distribution in *Aplysia* and support the conclusion that there are striking similarities in the distribution of catecholamines in gastropod molluscs.

Unlike many of the gastropod molluscs studies so far, the level of autofluorescence in all *Aplysia* tissues is relatively high. The reason for this difference is not clear, but has also been described in the annelid, *Lumbricus terrestris* (22). This autofluorescence has hampered the localization of amines in *Aplysia*, which has otherwise proven to be an extremely useful animal for studies of the physiology of the nervous system, particularly of amines in synaptic transmission (10). Some fluorescence histochemical studies of *Aplysia* were done in which serotonin was localized in nerve fibers in the heart (30) and cells in the gill (27). Results were obtained in the heart by air drying the intact heart and in the gill by using frozen sections. Air drying is only feasible for very thin specimens (2, 9) such as the heart which did not appear to contain much autofluorescence. We found that with slight modifications of the original histochemical method (9) that included using a lower reaction temperature, as well as a microscope with incident illumination, and a number of specificity tests, catecholamines could be clearly localized in many parts of the nervous system.

The results of the specificity tests showed that the fluorescence was due to a specific reaction of a catecholamine with formaldehyde. Other investigators have shown that the formaldehyde reaction as used in this study is specific.
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for catecholamines (dopamine, noradrenaline and adrenaline) and serotonin and does not produce an observable fluorescent product with other amines such as glutamine and octopamine which are also found in nerve tissue (2). The catecholamine in the Aplysia eye is probably dopamine since dopamine is the predominant catecholamine in most molluscs (3, 24, 25, 29, 32) and in Aplysia (5, 21).

The effects of reserpine and a-mpt observed in this study were similar to those observed in other studies. Reserpine is known to deplete both catecholamines and serotonin. It has been used to deplete these amines in several molluscs including Planorbis (24), Helix aspersa (28), Helix pomatia (8) and Limax (23). a-mpt is known to inhibit specifically the synthesis of catecholamines and has been shown to decrease green catecholamine fluorescence in Helix pomatia (8) and Planorbis (24). In previous studies reserpine and a-mpt were injected into whole animals whereas in our study these drugs were applied to isolated tissue in a culture medium. This enabled us to use one eye from the same animal as a control for the other since seasonal variations in catecholamine levels in Helix (4) and daily variations in the physiology of the eye (11-13) have been reported. Thus, although similar results were obtained, the dosage and time of drug application that we used are not directly comparable with other studies.

We also measured the emission spectrum of the fluorescence to characterize further the formaldehyde-induced fluorescence. Although the shape of the emission spectrum may be influenced by such factors as autofluorescence, cellular inclusions, optics and the photomultiplier characteristics, the peak value of the emission is constant and characteristic of the fluorophore (e.g. CA 475 nm; 5-hydroxy tryptamine, 525 nm) (2). The peak emission of the secondary cell and nerve fluorescence at 470 nm coincides with that of the catecholamines and is the same as the peak emission of cells and nerve fibers in the pedal ganglion which has 4 to 5 times more dopamine (5, 21) than other Aplysia ganglia. Measurements of the yellow autofluorescent receptor cell cytoplasm were also made since serotonin produces a yellow fluorescence and catecholamines can produce a fluorescence that appears yellow in the microscope if intense reaction conditions are used or if the concentration of catecholamines is high (2).

These measurements showed a broad emission with a peak at about 500 nm that was clearly different from the narrow emission spectrum and peak of the secondary cell fluorescence. Besides, similar emission spectra were obtained from receptor cells of eyes not exposed to formaldehyde. Thus the results of this study indicate that the secondary cells and nerve fibers in the eye and optic nerve contain a catecholamine.

In addition, the results of this study suggested that an ultrastructural study of the secondary cells and nerve fibers in the eye and optic nerve would reveal the presence of dense core vesicles as shown for many other aminergic cells (32) such as the giant dopamine cell in Planorbis (26). We have recently found (19) that dense core vesicles occur in the same regions as the green catecholamine fluorescence in the eye and optic nerve. Electron microscopic histochemical studies have confirmed that dense core vesicles in the secondary cells and nerve fibers in the eye contain an amine. Thus, at least some of the catecholamine in the eye is stored in vesicles, and may be transported toward optic nerve endings found in the cerebral ganglion (17, 18).

ACKNOWLEDGMENTS

We wish to thank Dr. R. Cowden for generous assistance and the use of his equipment and Drs. T. Fitzharris, S. S. Spicer and Patrick L. Moore for comments on the manuscript.

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