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COEXISTENCE OF SEVERAL PUTATIVE NEUROTRANSMITTERS IN SINGLE IDENTIFIED NEURONS OF APLYSIA

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COEXISTENCE OF SEVERAL PUTATIVE NEUROTRANSMITTERS IN SINGLE IDENTIFIED NEURONS OF APLYSIA

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ABSTRACT

In identified neurons of Aplysia, measurable amounts of serotonin, histamine, octopamine, acetylcholine, glutamine and glutamate are present in the same cell.

I. INTRODUCTION

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.[13] The neurons can be distinguished by the patterns of spontaneous discharge and spontaneous synaptic inputs as well as by the type 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 identified neurons, L10, L11, and R2 in the abdominal ganglion and the left pleural giant cell (PGC), were found to contain choline acetyl transferase.[15, 20] This observation suggests that only these neurons contain acetylcholine (Ach), and the recent measurements of Ach in single cells have confirmed its presence in these four neurons but no others.[22] In a study of content of 5-hydroxytryptamine (5-HT, serotonin), Weinreich et al.[30] found that only the paired giant cerebral cells (C1) contained measurable 5-HT (6.2 pmoles/cell) while all other neurons contained less than 0.4 pmole/cell. Furthermore, Eisenstadt et al.[11] have demonstrated that among the large identified cells only cells R2, L10 and L11 synthesize Ach from ^{3}H-choline while only cell C1 synthesizes 5-HT from L-^{3}H-tryptophan.

In contrast to these results the concentrations of glutamate and glutamine, considered to be putative neurotransmitters, have been found to be relatively constant from cell to cell.[2] This may imply that these two compounds do not act as neurotransmitters in Aplysia. There is a similar widespread presence of L-aromatic amino acid decarboxylase,[29] acetylcholinesterase and catechol-O-methyl transferase.[21]

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 5-HT, histamine, octopamine, dopamine and norepinephrine in the large identified cells from the various ganglia. In this communication we report studies on four neurons: R2, R14, L11 and C1. These neurons and others have been found to contain more than one putative neurotransmitter.

II. MATERIALS AND METHODS

Aplysia californica (200-400 g) were obtained from Pacific Biomarine Supply Company, Venice, California, and were housed in tanks of artificial seawater at 15°C. 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.[13] The giant cerebral cell C1 was identified according to the description of Weinreich et al.[30] 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 which 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.[7] Cells from two or three animals were pooled for most assays.

For norepinephrine and dopamine assays, cells were homogenized in micro-homogenizers (Micrometric Instrument Company, Cleveland, Ohio) containing 35 μl of 0.1 N 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[23] of the method of Coyle and Henry.[9]

Serotonin (5-HT) was assayed according to a modification[25] of the method of Snyder et al.[27] This method is based on the ability of an enzyme, hydroxyindole-O-methyl transferase (HIOMT) (EC 2.1.1.4), present in the pineal gland, to transfer the [^{3}H] methyl group of [^{3}H]-methyl-S-adenosyl-1-methionine (^{3}H-SAM) to the hydroxyl group of N-acetyl serotonin. Prior to the O-methylation reaction, serotonin is acetylated enzymatically with N-acetyl transferase from rat liver (NAT) (EC 2.3.1.5) and acetyl coenzyme A (AcCoA). Thus, in the course of an assay, serotonin is converted to N-acetyl serotonin, which is converted in turn to radiolabelled melatonin. This radioactive product is extracted from the aqueous phase into toluene. In order to identify this product, aliquots of the toluene extract were dried at 40°C under vacuum for 1 hour. The residue was taken up in 50 μl of ethanol and spotted on chromatography sheets. Two micrograms each of melatonin, O-methyltryptophol, O-methyltryptamine and O-methyl-N, N-dimethyltryptamine were run simultaneously on the same plate. Two solvent systems were used: methyl acetate-isopropanol-ammonium hydroxide 10 percent (45:35:20) and toluene-acetic acid-ethyl acetate-water (80:40:20:5). More than 90 percent of the radioactive product extracted was found to have the same R_F 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-acetyl serotonin or other indoles which might be O-methylated by HIOMT was ruled out by use of tissue blanks prepared by omitting AcCoA from the incubation mixture. Under these circumstances about the same number of counts were obtained as when the acidic tissue extract was replaced by 0.1 N HCl.

Histamine was assayed according to a modification of the method of Snyder et al.[27] 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^{o}C after 4 μl of histamine methyl transferase and 1 μl of ^{3}H-methyl-S-adenosyl-1-methionine (0.11 nmole, specific activity 4.5 mCi/mole; New England Nuclear Corporation, Boston, Massachusetts) were added to each tube. The reaction was stopped by addition of 20 μl of 0.1 N perchloric acid containing 20 μg of 1-methyl histamine; and 0.5 ml of 3 N NaOH was added to each tube followed by 6 ml of chloroform. The aqueous and organic phases were mixed on a Vortex Genie mixer for 30 seconds 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 N 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.[27]

For octopamine analysis, cells were homogenized in 65 μl of cold 0.2 M Tris HCl buffer, pH 8.6, containing the monoamine oxidase inhibitor, iproniazid, 1×10^{-3} M. The samples were assayed according to the method of Saavedra.[24] The product formed in the course of the incubation was identified as synephrine, as described earlier.

III. RESULTS

The concentrations of 5-HT, histamine, dopamine, norepinephrine and octopamine were measured in identified neuronal cell bodies of Aplysia and the results are shown in Table I. Also shown are values for acetylcholine,[22] glutamate, and glutamine,[2] reported by others.

Table I. Putative Neurotransmitters in Identified Neurons of Aplysia*

	R2	R14	L11	C1
Serotonin	$1.8 \times 10^{-5} \pm 0.3$ (8)	$3.4 \times 10^{-5} \pm 1.2$ (5)	$1.1 \times 10^{-5} \pm 0.3$ (4)	$9.4 \times 10^{-4} \pm 3.1$ (10)
Histamine	$3.0 \times 10^{-6} \pm 0.5$ (5)	$7.0 \times 10^{-6} \pm 3.0$ (4)	$4.5 \times 10^{-6} \pm 0.7$ (4)	$1.4 \times 10^{-5} \pm 0.1$ (5)
Octopamine	$2.5 \times 10^{-6} \pm 1.0$ (5)	$1.5 \times 10^{-4} \pm 0.5$ (5)	$1.4 \times 10^{-5} \pm 0.6$ (5)	N. D. (5)
Dopamine	N. D. (6)	N. D. (6)	N. D. (3)	--
Norepinephrine	N. D. (6)	N. D. (6)	N. D. (3)	--
Acetylcholine†	3.9×10^{-4}	N. D.	3.3×10^{-4}	N. D.
Glutamate‡	7.6×10^{-3}	3.2×10^{-3}	6.0×10^{-3}	3.5×10^{-3}
Glutamine‡	3.5×10^{-3}	2.0×10^{-3}	2.1×10^{-3}	2.0×10^{-3}

* Results are reported as molarity ± SEM. 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 Borys, Weinreich and McCaman,[2] who expressed their data as μmole/g protein. For conversion to molarity, values for R2 and L11 were obtained as proportions from values given for acetylcholine as both μmole/g protein and molarity by McCaman, Weinreich and Borys.[22] For R14, concentrations were calculated using a cell volume of 22 nl reported by Giller and Schwartz[15] and a protein content of 2.08 μg/cell reported by McCaman and Dewhurst.[21] For C1, concentrations were approximated using a cell volume of 8.2 nl (calculated from average diameter observed in these experiments) and assuming a protein concentration of 0.8 μg/cell (given by McCaman and Dewhurst[21] for cell R1, which is similar in size to C1).

† From McCaman, Weinreich and Borys[22]

‡ From Borys, Weinreich and McCaman[2]

5-HT is present in cell C1 as well as in R2, R14 and L11 where its presence has not been previously demonstrated. The 5-HT content of C1 (9.4×10^{-4} M $\pm$ 3.1) is much greater than that of R2, R14 and L11. However, 5-HT is present in concentrations greater than 10^{-5} M in these other neurons. The presence of 5-HT has also been reported in C1 by Weinreich et al.[30] using a fluorescent assay, and their value is in good agreement with ours.

It has not escaped our attention that the addition of 5-hydroxytryptophan (5-HTP) to the incubation mixture results in a significant formation of radioactive melatonin; in fact, we have recently developed an extremely sensitive assay for tryptophan hydroxylase based on this finding (Brownstein, Kiser and Axelrod, unpublished observations). 5-HTP is decarboxylated to serotonin by the L-aromatic amino acid decarboxylase present in our NAT preparation. The addition of MK-486 (5×10^{-4} M) (Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey) to the incubation mixture completely inhibits the decarboxylase present there, and prevents endogenous 5-HTP from interfering with the assay of serotonin. In R2, R14 and L11, as well as in C1, all of the melatonin formed during the assay procedure appeared to be derived from serotonin present in these neurons. It was not possible to demonstrate the presence of any 5-HTP in the cells. This suggests that, as in warm-blooded species, the activity of L-aromatic amino acid decarboxylase is considerably greater than that of tryptophan hydroxylase in 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 I in concentrations between 10^{-6} and 10^{-5} M. The concentration of histamine in cell C1 is nearly two orders of magnitude less than that of 5-HT.

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 R2 and L11 (which are known to contain acetylcholine) are actually present in either satellite neurons or glial cells. It proved impossible to aspirate the cytoplasm from single cells following impalement with blunt micropipettes. Therefore, we elected to separate cytoplasm from membrane fragments mechanically under the dissecting microscope. The cells were removed, opened and their cytoplasm carefully blotted onto the pestle of one microhomogenizer. The membrane was then placed on the pestle of a second microhomogenizer. The two samples were analyzed as above. In eight separate determinations using R2, 75 percent of the total of both serotonin and histamine was found in the cytoplasm, while only 25 percent remained in the membrane fraction. Clearly most of the serotonin and histamine we measure must be intracellular.

Although dopamine is known to be present in the nervous system of Aplysia,[4] we have not detected dopamine in any identified cell body. Norepinephrine was not found in single neurons either, confirming the previous finding that norepinephrine is not present in these ganglia.[4]

Octopamine was found in some but not all of the large identified neurons.[26] It is present in relatively high concentration in cell R14 (1.5×10^{-4} M $\pm$ 0.5) but was detectable in R2 and L11 also.

IV. DISCUSSION

Because each of the substances listed in Table I (except norepinephrine) is present in the nervous system of Aplysia, and because specific postsynaptic receptors for these substances have been demonstrated,[5,6,14] they must be considered to be putative neurotransmitters.

Serotonin, octopamine and acetylcholine are unequally distributed among the single neurons studied, while Table I indicates that histamine, glutamine and glutamate are about evenly distributed. Acetylcholine[22] and choline acetyl transferase[15,20] are present in R2 and L11 but not in C1 or R14. Octopamine is in moderate concentration in R14 but is present also in R2 and L11. Serotonin is present in R2, R14 and L11 as well as in C1. We have recently demonstrated tryptophan hydroxylase activity in R2 as well as in C1 (Brownstein, Kiser, Carpenter and Axelrod, unpublished observations) suggesting that serotonin is not only present but is also synthesized by neurons which are known to contain and synthesize acetylcholine. Gamma-aminobutyric acid (GABA) has recently been demonstrated by dansyl chloride microchromatographic techniques to be present in most single cells of Aplysia studied, including C1 and R2.[8]

The observation that these putative neurotransmitters are not uniformly distributed among the nerve cells of Aplysia suggests biochemical specificity. Thus, we should like to be able to call R2 and L11 "cholinergic", R14 "octopaminergic" and C1 "serotonergic". 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 by 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 could be that a single neuron is capable of synthesizing only one transmitter but has uptake systems for other substances which have no function in that neuron. This suggestion is supported by the observations of Eisenstadt et al.[11] that R2 and L11 synthesize Ach and that C1 synthesizes 5-HT, 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 levels of 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.[19] 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 be 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.[31] Furthermore, many mammalian neurons also have a considerably greater DNA content than the diploid amount[19] suggesting that, if this be 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 a postsynaptic receptor. Postsynaptic receptors for most putative transmitters other than Ach do appear to be very discrete and localized only to specific neurons. Although most Aplysia neurons do not appear to have receptors for all of these substances, many do have specific receptors for several of them.[8] Thus more than one transmitter might be released and subsequently activate postsynaptic receptor sites.

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[3] or inhibition of release.[12,18,28] These neurotransmitters might act on the same or different postsynaptic receptors. They could modulate one 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[1] have demonstrated that neurons of the dorsal root ganglion of cat which are exposed to labelled leucine synthesize and distribute, by fast axoplasmic transport, different proteins to the two major branches of the axon. The possibility that a neuron might release Ach at one terminal, 5-HT at a second, and octopamine at a third cannot be excluded. Furthermore, Grossman et al.[17] 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 useful purposes. Thus they might regulate one another's biosynthesis, storage, metabolism or release. For example, histamine[16] and 5-HT,[10,16] at millimolar concentrations, are known to inhibit dopamine-β-hydroxylase. 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.

The suggestions which we have made would allow for the possibility that each nerve cell plays a broader role in integrative processing of information than was heretofore thought possible.

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