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Phenyliminoimidazolidines

Characterization of a Class of Potent Agonists of Octopamine-Sensitive Adenylate Cyclase and Their Use in Understanding the Pharmacology of Octopamine Receptors

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SUMMARY

Octopamine, a major aminergic neurotransmitter in invertebrates, exerts many of its actions through receptors which are associated with the activation of adenylate cyclase. The present study defines and characterizes a new class of potent octopamine agonists, the substituted phenyliminoimidazolidines (PIIs). Approximately 30 of these derivatives were examined for agonist and antagonist effects on the highly enriched and specific octopamine-sensitive adenylate cyclase present in the firefly light organ, as well as on adenylate cyclases present in other invertebrate and vertebrate tissues. Several derivatives were extremely active and some (*e.g.* 2,6-diethyl-PII) had potencies exceeding those of any previously described agonists of octopamine-sensitive adenylate cyclase. Stimulation by the potent PIIs was reversible, nonadditive to that caused by octopamine, and could be antagonized by antagonists such as cyproheptadine ($K_i = 4$ μM), phentolamine ($K_i = 23$ μM), and propranolol ($K_i = 72$ μM). These inhibitory constants agreed well with those for inhibiting octopamine stimulation. Certain PII derivatives acted as partial agonists and some as antagonists of octopamine stimulation. Structure-activity relationships revealed, among other things, that short-chain alkyl substitution in the 2- and 6-phenyl positions enhanced activity, as did further substitution of 4-halo, 4-methyl, or 4-hydroxy substituents. 4-Amino or *N*-alkyl substitution decreased activity. Structurally related benzylimidazoline derivatives such as tolazoline and naphazoline were partial octopamine agonists, generally less active than the PIIs. Comparison, in three invertebrate species, of the effects of the PIIs and two other chemical classes of octopamine agonists demonstrated clearcut differences in species responsiveness. Other comparative studies revealed that the agonist activity of the potent PIIs was specific for tissues containing an octopamine-sensitive adenylate cyclase; adenylate cyclases activated by dopamine or by β_1- or β_2-adrenergic agonists were unaffected by these compounds. Evaluation of the relative binding affinities of various PIIs for mammalian α-adrenergic receptors, as well as the ability of various antagonists to block PII binding, strongly suggested that the active PIIs are affecting a class of octopamine receptors distinct from mammalian α_1- or α_2-adrenergic receptors. These octopamine receptors also appeared distinct from mammalian 5-HT_1 and 5-HT_2 receptors. Correlative physiological studies in insects revealed that the active PIIs mimicked octopamine and were potent activators of light emission in the firefly light organ. The PIIs also caused disruption of motor and feeding behavior in tobacco hornworms, leading to insect death, an effect which was markedly potentiated by phosphodiesterase inhibitors. These data have relevance to understanding the structural requirements of agonists necessary for activation of octopamine-sensitive adenylate cyclase. In addition, the compounds described should be useful in elucidating the physiological actions of octopamine in invertebrates, as well as in developing selective pesticidal agents with decreased toxicity for vertebrates.

INTRODUCTION

Substantial biochemical and physiological evidence indicates that octopamine is a major aminergic neurotransmitter in invertebrates, having both neurohumoral and neurotransmitter roles (1–3). Octopamine exerts its effects through interaction with at least two classes of receptors which, on the basis of pharmacological criteria, have been designated octopamine-1 and octopamine-2 (4). Physiological studies suggest that many of the effects

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of octopamine at octopamine-2 receptors may be mediated by cyclic AMP (5). An adenylate cyclase specifically activated by octopamine was first identified, several years ago, in insect nerve cord (6) and, subsequently, in the tissues of a number of other invertebrate classes (reviewed in Refs. 2 and 3). Pharmacological characterization of octopamine-sensitive adenylate cyclase has been aided, recently, by the identification of an extremely enriched source of the enzyme in the firefly light organ (7).

Compared with other cyclic AMP-associated amine receptors (*e.g.*, α_2-adrenergic, β-adrenergic, D1-dopaminergic), there are relatively few synthetic agents which are potent agonists of octopamine-sensitive adenylate cyclase. The only compounds more potent than octopamine itself are *N*-methyloctopamine, with a K_a of about one-half that of octopamine (8), and a group of formamidine derivatives with from one-third to six times the potency of octopamine (9–11). In some tissues (*e.g.*, firefly light organ and cockroach nerve cord), however, the formamidines are partial rather than full agonists, capable of exerting inhibitory effects on octopamine responses.

As an aid in determining the physiological functions of octopamine in intact cell systems, it would be advantageous to define additional compounds with potent agonistic effects at octopamine receptors associated with the activation of adenylate cyclase.[1] Preferably, a series of chemically related derivatives with a range of potencies would be desirable. In the present paper, we identify and characterize a new chemical class of octopamine agonists, the substituted PIIs.[2,3] Biochemical structure-activity data on approximately 30 chemically related derivatives with a wide range of octopaminergic potencies are described. Some of these derivatives are full octopamine agonists with potencies up to almost 20 times that of octopamine itself. As described below, these active derivatives exert potent physiological effects in insects, including stimulation of light emission in the firefly and disruption of feeding in the tobacco hornworm.

MATERIALS AND METHODS

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 (10). After thawing at 4°, tail sections were opened, the light organs removed from the ventral cuticle, cleaned of any adhering nonlantern tissue, and homogenized, 10 mg/ml, in 6 mM Tris-maleate buffer, pH 7.4. A washed particulate (P_2) fraction was prepared as previously described (10) and maintained at 0° until use. Adenylate cyclase activity was also measured in P_2 fractions prepared from homogenates (15 mg/ml) of ventral nerve cords of adult cockroaches (*Periplaneta americana*) obtained from a laboratory colony. In some experiments, adenylate cyclase activity was also measured in P_2 fractions of ventral nerve cords (including head and tail ganglia) from 50-mm-long tobacco hornworms (*Manduca sexta*) raised on artificial media (Carolina Biological Supply). In other experiments, P_2 fractions were prepared from the caudate nucleus (10 mg/ml), liver (50 mg/ml), and heart (50 mg/ml) of 3-month-old male Sprague-Dawley rats.

Adenylate cyclase assay. Adenylate cyclase activity of all tissues was measured 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 (10). The various reaction components (minus ATP and GTP) were mixed and allowed to stand for 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 (13). 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 (14)) was nearly completely inhibited. Protein concentration was determined by the Lowry method.

For reversibility studies (Table 6), tissue homogenate was first preincubated with vehicle or drug, using the assay conditions described above, except that ATP was omitted. Homogenates were then diluted 100-fold with 6 mM Tris maleate (pH 7.4) and centrifuged at 100,000 × *g* for 30 min. The pellet was resuspended in 20 cc of buffer and respun. The final P_2 pellet was resuspended (5 mg, wet weight/ml) and assayed for basal and hormone-stimulated adenylate cyclase activity, as described under Results and Discussion.

In some cases, the incubation medium for the adenylate cyclase assay was modified as follows to allow solubilization of hydrophobic compounds, such as DDCDM. Stock solutions (10×) of compounds were initially dissolved in 100% polyethylene glycol 400 (Baker), then diluted to a final concentration of 10% polyethylene glycol, which was incorporated into the buffer. Extensive preliminary testing with a variety of solvents (polyethylene glycol, methanol, dimethyl sulfoxide, propylene glycol), at concentrations from 1–50%, had established that polyethylene glycol caused the least change of 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 experiments, the activity of agonists was always measured relative to the activity of octopamine run under the same solvent conditions.

Activation constants (K_a) were calculated from dose-response curves containing 12–16 data points, each assayed for cyclic AMP content in duplicate. For the more potent compounds shown in Tables 1 and 2, dose-response curves were repeated from 2–5 times. In these tables, the relative potency of a particular agonist for activating adenylate cyclase is expressed through a potency ratio, which was calculated by dividing the K_i value for octopamine (run in the same experiment) by the K_i value for the agonist. A ratio greater than 1 indicates greater potency than octopamine and vice versa. The relative maximum efficacy (V_{max}) for a particular agonist is expressed as a percentage of the maximum enzyme activation caused by optimal concentrations of octopamine.

For inhibition curves, various concentrations of antagonist were tested against a fixed concentration of octopamine agonist (usually 1 or 10 μM). Stimulation in the presence of octopamine 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 (15). Inhibitory constants (K_i) for antagonists were calculated from the equation (16), $K_i = (IC_{50})/(1 + S/K_a)$, where IC_{50} was the concentration of antagonist necessary

[1] Because current pharmacological classification schemes (4) place octopamine receptors coupled to adenylate cyclase under the octopamine-2 subtype, for convenience this terminology will be used in the present article. However, the possibility should be borne in mind that not all octopamine-2 receptors may be coupled to adenylate cyclase and that not all octopamine-stimulated cyclic AMP responses may pharmacologically fit the octopamine-2 subtype.

[2] A preliminary report describing some of these compounds has recently been published (12).

[3] The abbreviations used are: PII, 2-(phenylimino)imidazolidine; DCDM, *N*-demethyl-chlordimeform; DDCDM, *N,N*-didemethylchlordimeform; IBMX, 3-isobutyl-1-methylxanthine; NC-5, 2,6-diethyl-PII; NC-7, 2-chloro-4-methyl-PII; NC-9, 2,4-dimethyl-PII; NC-13, 2,4,6-trimethyl-PII; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

to give 50% inhibition of activity in the presence of octopamine agonist, S was the concentration of octopamine agonist present (usually 1 or 10 μM), and K_a was the concentration of octopamine 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 3 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 (12). Drug (dissolved in insect saline (17)) was injected (in 3–5 μl) into the abdominal cavity dorsal to the lantern and light emission recorded for 45 min or until it peaked, following which the next (larger) dose of drug was injected. In the case of animals injected with drugs other than octopamine, following the last dose, 10 nmol of octopamine (a maximally effective dose) was injected. Light production is expressed as radiance, each unit approximately equal to 1.6 nanowatts, as measured in a solid angle of 0.033 steradiants.

Effects on 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 an aerosal to isolated hydrated tomato leaves (each maintained in a closed plexiglass container) and allowed to dry (12, 18). 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 "antifeeding activity" observed was the net result of motor-behavioral disruption and not necessarily due to a specific effect on feeding (see below). As described in the text, the phosphodiesterase inhibitor, IBMX (0.1%), was mixed with drug and applied to all (both control and experimental) leaves. At the dose used, IBMX itself had little or no effect on the rate of leaf consumption.

Boehringer Ingelheim, Pfizer, American Cyanamid, Professor W. Soudijn (Leiden University), and Professor G. LeClerc (Université Louis Pasteur, Strasbourg) kindly supplied many of the PII analogs. DCDM and DDCDM (as the hydrochloride salt) was supplied by Dr. H. M. LeBaron. Phentolamine were supplied by Ciba-Geigy and cyproheptadine by Merck. Most common reagents were obtained from Sigma.

RESULTS AND DISCUSSION

Stimulatory effects of PIIs on light organ adenylate cyclase. Previous studies have shown that the firefly light organ contains an octopamine-sensitive adenylate cyclase of high specific activity (7, 10). This enzyme is selective for octopamine and structurally related phenylethanolamines. Additivity and antagonist studies fail to support the presence, in the light organ, of other amine-stimulated adenylate cyclases, such as those activated by dopamine, norepinephrine, serotonin, or histamine. Because of the selectivity of the light organ enzyme, the PIIs were initially screened and characterized for octopaminergic activity by measuring their effect on adenylate cyclase in P_2 fractions of *P. pyralis* light organs.

Fig. 1 shows the potent stimulation of light organ adenylate cyclase by three PII derivatives. In this and other experiments, NC-5 (2,6-diethyl PII) and NC-13 (2,4,6-trimethyl PII) were full agonists, with V_{max} values not significantly different from the enzyme V_{max} produced by octopamine. In the experiment shown, the K_a for adenylate cyclase activation by NC-5 was 1.5 μM and for NC-13, 8 μM. In various experiments, the potency of NC-5 varied from 13–28 times that of octopamine (mean ± S.E. = 19 ± 3.5) and that of NC-13 varied from 3–5 times that of octopamine (mean = 4.3 ± 0.2). Compared with other chemical classes of octopamine agonists (*e.g.*, see Fig. 2), NC-5 is the most potent activator of firefly octopamine-sensitive adenylate cyclase yet reported.

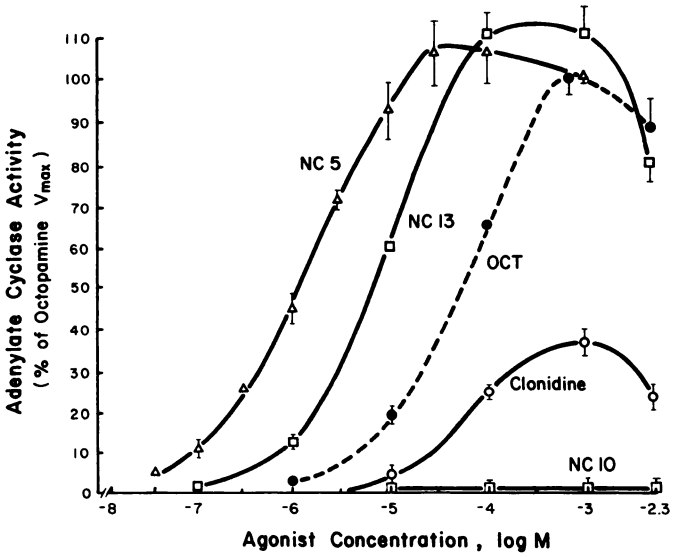


FIG. 1. *Effects of octopamine (OCT) and four PII derivatives on firefly light organ adenylate cyclase activity*

In both this figure and in Figs. 2–4, enzyme activity is expressed as a percentage of the maximal stimulation caused by octopamine in the same experiment. Values shown are the 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 10–25 pmol/mg protein/min, and octopamine, at optimal concentrations, stimulated this activity about 50-fold. The results shown in these experiments are typical of those seen in other experiments with the same compounds. Kinetic constants are summarized in Tables 1 and 2.

A number of PII derivatives were partial agonists of light organ adenylate cyclase, with V_{max} values which ranged from 1–85% that of octopamine and with potencies which varied from 0.1–10 times that of octopamine. Fig. 1 shows, for example, the effect of 2,6-dichloro-PII (clonidine), which demonstrated a V_{max} of 35% and a potency ratio compared to octopamine (K_a octopamine/K_a test compound) of 0.95. Although there was some variability among different experiments, in the absolute K_a values of various derivatives, the potency ratio of a given compound (relative to octopamine) remained quite reproducible. Fig. 1 shows that the unsubstituted PII derivative, NC-10, was without agonist activity.

Stimulatory effects of octopamine and other octopamine agonists. For purposes of comparison, Fig. 2 shows the activation of light organ adenylate cyclase by octopamine, chemically a phenylethanolamine. In the experiment shown, the K_a of octopamine for stimulation of the enzyme was about 30 μM and in various experiments ranged from 15–40 μM. At maximally effective concentrations of octopamine, enzyme activity was stimulated, typically, about 50-fold over basal levels. We have previously reported that, in the same tissue at concentrations up to 2000 μM, dopamine, serotonin, and isoproterenol are less than 2% as effective as octopamine in activating adenylate cyclase (10, 12). At 5000 μM, isoproterenol stimulation is less than 10% that of octopamine.

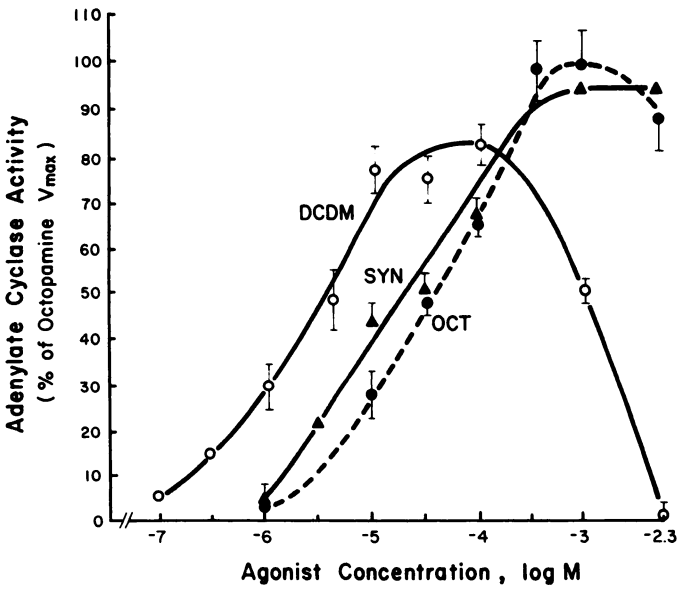


FIG. 2. *Effects of the formamidine (DCDM) and the phenylethanolamine (synephrine (SYN)), compared with octopamine (OCT), on light organ adenylate cyclase activity*

Activity is as described in the legend to Fig. 1.

The effects of synephrine (*N*-methyloctopamine), the most potent phenylethanolamine we have yet tested, is also shown in Fig. 2. In most experiments, this compound was about 1.5–2 times as potent as octopamine, with a comparable V_{max}. Stimulation by maximally effective concentrations of synephrine was nonadditive with that produced by maximally effective concentrations of octopamine.

Fig. 2 also shows the effects of the formamidine derivative, monodemethylchlordimeform (DCDM), the most potent (in terms of adenylate cyclase activation) formamidine that we or others have yet reported (9, 10).[4] As described previously, DCDM is a partial agonist of light organ adenylate cyclase activity (10). When increasing doses of DCDM are added to a maximally effective dose of octopamine, there is a progressive partial inhibition of the octopamine response, with an EC_{50} of about 200 μM (data not shown).

Structure-activity relationships of the phenyliminoimidazolidines. Table 1 lists the kinetic data obtained for a series of mono-, di-, and tri-substituted PII derivatives. Table 2 lists similar data for mono-substituted derivatives of the phenyliminoimidazolidine, clonidine (2,6-dichloro-PII). Shown for each compound is the V_{max} (relative to octopamine = 100%) for activating light organ adenylate cyclase, as well as the potency ratio, the calculation of which is described under Materials and Methods. As can be seen in Table 1, in addition to NC-5 and NC-13, other potent PII analogs included 2-methyl-4-chloro PII (NC-7) (potency ratio = 8.6 ± 1.1), 2,4-dimethyl PII (NC-9) (ratio = 5.1), 2,4-dichloro (ratio = 3.7), 2,4,5-trichloro (ratio = 2.8 ± 0.1), and 2-methyl-3-bromo (ratio = 2.9).

Greater enzyme activation (V_{max}) appeared to be favored by short chain alkyl (methyl or ethyl) substitutions in the 2-, 4-, or 6-positions of the aromatic ring. Halogen substitutions were less effective. For example, the 2,4-dimethyl derivative had almost twice the V_{max} and potency as the 2,4-dichloro derivative. Among di-substituted derivatives, the presence of alkyl groups on the 2- and 6-positions near the imidazolidine side chain seemed particularly important. Thus, the 2-methyl-4-chloro derivative had almost twice the V_{max} and was 10 times more potent than the positional isomer, 2-chloro-4-methyl-PII. Also, the V_{max} of the 2,6-dimethyl derivative was almost 2.5 times that of the 2,6-dichloro derivative (Table 2), and the V_{max} of the 2-chloro-6-methyl derivative was intermediate.

The above data suggest that the phenyl ring substitution requirements for PII derivatives active at octopamine-2 receptors differ substantially from the substitution requirements for phenylethylamines active as octopaminergic agonists. Further evidence for this difference is seen in the extremely low activity of the catechol analog, ST-1943, a PII derivative with ring substitutions like those of norepinephrine. Thus, whereas norepinephrine is a full agonist of light organ adenylate cyclase with a potency ratio of about 0.1 (12), ST-1943 is almost completely devoid of activity.

Effects of alkyl substitution size on activity. Increasing chain length from methyl to ethyl for the 2,6-disubstituted derivative caused a small increase in V_{max} and a marked increase in potency. Indeed, as mentioned above, NC-5 is the most potent agonist of octopamine-sensitive adenylate cyclase yet reported. It was of interest, therefore, to determine if an additional increase in alkyl chain size would further enhance octopaminergic activity. Although we were unable to obtain the 2,6-di-*N*-propyl derivative, we were able to obtain and test 2,6-di isopropyl-PII. As shown in Table 1, this derivative has a V_{max} of only 28% that of octopamine, which is considerably less than that of both the 2,6-diethyl derivative and 2,6-dimethyl derivative. The potency of the diisopropyl compound is also much less than that of NC-5, although greater than that of the dimethyl derivative. Thus, excessive bulkiness at the 2- and 6-positions of the phenyl ring appears to create interference both with binding and with activation of the adenylate cyclase enzyme. Because of a limitation in the availability of certain derivatives, we have not yet determined the optimal size of alkyl 2,6-substitutions. It is possible that asymmetrical substituents (*e.g.*, 2-methyl-6-ethyl or 2-ethyl-6-*N*-propyl) could have more activity than the 2,6-diethyl derivative.

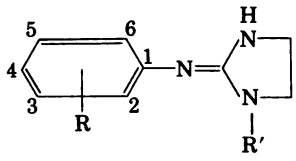
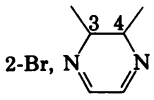
Tri-substituted derivatives. Experiments with tri-substituted derivatives revealed that, in compounds already substituted at the 2- and 6-phenyl positions, additional substitution of either an alkyl or halo group at the 4-position substantially increased activity. Thus, adding a 4-methyl group to the 2,6-dimethyl derivative further increased V_{max} and increased potency almost 10-fold. Addition of a 4-chloro group to the 2,6-dichloro deriva-

[4] In preliminary tests, we have found that certain difunctional formamidines of the general form (*R*-phenyl-HN=C)$_2$ may exceed the potency of DCDM. However, it is likely that such difunctional compounds are not acting directly but, instead, are undergoing hydrolysis to two monofunctional forms.

TABLE 1

Octopamine receptor activity of 2-(phenylimino)imidazolidines

Activity (stimulation of light organ adenylate cyclase) is expressed relative to the activity of octopamine (V_{max} = 100%; K_a ratio = 1) run in the same experiment. Typically, basal adenylate cyclase activity was 15 ± 5 pmol/mg of protein/min, and octopamine (OCT) stimulation at V_{max} was 50-fold. Thus, a V_{max} of only 9% (*e.g.*, NC-2) still represents about a 4.5-fold stimulation of basal activity. Results here and in Table 2 are a compilation of a number of experiments. When an individual compound was tested more than once, values shown are mean (±S.E.M.). NA, no activity.

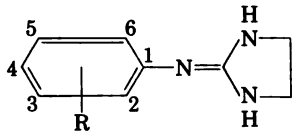
Compound		(R′)	V_{max} (% OCT)	$\frac{K_a \text{ (OCT)}}{K_a \text{ compound}}$
NC-10		H	NA	NA
NC-12	4-Br	H	34	3.0
DS-22[a]	4-Cl	H	17	2.4
ST-608	2-Cl, 3-CH_3	H	43	0.15
ST-606	2-CH_3, 3-Br	H	45	2.9
NC-8	2-Cl, 4-Cl	H	47	3.7
ST-375	2-Cl, 4-CH_3	H	41	0.62
NC-7	2-CH_3, 4-Cl	H	69 ± 6	8.6 ± 1.1
NC-9	2-CH_3, 4-CH_3	H	78	5.1
ST-600	2-CH_3, 5-F	H	16	0.50
NC-2	2-Cl, 5-Cl	H	9	1.1
NC-6	2-Br, 6-Br	H	35 ± 1	2.1 ± 0.1
Clonidine	2-Cl, 6-Cl	H	35	0.95
ST-93	2-Cl, 6-CH_3	H	69	0.32
NC-4	2-CH_3, 6-CH_3	H	80	0.41
NC-5	2-CH_2CH_3, 6-CH_2CH_3	H	97 ± 6	19.0 ± 3.5
AC-82-1	2-CH_2CH_3, 6-CH_2CH_3	CH_3	98	0.95
NC-20	2,6-diisopropyl	H	28	1.95
ST-1943	3-OH, 4-OH	H	NA	NA
NC-3	2-Cl, 4-Cl, 5-Cl	H	80 ± 3	2.8 ± 0.1
NC-13	2-CH_3, 4-CH_3, 6-CH_3	H	108 ± 6	4.3 ± 0.1
UK-14304-18		H	22	0.12

[a] DeJong and Soudijn (25), compound 22.

TABLE 2

Octopamine receptor activity of clonidine analogs

Activity (stimulation of light organ adenylate cyclase) is expressed relative to the activity of octopamine (V_{max} = 100%; K_a ratio = 1) run in the same experiment. Typically, basal adenylate cyclase activity was 15 ± 5 pmol/mg protein/min, and octopamine (OCT) stimulation at V_{max} was 50-fold.

Compound		V_{max} (% OCT)	$\frac{K_a \text{ (OCT)}}{K_a \text{ compound}}$
Clonidine	2-Cl, 6-Cl	35	0.95
NC-11	2-Cl, 4-Cl, 6-Cl	60	1.9
NC-14	2-Cl, 4-NH_2, 6-Cl	29	0.7
ST-666	2-Cl, 4-OH, 6-Cl	34	1.7
NC-15	2-Cl, 4-$N(CH_3)_2$, 6-Cl	38 ± 13	1.8 ± 0.5
NC-16	2-Cl, 4-$NCH_3(CH_2CH_2Cl)$, 6-Cl	6 ± 1	4.8 ± 2.9
NC-17	2-Cl, 4-$CH_2NCH_3(CH_2CH_2Cl)$, 6-Cl	NA[a]	NA

[a] NA, no activity.

tive doubled both V_{max} and potency (Table 2). Addition of a 4-chloro group to the relatively inactive 2,5-dichloro derivative increased V_{max} by almost 9-fold. Because of the high potency of the 2,6-diethyl derivative, it is possible that 2,6-diethyl derivatives substituted with a halo or alkyl group in the 4-position could be quite active.

Addition of a 4-hydroxy group to the 2,6-dichloro derivative also increased activity, with almost a doubling of potency (Table 2). However, additional derivatives will have to be tested before it can be concluded that this enhancing effect of 4-hydroxylation is similar to the enhancing effect of 4-substitution of a halo or alkyl group.

Unlike *p*-substitution of halo, alkyl, or hydroxy groups, addition of a 4-amino group to 2,6-dichloro-PII (clonidine) (Table 2) resulted in a decrease in potency. This decreased potency of 4-amino-clonidine compared with clonidine is of interest, since it is one of the structure-activity relationships (see below) that helps to distinguish PIIs with potent α_2-adrenergic agonist activity from those derivatives with potent octopaminergic activity. Thus, 4-amino-substitution of clonidine markedly increases potency at α_2-adrenergic receptors (see Table 8) but, as shown, decreases ability to activate octopamine-sensitive adenylate cyclase.

Effects of bromo-substitution. Substituting a bromo for a chloro group increased octopamine agonist activity. Thus, 4-bromo-PII had about twice the V_{max} of the 4-chloro derivative. Also, 2,6-dibromo-PII had about 1.5 times the potency of the 2,6-dichloro derivative. In future studies, it will be of interest to determine if 2-methyl-4-bromo-PII (which could not be obtained for the current studies) is more active than the already potent 2-methyl-4-chloro derivative.

Effects of N-methylation. Among the phenylethanolamines and the formamidines, addition of an *N*-methyl group to the terminal nitrogen increases potency at octopamine-2 receptors. Thus, *N*-methyloctopamine (synephrine) is almost twice as potent as octopamine (Fig. 2), and the corresponding *N*-methylformamidine derivative (DCDM) is about twice as potent as the demethylated derivative, DDCDM (10). To determine how methylation of one of the imidazolidine ring nitrogens might alter activity, we examined the effect of adding an *N*-methyl group to the already potent 2,6-diethyl derivative. As shown in Table 1, there was no change in V_{max} of the methylated derivative but there was a marked (over 10-fold) decrease in potency. We observed a similar decrease in octopaminergic activity following *N*-methylation of 2-(2,6-dichloro)-benzylimidazoline (Table 3), a compound which, unlike the PIIs but like octopamine, has a carbon rather than a nitrogen adjacent to the substituted phenyl ring (see below).

Because the 2-benzylimidazolines do have an imidazoline ring, the observed decrease in activity seen with *N*-methylation suggests that the N-terminal ring structure is the key feature distinguishing the enhancing effects on potency observed following *N*-methylation of the phenylethanolamines and formamidines from the negative effects observed following *N*-methylation of the PIIs and 2-benzylimidazolines. Possibly, this indicates that a certain degree of steric bulkiness (created by either a ring structure or by *N*-methylation) is favorable for binding but that excessive bulkiness (created by the combination of a ring structure and *N*-methylation) is not.

TABLE 3

Octopamine-2 receptor activity of 2-benzylimidazolines

Activity measurements as described in Tables 1 and 2.

Compound			V_{max} (% OCT)	K_a (OCT) / K_a compound
Tolazoline		H	14	<0.07
ST-1913	2-Cl, 6-Cl	H	17	1.9
DS-10[a]	2-Cl, 6-Cl	CH_3	NA[b]	NA
ST-71	2,4,6-trimethyl	H	22	5.5
Naphazoline		H	41	3.1

[a] DeJong and Soudijn (25), Compound 10.

[b] NA, no activity.

2-Benzylimidazolines. The benzylimidazolines are related to the PIIs but differ in having a carbon rather than a nitrogen atom adjacent to the substituted phenyl ring (see Table 3). Because the benzylimidazolines have structural similarities to both PIIs and to phenylethanolamines (such as octopamine), it was of interest to determine whether these compounds would act as agonists of octopamine-sensitive adenylate cyclase. The benzylimidazolines are of additional interest since, on the basis of physiological studies, two of them (naphazoline and tolazoline) have been proposed as being key compounds in distinguishing among various octopamine receptor subtypes (4).

However, Table 3, which summarizes the kinetic data from various dose-response curves, shows that the benzylimidazolines were only partial agonists of light organ adenylate cyclase. Of particular interest was the comparison between NC-13 (Table 1) and the corresponding benzylimidazoline compound, ST-71 (2(2,4,6-trimethyl)imidazoline) (Table 3). Substitution of a side chain carbon for a nitrogen caused little change in potency ratio but resulted in a marked decrease in V_{max}, from 100 to 22%. Thus, ST-71 appears to be able to bind to the light organ octopamine receptor but is not able to cause full activation of adenylate cyclase. Consistent with this was the observation that, in additivity studies with octopamine, ST-71 was able to act as a potent partial antagonist of octopamine-stimulated enzyme activity (data not shown).

Naphazoline (2[1-naphthylmethyl]imidazoline) has been reported by Evans to be the most effective of several agonists affecting locust muscle (SETi) twitch tension, a putative octopamine-modulated response (4). In the locust, naphazoline was a full agonist with a potency ranging from 11-fold (on tension) to 200-fold (on relaxation rate) that of octopamine, itself. In contrast, in the

present experiments, naphazoline was a partial agonist of light organ adenylate cyclase, with a V_{max} of 41%. Although its K_a ratio was 3.1 (Table 3), absolute stimulation at any concentration was always less than that of octopamine (Fig. 3).

Also interesting (in terms of differential physiological and biochemical effects) was the effect of tolazoline (2-benzyl-2-imidazoline). In physiological experiments on locust muscle, tolazoline has been reported to be a full agonist which is either equipotent (octopamine$_{2A}$ response), somewhat more potent (octopamine$_{2B}$ response), or substantially more potent (octopamine$_1$ response) than octopamine itself (4). However, in the light organ, tolazoline was almost devoid of activity, with a potency ratio of less than 0.07 and a V_{max} of only 14% of octopamine (Table 3 and Fig. 3).

It is interesting that the above differences in potency may not entirely be due to species variation (see also below), since recent studies of cyclic AMP accumulation in intact locust muscle yield data more nearly similar to that in Table 3 than to previously reported physiological data from locust muscle (4). Thus, in contrast to their potent physiological effects on twitch tension, naphazoline and tolazoline are both weak partial agonists of cyclic AMP accumulation in isolated locust muscle (5).

The above, as well as our own results, do not necessarily imply that the benzylimidazolines are poor agonists of all octopamine receptors. It is possible that the potent *physiological* effects of benzylimidazolines on locust muscle could be due to the presence of spare receptors or to the stimulation of octopamine receptors not associated with a cyclic AMP mechanism. On the other hand, the greater potency of benzylimidazolines (relative to octopamine) in physiological studies could also be due to other factors, such as differential metabolism (*e.g.*, reuptake, conjugation, oxidation, and *N*-acetylation (1)). This latter explanation would be consistent with other data (such as the use of forskolin and IBMX) which suggest that at least some of the effects of the benzylimidazolines on locust muscle tension may be mediated by octopamine receptors linked to adenylate cyclase (5, 19).

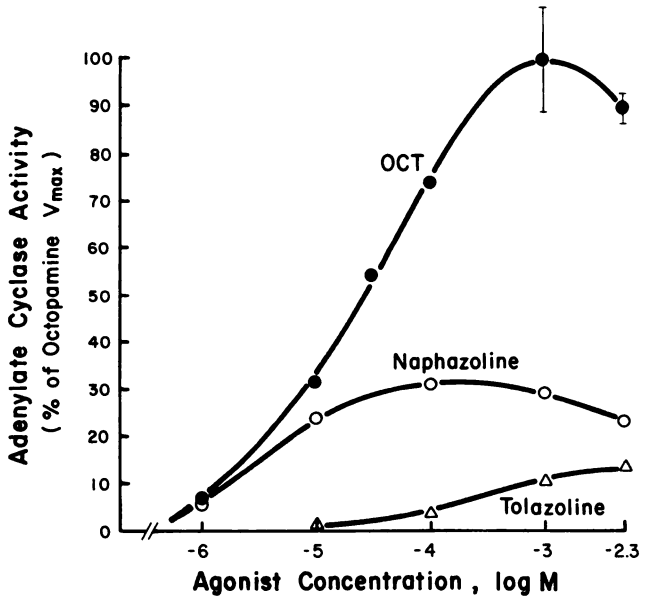


FIG. 3. *Effects of the 2-benzylimidazolines, naphazoline, and tolazoline, compared with octopamine (OCT), on light organ adenylate cyclase activity*

Details of data presentation are described in the legend to Fig. 1.

TABLE 4

Nonadditive effects of octopamine, phenyliminoimidazolidines, and formamidines on light organ octopamine-activated adenylate cyclase

Values shown are the means (± ranges) for two determinations, each assayed for activity in duplicate.

Compound	Adenylate cyclase activity
	pmol/mg protein/min
None	10 ± 1
Octopamine (1 mM)	540 ± 95
NC-5 (100 μM)	470 ± 80
DCDM (100 μM)	440 ± 15
Octopamine + NC-5	485 ± 10
Octopamine + DCDM	425 ± 40
NC-5 + DCDM	430 ± 15

In addition to the above structure-activity experiments, other studies (described below) were run to investigate the relationship between the activation of light organ adenylate cyclase by the PIIs and by octopamine.

Additivity studies. At maximally effective concentrations, the PIIs, formamidines, and octopamine were nonadditive in their effects on light organ adenylate cyclase activity. An example of a typical additivity study is shown in Table 4, with NC-5, DCDM, and octopamine. Lack of additivity was also shown by PIIs tested in a series of doses against a submaximal dose of octopamine. An example of such an experiment, with NC-13 and octopamine, is shown in Fig. 4A. These types of data, coupled with antagonist and physiological studies (below), suggest that the PIIs and octopamine are affecting a similar class of receptors.

Inhibition of PII stimulation by receptor antagonists. To supply additional evidence that the PIIs may be activating light organ adenylate cyclase through interaction with a class of octopamine receptors, experiments were carried out to block agonist activation through the use of compounds known to competitively block octopamine-sensitive adenylate cyclase. Because no single antagonist has yet been characterized as being specific for octopaminergic responses, the three inhibitors tested (cyproheptadine, phentolamine, and propranolol) are those which have been most frequently utilized in previously published biochemical and physiological studies of octopamine receptor blockade (1–3). These antagonists display a range of potencies against octopamine-sensitive adenylate cyclase (10).

Table 5 lists the K_i values determined from inhibition experiments in which the concentration of PII agonist (NC-5) was held constant and the concentration of antagonist was varied. The observed IC_{50}, together with the agonist substrate concentration and the K_a for enzyme activation by the agonist (determined separately), were used to calculate the K_i values shown. (In other experiments, below, we determined that the PIIs cause activation of octopamine-sensitive adenylate cyclase through a reversible binding to octopamine receptors.)

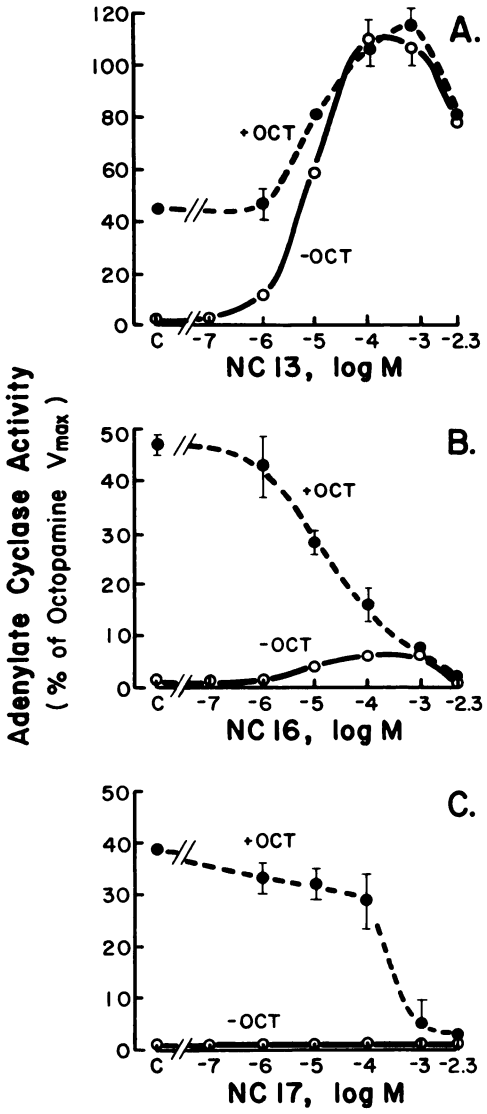


FIG. 4. *Effects of PII derivatives on light organ adenylate cyclase activity, in the absence or presence of a fixed (10 μM) concentration of octopamine (OCT)*

Part A shows the full agonist effect of NC-13; Part B, the partial agonist effect of NC-16; and Part C, the antagonist effect of NC-17, a derivative which lacks any agonist activity.

TABLE 5

Calculated K_i values for various antagonists in inhibiting the stimulation of light organ adenylate cyclase activity by either NC-5 (1 μM), octopamine (OCT) (10 μM), or DCDM (10 μM)

Compound	K_i		
	NC-5	OCT	DCDM
		μM	
Cyproheptadine	4	6	2
Phentolamine	23	46	18
Propranolol	72	175	50

Also shown in Table 5 are the K_i values for the same antagonists determined from their ability to inhibit light organ enzyme activation either by octopamine or by the formamidine, DCDM. Comparison of these three sets of values reveals a substantial correspondence both in absolute and relative potencies. Thus, among the three agonists, the ratio of K_i values for phentolamine *versus* cyproheptadine falls within a fairly narrow range, from 6–9. Furthermore, the ratio of K_i values for propranolol *versus* phentolamine ranges only from 2.8–3.8. These results, together with other data (below), support the possibility that the stimulatory action of the PIIs on light organ adenylate cyclase occurs through a reversible binding to receptors similar to those of the octopamine-2 class.

Reversibility of stimulation. Light organ adenylate cyclase activation by octopamine is known to be reversible (10). Therefore, the above described similarity of K_i values for various antagonists in inhibiting light organ adenylate cyclase stimulation by octopamine *versus* NC-5 suggests that enzyme activation by the PIIs may also be reversible. More direct evidence for reversibility was obtained from other experiments in which light organ homogenates were first preincubated with a PII, then washed, and enzyme activity subsequently determined both in the absence and presence of agonist. An example of such an experiment is shown in Table 6 for NC-16. The degree of reversibility of this derivative was of particular interest, since the *p*-chlorethyl substitution on this compound is similar to that on certain other compounds (such as phenoxybenzamine and some alkylating agents) which are known to exhibit irreversible binding to membrane proteins (20). Table 6 (first data column) shows that preincubation of the enzyme with NC-16, followed by two washings with buffer, resulted in no significant residual activation of adenylate cyclase. As shown in the next two columns, the enzyme could be restimulated by either NC-16 or by octopamine. These data suggest that NC-16 and octopamine bind reversibly to the light organ octopamine-2 receptor. Similar reversibility has also been reported for the formamidines (10).

Partial agonism and antagonism. As shown in Tables 1 and 2, a number of PII derivatives were partial agonists, failing to fully activate adenylate cyclase even at high concentrations. Among the partial agonists, some bound with high affinity, such as NC-7 (potency ratio = 8.6 ±

TABLE 6

Reversibility of receptor-mediated stimulation of light organ adenylate cyclase by octopamine or by NC-16 (4-[chlorethylmethylaminol] clonidine)

Light organ homogenate was preincubated for 5 min at 30° with (left column) octopamine, NC-16, or no drug. Homogenates were then washed twice as described in text and P_2 fraction retested (right-hand three columns) for basal, octopamine-stimulated, or NC-16-stimulated adenylate cyclase activity. Values are the means (± range) of two determinations, each assayed for activity in duplicate.

Pretreatment with	Post-washing adenylate cyclase activity		
	Basal	OCT (1 mM)	NC-16 (100 μM)
	pmol/mg protein/min		
No drug	23 ± 1	1150 ± 250	270 ± 5
Octopamine (1 mM)	69 ± 2	1250 ± 30	260 ± 25
NC-16 (1 mM)	35 ± 5	1025 ± 35	170 ± 10
No pretreatment	39 ± 2	1120 ± 60	210 ± 5

1.1; V_{max} = 69 ± 6%) and NC-16 (potency = 4.8 ± 2.9; V_{max} = 6 ± 1%). Other partial agonists bound with low affinity, such as ST-608 (potency ratio = 0.15; V_{max} = 43%) and UK 14304-18 (potency ratio = 0.12; V_{max} = 22%).

Those partial agonists binding with high affinity were able to act as effective partial antagonists. Fig. 4B, for example, shows the effect of increasing concentrations of NC-16 on a fixed 10 μM concentration of octopamine. NC-16 progressively inhibited octopamine stimulation to a maximum inhibition of greater than 90%. The IC_{50} for this effect was about 15 μM and the calculated K_i, about 10 μM. Above 1 mM, NC-16 alone showed a negative cooperative effect on adenylate cyclase activation, with almost no enzyme stimulation occurring at 5 mM. This negative cooperativity at higher concentrations was characteristic of a number of the other PIIs which also showed partial agonist effects.

Fig. 4C shows the effects of NC-17, a derivative which showed no significant agonist activity (see Table 2). At 1 mM, however, NC-17 was able to nearly completely block the effects of 10 μM octopamine. Thus, NC-17 displayed characteristics suggesting that it is an antagonist of octopamine-activated adenylate cyclase (calculated K_i = 225 μM) lacking any significant agonist activity.

Effects of PIIs on adrenergic- and dopaminergic-stimulated adenylate cyclase. To determine the selectivity of the PIIs for activation of octopamine-sensitive adenylate cyclase, NC-5, the most potent agonist of the light organ enzyme, was tested for its effects in tissues known to contain adenylate cyclases activated by other phenylethylamines but not by octopamine. These tissues included the rat caudate nucleus, known to contain a dopamine-sensitive adenylate cyclase (21); rat heart ventricle, known to contain a predominately β_1-adrenergic-sensitive adenylate cyclase (22); and the rat liver, known to contain a β_2-adrenergic-sensitive adenylate cyclase (22). In addition to NC-5, octopamine and the formamidine, DCDM, were also tested in each tissue. The results from dose-response studies, which have been reported in detail elsewhere (10, 12, 23), showed that NC-5 is an extremely weak agonist of dopaminergic- and β-adrenergic-sensitive adenylate cyclases. Table 7, which summarizes some of this data, compares in four tissues the relative ability of NC-5, DCDM, octopamine, isoproterenol, and dopamine to activate adenylate cyclase. At the particular doses shown, NC-5 was 15 times more potent than octopamine in the light organ but less than 1% as potent as dopamine in stimulating adenylate cyclase in the caudate and less than 1% as potent as isoproterenol in activating enzyme activity in the heart or liver.

TABLE 7

Selectivity of octopamine (OCT), phenyliminoimidazolidines (NC-5), formamidines (DCDM), and catecholamines (dopamine (DA) and isoproterenol (ISO)) for activating adenylate cyclase in firefly light organ, rat heart, rat liver, or rat caudate nucleus

Agonist[a]	Adenylate cyclase activity expressed as a percentage of the stimulation by			
	OCT (1 μM) in light organ	ISO (1 μM) in heart	ISO (1 μM) in liver	DA (10 μM) in caudate
OCT	100	17	<1	<1
NC-5	1500	<1	<1	<1
DCDM	400	<1	5	30
ISO	<1	100	100	10
DA	<1	<1	<1	100

[a] Tested at concentrations shown at right.

Relationship to α-adrenergic receptors. A number of PIIs are known to bind to mammalian α_2-adrenergic receptors (24, 25). The question arises, therefore, as to whether the potency of PIIs as agonists of octopamine-sensitive adenylate cyclase could be due to the fact that they are affecting a class of octopamine receptors similar in binding characteristics to mammalian α_2-adrenergic receptors. Several lines of evidence suggest that this is not the case and that the light organ octopamine receptors can be clearly distinguished from α_2-receptors. Most obviously, mammalian α_2-adrenergic receptors are known to couple in a negative fashion to adenylate cyclase, causing an inhibition of enzyme activity (26), whereas in the light organ the PIIs are positively coupled to adenylate cyclase activity, causing a stimulation of enzyme activity.

Furthermore, we have compared the relative binding affinities (K_D) of various PIIs for mammalian α_2-adrenergic receptors to their relative potencies (K_a) as agonists of octopamine-sensitive adenylate cyclase and have found a very poor relationship between these two characteristics. Table 8 lists such data. For over 20 derivatives, the calculated correlation coefficient between data for α_2-adrenergic binding and data for activation of octopamine-sensitive adenylate cyclase was −0.18, which was without statistical significance. Table 8 reveals that several derivatives, such as UK-14304-18 or *p*-aminoclonidine (NC-14), which have high affinity for α_2-adrenergic receptors, were quite weak at the light organ octopamine receptor. Conversely, derivatives such as NC-5 or NC-7, with relatively low affinity for α_2-adrenergic receptors, were quite potent as agonists of octopamine-sensitive adenylate cyclase.

In other studies, we evaluated the ability of a number of known antagonists to block activation of light organ adenylate cyclase by octopamine. We then compared the calculated K_i values of such compounds with the K_i values of these same compounds as antagonists of mammalian α_2-adrenergic receptor sites, as determined from published [^{3}H]clonidine-binding experiments (24). Table 9 shows this data. For the nine compounds shown, the calculated correlation coefficient was −0.24, which was not significant. Table 9 also lists the relative potencies for the same compounds as antagonists at mammalian α_1-adrenergic receptor sites, as measured by displacement of [^{3}H]WB4101 binding (24). The calculated correlation coefficient between the data for blockade of octopamine activation and inhibition of [^{3}H]WB4101 binding was 0.13, again without significance.

Taken together, the above observations concerning agonist and antagonist binding, as well as the positive coupling of PII binding to adenylate cyclase activation, strongly suggest that the active PIIs shown in Tables 1

TABLE 8

Potency of phenyliminoimidazolidines for mammalian α_2-adrenergic receptor versus potency as agonists for octopamine-sensitive adenylate cyclase

Potency values (relative to clonidine) for the α_2 receptor represent the mean from several published studies using [^{3}H]clonidine (24, 25, 38–41). In a few cases, in which binding data were unavailable, physiological measurements of potency (relative to clonidine) were utilized (42–46). Octopamine-2 potency (relative to clonidine) was recalculated from data shown in Tables 1 and 2 and (for tyramine and norepinephrine) from other dose-response curves not shown. Correlation coefficient between the two measures was −0.18 (not significant).

Compound	Potency relative to clonidine (=1) for	
	OCT-2 receptor	α_2 receptor
NC-5	20.0	0.15
NC-7	9.1	0.28
NC-9	5.4	1.2
NC-16	5.3	0.22
NC-8	3.9	0.76
NC-12	3.2	0.028
ST-606	2.9	0.08
NC-18	2.4	0.084
NC-11	2.0	0.15
NC-20	2.0	0.028
NC-15	1.9	0.39
ST-666	1.7	0.15
NC-6	1.9	0.41
NC-2	1.2	0.33
Octopamine	1.1	0.0037
Clonidine	1.0	1.0
NC-14	0.74	4.6
ST-606	0.50	0.78
NC-4	0.43	0.48
ST-93	0.32	0.78
Tyramine	0.15	0.0009
Norepinephrine	0.13	0.33
UK-14304-18	0.12	4.5
NC-10	0.02	0.02

and 2 are affecting a class of octopamine receptors in the light organ which are distinct from α_2- or α_1-adrenergic receptors and are associated with activation of adenylate cyclase.

Relationship to octopamine-1 receptors. The present experiments do not preclude the possibility that the PIIs may also affect octopamine receptors not associated with adenylate cyclase. Unfortunately, the potent derivatives described in this paper have not been tested previously in physiological experiments investigating such octopamine-1 receptors. However, Evans has reported that the partial octopamine-2 agonist, clonidine, is a full agonist of the octopamine-1-mediated alteration of locust extensor tibiae (ETi) myogenic rhythm, with a potency more than 10 times that of octopamine (4). This contrasts with the partial and weak (½–1/10 the potency of octopamine) agonist effect of clonidine on the twitch tension of the ETi, an octopamine-2 response (4). (Consistent with the present results, Evans has reported that clonidine is quite weak (relative to octopamine) as a stimulator of cyclic AMP accumulation in this muscle (5).) In future neurophysiological studies, it will be of interest to

TABLE 9

Relative potency of antagonists in blocking activation of light organ adenylate cyclase by octopamine (OCT), in inhibiting [^{3}H]clonidine binding, and in inhibiting [^{3}H]WB4101 binding

Data for inhibiting octopamine-sensitive adenylate cyclase were calculated from K_i values determined from inhibitory experiments in which [octopamine] (10 μM) was held constant and [antagonist] was varied (see Materials and Methods). Data for [^{3}H]clonidine and [^{3}H]WB4104 were calculated from Ref. 24. Calculated correlation coefficient for column A versus B was −0.24 (not significant) and for A *versus* C was 0.13 (not significant).

Antagonist	Potency relative to phentolamine (=1) for inhibiting		
	OCT-sensitive adenylate cyclase (A)	[^{3}H] Clonidine binding (B)	[^{3}H]WB4101 binding (C)
Chlorpromazine	11.0	0.037	0.58
Imipramine	8.8	0.0076	0.07
Phentolamine	1.0	1.0	1.0
Phenoxybenzamine	0.76	0.37	0.90
Diphenhydramine	0.40	0.0063	0.001
Piperoxan	0.31	0.23	0.020
Propranolol	0.30	0.0037	0.00052
Dihydroergotamine	0.17	9.2	1.0
Yohimbine	0.086	0.15	0.0075

investigate the effect of PIIs such as NC-5 and NC-13 on the ETi myogenic rhythm. Such studies should help determine the selectivity of the PIIs for octopamine-2 *versus* octopamine-1 receptors.

Published studies with various receptor antagonists do suggest, however, that the pharmacological characteristics of octopamine-1 receptors (at least as determined by physiological responses of the locust muscle) differ significantly from the characteristics of light organ octopamine-2 receptors (as determined by the activity of adenylate cyclase). Thus, as an antagonist of the octopamine-induced change in ETi myogenic rhythm, phentolamine is more than 5-fold more potent than chlorpromazine (4), whereas, in the light organ, chlorpromazine is 10 times more potent than phentolamine in blocking octopamine enzyme activation. Likewise, in the ETi, clozapine and yohimbine are about equipotent (4), whereas in the light organ, clozapine is 75-fold more potent. Other differences involving the agonist potencies of the benzylimidazolines were described above (see discussion related to Table 3).

The light organ octopamine-2 receptor also appears to differ from the octopamine receptor characteristics which Dudai (27) described using inhibition of [^{3}H]octopamine binding in membrane preparations of *Drosophila* heads. Thus, similar to the situation in locust muscle, phentolamine is more than 5-fold more potent than chlorpromazine in *Drosophila*, whereas the converse is true in the light organ. Furthermore, whereas dihydroergotamine is 60-fold more potent than chlorpromazine in *Drosophila*, chlorpromazine is more than 50-fold more potent than dihydroergotamine in the light organ.[5]

[5] There also exist some pharmacological differences between octopamine-2 physiological responses in locust muscle and octopamine-2

TABLE 10

Relative potency of compounds in inhibiting [^{3}H]serotonin binding (5-HT$_1$ sites), in interacting with light organ octopamine-sensitive adenylate cyclase, and in inhibiting [^{3}H]spiroperidol binding (5-HT$_2$ sites)

Data for inhibiting octopamine-sensitive adenylate cyclase were calculated from K_i values as described in Table 9. (Data for serotonin and norepinephrine only were derived from K_a values.) Data for [^{3}H] serotonin and [^{3}H]spiroperidol binding were calculated from Refs. 47–52. Calculated correlation coefficient for column A *versus* B was −0.38 (not significant) and for B *versus* C, 0.35 (not significant). LSD, lysergic acid diethylamide.

Compound	Potency relative to LSD (=1) for inhibiting		
	[^{3}H]Serotonin binding (A)	Octopamine-sensitive adenylate cyclase (B)	[^{3}H]Spiroperidol binding (C)
Serotonin	2.3	0.2	0.0022
LSD	1.0	1.0	1.0
Methysergide	0.083	1.3	0.92
Clozapine	0.013	25.0	0.34
Phenoxybenzamine	0.0085	2.9	0.10
Cyproheptadine	0.0071	33.0	1.1
Fluphenazine	0.0053	11.0	0.22
Chlorpromazine	0.0024	40.0	0.58
Haloperidol	0.0010	4.7	0.15
Norepinephrine	0.00005	1.5	0.00001

Relationship to 5-HT$_1$ and 5-HT$_2$ receptors. In the present experiments, serotonin did not activate light organ adenylate cyclase, suggesting that the observed stimulation by the PIIs was not due to their activation of a serotonin-sensitive adenylate cyclase. Further evidence that the octopamine receptors present in the light organ are distinct from mammalian 5-HT$_1$ and 5-HT$_2$ receptors is provided by the data in Table 10. This table compares the ability of several antagonists (and two agonists) to block (or, for the agonists, to stimulate) light organ octopamine-sensitive adenylate cyclase with the ability of the same compounds to displace [^{3}H]serotonin (a measure of 5-HT$_1$ sites) or [^{3}H]spiroperidol (a measure of 5-HT$_2$ sites) from mammalian brain. For the 10 compounds shown, the calculated correlation coefficient for effects on octopamine-sensitive adenylate cyclase *versus* effects on 5-HT$_1$ receptors was −0.38. This value was without statistical significance and, therefore, fails to support any similarity in the pharmacology of these two classes of receptors. Similarly, using the same compounds, the correlation coefficient for octopamine-sensitive adenylate cyclase *versus* effects on 5-HT$_2$ receptors was 0.35, which was again without statistical significance.

Species-dependent effects. In vivo, it has been shown that the pesticidal potency of the formamidines, whose toxic effects are thought to be due to their octopaminergic agonist activity (9–11), varies considerably among different insect species (28, 29). In order to investigate possible species differences in the effects of the PIIs and formamidines at the receptor level, the activation of adenylate cyclase by NC-5 and NC-7, octopamine, and the formamidine, DDCDM, was evaluated in nerve tissue from the cockroach, *P. americana*, and from the tobacco hornworm, *M. sexta*. These two species were chosen because of their known difference in sensitivity to the octopaminergic formamidine pesticides (below). For each agonist, dose-response curves were run on P_2 fractions of ventral nerve cord from each of these two species, as well as on P_2 fractions from firefly light organ. Table 11 summarizes the results. In each tissue, the V_{max} for each agonist is expressed as a percentage of maximum hormone stimulation observed with octopamine. Similarly, in each tissue, K_a values are expressed relative to the K_a for octopamine in that tissue (K_a(octopamine)/K_a(agonist)).

NC-5 was a full and potent agonist in all three tissues, potencies ranging from 8–19 times that of octopamine. In sharp contrast, DDCDM displayed substantially different species-dependent responses. In *Manduca* nerve cord, DDCDM was a full agonist with 20 times the potency of octopamine. However, in the cockroach nerve cord, this formamidine was much less potent (only three times that of octopamine) and, more strikingly, was a poor partial agonist with only 12% of the activity of octopamine itself. Activity in the light organ was intermediate. These results are consistent with reports that the related formamidine, DCDM, is considerably more potent in activating adenylate cyclase in *Manduca* nerve cord (9) than in the firefly light organ (9, 10), cockroach nerve cord (30), or locust brain (31). Also consistent, Evans and Gee have reported that DCDM is less potent than octopamine in altering octopamine-2 physiological responses in locust muscle (11).

In the present experiments, it was of interest that NC-7, which has phenyl substituents identical to those of DDCDM, displayed agonist properties somewhat similar to those of the formamidine. In *Manduca* nerve cord, NC-7 was a full agonist, more potent than NC-5, while in cockroach nerve cord, NC-7 was a partial agonist. Activity in the light organ was intermediate. However, although a partial agonist, NC-7 was significantly more

TABLE 11

Effects of phenyliminoimidazolidines (NC-5 and NC-7), octopamine, and formamidines (DDCDM) on activating adenylate cyclase in three invertebrate species

Activity is expressed relative to octopamine (V_{max} = 100%; K_a ratio = 1). V_{max} values shown are the mean (± range) for quadruplicate cAMP determinations for a single dose-response curve. Curves for each compound were run at least twice in each tissue, and the data shown are typical of those seen in these other experiments.

Compound	Firefly light organ		Cockroach nerve cord		*Manduca* nerve cord	
	V_{max}	K_a ratio	V_{max}	K_a ratio	V_{max}	K_a ratio
NC-5	97 ± 5	19	91 ± 3	11	96 ± 4	8
NC-7	68 ± 10	10	45 ± 5	12	100 ± 3	16
Octopamine	100 ± 5	1	100 ± 5	1	100 ± 5	1
DDCDM	68 ± 5	4	12 ± 1	3	97 ± 3	20

biochemical responses in the light organ. However, before definitive conclusions can be drawn about differences in octopamine-2 receptor subtype between these two tissues, direct adenylate cyclase studies need to be performed in locust muscle (see discussion below).

active than DDCDM in the cockroach nerve cord (45% of octopamine V_{max}).

Relevant to the above biochemical experiments are the physiological observations that the formamidines are potent pesticides against *Manduca* but are very weak against the cockroach. Furthermore, as will be shown below, NC-7 is more toxic than NC-5 as an antifeedant in *Manduca*, a physiological observation which is consistent with the biochemical receptor activity of the PIIs in *Manduca* but not in the light organ. For this latter tissue, in which NC-5 is more potent than NC-7, NC-5 is similarly more active as a stimulator of physiological light emission (see Table 12).

It is possible that the species differences in sensitivity to the pesticidal effects of the formamidines and NC-7 could be due to differences in metabolism. With the formamidines, such differences, particularly in *N*-demethylation, have been previously observed, *in vivo*, over a period of many minutes (29, 32). However, we have previously shown that, in broken cell preparations, such demethylation does not appear to occur, particularly during the very short incubation times used in the present study (10). Not only that, but in the present experiments we utilized the (already) *N*-demethylated derivative, DDCDM, to avoid this possibility. In other studies, we have observed that, under the present incubation conditions, hormone response to both formamidines and phenylethanolamines remains linear over the incubation period (8, 10). This suggests that oxidation or metabolism of the hormone to a less (or more) active form is unlikely. (*In vivo*, species differences to the effects of the formamidines or PIIs might be affected by differences in penetrability through the barrier systems surrounding insect nerve tissue. However, the use of broken cell preparations in the present experiments makes this possibility unlikely.)

Another explanation for the observed species differences of the formamidines and NC-7 could relate to possible effects of the formamidines (*e.g.*, Ref. 33) and PIIs on differing populations of nonoctopaminergic cyclic AMP-associated receptors. Still another possible reason for the observed species differences could be that these compounds are affecting, not a homogenous class of octopamine-2 receptors but, rather, two or more receptor subtypes whose distribution varies among the species. Clearly, however, additional comparative pharmacological data are needed before speculating about the existence of octopamine-2 subtypes.

TABLE 12

Summary of potencies of octopamine agonists in stimulating firefly light emission and in exerting antifeeding toxicity in Manduca sexta

Compound	EC_{50}	
	Light emission	Antifeeding
	μM injected	*mg % spray*
NC-5	60	150
NC-7	130	15
Formamidine	220	20
Octopamine	1000	300
NC-10	NA[a]	NA

[a] NA, no activity.

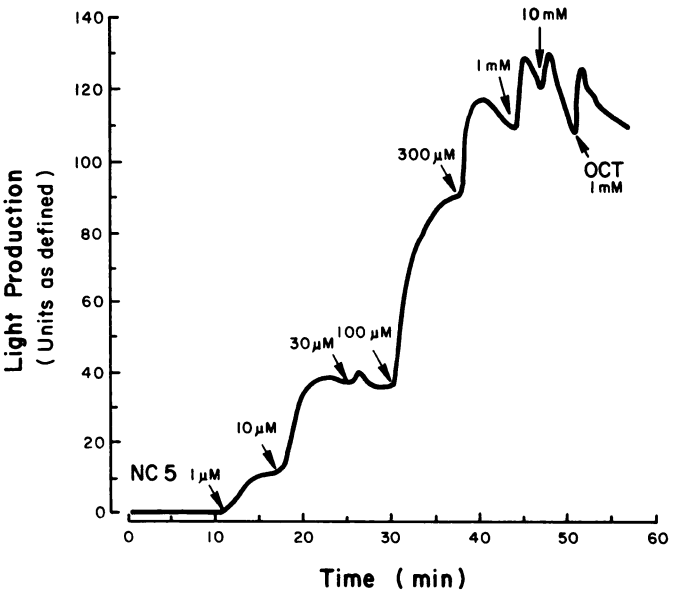


FIG. 5. *Effect of NC-5 on eliciting light emission in the isolated firefly tail*

Compound (at the concentrations shown) was injected (in 3–5 μl) into the haemolymph dorsal to the light organ, and light emission measured as described in text. Following the last dose of NC-5, 1 mM octopamine (*OCT*) (a maximally effective dose) was injected.

Physiological effects on firefly light emission. The initiation of light emission in the firefly light organ is a neurally mediated event (34). Octopaminergic neurons from the terminal abdominal ganglia are known to synapse in the light organ (35) and, in isolated denervated light organs, octopamine mimics the effect of nerve stimulation in eliciting light emission (reviewed in Refs. 3 and 34). In previous studies, we have shown that the potency of various phenylethanolamines in eliciting light emission in isolated light organs is correlated with the ability of such amines to activate light organ adenylate cyclase (7, 8). Furthermore, because cholera toxin, forskolin, and cyclic AMP analogs mimic the physiological effect of octopaminergic agonists in the firefly, it appears that light emission is associated with the activation of adenylate cyclase (36).

In the present experiments, light output from isolated mounted tails was measured following injection of increasing doses of test compound, dissolved in insect saline, into the hemolymph dorsal to the lantern. The saline solution lacked calcium and contained 20 mM manganese, a salt composition which, in other experiments, was shown to block neuronal release of octopamine (36). Thus, the responses recorded are thought to be due to direct postsynaptic effects of the injected test compounds. Fig. 5, a typical experiment, shows that NC-5 was a potent stimulator of light emission. In the particular experiment shown (total $N = 6$), injected concentrations of the PII as low as 1 μM initiated light production. This makes NC-5 the most potent light-generating compound yet reported in the firefly. Higher concentrations of NC-5 caused a progressive increase in light output to a maximum equal to that induced by optimal concentrations of octopamine.

Dose-response experiments were also carried out for octopamine, NC-7, NC-10, and the formamidine, DCDM. Octopamine elicited similar effects to NC-5 but was less potent, particularly at lower concentrations. Typically, 100 μM octopamine was the lowest concentration which was able to elicit a light response. Table 12 summarizes the calculated EC_{50} concentrations necessary for eliciting light emission for each of the four agonists. This table is based upon 5–10 dose-response curves for each compound. It will be noted that the rank order physiological potency of the various compounds corresponds to their rank order potency as activators of light organ adenylate cyclase (Tables 1 and 11). Furthermore, the absolute ratio of the physiological potencies of NC-5 to octopamine (about 16) closely matches the ratio (about 19) of biochemical potencies of the two compounds as agonists of light organ adenylate cyclase.

It was of interest, too, that the partial agonistic effect of NC-7 and the formamidine, DCDM, seen *in vitro* (Table 1, Fig. 2) was also observed when these compounds were used to elicit light emission. Thus, at optimal concentrations, neither of these compounds caused as much light output as did octopamine. Furthermore, with the increasing dose of agonist, there was frequently a biphasic response curve similar in appearance to the biphasic enzyme activity curve frequently observed in broken cell preparations (Fig. 2). In both situations, higher concentrations of agonist caused a partial inhibition of response. An example of a biphasic light response to DCDM is shown in Fig. 6. It will be noted that, following application of high concentrations of DCDM, application of octopamine failed to restore light production. This result is consistent with prior biochemical studies (10) indicating that, at high concentrations, DCDM can act as an octopamine receptor antagonist. Similar biphasic effects were seen with NC-7, although maximal response with NC-7 was less than that elicited by DCDM.

Antifeeding effects. The formamidines are used commercially as pesticides against ticks, mites, and a limited

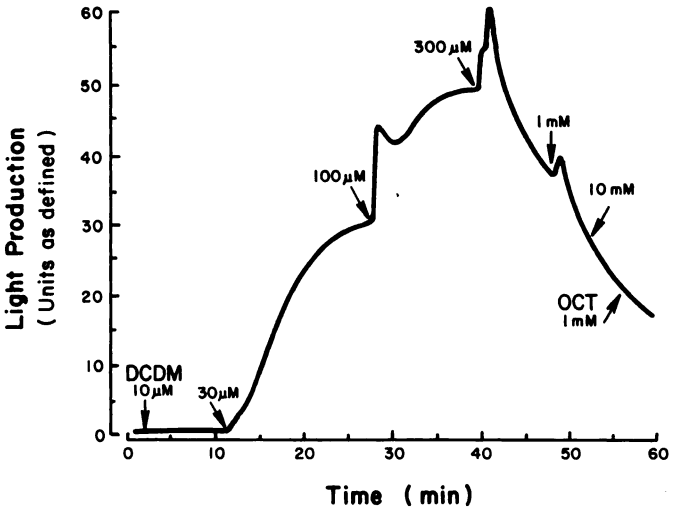


FIG. 6. *Effect of the formamidine, DCDM, on eliciting light emission in the isolated firefly tail*

Details are as described in the legend to Fig. 5 and in the text.

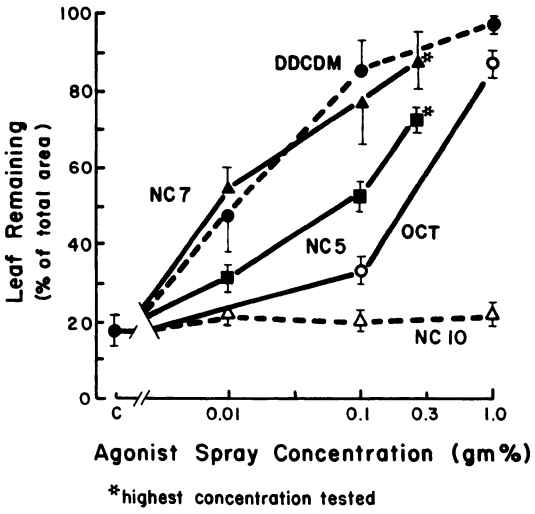


FIG. 7. *Effect of various compounds on disruption of feeding in the tobacco hornworm*

Compounds were applied as a spray to isolated tomato leaves, allowed to dry, and the leaves then exposed to hornworm larvae. The percentage of leaf area remaining after 72 h, measured in duplicate for each concentration in each experiment, indicates the degree to which feeding was disrupted (and the degree to which the leaves were "protected" by the compound (see text for details)). The data for NC-10 are from a single experiment. The values shown for the other compounds represent the mean (± range) for two to three separate experiments. *OCT*, octopamine.

number of insect species, including certain lepidoptera, such as *M. sexta* (the tobacco hornworm) (28, 29). These compounds appear to exert their toxic effects primarily through motor-behavioral disruption, including induction of tremors, hyperactivity, incoordination, and an inability to feed (28). Because the formamidines can activate octopamine-sensitive adenylate cyclase (9, 10, 30) and because the toxicity of these compounds can be enhanced by phosphodiesterase inhibitors (18), it has been proposed that the pesticidal effects of the formamidines are mediated through cyclic AMP increases (9, 10, 18) (however, see also Ref. 33).

Because the PIIs were able to activate adenylate cyclase in *Manduca* nerve cord (Table 11), it was of interest to determine if compounds such as NC-5 and NC-7 could exert any toxic effects against this species. These experiments were carried out with young (3-day-old) first instar *Manduca* larvae, which are sensitive to the effects of the formamidines. Tomato leaves were spray treated with various doses of NC-5, NC-7, NC-10, DDCDM, or octopamine, and larvae allowed to feed on the leaves for several days. To enhance toxic effects, all leaves were also treated with the phosphodiesterase inhibitor, IBMX, applied at a low dose which, by itself, did not exert any toxic effect. (In other studies (below), we have shown that the synergistic effect of IBMX on octopamine agonists is due to its action as a phosphodiesterase inhibitor and not as an adenosine antagonist (18).)

Fig. 7 shows the results of dose-response studies for the four octopamine agonists and the inactive PII derivative, NC-10. The graphs plot the amount of leaf remaining (as a percentage of starting area) after larvae had fed on the treated leaves for 72 hr. The greater the

leaf area remaining, the greater the toxic effect of the compound applied. Typically, by 72 hr, untreated leaves or leaves treated only with 0.1% IBMX showed less than 20% of leaf area remaining.

With increasing concentrations of NC-5, there was a progressive inhibition of leaf feeding to a maximum inhibition of about 70%. At the higher concentrations of this PII, larvae manifested tremors, hyperactivity, and leaf walk-off behavior. Animals showed particular difficulty during molting (a task demanding of motor coordination), frequently being unable to shed their old cuticle, and subsequently dying. These effects persisted so long as larvae continued to feed on the treated leaves.

Unlike its effects on light emission (in which NC-7 was weaker than NC-5), NC-7 was significantly more potent and more effective than NC-5 as a toxic agent for *Manduca*. This relative *in vivo* potency corresponds quite well to the potency seen *in vitro* for the two compounds. Thus, as shown in Table 11, in broken cell preparations of *Manduca* nerve cord, NC-7 was about twice as potent as NC-5, whereas, in the light organ, NC-5 had the greater potency (see previous discussion of species differences). NC-10, the unsubstituted PII derivative which lacked *in vitro* activity as an octopamine-2 agonist (Table 1, Fig. 1) also lacked antifeeding activity.

A similar correspondence between *in vivo* toxicity and *in vitro* octopaminergic agonist activity was seen for the formamidine, DDCDM, and for octopamine itself. Thus, in *Manduca*, the rank order potency for the four compounds (DDCDM ≃ NC-7 > NC-5 > octopamine > NC-10) was the same *in vitro* (Tables 1 and 11) and *in vivo* (Table 12). The four active compounds also exerted qualitatively similar effects on behavior. These observations serve to support further the possibility that the observed physiological actions of the PIIs were due to an effect on octopamine-2 receptors *in vivo*.

Synergistic effects of phosphodiesterase inhibitors on PII toxicity. Additional evidence that the antifeeding effects of the PIIs observed *in vivo* were mediated through an activation of adenylate cyclase came from the observed synergistic effects of phosphodiesterase inhibitors on PII toxicity. Fig. 8 shows the dose-dependent antifeeding effects of NC-7 in the absence or presence of IBMX (0.1% spray). At the dose used, IBMX alone caused only a small inhibition of feeding. However, when combined with increasing doses of NC-7, IBMX caused a marked enhancement both of the efficacy and potency of the PII. A similar enhancement was seen when IBMX was combined with NC-5 or with octopamine (data not shown). Indeed, in the absence of IBMX, octopamine showed almost no activity as an antifeedant. As previously described, IBMX also enhances the activity of various formamidine derivatives (18).

Other methylxanthine derivatives, such as caffeine or theophylline, which were able to inhibit *Manduca* phosphodiesterase activity, also exhibited synergism with various octopamine agonists (data not shown). However, derivatives such as 8-phenyl-theophylline or 8-phenyl-1,3-dipropylxanthine, which were poor antagonists of nerve cord phosphodiesterase activity, were, similarly, poor synergists. Since these latter compounds are known to be potent adenosine receptor antagonists (37), it appears that phosphodiesterase inhibition, rather than adenosine receptor blockade, plays a role in the synergistic effects of the methylxanthines.

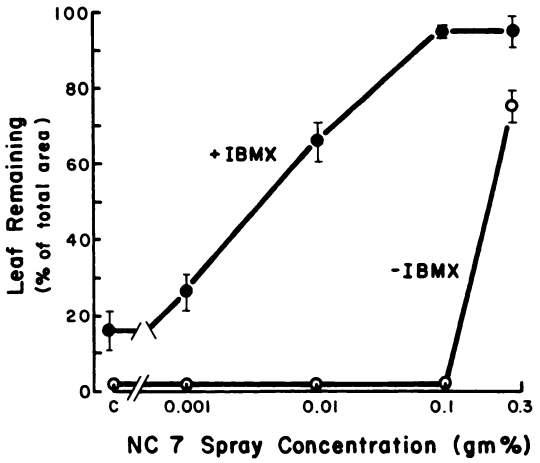


FIG. 8. *Synergistic effect of a fixed dose (0.1% spray) of the phosphodiesterase inhibitor, IBMX, on the ability of NC-7 to disrupt feeding of tobacco hornworm larvae*

Procedure is as described in Fig. 7 and in the text. The values shown represent the mean (± range) of duplicate area measurements from a single experiment and are typical of those seen in other experiments.

Furthermore, the enhancements of pesticidal potency of the various octopaminergic agonists, seen with IBMX, are paralleled by corresponding increases in the cyclic AMP content of *Manduca* nerve cord (18). Thus, the rank order antifeeding potency of various primary agonists with or without IBMX (DDCDM + IBMX = NC-7 + IBMX > octopamine + IBMX = DDCDM alone > NC-7 alone > octopamine alone (almost inactive)) matches the rank order potency of the same combinations in elevating cyclic AMP when incubated with intact segments of ventral nerve cord.

CONCLUSION

Taken together, the above physiological studies of light emission, antifeeding activity, and synergism supply additional evidence that substituted PIIs can be potent agonists of octopamine receptors associated with the activation of adenylate cyclase. In future studies, these compounds should prove to be helpful in delineating the pharmacology of octopamine receptors (and receptor subtypes) in invertebrates. They may also help to point the way toward developing extremely potent and relatively specific octopamine agonists which could be useful as pesticidal agents with reduced toxicity for vertebrates (12).

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