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PHOTOAFFINITY LABELING OF SEROTONIN-BINDING PROTEINS

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Summary

A photosensitive arylazide derivative of serotonin (nitroarylazidophenyl serotonin, NAP-serotonin) has been synthesized for use in studying the biochemical nature of serotonin binding sites. [^{3}H]-NAP-serotonin possesses a similar ability to bind to the crude membranes of rat brains does [^{3}H]-serotonin and therefore seems suitable for use as a photoaffinity labeling probe for serotonin binding sites. Upon irradiation with ultraviolet light, [^{3}H]-NAP-serotonin covalently attaches to protein components of the brain homogenate. Several distinct radioactively labeled proteins have been separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Their apparent molecular weights were 80,000, 49,000, and 38,000 (±5%). When 1 μM of unlabeled serotonin or d-lysergic acid diethylamide (d-LSD) was added prior to photolysis, the incorporation of [^{3}H]-NAP-serotonin into these proteins was inhibited significantly. No inhibitory effect was observed when dopamine was used. These observations suggest that the photoaffinity labeled proteins are specific for serotonin binding.

The binding of radioactive serotonin to membrane receptors has been intensively studied in recent years (1-5). This radioligand is known to bind to protein, but the exact nature of the protein is poorly understood. Previous work in our laboratory has led to the isolation of two types of serotonin-binding proteins from both rat hypothalamus and spinal cord (6). Highly purified serotonin binding proteins were obtained and their interactions with LSD were studied (7). However, the low yield of the purified serotonin-binding proteins prevented thorough biochemical characterizations. A detergent was required to solubilize the membrane proteins prior to purification so that some properties of the serotonin-binding proteins may have been altered before the binding studies were performed.

We are now reporting a new approach to the study of serotonin-binding proteins. An arylazide derivative of serotonin has been synthesized as a photoaffinity labeling probe (8,9). This derivative, [^{3}H]-nitroarylazidophenyl serotonin ([^{3}H]-NAP-serotonin), binds to protein components of a rat brain homogenate and becomes covalently attached to the protein through an active nitrene group upon irradation (8). The radioactively labeled proteins are then separated on sodium dodecyl sulfate polyacrylamide gels. Since the binding of the serotonin derivative to individual proteins can be demonstrated, this technique provides more specific information than do conventional radioligand binding assays with membrane preparations. This method does not require detergent treatment to solubilize the protein prior to binding the radioligand so the binding probably reflects more accurately the physiological state of the receptor.

Methods

Synthesis of NAP-serotonin

The oxalate salt of 5-hydroxytryptamine (serotonin, 0.15 mmoles) was reacted with 0.3 mmoles of 4-fluoro-3-nitro-phenylazide (FNPA) in 1 ml of 233 mM Na_2CO_3 for 16 hours. The reaction was performed in the dark under a nitrogen atmosphere. The reaction mixture was then brought to dryness with a rotary evaporator and then redissolved in 0.5 to 1 ml of $CHCl_3$:CH_3OH (1:1; v/v). The products were separated on silica gel plates developed with $CHCl_3$. As shown in Fig. 1-A, the major product (b), nitroarylazidophenyl serotonin (R_f = 0.1), was well separated from serotonin (a), FNPA (d), and a minor product (c). NAP-serotonin was eluted from the plates with methanol and the silica gel was removed by centrifugation. The solvent was evaporated and the residue was rechromatographed on silica gel plates developed with $CHCl_3$:CH_3OH (9:1; v/v). The synthesis afforded a yield of 60 to 70 percent.

Synthesis of [^{3}H]-NAP-serotonin

Five millicuries of [1,2-[^{3}H](N)]-serotonin binoxalate (specific activity 24 to 29 Ci/mmole, from New England Nuclear) was used as received, lyophilized, and taken up in 200 µl of 233 mM Na_2CO_3. A two to three-fold molar excess of FNPA was reacted with [^{3}H]-serotonin as described above. The major product, [^{3}H]-NAP-serotonin co-migrated with cold NAP-serotonin on the silica gel plates, and was eluted from the plates for this study. Approximately 5 to 10 percent of the total counts were recovered. All of the procedures were performed under dim light.

Preparation of 50,000 x g Pellet of Rat Brain Homogenate

One gram of whole rat brain (with the cerebellum removed) was homogenized with 40 volumes of 50 mM Tris-HCl buffer, pH 7.4. The homogenate was centrifuged for 10 minutes at 1000 x g in order to remove the debris. The supernatant was then centrifuged for 15 minutes at 50,000 x g. The pellet was resuspended in 40 volumes of the same buffer, incubated for 15 minutes at 37°C, and centrifuged for 15 minutes at 50,000 x g in order to remove the endogenous serotonin (10). The resultant pellet was then resuspended in 5 volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 4 mM $CaCl_2$, 10 µM pargyline, and 0.1 percent ascorbic acid.

Radioligand Binding Assay

Aliquots of the 50,000 x g pellet containing approximately 500 µg protein were incubated in triplicate at 37°C for 10 minutes following the addition of tritiated and unlabeled ligands. The samples were cooled on ice and then rapidly vacuum-filtered through Whatman GF/B filters. The filter wells were rapidly rinsed with 15 ml of ice-cold buffer. The specific binding was defined as the difference between the total counts trapped on the filter in the absence and the presence of 10 µM unlabeled serotonin. To determine the ability of NAP-serotonin to displace [^{3}H]-serotonin binding, various concentrations of NAP-serotonin (10^{-8}M to 10^{-3}M) was incubated with 2 nM [^{3}H]-serotonin.

Photoaffinity Labeling of 50,000 x g Pellet with [^{3}H]-NAP-serotonin

Proteins from the 50,000 x g pellet at a concentration of 500 µg/ml were incubated in the dark with 0.3 µCi/ml [^{3}H]-NAP-serotonin at 37°C for 10 minutes and then cooled on ice. The final concentration of [^{3}H]-NAP-serotonin was 10 nM. The samples were then irradiated at 0°C for 5 minutes with a UVS-11 Mineralight hand lamp at a distance of 5 cm. In order to remove the unreacted [^{3}H]-NAP-sero-

tonin, the pellets were washed three times with 50 mM sodium phosphate buffer, pH 7.6, and precipitated again by using an Eppendorf centrifuge Model 5412. Approximately 200 to 250 μg protein was dissolved in 50 mM sodium phosphate buffer, pH 7.6, containing 1 percent SDS and 2 percent mercaptoethanol, and then was applied to sodium dodecyl sulfate polyacrylamide gels for electrophoresis (11). At the end of the electrophoresis, the gel was sliced and dissolved in PPO-POPOP-toluene scintillation counting solution containing 10 percent NCS and 1 percent H_2O. The samples were then counted in a Beckman LS 335 liquid scintillation counter.

Results and Discussion

NAP-serotonin as a Probe for Studying Serotonin Binding Sites

As shown in Fig. 1, NAP-serotonin (b), the major product, was well separated from the parent compounds serotonin (a) and FNPA (d), a minor product (c), and the photolyzed products (e) by thin-layer chromatography on silica gel plates. The precise linkage between the nitroarylazidophenyl group and serotonin is currently being studied by infrared and UV absorption spectroscopy.

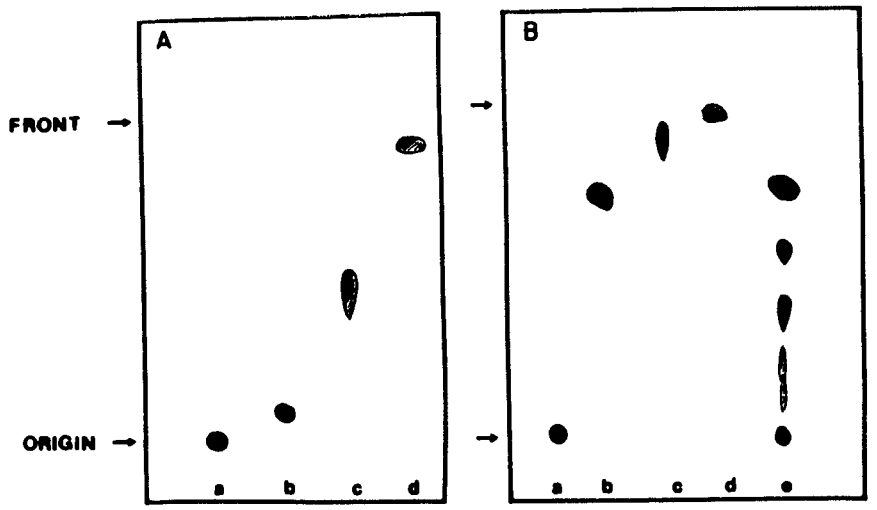

FIG. 1

Silica gel thin layer chromatography of NAP-serotonin. The plate was developed with (A):$CHCl_3$, (B):$CHCl_3$:CH_3OH (9:1; v/v).

(a): unreacted serotonin, (b): NAP-serotonin, major product, (c): minor product (d): unreacted FNPA and (e): photolyzed products of NAP-serotonin, NAP-serotonin was irradiated with UV light for 5 minutes before chromatograph.

[^{3}H]-NAP-serotonin binding to the crude membranes from rat brains is depicted in Fig. 2. Scatchard analysis indicated that [^{3}H]-NAP-serotonin exhibits a high-affinity binding site similar to that of [^{3}H]-serotonin. The dissociation constant and the maximum number of the high-affinity [^{3}H]-NAP-serotonin binding is the same as that of [^{3}H]-serotonin binding. However, the low-affinity [^{3}H]-NAP-serotonin binding was not saturable probably due to the diffusion of [^{3}H]-NAP-serotonin into lipid moiety of the membrane since aryl group introduced higer hydrophobicity.

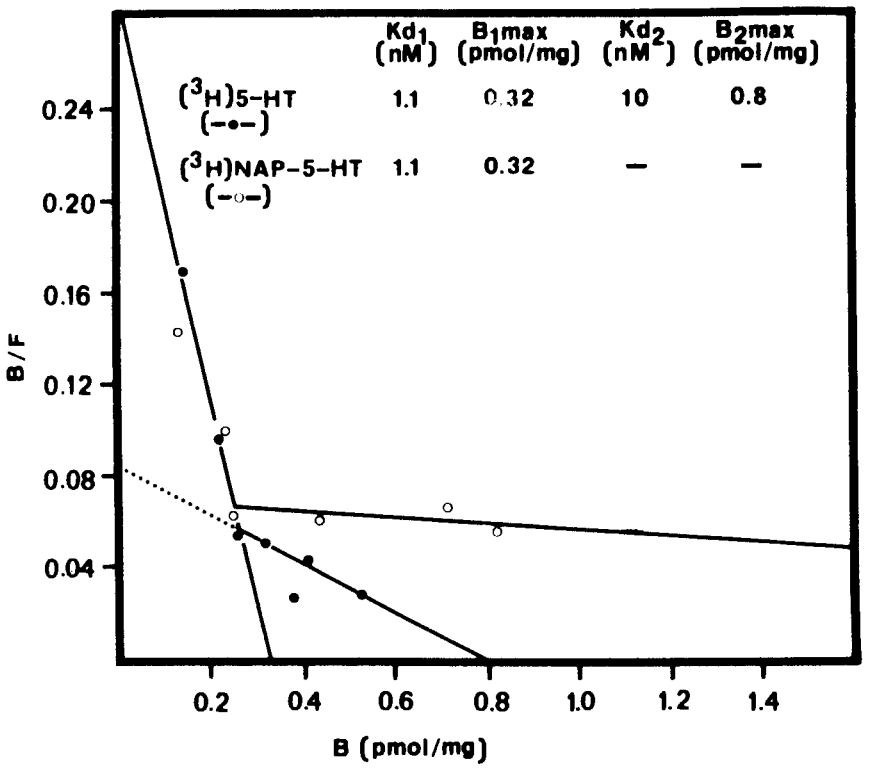

FIG. 2

Scatchard analysis of [^{3}H]-NAP-serotonin and [^{3}H]-serotonin binding to the crude membranes of rat brain.

Crude membrane proteins of 500 µg were incubated in triplicate with [^{3}H]-serotonin or [^{3}H]-NAP-serotonin in the absence and the presence of 10 µM unlabeled serotonin. The concentrations of the radioligands were 1 to 20 nM. The binding assays of [^{3}H]-NAP-serotonin were carried out under dim light. Key to the plot: (·——·): [^{3}H]-serotonin; (o——o): [^{3}H]-NAP-serotonin.

The ability of unlabeled NAP-serotonin to displace membrane-bound [^{3}H]-serotonin is shown in Fig. 3. Although displacement potency of NAP-serotonin was considerably less than that of serotonin itself (by a factor of 100), NAP-serotonin was still able to displace 90 percent of the [^{3}H]-serotonin binding. Under the same conditions, d-LSD inhibited [^{3}H]-serotonin binding almost as effectively as serotonin itself. Dopamine, on the other hand, did not inhibit [^{3}H]-serotonin binding even at a concentration as high as 10^{-4}M. These observations suggest that NAP-serotonin may only bind to the [^{3}H]-serotonin binding sites and that it may therefore be a suitable probe for locating specific serotonin binding sites. The lower displacement potency of NAP-serotonin compared to serotonin itself may be explained by the higher non-specific binding of NAP-serotonin compared to serotonin. However, the total amounts of [^{3}H]-NAP-serotonin and [^{3}H]-serotonin displaceable by 10 µM unlabeled serotonin are similar (Fig. 2).

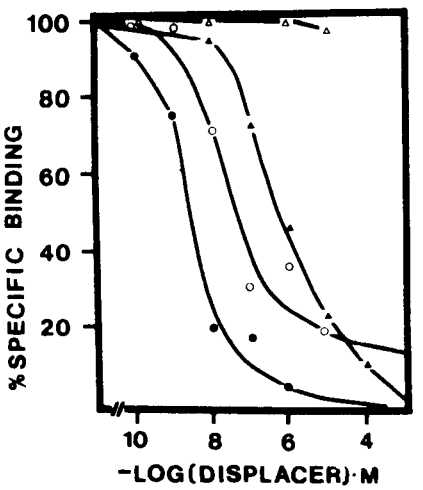

FIG. 3

Displacement of [^{3}H]-serotonin binding by unlabeled NAP-serotonin. [^{3}H]-serotonin was incubated with increasing concentrations of unlabeled NAP-serotonin (▲—— ▲), serotonin (• —— •), d-LSD (o —— o), and dopamine (Δ —— Δ). See text for details of the assay. With NAP-serotonin the assay was performed under dim light.

Photoaffinity Labeling of Serotonin-Binding Proteins

[^{3}H]-NAP-serotonin was incubated with the 50,000 x g pellet in the dark at 37°C for 10 minutes and then the mixture was cooled on ice. Irradiation with ultraviolet light resulted in the irreversible binding of the tritium-label to protein components of the homogenate. When the proteins were solubilized and subjected to SDS polyacrylamide gel electrophoresis, three distinct bands of radioactively labeled proteins were separated (Fig. 4). The apparent moleculare weights of these proteins based on their mobility in the gels were 80,000, 49,000, and 38,000 (±5%). Bovine serum albumin (mol. wt. 65,000), ovalbumin (mol. wt. 45,000), chymotrypsinogen A (mol. wt. 25,000), and ribonuclease (mol. wt. 13,800) were used as reference standards for the molecular weight determinations.

Evidence that the attachment of tritium-label to these proteins represented specific serotonin binding was obtained by incubating unlabeled serotonin at a concentration of 1 μM along with the [^{3}H]-NAP-serotonin and the 50,000 x g pellet prior to photolysis. Under these conditions, the amount of radioactivity incorporated into the proteins with molecular weights of 80,000, 49,000 and 38,000 was significantly decreased (Fig. 4). This findings suggests that the incorporation of [^{3}H]-NAP-serotonin into proteins is displaceable by excess cold serotonin. The amount of radioactivity incorporated into each protein in the absence and the presence of 1 μM cold serotonin allowed us to estimate the percent displacement of total serotonin binding into each protein. As shown in Table 1, 60 percent of the total [^{3}H]-NAP-serotonin binding to the protein with molecular weight 49,000 was displaced by cold serotonin, and 50 percent of the [^{3}H]-NAP-serotonin incorporation into the proteins with molecular weights of 80,000 and 38,000 was displaced. When 1 μM LSD was added prior to photolysis instead of 1 μM cold serotonin, the incorporation of radioactivity into these proteins was inhibited by about 50 percent (Table 1). The presence of 1 μM of dopamine in the incubation mixture prior to photolysis did not affect the incorporation of [^{3}H]-NAP-serotonin into any of the labeled proteins. These results suggest that the incorporation of [^{3}H]-NAP-serotonin into protein components may

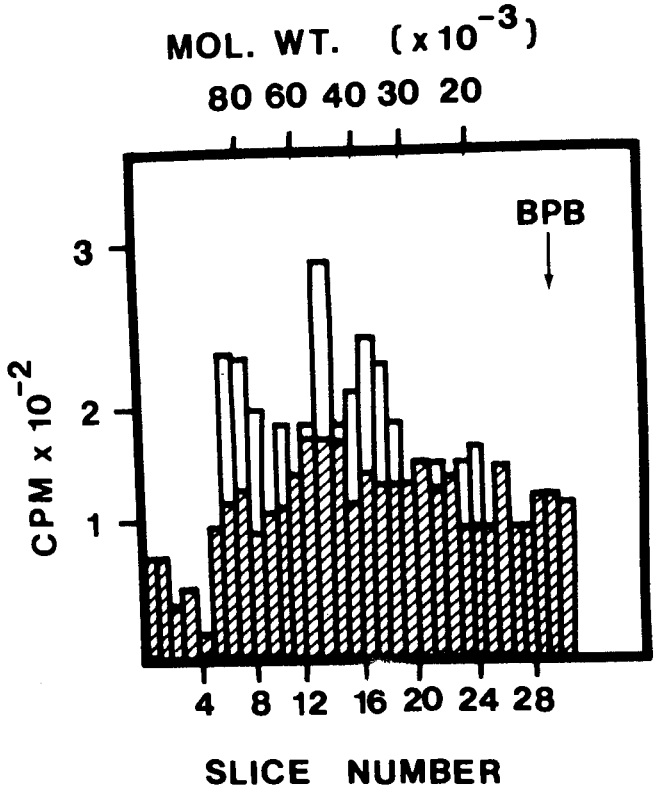

FIG. 4

Radioactivity profile of [^{3}H]-NAP-serotonin labeled membrane proteins. 50,000 x g pellet containing 200 µg protein were incubated with 10 nM [^{3}H]-NAP-serotonin (about 0.3 µCi/ml) in the absence and the presence of 1 µM unlabeled serotonin at 37°C for 10 minutes in the dark. The mixtures were then irradiated with ultraviolet light for 5 minutes at 4°C. [^{3}H]-NAP-serotonin labeled membranes were subjected to SDS polyacrylamide gel electrophoresis. Following the electrophoresis the gels were sliced and counted. ▭ indicate specific binding, ▨ indicate non-specific binding. See text for details.

specifically label serotonin binding sites. The physiological significance of these photoaffinity labeled serotonin binding sites is suggested by the fact that the incorporation of [^{3}H]-NAP-serotonin into membrane proteins increased in a parallel fashion to the increase in specific [^{3}H]-serotonin binding in the crude membranes from the brains of p-chlorophenylalanine treated rats. This data has been submitted elsewhere for publication.

In summary, the present study provides a new approach to the study of the biochemical nature of serotonin binding sites. Using the photoaffinity labeling technique we demonstrated that there may be three proteins involved in specific serotonin binding. The possible function of each of these three proteins in relation to the serotonin receptor is currently under investigation.

TABLE 1

Effect of Various Drugs on the Photocatalyzed Covalent Incorporation of [^{3}H]-NAP-serotonin to 50,000 x g Pellet of Rat Brain Tissue Homogenate

Drug	% Displacement Molecular Weight of Protein		
	80,000	49,000	38,000
None (Control)	0	0	0
Serotonin	50	60	50
d-LSD	50	56	40
Dopamine	0	0	10

[^{3}H]-NAP-serotonin was incubated with 50,000 x g pellet in the absence and in the presence of 1 μM unlabeled serotonin in the dark. At the end of incubation, the mixture was irradiated with UV light and then applied to SDS gel electrophoresis. The percent displacement was estimated by the difference in [^{3}H]-NAP-serotonin incorporation in the absence and the presence of 1 μM of each drug. These data represent the average of three experiments. The error was less than 10 percent.

Acknowledgments

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