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Development of a Photoaffinity Ligand for Octopamine Receptors

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SUMMARY

Octopamine receptors are widely distributed in invertebrate species, yet little is known about their biochemical structure or tissue localization, in part because there exist no high affinity or irreversible ligands for these receptors. This paper characterizes 2-(2,6-diethyl-4-azidophenylimino)imidazolidine (NC-5Z), a new, high affinity octopamine receptor probe that binds reversibly and, under photolyzing conditions, irreversibly to membrane-associated octopamine receptors. Under reversible conditions NC-5Z is a full agonist, 50–100 times more potent than octopamine in activating the highly enriched and specific octopamine-sensitive adenylate cyclase of the firefly light organ. NC-5Z shows a similar potency in cockroach muscle and thoracic ganglia and in tobacco hornworm nerve cord. Activation of light organ adenylate cyclase by NC-5Z is nonadditive to that caused by octopamine and can be blocked by antagonists, including mianserin (K_i = 0.9 μM), cyproheptadine (K_i = 5 μM), phentolamine (K_i = 20 μM), and propranolol (K_i = 75 μM). These constants agree well with those for the same antagonists in inhibiting stimulation due to octopamine. In physiological studies, NC-5Z mimics the action of, but is more potent than, octopamine in stimulating light emission in intact firefly tails and in disrupting motor behavior and feeding of tobacco hornworms. Under reversible conditions, [^{3}H]NC-5Z, the tritiated derivative of NC-5Z, binds to light organ membranes with an apparent affinity (0.59–0.7 μM) similar to that (0.35–0.7 μM) for NC-5Z in activating adenylate cyclase. Under photolyzing conditions, NC-5Z irreversibly activates light organ adenylate cyclase, and this can be blocked by an excess of octopamine. Under similar conditions, [^{3}H]NC-5Z binds irreversibly to light organ membranes and to membranes from tobacco hornworm nerve ganglia, fat body, and gut. This binding is reduced by prior incubation with octopamine agonists, including octopamine, demethyl-chlordimeform, and 2-(phenylimino)imidazolidines, but not by norepinephrine, dopamine, serotonin, or histamine. Irreversible binding is also reduced by prior incubation with antagonists, most effectively (55% of total binding) by mianserin. The apparent affinity of [^{3}H]NC-5Z for membrane binding, as reflected by its ability to be displaced by mianserin, is altered by GTP. In autoradiographic studies of whole tissue, [^{3}H]NC-5Z shows irreversible, mianserin-displaceable labeling of intact firefly light organs. Taken together, these data indicate that NC-5Z and [^{3}H]NC-5Z are potent and selective agonists of octopamine receptors in a variety of tissues. Under reversible conditions, NC-5Z should be useful for physiological studies of octopamine receptor action. Under photolyzing conditions, NC-5Z and [^{3}H]NC-5Z bind irreversibly and should be useful both for anatomical studies of octopamine receptor localization and for biochemical studies characterizing and isolating octopamine receptor proteins.

In many invertebrate species, norepinephrine is present at a concentration much lower than that found in vertebrates, and, in these lower phyla, firm evidence for the existence of α- or β-adrenergic receptors is lacking (1, 2). By contrast, octopamine, the 4-hydroxy analog of norepinephrine, is present in most invertebrates at much higher concentrations than in vertebrates (3, 4), and unique receptors for octopamine have been identified in the lower phyla (5, 6). Physiologically, octopamine functions in invertebrates as a neurotransmitter, neurohormone, and neuromodulator (1, 2, 7), and it has been proposed that octopamine may fulfill a role, in invertebrates, analogous to that which norepinephrine fulfills in vertebrates (8, 9).

The existence of octopamine receptor subtypes has been postulated on the basis of physiological studies (6). However, biochemical research on octopamine receptors has been hampered by a paucity of potent octopamine agonists and antagonists and by the lack of any irreversible octopamine receptor ligands. This lack of irreversible ligands is particularly relevant to the lower affinity octopamine receptor subtype(s) that are associated with adenylate cyclase activation. Because of the extremely rapid off-time of existing ligands for such receptors, it has not been possible to carry out effective studies involving

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ABBREVIATIONS: NC-5Z, 2-(2,6-diethyl-4-azidophenylimino)imidazolidine; PII, 2-(phenylimino)imidazolidine; NC-5, 2-(2,6-diethyl-phenylimino)imidazolidine; NC-7, 2-(2-chloro-4-methyl-phenylimino)imidazolidine; DCDM, *N*-demethylchlordimeform; DDCDM, *N,N*-didemethylchlordimeform; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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binding, histological localization of receptors, or receptor protein isolation.

In this paper, I describe the development and characterization of a new octopamine agonist that is the most potent reversible activator of octopamine-activated adenylate cyclase yet described. Of particular relevance to studies of receptor localization and isolation is the fact that this compound also functions as a photoaffinity analog that can bind irreversibly to octopamine receptor proteins both in membrane preparations and in intact tissues.

Materials and Methods

Synthesis. NC-5Z (Fig. 1) was developed as a result of recent work on a reversible class of potent and selective octopamine agonists, the substituted PIIs. These PIIs have been shown to activate octopamine-sensitive adenylate cyclase in several species (10), to exert octopamine-like antifeeding effects in insects (11), and to mimic the electrophysiological effects of octopamine at octopamine$_1$, octopamine$_{2A}$, and octopamine$_{2B}$ receptor subtypes (12). NC-5Z was synthesized with the goal of creating a PII derivative that would maintain its potency and would contain a photoactivatable azido group capable of covalently linking to octopamine receptor sites. As described below, NC-5Z fulfilled these criteria and proved, under reversible conditions, to be significantly more potent than previously described octopamine agonists.

Synthesis of NC-5Z is described in detail elsewhere.[1] Synthesis of [^{3}H]NC-5Z (the tritiated derivative of NC-5Z), is also described.[1] [^{3}H]-NC-5Z could not be prepared directly from NC-5Z because of the lability of the azido group. Instead, it was made by first synthesizing 2-(2,6-diethyl-3,5-dibromo-4-aminophenylimino)imidazolidine, reducing this with tritium gas at the 3- and 5-positions, and then converting the amino derivative to the azido compound. The activity of the labeled compound was confirmed by two separate procedures which showed (a) that it was as potent as NC-5Z in activating the octopamine-sensitive adenylate cyclase of the firefly light organ and (b) that it was as potent as NC-5Z in stimulating light emission from intact firefly tails.[1]

[^{3}H]NC-5Z was stored as a solution in ethanol under argon at −85° and was protected from light. At intervals, it was checked for decomposition by thin layer chromatography on Analtech silica gel GF plates, using two separate solvent systems, $CHCl_3/CH_3OH/NH_4OH$ (9:1:0.1) and $(C_2H_5)_3N/CH_3OH$ (2:98). After chromatography, the dried thin layer plates were exposed to standard X-ray film and the resulting autoradiograms were compared with the position (seen by UV shadowing) of NC-5Z standards (made up fresh from crystalline NC-5Z), which had been run in parallel. Under these conditions, [^{3}H]NC-5Z showed no evidence of chemical decomposition for a period in excess of 1 year.

Tissue preparation. Specimens of firefly, *Photinus pyralis*, were collected in summer, frozen on dry ice, and stored in liquid N_2. Under these conditions, octopamine-sensitive enzyme activity remains stable for 6 months or longer (13). After thawing at 4°, tail sections were opened and the light organs were removed from the ventral cuticle, cleaned of any adhering, nonlantern tissue, and homogenized in 6 mM Tris-maleate buffer, pH 7.4. A washed particulate (P_2) fraction was prepared by diluting the homogenate with 30 ml of 6 mM Tris-maleate (pH 7), centrifuging at 120,000 × *g* for 30 min, rehomogenizing the pellet in 30 ml of buffer, and recentrifuging. The final pellet was resuspended in a volume of 6 mM Tris-maleate equivalent to the starting amount used for homogenization, and the tissue was maintained at 0° until use. For adenylate cyclase measurements, the initial tissue homogenate concentration was 5 mg of wet weight/ml; for reversible binding studies under dim illumination, the initial concentration was 0.2–2.0 mg/ml; and for photolysis experiments, the initial concentration of tissue was 20–50 mg/ml. From adult cockroaches (*Periplaneta americana*), P_2 fractions were prepared from homogenates (8–10 mg/ml) of combined pro-, meso-, and metathoracic ganglia and from homogenates (35 mg/ml) of metathoracic leg muscle (main depressor plus posterior coxal depressor). Three additional P_2 fractions were also prepared from ventral nerve cords (including head and tail ganglia), fat body, and cleaned gut muscle segments from 50-mm long tobacco hornworms (*Manduca sexta*) that were raised on artificial media (Carolina Biological Supply, Burlington, NC). For adenylate cyclase assay, 10–20 mg/ml of *Manduca* tissue was initially homogenized; for photolysis binding experiments, 100–200 mg/ml of tissue was initially homogenized.

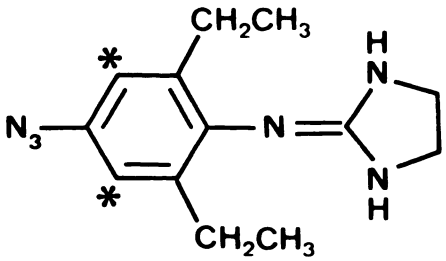

Fig. 1. Structure of NC-5Z. [^{3}H]NC-5Z is labeled (*) at the 3- and 5-positions of the phenyl ring.

Adenylate cyclase assay. Adenylate cyclase activity of all tissues was measured in dim light in test tubes containing (in 0.3 ml) 80 mM Tris-maleate, pH 7.4; 10 mM theophylline; 8 mM $MgCl_2$; 0.1 mM GTP; 0.5 mM EGTA; 2 mM ATP; 0.06 ml of P_2 fraction; and various compounds to be tested. Prior experiments had determined that, under these conditions, adenylate cyclase activity was optimized (13). The various reaction components (minus ATP and GTP) were mixed and allowed to stand for 5–10 min at 0°. The enzyme reaction (4 min at 30°) was then initiated by addition of ATP and GTP, stopped by heating to 90° for 2 min, and then centrifuged at 1000 × *g* for 15 min to remove insoluble material. Cyclic AMP in the supernatant was measured by protein binding assay (14). Under these assay conditions, enzyme activity was linear with respect to time and enzyme concentration, and phosphodiesterase activity [measured by the method of Filburn and Karn (15)] was nearly completely inhibited. Protein concentration was determined by the Lowry method. Activation constants (K_a) were calculated from dose-response curves that contained 12–16 data points, each assayed for cyclic AMP content in duplicate. In most cases, dose-response curves were repeated two to four times.

To allow solubilization of hydrophobic compounds, including NC-5, NC-5Z, and the formamidines DCDM and DDCDM, stock solutions (10×) were initially dissolved (in dim light) in 50% polyethylene glycol 400 (J. T. Baker, Phillipsburg, NJ) and then diluted to a final concentration of 5% polyethylene glycol in the final assay buffer. Extensive preliminary testing with a variety of solvents (polyethylene glycol, methanol, dimethylsulfoxide, and propylene glycol), at concentrations from 1 to 50%, had established that polyethylene glycol caused the least change in enzyme activity. Thus, at concentrations of polyethylene glycol up to 15%, there was almost no alteration in the V_{max} and K_a for activation of light organ adenylate cyclase by octopamine. Nonetheless, in all adenylate cyclase experiments, the activity of agonists was always measured relative to the activity of octopamine run under the same solvent conditions. For both reversible and irreversible binding studies involving [^{3}H]NC-5Z, a stock solution (either 54 or 114 μM) in absolute ethanol was diluted directly into the final aqueous binding buffer. Under these conditions, [^{3}H]NC-5Z remained soluble.

For reversibility studies (Fig. 6), tissue homogenate was first preincubated in dim light at room temperature for 5 min with vehicle or drug, using the assay conditions described above, except that ATP was omitted. Homogenates were transferred to 15-mm open wells in a

[1] J. A. Nathanson and G. Kaugars. A probe for octopamine receptors: synthesis of 2-(2,6-diethyl-4-azidophenylimino)imidazolidine and its tritiated derivative, a potent reversible and irreversible activator of octopamine-sensitive adenylate cyclase. Submitted for publication.

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plastic tissue culture dish and irradiated for 10 min at a distance of 2 cm from a bank of three 15 W Sylvania GTE germicidal lamps (G15T8). Homogenates were transferred to centrifuge tubes, diluted 100-fold with 6 mM Tris-maleate (pH 7.4), and centrifuged at 130,000 × *g* for 30 min. The pellet was resuspended in 20 ml of buffer and was recentrifuged and washed twice more. The final pellet was resuspended (2–3 mg of wet weight/ml) and assayed for basal adenylate cyclase activity, as described above.

For antagonist studies, various concentrations of antagonist were tested against a fixed concentration of agonist (usually 1 μM for NC-5Z and 10 μM for octopamine). Stimulation in the presence of agonist plus antagonist was calculated as the increase over that seen in the presence of antagonist alone. When necessary, a computerized sigmoidal curve-fitting program was utilized for curve fitting (16). Inhibitory constants (K_i) for antagonists were calculated from the equation (17) $K_i = (IC_{50})/(1 + S/K_a)$, where IC_{50} is the concentration of antagonist necessary to give 50% inhibition of activity in the presence of agonist, S is the concentration of agonist present, and K_a is the concentration of agonist necessary for half-maximal activation of adenylate cyclase activity in the particular tissue used.

Effects on light emission. To measure the effects of drugs on firefly light emission, an isolated tail (terminal three abdominal segments containing the light organ) of a fresh adult *P. pyralis* male was mounted on a 30 g stainless steel needle and placed at the focal point of an optical system connected to a photometer-photomultiplier-chart recorder combination (11). Stock solutions (10×) of drugs in 50% polyethylene glycol were dissolved in insect saline (18) modified to contain no calcium and 20 mM manganese, a salt composition that increased the specificity of the bioassay by blocking any potential effects of drugs on endogenous release of octopamine from nerve terminals (19). Three to five microliters of drug solution were injected into the abdominal cavity dorsal to the lantern and light emission was recorded for 45 min or until it peaked, after which the next (larger) dose of drug was injected. Light production is expressed as radiance, each unit approximately equal to 1.6 nW, as measured in a solid angle of 0.033 steradiants.

Effects of insect behavior. To measure the effects of drugs on motor and feeding behavior of tobacco hornworms (*M. sexta*), drugs, dissolved in 50% methanol/water, were applied as a fine aerosol to isolated tomato leaves (each maintained in a closed Plexiglass container) and allowed to dry (11, 20). (Leaves were kept hydrated by placing their stems through a small hole in the cap of a water-filled 4-ml scintillation vial). A group of six, 3-day-old *M. sexta* larvae (reared on artificial media) were then placed on each leaf and the amount of leaf remaining 72 hr later was measured. The "anti-feeding activity" observed was the net result of motor-behavioral disruption and was not necessarily due to a specific effect on feeding (see below). The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.1%), was mixed with drug and applied to all (both control and experimental) leaves. At the dose used, 3-isobutyl-1-methylxanthine itself had little or no effect on the rate of leaf consumption.

Binding experiments. To measure reversible binding under dim light, various concentrations of [^{3}H]NC-5Z (specific activity, 17.4 or 34.4 Ci/mmol), in the absence or presence of 500 μM mianserin, were incubated in the cold for 2 hr using the incubation medium described above for adenylate cyclase, including ATP, GTP, and the light organ P_2 fraction. As determined by prior experiment, a tissue concentration range of 0.2 to 2 mg (wet weight)/ml of starting homogenate gave linear binding. Also, as determined experimentally, by 2 hr binding had reached a maximum. At the end of the incubation, fresh ATP and GTP were added and bound label was separated from free ligand by one of two methods. In some experiments, the contents of each tube were transferred to a GF/C filter on an Amicon filtration head, washed four times with cold 6 mM Tris-maleate (pH 7.4), dried, solubilized in 0.5 ml of Protosol, and counted for radioactivity using Econofluor. (To reduce nonspecific binding, filters were pretreated by overnight soaking in 3% polyethylenimine.) In other experiments, contents of each tube were transferred to siliconized microfuge tubes, and bound label was separated from free by centrifugation in the cold (12,000 × *g*) for 10 min. Supernatant was aspirated using a fine-bore heat-pulled Pasteur pipette, and the pellet was solubilized in Protosol overnight and counted in Aquasol. In both filtration and centrifugation assays, tubes were run in quadruplicate.

Photoaffinity labeling. For affinity labeling, tissue membranes were added to tubes that contained Tris-maleate, theophylline, $MgCl_2$, EGTA, ATP, and GTP, prepared as described above for adenylate cyclase assay. Displacing agents were added as described in Results, the mixture was incubated at 4° for 15 min, after which [^{3}H]NC-5Z (17.4 or 34.4 Ci/mmol) was added to a final concentration of 0.5 or 1 μM, and tubes were incubated for an additional 60–120 min in the cold. New ATP and GTP was then added and the contents of the tubes were transferred to quartz (far UV) spectrophotometry minicuvettes (1-mm path length; total volume, 0.30 ml). Photolysis was carried out for 15 min at 4° under a bank of three 15 W Sylvania GTE germicidal lamps (G15T8). Cuvettes were turned over every 2 min during the exposure and, after photolysis, the contents were transferred to tubes that contained 10 ml of 6 mM Tris-maleate, pH 7.4. Tubes were centrifuged at 120,000 × *g* for 30 min and the pellet was resuspended in 10 ml of buffer and washed twice more. The final pellet was solubilized in NaOH and aliquots were taken for measurement of radioactivity by scintillation counting. In some experiments, photolyzed membranes were washed, instead, by five repeated cycles of precipitation with 7.5% cold trichloroacetic acid and solubilization of the pellet with 0.2 ml of 1 N NaOH. (In these cases, 0.2 ml of 0.63% bovine serum albumin was added as a carrier protein.)

Tissue autoradiography. Light organs were dissected from previously frozen fireflies, cleaned, bisected, and placed in plastic tissue culture wells in 0.3 ml of adenylate cyclase incubation mix (above) in which the Tris-maleate buffer concentration had been increased to 140 mM. Mianserin (1 mM) was added to some wells and the light organs were incubated for 15 min at 4°, after which [^{3}H]NC-5Z (17.4 Ci/mmol) was added to a final concentration of 2.5 μM and the incubation was continued for 60 min at 4°. Light organs and incubation mix were then transferred to quartz spectrophotometry minicuvettes (1-mm path length) and photolyzed, as described above, for 15 min. Light organs were then removed, washed twice for 15 min with 1 ml of cold phosphate-buffered saline, and fixed overnight in the cold in 2% paraformaldehyde. The light organs were then washed for 30 min three times with phosphate-buffered saline, slide mounted, dried, dehydrated sequentually through 50, 80, 95, 100, and 100% ethanol, dried again, and placed in contact with LKB Ultrofilm [^{3}H] (No. 2208-190) for 24–48 hr.

Most common reagents were from Sigma Chemical Co. (St. Louis, MO). DDCDM and phentolamine were supplied by Ciba-Geigy (Summit, NJ), 2-(2-methyl-4-chlorophenylimino)imidazolidine by G. Leclerc (Universite Louis Pasteur, Strasbourg) or Boehringer Ingelheim (Indianapolis, IN), cyproheptadine by Merck (Rahway, NJ), and mianserin by Organon (Nijmegan, The Netherlands).

Results and Discussion

Effect of NC-5Z on Reversible Activation of Light Organ Adenylate Cyclase

The firefly light organ contains an octopamine-activated adenylate cyclase that is of high specific activity and is selective for octopamine and structurally related phenylethanolamines. In this tissue, there is no evidence for receptor-linked adenylate cyclase activation by norepinephrine, dopamine, serotonin, histamine, or adenosine (10, 21, 22). Accordingly, activation of adenylate cyclase in light organ membranes was used for the initial evaluation of the octopaminergic activity of NC-5Z. NC-5Z was first tested under nonphotolyzing conditions (in dim

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light) in which the azido group remains intact and in which (see later sections) binding remains reversible.

Fig. 2 shows that NC-5Z was a full agonist of light organ adenylate cyclase and was extremely potent, indeed, the most potent agonist yet described in this system. Maximal stimulation of enzyme activity occurred at an NC-5Z concentration of 10–100 μM, and significant stimulation (260% of control) was detectable at concentrations of NC-5Z as low as 10 nM. In various experiments, the calculated K_a for activation of adenylate cyclase ranged from 0.35 to 0.7 μM, a figure about 50- to 100-fold more potent than that of octopamine itself.

In prior studies, we have shown that clonidine, a PII derivative with α_2-adrenergic activity in vertebrates, has relatively little activity in the octopaminergic firefly lantern. Consistent with this, Fig. 2 shows that the *p*-azido deriviative of clonidine (an affinity ligand for α_2-receptors) also had relatively little octopaminergic activity, being a poor partial agonist, nearly 1000-fold weaker than NC-5Z.

In additivity experiments (not shown), a combination of a maximally effective concentration of octopamine (5000 μM) plus a maximally effective concentration of NC-5Z (100 μM) stimulated enzyme activity to the same degree (99 ± 6% of octopamine V_{max}) as octopamine alone, suggesting that the two compounds were affecting the same receptor.

Antagonists

Stimulation of light organ adenylate cyclase activity by NC-5Z could be inhibited by several antagonists, including phentolamine, cyproheptadine, and mianserin, which have shown selectivity for octopamine receptors (5, 23, 24). The β-adrenergic antagonist propranolol was less potent. Table 1 shows that the calculated K_i values and relative potencies of these various blockers in inhibiting NC-5Z stimulation of light organ adenylate cyclase were quite similar to those for these same blockers in inhibiting stimulation of adenylate cyclase by octopamine or by the structurally distinct octopaminergic agonist DCDM. These data support the evidence described above showing that NC-5Z is a potent and selective agonist of octopamine-activated adenylate cyclase.

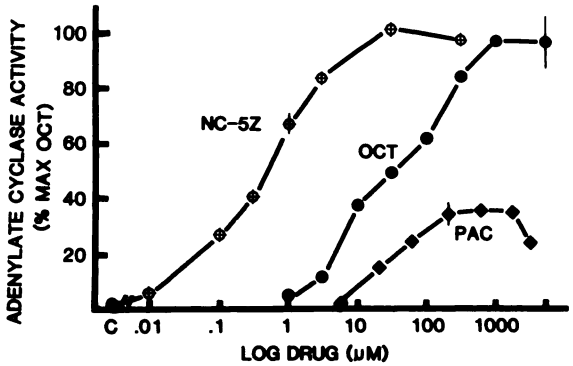

Fig. 2. Effects of NC-5Z, octopamine (*OCT*), and *p*-azido-clonidine (*PAC*) on firefly light organ adenylate cyclase activity. In both this figure and Fig. 3, enzyme activity is expressed as a percentage of the maximal stimulation caused by octopamine in the same experiment. Values shown are mean (± range) of replicate samples, each assayed for cyclic AMP content in duplicate. The absence of error bars at certain points indicates that the size of the error is smaller than the size of the symbol. Typically, basal enzyme activity was 20–50 pmol/mg of protein/min in the light organ, and, at optimal concentrations, enzyme activity was stimulated by octopamine 30- to 60-fold.

TABLE 1

Calculated k_i values for antagonists inhibiting stimulation of light organ adenylate cyclase by either octopamine, DCDM (a formamidine), or NC-5Z

Some of the data for octopamine and DCDM have been reported previously in Ref. 10.

Antagonist	K_i		
	Octopamine	DCDM	NC-5Z
	μM		
Mianserin	0.6		0.9
Cyproheptadine	6	2	5
Phentolamine	46	18	20
Propranolol	175	50	75

Other Tissues and Species

NC-5Z was potent not only in the firefly light organ but also in other tissues and species known to contain octopamine receptors (1, 2, 10). In broken cell fractions of cockroach (*P. americana*) thoracic ganglia, NC-5Z was a full agonist, about 70-fold more potent than octopamine (Fig. 3A). In cockroach leg muscle, NC-5Z was over 100-fold more potent than octopamine and was a partial agonist showing about 70% of the maximal activity of octopamine (Fig. 3B). In each of these two tissues, NC-5Z caused significant stimulation of activity at concentrations as low as 10 nM. In the nerve cord of *M. sexta* (tobacco hornworm) larvae, NC-5Z caused (in two separate assays) an apparent biphasic response (Fig. 3C). There was a higher affinity component causing partial stimulation of adenylate cyclase at a K_a of about 4 nM and a lower affinity component causing additional stimulation at a K_a of about 3 μM. In the same tissue, the K_a for octopamine activation of adenylate cyclase was about 80 μM. Of interest, previous studies with the formamidines, DCDM and DDCDM, and with the PIIs, NC-5 and NC-7, have suggested that *Manduca* nerve cord contains both a high affinity octopamine-activated adenylate cyclase, stimulated preferentially by formamidines and NC-7, and a lower affinity octopamine-activated adenylate cyclase, stimulated preferentially by NC-5 (10, 25). In studies of locust, Evans (12) has also detected a high affinity (about 10 nM) and low affinity (about 5 μM) component to octopamine stimulation of cyclic AMP levels in intact leg muscle. The structural similarities of NC-5Z (a 2,4,6-trisubstituted derivative) to both NC-5 (a 2,6-disubstituted derivative) and NC-7 (a 2,4-disubstituted derivative) may account for the ability of NC-5Z to stimulate both a high affinity and a low affinity component to receptor-activated adenylate cyclase in *Manduca* nerve cord. Taken together, the above studies in firefly, cockroach, and tobacco hornworm indicate that NC-5Z should be useful for activating octopamine receptors from a wide variety of tissues and species.

In Vivo Activity

Stimulation of light emission. In the firefly, initiation of light emission from the light organ is under neural control (26, 27). Octopaminergic nerves innervate the light organ and a variety of evidence indicates that octopamine, released from these nerves, acts upon octopamine receptors coupled to adenylate cyclase to initiate a series of reactions ending in luciferin/luciferase interaction and light emission (19, 28, 30).

As has been reported previously, octopamine, injected into intact isolated firefly tails, caused a dose-dependent stimulation of light production from the lantern (Fig. 4). NC-5Z, injected

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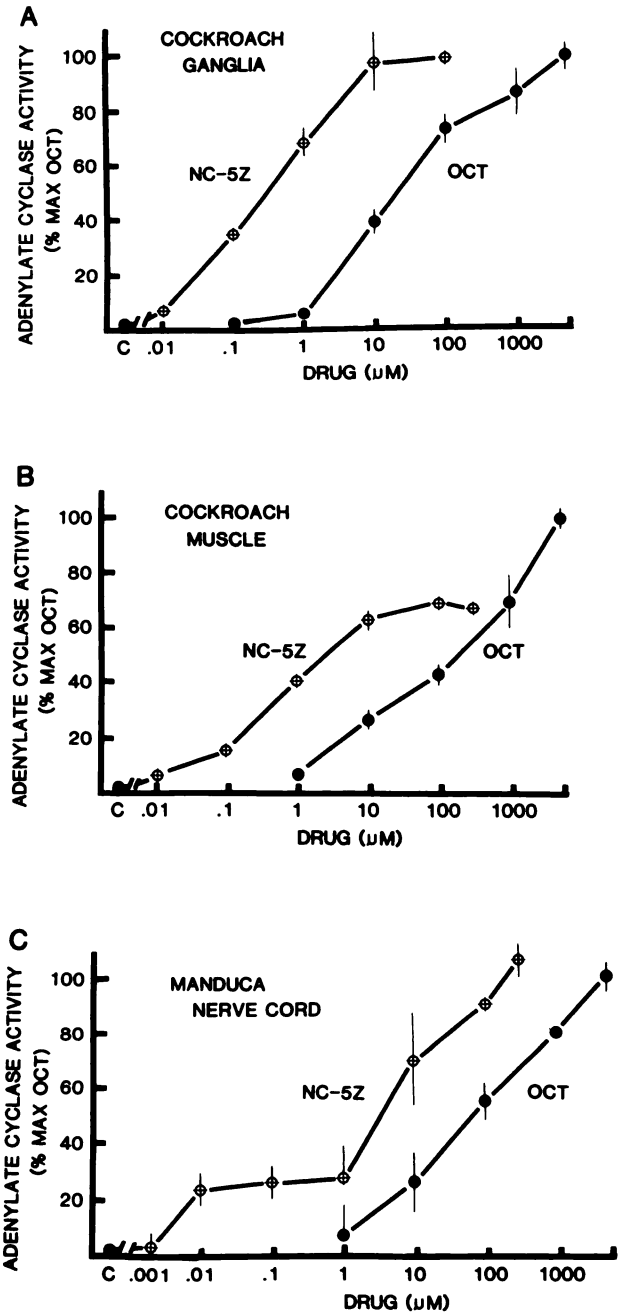

Fig. 3. Comparative effects of NC-5Z and octopamine (*OCT*) on adenylate cyclase activity in cockroach thoracic ganglia (A), cockroach leg muscle (B), and tobacco hornworm nerve cord (C). Basal enzyme activities were 37 ± 3 pmol/mg of protein/min for A, 2.1 ± 0.1 pmol/mg of protein/min for B, and 19 ± 0.4 pmol/mg of protein/min for C. Maximal stimulation by octopamine was 6.5-fold in A, 15-fold in B, and 2.1-fold in C.

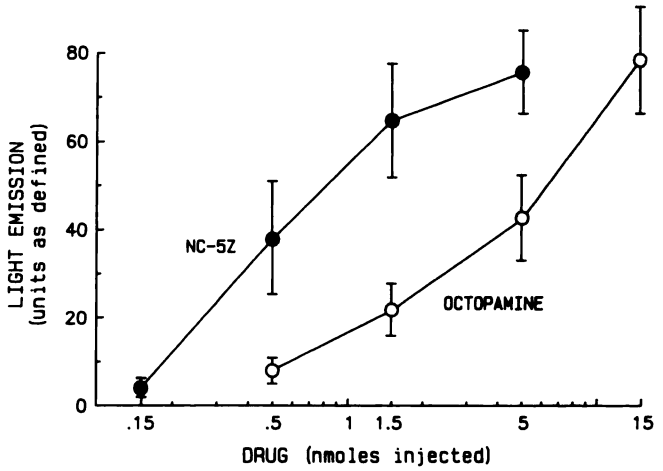

Fig. 4. Effects of NC-5Z and octopamine on eliciting light emission in the isolated firefly tail. Shown is the maximal illumination (in radiance, as defined in Materials and Methods) resulting from injection of the indicated dose of drug. Values are the mean (± standard error) of six animals for NC-5Z and five animals for octopamine.

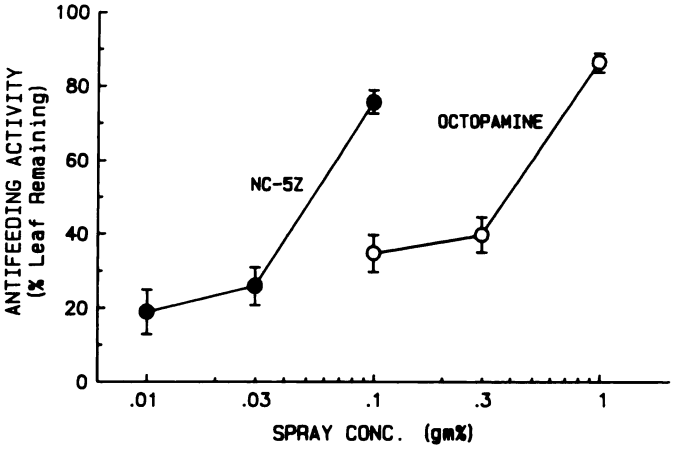

Fig. 5. Comparative effects of NC-5Z and octopamine on disruption of feeding in the tobacco hornworm. Compounds were applied as a spray to isolated tomato leaves and allowed to dry, and the leaves were then exposed (in dim light) to hornworm larvae. The percentage of leaf area remaining after 72 hr, measured in duplicate for each concentration, indicates the degree to which feeding was disrupted (and the degree to which the leaves were "protected" by the compound).

in a similar fashion, mimicked the action of octopamine. The maximal degree of light production elicited by NC-5Z was equivalent to the maximal degree of light production elicited by octopamine, but NC-5Z was about 15-fold more potent. This 15-fold greater potency compares with a 70-fold greater potency of NC-5Z in activating light organ adenylate cyclase (Fig. 2). Such quantitative comparisons between the *in vitro* and *in vivo* assay systems may not be entirely valid because of the possible differences in penetration and metabolism of compounds *in vivo* and because of the physiological complexity of light production regulation, which appears to involve several biochemical steps beyond the activation of adenylate cyclase.)

Pesticidal and pestistatic effects. Prior work has shown that, in certain insect species, large doses of octopaminergic agonists, including octopamine, the formamidines, and the PIIs, cause behavioral and motor abnormalities that interfere with feeding and sometimes result in insect death (10, 11, 30). In the present studies, NC-5Z showed the same effect. The azido derivative was about 10-fold more potent than octopamine in inhibiting the consumption of tomato leaves by *Manduca larvae*, as measured by the percentage of leaf area remaining after 72 hr of feeding (in dim light) on leaves sprayed with various concentrations of the compound (Fig. 5). Visual observations indicated that NC-5Z elicited behavioral changes (hyperactivity, tremor, rearing, leaf "walk off") similar to those seen with octopamine and other octopaminergic agonists such as the formamidines.

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Irreversible Activation of Adenylate Cyclase by NC-5Z

The UV absorption spectrum of NC-5Z showed peaks at 210 and 255 nm. As with other azido-substituted ligands, it was expected that, following preincubation of membranes with NC-5Z, exposure of the PII to intense UV light at these wavelengths would yield an active nitrene compound that could then react covalently with sites on nearby proteins (31). If NC-5Z were occupying an octopamine receptor at the time of photolysis, then the ligand should bind to a site on or near the receptor protein. *A priori*, it was not possible to predict whether this covalent binding would mimic the effect of reversible binding and lead to activation of adenylate cyclase. To determine this, experiments were carried out in which firefly membranes were preincubated with or without NC-5Z and then photolyzed. The membranes were then washed extensively to remove free ligand and ligand that was reversibly bound. After this, adenylate cyclase activity was assayed. Fig. 6 shows that prior photolysis with NC-5Z led to a 3-fold increase in adenylate cyclase activity, suggesting that a proportion of the octopamine receptors had been irreversibly bound and were being persistantly activated. That this was due to receptor activation was further supported by the fact that persistant adenylate cyclase activation could be blocked if, during the preincubation, NC-5Z was incubated together with an excess of octopamine (Fig. 6). Presumably, the excess octopamine displaced NC-5Z so that, during later photolysis, octopamine (and not NC-5Z) occupied the receptor. After photolysis, this octopamine was removed during washing, so that the receptor-activated adenylate cyclase returned to its unstimulated state. As would be expected, preincubation and photolysis with octopamine alone (Fig. 6) failed to cause persistent activation of adenylate cyclase.

Reversible Binding of [^{3}H]NC-5Z to Membranes

In order to carry out both reversible and irreversible receptor binding experiments, a radioactively labeled derivative of NC-5Z, tritiated in the 3- and 5-positions, was synthesized as described in Materials and Methods and elsewhere[1] (Fig. 1).

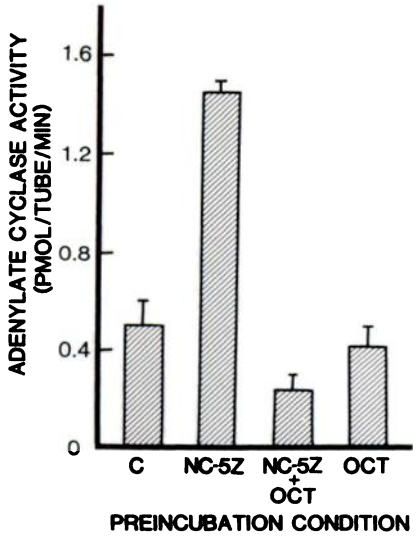

Fig. 6. Irreversible activation of light organ adenylate cyclase by NC-5Z. Light organ membranes were preincubated without drug (*C*), with NC-5Z (3 μM), with NC-5Z (3 μM) plus octopamine (*OCT*) (300 μM), or with octopamine (300 μM) alone. Membranes were then photolyzed with UV light, after which they were washed extensively and then assayed for adenylate cyclase activity.

[^{3}H]NC-5Z was first used to measure binding to light organ membranes under reversible conditions. This was done in order to determine whether there existed, in the highly homogeneous light organ tissue, any high affinity binding sites in addition to the lower affinity sites associated with adenylate cyclase activation. Such high affinity octopamine sites have previously been demonstrated using [^{3}H]octopamine binding to membranes prepared from *Drosophila* heads (32).

To measure reversible binding, light organ membranes were incubated, as described in Materials and Methods, with various concentrations of [^{3}H]NC-5Z in the absence or presence of an excess of mianserin. [Mianserin was used because of its potency in inhibiting octopamine-activated adenylate cyclase in the light organ (Table 1)]. Initially, a filtration assay was used to separate bound from free ligand in an effort to detect high affinity mianserin-displaceable [^{3}H]NC-5Z binding sites. However, there was no detectable specific binding at picomolar or low nanomolar concentrations, indicating either that [^{3}H]NC-5Z does not bind to such high affinity receptor sites or that the light organ does not contain enough sites to be detected. We were able to detect specific binding of [^{3}H]NC-5Z at higher concentrations of the ligand (data not shown). This low affinity binding presumably represented binding to the receptors associated with adenylate cyclase activation. By filtration assay, the concentration of [^{3}H]NC-5Z resulting in 50% maximal binding was about 0.7 μM. However, because, in general, ligands binding in the high nanomolar range show a short half-life of receptor off-time (33), it is likely that some bound counts were lost during filtration, making this separation technique inadequate to assess low affinity binding.

Accordingly, additional experiments were carried out in which bound and free ligand were separated by centrifugation (see Materials and Methods). Fig. 7A shows a Scatchard plot of binding data from such an experiment. (In this experiment, mianserin-displaceable specific binding represented 50.2% of the total binding.) Assuming a single site, the data yielded a calculated K_D of 0.59 μM, similar to the K_a of 0.35–0.7 determined for activation, by NC-5Z, of light organ adenylate cyclase (Figs. 2 and 7B).[2] Fig. 7A, *inset*, shows the Hill plot for the binding data. A least-squares linear regression of the points yielded a slope (n_H) (Hill coefficient) of 1.01, suggesting a single binding site. The calculated K_D from the Hill plot was 0.63 μM. In this and another experiment, the calculated total octopamine receptor number ranged from 33 to 200 nmol/g of protein. This figure is comparable to the value (100 nmol/g of protein) obtained for nicotinic receptor number in membranes from *Torpedo* (34).

The high receptor number in the light organ may not be unreasonable, given the fact that the octopamine-activated adenylate cyclase in the light organ has one of the highest specific activities yet reported for any excitable tissue in the animal kingdom. The tremendous activity and selectivity of NC-5Z for the light organ is illustrated in Fig. 7B, which compares the specific activity of NC-5Z activation of adenylate cyclase in the firefly light organ with that in hornworm nerve cord and cockroach muscle. Although basal activities are low in all three tissues, NC-5Z-stimulated activity in the light organ is about 100-fold greater than that seen in the insect nerve cord

[2] In some experiments, the saturation curve suggested the possible existence of another, lower affinity component of mianserin-displaceable binding.

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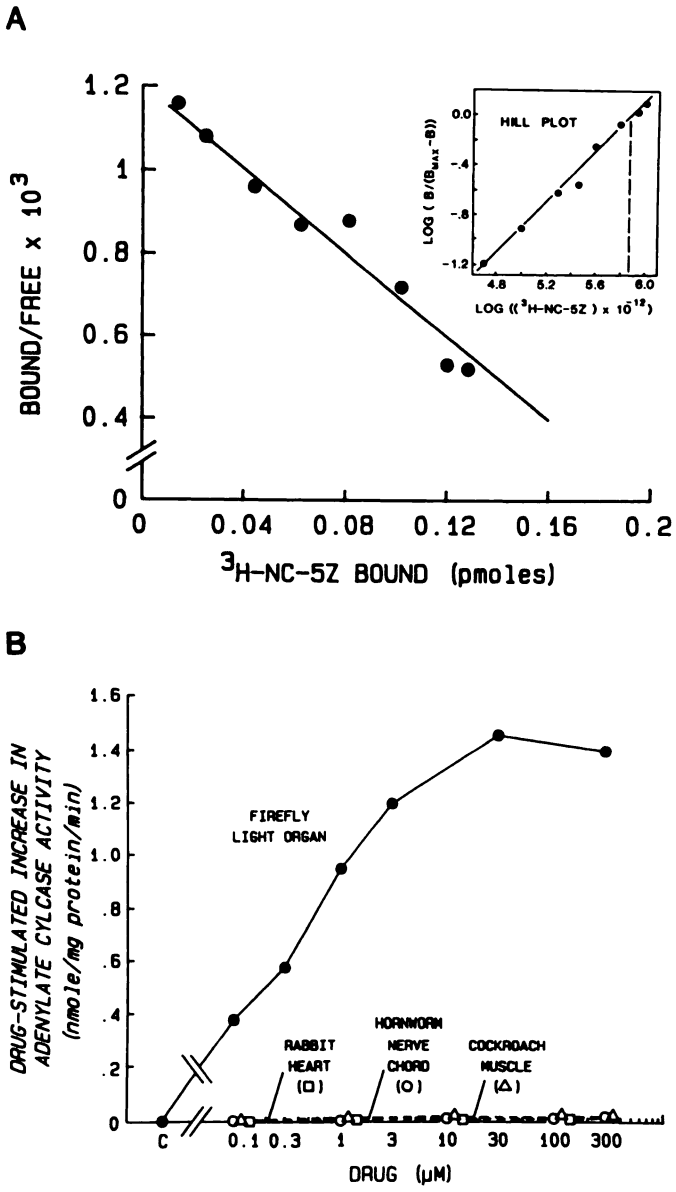

Fig. 7. A, Scatchard plot of binding of [^{3}H]NC-5Z to light organ membranes under reversible conditions. B, Comparative receptor-stimulated adenylate cyclase activity of NC-5Z in firefly light organ, tobacco hornworm nerve cord, and cockroach muscle and of isoproterenol in rabbit heart. In A, values shown are the mean ± standard error of quadruplicate samples and represent specific binding, *i.e.*, the amount of binding displaced by a 1000-fold excess of mianserin, as determined by centrifugation assay. Specific displaceable binding averaged 50.2% of total binding. *Inset*, Hill plot for the same data. Specific activities in B were calculated from activation of adenylate cyclase in membrane preparations from the four tissues. For the insect tissues, NC-5Z was the activator; for rabbit heart, isoproterenol was used. Basal activities (in pmol/mg of protein/min) were 49 in the light organ, 11 in rabbit heart, 19 in hornworm nerve cord, and 2.1 in cockroach muscle.

or muscle. Also shown in Fig. 7B, for comparison, is the activation, by isoproterenol, of the β-adrenergic receptor-coupled adenylate cyclase in rabbit heart, which is about 1/150 of the activation caused by NC-5Z in the light organ. Interestingly, this ratio of adenylate cyclase activities (1/150) is similar to the ratio (range, 1/70 to 1/450) between the reported concentrations of β-adrenergic receptors in mammalian tissues (as high as 475 pmol/g of protein) (35) and the concentration of NC-5Z binding sites (33-200 nmol/g protein) we have calculated for the light organ.

Photoaffinity Labeling of [^{3}H]NC-5Z

To carry out photoaffinity labeling, light organ membranes were first incubated with [^{3}H]NC-5Z under reversible conditions (in dim light) in the cold for 2 hr and then photolyzed for 5 to 15 min with short-wavelength UV light (see Materials and Methods). Displacing agents were added 15 min before the addition of [^{3}H]NC-5Z. Membranes were then extensively washed and the remaining bound ligand was measured. Fig. 8 shows that exposure to UV light caused a 14-fold increase in the amount of label bound to membranes. This increase (which was as high as 20-fold in other experiments) was seen both after washing by repeated high speed pelleting and resuspension and after washing using repeated cycles of NaOH solubilization and trichloroacetic acid precipitation (see Materials and Methods).

The presence, during the incubation period, of a 1000-fold excess of octopamine significantly reduced photolyzed [^{3}H]NC-5Z binding (Fig. 8). In five separate experiments, this specific octopamine-displaceable binding averaged 36 ± 3% (mean ± SE) of the total binding seen following photolysis. The PII octopamine agonist NC-7 (which is structurally distinct from octopamine), also reduced binding (39 ± 3%) (mean ± SE; four experiments), whereas, at identical concentrations, serotonin, dopamine, norepinephrine, and histamine did not reduce binding.[3] Cyproheptadine, an antagonist that inhibits octopamine-activated adenylate cyclase (Table 1), also reduced binding (35 ± 2%) (range; two experiments) (Fig. 8). NC-5, another PII octopamine agonist, reduced [^{3}H]NC-5Z binding by 20 ± 2% (range; two experiments). In additivity studies, a combination of NC-5, NC-7, and octopamine displaced [^{3}H]NC-5Z binding to a degree that did not exceed that displaced by each agent individually (Table 2).

Mianserin, which was the most potent inhibitor of octopamine-activated adenylate cyclase under reversible conditions

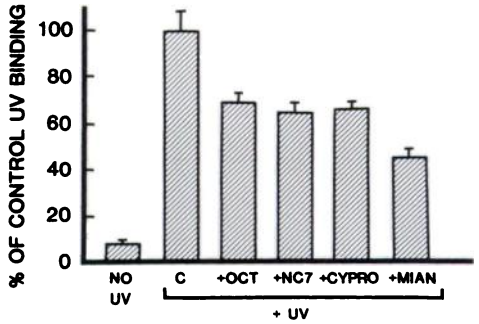

Fig. 8. Photoaffinity labeling of [^{3}H]NC-5Z to light organ membranes. Values (mean ± standard error of quadruplicate samples) are for irreversible binding of 1 μM [^{3}H]NC-5Z and are expressed as a percentage of UV binding seen in the absence of displacing agents. Procedure here and in Figs. 9 and 10 was as described in Materials and Methods and in Table 2. Control binding was 51.4 pmol/mg of protein. *C*, control; *OCT*, octopamine (1 mM); *CYPRO*, cyproheptadine (1 mM); *MIAN*, mianserin (1 mM).

[3] In some experiments, dopamine and serotonin were noted to increase the apparent binding of [^{3}H]NC-5Z. The reason for this increase (which was mianserin displaceable) is unclear, although possibilities include an increase in the amount of [^{3}H]NC-5Z available for octopamine sites through either inhibition of metabolism of [^{3}H]NC-5Z or through displacement, by the monoamines, of [^{3}H]NC-5Z from low affinity, nonspecific binding sites.

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TABLE 2

Nonadditive effects of octopamine, NC-5, and NC-7 in reducing photolyzable [^{3}H]NC-5Z binding to firefly light organ membranes

Light organ membranes were preincubated for 15 min with the displacing agent, then 1 μM [^{3}H]NC-5Z was added, and the incubation was continued for 60 min. Membranes were then photolyzed for 15 min and subsequently washed to remove soluble and reversibly bound [^{3}H]NC-5Z by five cycles of trichloroacetic acid precipitation and NaOH solubilization. Values (mean ± range for replicate samples) show the per cent displacement relative to the binding seen in the presence of [^{3}H] NC-5Z alone. This control binding was 21.9 pmol/mg of protein.

Displacing Agent	[^{3}H]NC-5Z Displaced
	%
Octopamine (3 mM)	34 ± 1
NC-5 (1 mM)	22 ± 2
NC-7 (1 mM)	48 ± 8
Octopamine + NC-5 + NC-7	43 ± 1

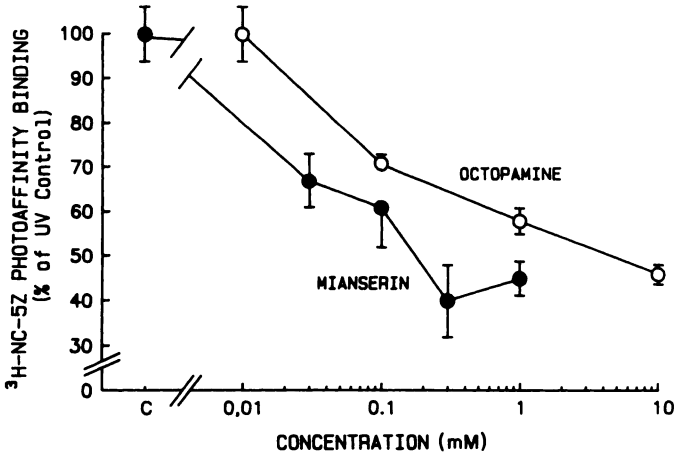

Fig. 9. Inhibition of [^{3}H]NC-5Z photoaffinity labeling by preincubation of light organ membranes with increasing concentrations of octopamine or mianserin. Methods are as described in Fig. 8 and in the text. [^{3}H]NC-5Z concentration was 1 μM.

(Table 1), was also the most effective and potent agent in displacing photolyzed [^{3}H]NC-5Z binding (Fig. 8). In seven separate experiments, a 1000-fold excess of mianserin displaced binding by an average of 55 ± 5% (mean ± SE). Fig. 9 shows that, in dose-response experiments, mianserin was about 10-fold more potent than octopamine in displacing [^{3}H]NC-5Z binding under photolyzing conditions. This compares with about a 40-fold difference in potency between mianserin (K_i = 0.6–0.9 μM) and octopamine (K_a = 25–35 μM) in interacting with octopamine-activated adenylate cyclase under reversible conditions.

[^{3}H]NC-5Z binding under photolyzing conditions was also observed in tissues other than the light organ. Fig. 10 shows mianserin-displaceable binding in tobacco hornworm nerve ganglia, fat body, and gut. In the latter tissue, the rank order of effectiveness of mianserin, octopamine, NC-5, and the formamidine octopamine agonist, DDCDM, in displacing photolyzable [^{3}H]NC-5Z binding was the same as the rank order of potency of the four agents in interacting with octopamine-activated adenylate cyclase under reversible conditions (Fig. 10).

Effect of GTP on Irreversible [^{3}H]NC-5Z Binding

In previous studies, we have shown that activation of octopamine-sensitive adenylate cyclase in light organ membranes is (as is the case with mammalian receptors coupled to adenylate cyclase) GTP dependent and cholera toxin sensitive (19, 22). To determine further whether irreversible [^{3}H]NC-5Z binding was octopamine receptor-related, additional experiments were carried out to measure the effect of GTP on [^{3}H] NC-5Z binding to washed light organ membranes. Table 3 shows that incubation and photolysis of membranes and [^{3}H] NC-5Z in the absence of GTP substantially reduced the ability of mianserin to displace binding. This result is consistent with studies from mammalian G_s adenylate cyclase systems showing that, in the absence of GTP, the affinity of the receptor for agonist is greatly increased, presumably due to an accumulation of receptor-G_s in a high affinity state (36). Under conditions of increased affinity for agonist, it thereby becomes more difficult for an antagonist to displace binding. Such an observation in the present studies further supports the conclusion that the displaceable portion of [^{3}H]NC-5Z binding is receptor related.

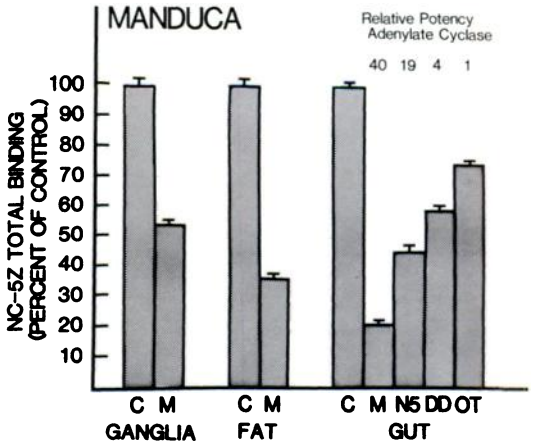

Fig. 10. Photoaffinity labeling by [^{3}H]NC-5Z (1 μM) of membranes from *M. sexta* (tobacco hornworm) nerve cord, fat body, and gut muscle. Shown is control binding (*C*) and that seen in the presence of a 1 mM concentration of the octopamine receptor antagonist mianserin (*M*) or one of the octopamine agonists, NC-5 (*N5*), DDCDM (*DD*), or octopamine (*OT*). At *right top* is the relative potency (determined from other experiments) of the four displacing agents in interacting with octopamine-activated adenylate cyclase under reversible conditions. Note the rank order correlation between the ability of agents to block photoaffinity labeling of membranes and the ability of agents to interact with octopamine receptors coupled to adenylate cyclase.

TABLE 3

GTP dependence of displacement of [^{3}H]NC-5Z by mianserin during photoaffinity labeling

Values (mean ± standard error of quadruplicate samples) show the cpm bound/15 μg of light organ membrane protein.

Condition	[^{3}H]NC-5Z bound		[^{3}H]NC-5Z Displaced
	−Mianserin	+Mianserin	
	cpm		%
−GTP	1850 ± 70	1590 ± 200	14
+GTP (0.1 mM)	2280 ± 180	1180 ± 70	48

Tissue Autoradiography

Because of the evidence described above indicating that [^{3}H] NC-5Z can bind irreversibly to octopamine receptors in isolated membranes, [^{3}H]NC-5Z could have potential use as an anatomical probe to localize octopamine receptors in tissues. To assess the feasibility of this, experiments were carried out to determine whether [^{3}H]NC-5Z could irreversibly label firefly light organs in the intact state (see Materials and Methods). Isolated intact

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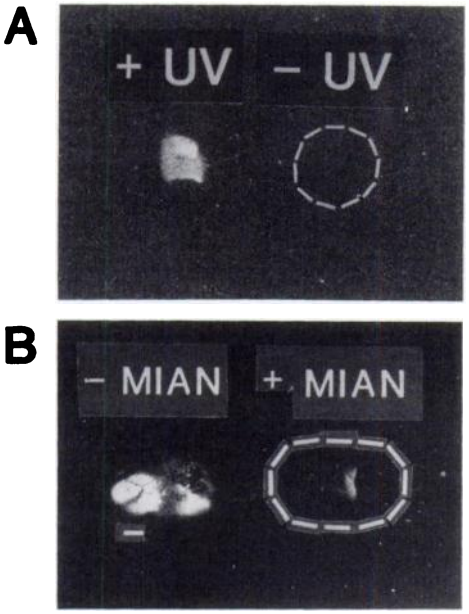

Fig. 11. Intact tissue autoradiography using photolyzed [^{3}H]NC-5Z. A, Isolated firefly light organs were incubated with 2.5 μM [^{3}H]NC-5Z, as described in the text. The light organ on the *left* was then photolyzed, whereas the one on the *right* was not. Both were then extensively washed, fixed, dehydrated, mounted, and exposed to tritium-sensitive X-ray film. The photolyzed lantern heavily exposed the film whereas the nonphotolyzed light organ (within *white dotted circle*) did not. B, Isolated firefly light organs were photolyzed with 2.5 μM [^{3}H]NC-5Z either in the absence or presence of the octopamine antagonist mianserin (*MIAN*) (1 mM). Mianserin (*right*) displaced a substantial amount (63% by densitometry) of the photoaffinity label. *Marker*, 0.5 mm.

light organs were incubated in dim light with [^{3}H]NC-5Z for 60 min and were then exposed either to no light or to intense UV light. After this, lanterns were extensively washed, fixed, slide mounted, dehydrated, and exposed to tritium-sensitive X-ray film. Fig. 11A shows that, in the absence of photolysis, no labeling was seen in the autoradiogram whereas, with UV exposure, extensive labeling was apparent. In another experiment, isolated lanterns were photolyzed with [^{3}H]NC-5Z, either in the absence or presence of 1 mM mianserin. Fig. 11B shows that mianserin displaced a significant amount of label. These experiments demonstrate the potential usefulness of [^{3}H]NC-5Z as an anatomical probe for localizing octopamine receptors in tissues.

Conclusion

The pharmacological and physiological data presented in this paper indicate that under, nonphotolyzing conditions, NC-5Z is a selective and extremely potent reversible agonist for octopamine receptors coupled to adenylate cyclase. Although we have not tested NC-5Z on non-cyclic AMP-associated octopamine receptors, Evans (12) has recently shown that the structurally related, reversible PII agonists, NC-5 and NC-7, are effective on a wide variety of octopamine receptor subtypes, including octopamine$_1$, octopamine$_{2A}$, and octopamine$_{2B}$. Thus, it is likely that NC-5Z, in addition to activating octopamine receptors associated with adenylate cyclase, will also be effective at other octopamine receptor subtypes.

Under photolyzing conditions, NC-5Z binds irreversibly to membranes and, presumptively, to octopamine receptor proteins. Because binding can be displaced by octopamine agonists and antagonists and because adenylate cyclase can be persistently activated by photolyzed NC-5Z, at least some of this irreversible binding appears to occur at the octopamine binding site of the receptor. Thus, under photolyzing conditions, NC-5Z and [^{3}H]NC-5Z should, in future work, be useful for localizing octopamine receptors in tissues and for biochemically isolating and characterizing octopamine receptor proteins.

Acknowledgments

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