Coexistence of Several Putative Neurotransmitters in Single Identified Neurons of Aplysia

(serotonin/histamine/octopamine/acetylcholine/micro-enzymatic assays)

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ABSTRACT By sensitive enzymatic micromethods several putative neurotransmitters were measured in four identifiable neurons of Aplysia californica (R-2, R-14, L-11, and C-1). Serotonin was found in all of these neurons, and octopamine in all but C-1. Acetylcholine has been previously reported to be present in R-2 and L-11. The catecholamines, norepinephrine and dopamine, were not detected in the four cells examined. The possible biological consequence of the presence of several putative neurotransmitters in single identifiable neurons is discussed.

The nervous system of Aplysia contains many exceptionally large nerve cell bodies, some of which approach 1 mm in diameter. A number of these cells have been identified and numbered on the basis of their position, size, and pigmentation, as well as their electrophysiologic properties (1). The neurons can be distinguished by the patterns of spontaneous discharge and spontaneous synaptic inputs, as well as by the nature of synaptic inputs elicited upon stimulation of each of the peripheral nerves and the projection of axons.

Recently it has become possible to distinguish biochemical differences among the identified cells. Only four neurons, L-10, L-11, and R-2 in the abdominal ganglion and the left pleural giant cell (PGC), were found to contain choline acetyltransferase, the enzyme that synthesizes the neurotransmitter, acetylcholine (2, 3). This observation suggests that only these neurons contain acetylcholine, and the recent measurements of acetylcholine in single cells have confirmed its presence in these four neurons but not others (4). In a study of content of 5-hydroxytryptamine (serotonin), it was found that only the paired giant cerebral cells (C-1) contained measurable serotonin (6.2 pmol per cell), while all other neurons contained less than 0.4 pmol per cell (5). Furthermore, Eisenstadt et al. (6) have demonstrated that among the large identified cells, only cells R-2, L-10, and L-11 synthesize acetylcholine from [3H]choline, while only cell C-1 forms serotonin from L-[3H]tryptophan.

In contrast to these results, the levels of glutamate and glutamine, considered to be putative neurotransmitters, have been found to be relatively constant from cell to cell (7). This may imply that these two compounds do not act as neurotransmitters in Aplysia. There is a similar widespread presence of L-aminonic acid decarboxylase (8), acetylcholinesterase, and catechol-O-methyl transferase (9), enzymes that form and metabolize neurotransmitters.

Recently our laboratory has developed very sensitive enzymatic assays for several neurotransmitters and putative transmitters which make it possible to assay these substances in single nerve cells of Aplysia. We have measured the concentrations of the putative transmitters serotonin, histamine, octopamine, and dopamine in the large identified cells from the various ganglia. In this communication we report studies on four neurons: R-2, R-14, L-11, and C-1. These neurons have been found to contain more than one putative neurotransmitter.

MATERIALS AND METHODS

Aplysia californica (200–400 g) were obtained from Pacific Biomarine Supply Co., Venice, Calif., and were housed in tanks of artificial sea water at 15°. The animals were pinned to dissecting trays and the ganglia were removed. Cells of the abdominal ganglion were identified on the basis of their size, position, and color according to the description of Frazier et al. (1). The giant cerebral cell, C-1, was identified according to the description of Weinreich et al. (5). Single cells were removed with watchmaker’s forceps after the connective tissue capsule was slit with a razor blade. The cells were removed with the aid of a stereomicroscope that was fitted with a graticule. The diameter(s) of the cells, and thus the volume, were determined at the time of their dissection. The cells were held at the region of the origin of the axon and gently plucked from the ganglia in order to avoid contamination with neuropile. The isolated cell bodies were covered by a multilayered glial investment, but were presumed to be free from contamination by nerve terminals since there are no axosomatic synapses in Aplysia (10). Cells from two or three animals were pooled for most assays.

For norepinephrine and dopamine assays, four to six cells were homogenized in microhomogenizers (Micrometric Instrument Co., Cleveland, Ohio) containing 35 μl of 0.1 M perchloric acid. The homogenizers were placed in adaptors and centrifuged for 20 min at 10,000 X g. Twenty-five microliters of the supernatant were removed for assay of norepinephrine and dopamine according to a micromodification (11) of the method of Coyle and Henry (12).

Serotonin was assayed according to the method of Saavedra et al. (13, 14). This method is based on the ability of an enzyme, hydroxyindole-O-methyltransferase (EC 2.1.1.4; S-adenosyl-L-methionine: N-acetylserotonin O-methyltransferase), present in the pineal gland, to transfer the [3H]methyl group of [3H]methyl-S-adenosyl-L-methionine to the hydroxyl group of N-acetylserotonin. Prior to the O-methylation reaction, serotonin is acetylated enzymatically with N-acetyltransferase from rat liver (EC 2.3.1.5; acetyl-
were added to each tube. The reaction was stopped by addition of 20 μl of 0.1 M perchloric acid containing 20 μg of 1-methyl histamine, and 0.5 ml of 3 M NaOH was added to each tube followed by 6 ml of chloroform. The aqueous and organic phases were mixed and the tubes were centrifuged at low speed to separate the phases. The aqueous phase was aspirated and discarded, and the chloroform was washed once with 1 ml of 3 M NaOH. Five milliliters of chloroform were transferred to counting vials and the chloroform was evaporated just to dryness under a stream of air. Blanks were prepared by adding 10 μl of phosphate buffer to tubes instead of tissue extract. Standards were also prepared in phosphate buffer. The product of the assay of tissue extracts was found to be isographic with genuine 1-methyl histamine in the chromatographic systems used by Snyder et al. (15).

For octopamine analysis, cells were homogenized in 65 μl of cold 0.2 M Tris·HCl buffer (pH 8.0), containing the monoamine oxidase inhibitor, iproniazid (1 mM). The samples were assayed according to the method of Saavedra (16). The product formed in the course of the incubation was identified as norepinephrine, as described earlier (16).

RESULTS

The concentrations of serotonin, histamine, dopamine, norepinephrine, and octopamine were measured in identified neuronal cell bodies of Aplysia, and the results are shown in Table 1. Also shown are values for acetylcholine (4), glutamate, and histamine (7) reported by others.

Serotonin is found in C-1 as well as in R-1, R-14, and L-11, where its presence has not been previously demonstrated. The serotonin content of C-1 (9.4 ± 3.1 X 10^-6 M) is much greater than that of R-2, R-14, and L-11. However, serotonin is present in concentrations greater than 10^-5 M in these other neurons. Serotonin has also been reported in C-1 by Weinreich et al. (5) using a fluorescent assay, and their value is in good agreement with ours.

The addition of 5-hydroxytryptophan to the assay system results in the formation of radioactive melatonin. 5-Hydroxy-

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**Table 1. Putative neurotransmitters in identified neurons of Aplysia**

<table>
<thead>
<tr>
<th></th>
<th>R-2</th>
<th>R-14</th>
<th>L-11</th>
<th>C-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotonin</td>
<td>1.8 X 10^-4</td>
<td>3.4 X 10^-4</td>
<td>1.1 X 10^-4</td>
<td>9.4 X 10^-4</td>
</tr>
<tr>
<td>± 0.3</td>
<td>± 1.2</td>
<td>± 0.3</td>
<td>± 3.1</td>
<td>± 3.1</td>
</tr>
<tr>
<td>Histamine</td>
<td>3.0 X 10^-4</td>
<td>7.0 X 10^-4</td>
<td>4.5 X 10^-4</td>
<td>1.4 X 10^-4</td>
</tr>
<tr>
<td>± 0.5</td>
<td>± 3.0</td>
<td>± 0.7</td>
<td>± 0.1</td>
<td>± 0.1</td>
</tr>
<tr>
<td>Octopamine*</td>
<td>2.5 X 10^-4</td>
<td>1.5 X 10^-4</td>
<td>9.1 X 10^-4</td>
<td>N.D.</td>
</tr>
<tr>
<td>± 1.0</td>
<td>± 0.5</td>
<td>± 0.6</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Dopamine</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Acetylcholine†</td>
<td>3.9 X 10^-1</td>
<td>N.D.</td>
<td>3.3 X 10^-1</td>
<td>N.D.</td>
</tr>
<tr>
<td>Glutamate‡</td>
<td>7.6 X 10^-3</td>
<td>3.2 X 10^-3</td>
<td>6.0 X 10^-3</td>
<td>3.5 X 10^-3</td>
</tr>
<tr>
<td>Glutamine‡</td>
<td>3.5 X 10^-2</td>
<td>2.0 X 10^-1</td>
<td>2.1 X 10^-3</td>
<td>2.0 X 10^-3</td>
</tr>
</tbody>
</table>

Results are reported as molarity ± SE. Molarity was calculated from volume estimations obtained by measurement of greatest and smallest cell diameters at time of dissection. Numbers in parentheses represent the number of samples. N.D. indicates none detected. Glutamate and glutamine values shown were calculated from the data of Borsy et al. (7), who expressed their data as μmol/g of protein. For conversion to molarity, values for R-2 and L-11 were obtained as proportions from values given for acetylcholine as both μmol/g of protein and molarity by McCaman et al. (4). For R-14, concentrations were calculated using a cell volume of 22 nl reported by Giller and Schwartz (3) and a protein content of 2.08 μg per cell reported by McCaman and Dewhurst (9). For C-1, concentrations were approximated using a cell volume of 8.2 nl (calculated from average diameter observed in these experiments) and assuming a protein content of 0.8 μg per cell [given by McCaman and Dewhurst (9) for cell R-1, which is similar in size to C-1].

* From Saavedra, Brownstein, Carpenter, and Axelrod (18).
† From McCaman, Weinreich, and Borsy (4).
‡ From Borsy, Weinreich, and McCaman (7).

CoA: arylamine N-acetyltransferase (N-acetylsertotonin) and acetyl coenzyme A. Thus, in the course of an assay, serotonin is converted to N-acetylsertotonin, which is converted in turn to radiolabeled melatonin. The radioactive product is extracted from the aqueous phase into toluene. To identify this product, aliquots of the toluene extract were dried at 40° under reduced pressure for 1 hr. The residue was taken up in 50 μl of ethanol and spotted on chromatography sheets. Two micromoles of each of serotonin, O-methylserotonin, O-methyltryptamine, and O-methyl-N,N-dimethyltryptamine were run simultaneously on the same plate. Two solvent systems were used: methyl acetate–isopropanol–10% ammonium hydroxide (45:35:20) and toluene-acetic acid–ethyl acetate–water (80:40:20:5). More than 90% of the radioactive product extracted was found to have the same Rf values as authentic melatonin in the two solvent systems, and the product isolated from cells in the course of our assay procedure was isographic with the product obtained after carrying authentic serotonin through the procedure.

The presence in tissue extracts of N-acetylsertotonin or other indoles that might be O-methylated by hydroxyindole-O-methyltransferase was ruled out by use of tissue blanks prepared by omitting acetyl coenzyme A from the incubation mixture. Under these circumstances, about the same number of counts was observed as when the acidic tissue extract was replaced by 0.1 M HCl.

Histamine was assayed according to a modification of the method of Snyder et al. (15). Cells were homogenized in 15 μl of 0.2 M sodium phosphate buffer (pH 7.8), and the samples were centrifuged. Ten microliters of the supernatant were removed from each homogenizer and added to 15-μl glass-stoppered centrifuge tubes. The tubes were incubated for 30 min at 37° after 4 μl of histamine methyltransferase and 1 μl of [3H]methyl-S-adenosyl-1-methionine (0.11 nmol, specific activity 4.5 mCi/mol; New England Nuclear Corp., Boston, Mass.) were added to each tube. The reaction was stopped by addition of 20 μl of 0.1 M perchloric acid containing 20 μg of 1-methyl histamine, and 0.5 ml of 3 M NaOH was added to each tube followed by 6 ml of chloroform.
tryptophan is decarboxylated to serotonin by the L-aromatic amino-acid decarboxylase present in the enzyme preparation. The addition of MK-486 (5 × 10⁻⁴ M) (Merck, Sharp and Dohme Research Laboratories, Rahway, N.J.) to the incubation mixture completely inhibits the decarboxylase and prevents endogenous 5-hydroxytryptophan from interfering with the assay of serotonin (14). In R-2, L-11, and R-14, as well as in C-1, all of the melatonin formed during the assay procedure appeared to be derived from serotonin present in these neurons as in other species. It was not possible to demonstrate the presence of 5-hydroxytryptophan in the Aplysia. Thus, the tryptophan was detected in the cytoplasm of single neurons of Aplysia.

Histamine has not been previously detected in nerve cells of Aplysia. It is present in each of the cells shown in Table 1 in concentrations between 10⁻⁴ and 10⁻⁸ M. The concentration of histamine in cell C-1 is nearly two orders of magnitude less than that of serotonin.

There is a heavy glial investment coating all Aplysia neurons, which contributes considerably to membrane surface area although very little to total volume. In addition, small satellite neurons are sometimes adherent to larger neurons. While these were always removed when seen, some may have been missed. Thus, it might be argued that the serotonin and histamine measured in neurons such as R-2 and L-11 (which are known to contain acetycholine) is present in either satellite neurons or glial cells. It proved impossible to aspirate the cytoplasm from single cells after impalement with blunt micropipettes. Therefore, the cytosol was separated from membrane fragments mechanically under the dissecting microscope. Cells were dissected and broken opened with forceps and their cytosol were carefully pressed on a pestle of a microhomogenizer. The membrane was then placed on the pestle of another microhomogenizer. In eight separate determinations using R-2, 75% of the total of both serotonin and histamine was found to be present in the cytosol, while only 25% remained in the membrane fraction. This clearly demonstrated that most of the serotonin and histamine measured is intracellular.

Although dopamine is known to be present in the nervous system of Aplysia (17), dopamine was not detected in any of the examined cells. Norepinephrine was found neither in whole ganglia nor in single neurons, confirming the previous finding that this catecholamine is absent from these ganglia (17). The sensitivity (twice background) of the dopamine and norepinephrine assays was 40 and 10 pg, respectively. Octopamine was found in some but not all of the large neurons (17). It is present in relatively high concentration in cell R-14 (1.5 × 10⁻⁴ ± 0.5 M), but was also detectable in R-2 and L-11 (18).

**DISCUSSION**

Because each of the substances listed in Table 1 (except norepinephrine) is present in the nervous system of Aplysia, and because specific postsynaptic receptors for these substances have been demonstrated (19, 20), they must be considered to be putative neurotransmitters.

Serotonin, octopamine, and acetycholine are unequally distributed among the single neurons studied, and histamine, glutamine, and glutamate are about evenly distributed. Acetycholine (4) and choline acetyltransferase (2, 3) are present in R-2 and L-11 but not in C-1 or R-14. Octopamine is present in moderate concentration in R-14 but is also found in R-2 and L-11 (18). Serotonin is present in R-2, R-14, and L-11 as well as in C-1. We have demonstrated tryptophan hydroxylase activity in R-2 as well as in C-1 (Brownstein, Kiser, Carpenter, and Axelrod, unpublished observations), suggesting that serotonin is not only present but is also synthesized by neurons that are known to contain and form acetycholine. ζ-Aminobutyric acid has recently been demonstrated to be present in most single cells of Aplysia studied, including C-1 and R-2 (21), by dansyl-chloride microchromatographic techniques.

The observation that putative neurotransmitters are not uniformly distributed among the nerve cells of Aplysia suggests biochemical specificity. Thus, we should like to be able to call R-2 and L-11 “cholinergic,” R-14 “octopaminergic,” and C-1 “serotoninergic,” but each of these neurons contains more than one putative neurotransmitter. Although there is no evidence presently available supporting the possibility that these neurons are releasing more than one transmitter, it appears that Dale’s principle, which implies that each nerve cell makes and releases only one transmitter, may be violated in the giant neurons of Aplysia.

The presence of multiple putative transmitters in one neuron may have no functional significance, if only one transmitter is released. If this be the case, the presence of the other substances is simply biologic noise, and the specificity must lie somewhere in the system transporting the transmitter down the axon, packaging it in synaptic vesicles, and releasing the contents of the vesicles. It is also possible that the single neuron is capable of synthesizing only one transmitter but has uptake systems for other substances that have no function in that neuron. This suggestion is supported by the observations of Eisenstadt et al. (6) that R-2 and L-11 synthesize acetycholine and C-1 synthesizes serotonin, but not vice versa. However, it is possible that the procedures used in these studies lacked sufficient sensitivity to detect small levels of enzymatic activity and that there is only a quantitative difference in synthetic activity.

The nuclei of the giant Aplysia neurons contain more than 200,000 times as much DNA as the haploid amount found in Aplysia sperm (22). It is possible that at least a part of this increased DNA reflects fusion of neurons in the course of development, giving in effect large somatic cell hybrids containing genetic material from many different neurons. If this were the case, Dale’s principle might be valid for a normal diploid neuron, but cells arising from fusion of many cells may contain all the apparatus for synthesis, uptake, packaging, and release of multiple transmitters. Fusion of nerve cells and their processes is well documented for other molluscs (23). Furthermore, many mammalian neurons also have a considerably greater DNA content than the diploid amount (see ref. 22), suggesting that, if this were the reason for the multiplicity of transmitters in Aplysia, the same situation might hold elsewhere.

Multiple transmitters may be released from a single neuron, with all but one being functionally inactive in that they do not have the appropriate postsynaptic receptor. Postsynaptic receptors for most putative transmitters other than acetycholine do appear to be discrete and localized only to specific neurons. Although most Aplysia neurons do not have receptors for all of these substances, many do have specific receptors for several of them (21). Thus, more than one transmitter might
be released and might subsequently activate postsynaptic receptors.

If more than one substance is released from presynaptic terminals, then it is possible that they might react with multiple receptors at both the presynaptic and postsynaptic membranes. Interaction of a neurotransmitter with the presynaptic membrane might result in either a release of the same or another transmitter (24) or inhibition of release (25, 27). These neurotransmitters might act on the same or different postsynaptic receptors. They could modulate another's actions if they act on the same receptor or produce antagonistic or synergistic effects on different receptors.

An alternative possibility of a functional role for these substances is that different branches of the same axon might release different neurotransmitters. Anderson and McClure (27) have demonstrated that neurons of the dorsal root ganglion of cat that are exposed to labeled leucine synthesize and distribute fast axoplasmic transport different proteins to the two major branches of the axon. The possibility that a neuron might release acetylcholine at one terminal, serotonin at a second, and octopamine at a third cannot be excluded. Furthermore, Grossman et al. (29) have recently demonstrated that there is a differential channeling of information into two branches of a single axon of the lobster.

Finally, it is possible that these compounds are not all functioning as neurotransmitters, but coexist for other purposes. Thus, they might regulate one another's biosynthesis, storage, metabolism, or release. For example, histamine (30) and serotonin (30, 31), at millimolar concentrations, are known to inhibit dopamine-β-hydroxylase, the enzyme that converts dopamine to norepinephrine. Changes in the level of histamine in nerve terminals could give rise to alterations in the rate of synthesis of octopamine, phenylethanolamine, and norepinephrine. Moreover, several of the putative transmitters share biosynthetic (L-aromatic amino-acid decarboxylase) and metabolizing (monoamine oxidase) enzymes. An excess of one precursor or transmitter might result in decreased formation or metabolism of a second compound. Alternatively, these various transmitters might share the same vesicle and compete for storage space.

Our findings suggest that nerve cells play a broader role in integrative processing of information than was heretofore thought possible.