

Characterization of octopamine-sensitive adenylate cyclase: Elucidation of a class of potent and selective octopamine-2 receptor agonists with toxic effects in insects

(phenyliminoimidazolidines/cyclic AMP/pesticides/firefly/tobacco hornworm)

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ABSTRACT Octopamine-2 receptors, associated with activation of adenylate cyclase, mediate a number of the important hormonal and neurotransmitter functions of octopamine in invertebrates. By utilizing the highly enriched octopamine-sensitive adenylate cyclase present in the firefly light organ, it has been possible to pharmacologically characterize octopamine-2 receptors and to define a new class of highly potent and selective octopamine-2 agonists. At low concentrations, these substituted phenyliminoimidazolidines stimulate light emission when injected into fireflies. At somewhat higher concentrations, these compounds, when ingested by tobacco hornworms, cause disruption of motor and feeding behavior, leading to insect death. The effects of these compounds are markedly potentiated by phosphodiesterase inhibitors and mimicked by other activators of octopamine-sensitive adenylate cyclase, including octopamine itself. Because octopamine-2 receptors appear to be present primarily in invertebrates, these findings, together with other data, raise the possibility that potent and selective octopamine agonists could be useful as insect toxins with low toxicity in vertebrates.

Biochemical, physiological, and anatomical studies have established that octopamine [1-(*p*-hydroxyphenyl)-2-aminoethanol] is an important neurotransmitter and neurohormone in invertebrates (reviewed in refs. 1-4). Many of the physiological functions of octopamine appear to be mediated by a class of octopamine receptors ["octopamine-2 receptors" (2)] specifically coupled to the activation of adenylate cyclase. Since octopamine-activated adenylate cyclase was described over a decade ago (5), there have been a number of efforts to characterize octopamine-2 receptors by studying the production of cyclic AMP in invertebrate nerve and muscle tissue (6-8). However, because several other hormone receptors (e.g., those for dopamine, serotonin, and proctolin) are also positively coupled to cyclic AMP production in these tissues (3, 4), it has been very difficult (due to the confounding effects of these other receptors) to define the pharmacological characteristics of octopamine-2 receptors.

Recently, studies from this and other laboratories have suggested that octopamine is the neurotransmitter involved in neural control of light emission in the firefly light organ, a tissue which, in certain respects, is analogous to the cholinergically innervated electroplax organ of electric fishes (9-12). The firefly light organ is highly enriched in octopamine-activated but not other amine-activated adenylate cyclases. Thus, octopamine can cause a >50-fold stimulation of adenylate cyclase in this preparation, which, unlike invertebrate nerve cord or brain, contains no significant amount of dopamine-, norepinephrine-, serotonin-, or

histamine-activated adenylate cyclase. For these reasons, the firefly light organ appears to be an excellent tissue with which to characterize octopamine-2 receptors.

Using this tissue in an attempt to develop better octopamine receptor agents, I have found that certain substituted 2-(phenylimino)imidazolidines are extremely potent and selective agonists of octopamine-activated adenylate cyclase, having greater activity than previously described compounds. I also report that these agents exert potent physiological and toxicological effects in insects, including stimulation of light emission in the firefly and disruption of motor activity (including feeding) in the tobacco hornworm.

MATERIALS AND METHODS

Specimens of *Photinus pyralis* were collected in summer, frozen on dry ice, and stored in liquid N₂. Under these conditions, octopamine-sensitive enzyme activity remains stable for 6 months or longer (13). After thawing at 4°C, tail sections were opened, and the light organs were removed from the ventral cuticle, cleaned of any adhering nonlantern tissue, and homogenized (10 mg/ml) in 6 mM Tris maleate, pH 7.4. A washed particulate (P₂) fraction was prepared as described (13) and kept at 0°C until used. In some experiments, adenylate cyclase was measured in P₂ fractions prepared from the ventral nerve cords (including head and tail ganglia) of adult cockroaches (*Periplaneta americana*) and of 50-mm-long tobacco hornworms (*Manduca sexta*). In other experiments, P₂ fractions were prepared from caudate nucleus (10 mg/ml), liver (50 mg/ml), and heart (50 mg/ml) of 3-month-old Sprague-Dawley rats.

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 MgCl₂; 0.1 mM GTP; 0.5 mM EGTA; 2 mM ATP; 0.06 ml of P₂ fraction; and various compounds to be tested. Prior experiments had shown that, under these conditions, adenylate cyclase activity was optimized (13). The enzyme reaction (4 min at 30°C) was initiated by addition of ATP and stopped by heating at 90°C for 2 min. The reaction mixture was 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 was nearly completely inhibited. Protein concentration was determined by the Lowry method. Activation constants (*K*_{act}) and inhibitory constants (*K*_i) were calculated as described (13).

To measure the effects of drugs on firefly light emission, an isolated tail (terminal three abdominal segments, contain-

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Abbreviations: DDCDM, didemethylchloridimeform; NC5, NC7, and NC9, the 2,6-diethyl, 2-methyl-4-chloro, and 2,4-dimethyl derivatives, respectively, of 2-(phenylimino)imidazolidine; *i*-BuMe-Xan, 3-isobutyl-1-methylxanthine.

ing the light organ) of a fresh adult *Photinus pyralis* male was mounted on a 30-gauge stainless-steel needle and placed at the focal point of an optical system connected to a photometer/photomultiplier/chart recorder combination. Drug [dissolved in insect saline (15)] was injected (in 3 μ l) into the abdominal cavity dorsal to the lantern and light emission was recorded for 45 min, after which the next (larger) dose of drug was injected. In the case of animals injected with drugs other than octopamine, 10 nmol of octopamine (a maximally effective dose) was injected after the last dose.

To measure the effects of drugs on motor and feeding behavior of tobacco hornworms, drugs (dissolved in water) were applied as an aerosol to isolated, hydrated tomato leaves and allowed to dry. A group of six 3-day-old *Manduca sexta* larvae (reared on artificial medium) were then placed on each leaf; 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 (16) (see below). In other biochemical experiments, isolated *Manduca* nerve cords treated with phenyliminoimidazolidines or octopamine showed increases in cyclic AMP content that were enhanced in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (*i*-BuMeXan). Thus, in these behavioral experiments, *i*-BuMeXan (0.1 mg per leaf) was included in both control and experimental leaves. (At the dose used, *i*-BuMeXan itself had little or no effect on the rate of leaf consumption.)

G. LeClerc, Boehringer Ingelheim, and Pfizer kindly supplied many of the phenyliminoimidazolidine analogs. Dimethylchloridimeform [DCCDM, *N*-(4-chloro-2-methylphenyl)formamide] was kindly supplied (as the hydrochloride salt) by H. M. LeBaron. Phentolamine (2-[*N*-(*m*-hydroxyphenyl)-*p*-toluidinomethyl]imidazoline) was sup-

plied by CIBA-Geigy, and cyproheptadine [4-(5*H*-dibenzo[*a,d*]cyclohepten-5-ylidene)-1-methylpiperidine], by Merck.

RESULTS AND DISCUSSION

To characterize octopamine receptors regulating cyclic AMP production, I first evaluated the hormone sensitivity of adenylate cyclase in P_2 fractions of *Photinus* light organs. Fig. 1A shows that the K_{act} of octopamine for stimulating enzyme activity in the light organ was $\approx 20 \mu$ M. In the same tissue, dopamine and the β -adrenergic agonist isoproterenol caused only a small degree of enzyme stimulation even at high concentrations. The α -adrenergic agonist phenylephrine (data not shown) was also of low potency ($K_{act} > 200 \mu$ M).

Table 1 lists the kinetic constants of receptor-mediated enzyme activation for several well-known phenylethylamines and phenylethylamine metabolites. With the exception of synephrine (*N*-methyloctopamine), which is slightly more potent than octopamine, no other phenylethylamine analog (among these and several dozen others that were examined) had more activity than octopamine. In all cases, those analogs that had partial activity satisfied criteria (data not shown) that strongly suggested that their effects on cyclic AMP production were due to their activation, at high concentrations, of the octopamine-specific adenylate cyclase. Thus, at optimally effective concentrations, none of the compounds showed activity that was additive to the activity of a maximally effective concentration of octopamine. Furthermore, the rank-order ability of certain antagonists (such as cyproheptadine, phentolamine, chlorpromazine [2-chloro-10-(3-dimethylaminopropyl)phenothiazine], and propranolol [1-(isopropylamino)-3-(1-naphthoxy)-2-propanol]) to block activation by these analogs was identical to the rank-order ability of the same antagonists to block enzyme activation by octopamine.

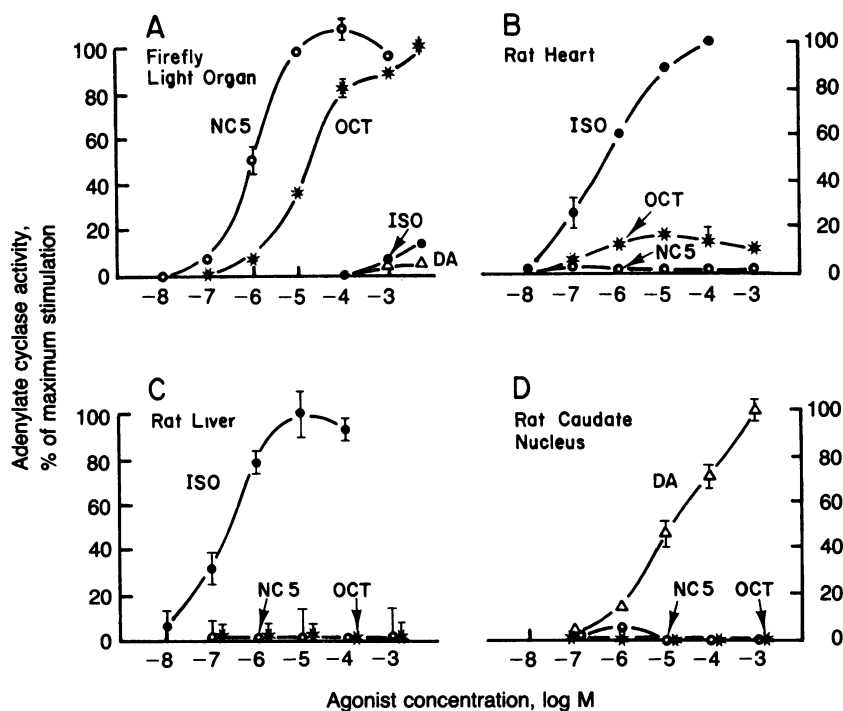


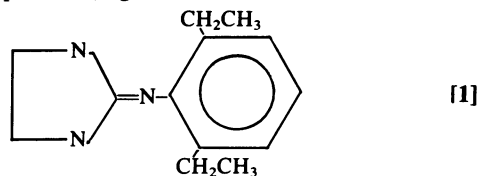
FIG. 1. Comparison of the relative effects of 2-(2,6-diethylphenylimino)imidazolidine (NC5), octopamine (OCT), isoproterenol (ISO), and dopamine (DA) in activating adenylate cyclase in the firefly (male *P. pyralis*) light organ (A), rat heart (B), rat liver (C), or rat caudate nucleus (D). Stimulation is expressed as a percentage of the maximal stimulation produced by the most active amine in that particular tissue. Values shown are means (\pm mean deviation) of replicate samples each assayed for cyclic AMP in duplicate. Basal and maximal activities (in pmol/mg of protein per min) are as follows: 7.0 ± 0.8 and 534 ± 22 in A; 6.8 ± 0.2 and 14.5 ± 0.5 in B; 1.7 ± 0.2 and 4.1 ± 0.2 in C; 50.8 ± 0.8 and 106 ± 2 in D.

Table 1. Structure-activity relationships of some phenylethylamines interacting with firefly light organ octopamine-sensitive adenylate cyclase

Compound					V_{\max} , % V_{\max} OCT	$\frac{K_{\text{act}} \text{ OCT}}{K_{\text{act}} \text{ compound}}$
	3	2	1	N		
β -Phenylethylamine	—	H	H	H ₂	13 ± 2	0.03
(±)-Phenylethanolamine	—	OH	H	H ₂	45 ± 1	0.11
(-)-Phenylephrine	3-OH	OH	H	HCH ₃	17 ± 1	<0.02
Tyramine	4-OH	H	H	H ₂	57 ± 1	0.15
(±)- <i>p</i> -Octopamine	4-OH	OH	H	H ₂	100 ± 1	1.0
(±)- <i>N</i> -Methyloctopamine	4-OH	OH	H	HCH ₃	108 ± 10	1.8
(±)- α -Methyloctopamine	4-OH	OH	CH ₃	H ₂	44 ± 2	0.05
Dopamine	3-OH,4-OH	H	H	H ₂	4 ± 1	0.26
(-)-Norepinephrine	3-OH,4-OH	OH	H	H ₂	72 ± 1	0.13
(-)-Epinephrine	3-OH,4-OH	OH	H	HCH ₃	75 ± 2	0.20
(-)-Isoproterenol	3-OH,4-OH	OH	H	HCH(CH ₃) ₂	19 ± 1	<0.03

Enzyme activity was determined as described in *Materials and Methods*. V_{\max} for stimulated adenylate cyclase is given as a % of the V_{\max} due to octopamine (OCT). The last column gives the calculated ratios of potency obtained in a particular experiment by dividing the K_{act} for octopamine activation of adenylate cyclase by the K_{act} of the compound for activating adenylate cyclase. Thus, values >1 indicate greater potency than octopamine.

In an effort to develop more potent agonists of octopamine-sensitive adenylate cyclase, a number of non-phenylethylamine structures were evaluated for activity. The ability to predict potentially active compounds was aided by the structure-activity relationships developed from the phenylethylamines and by previous work, from this and other laboratories, indicating that compounds containing an N-C-N side chain have partial octopamine agonist activity (13, 17, 18). Among several non-phenylethylamine groups that were found to be octopamine-2 agonists, the most potent were a series of substituted phenyliminoimidazolidines. Fig. 1A shows, for example, that 2-(2,6-diethylphenylimino)imidazolidine (NC5) [1] caused significant stimulation of light-organ adenylate cyclase at concentrations as low as 30 nM. The K_{act} was $\approx 1 \mu\text{M}$ (0.05 that of octopamine), and maximal stimulation was equal to that caused by octopamine (Fig. 1A).



In other experiments, NC5 had no activity as an octopamine antagonist, and, at maximally effective concentrations, stimulation by NC5 was nonadditive with that due to maximally effective concentrations of octopamine. Activation of enzyme activity by NC5 also was reversible and was inhibited by low concentrations of certain antagonists, such as cyproheptadine ($K_i = 5 \mu\text{M}$) and phentolamine ($K_i = 21 \mu\text{M}$), that are known to inhibit enzyme stimulation by octopamine (19) but only by higher concentrations of the β -adrenergic antagonist propranolol ($K_i = 75 \mu\text{M}$).

Among various 2-(phenylimino)imidazolidines investigated, the 2,6-diethyl derivative (NC5) had the greatest activity (potency relative to octopamine = 19). Other potent derivatives included 2-methyl-4-chloro (NC7) (potency ratio = 9.8); 2,4-dimethyl (NC9) (ratio = 5.1); 2,4,6-trimethyl (ratio = 4.3); 2,4-dichloro (ratio = 3.7); 2,4,5-trichloro (ratio = 3.0); and 2-methyl-3-bromo (ratio = 2.9). In prior physiological studies in insects, it has been reported that clonidine [2-(2,6-dichlorophenylimino)imidazolidine] can affect behavior (20) and stimulate octopamine receptors that may be distinct from those associated with adenylate cyclase (17).

However, as an agonist of cyclic AMP-associated octopamine-2 receptors, clonidine has been reported to be <10% as effective as octopamine (8). Similarly, in the present studies, clonidine was both less potent and less effective ($V_{\max} = 35\%$) than octopamine and also was a potent antagonist ($K_i = 20 \mu\text{M}$) of octopamine-stimulated enzyme activity. This contrasts markedly with the above described phenyliminoimidazolidine derivatives, which were full and potent octopamine-2 agonists lacking any significant antagonist activity. Thus, the structural configuration of compounds such as NC5 and NC7 is quite specific for both binding to and activation of this class of octopamine receptors.

To determine whether activation of octopamine-sensitive adenylate cyclase by the active phenyliminoimidazolidines was indicative of *in vivo* octopaminergic potency, some of the compounds were evaluated for their ability to stimulate light production when injected into transected, isolated tails of *Photinus* males. It is thought that the light-inducing effects of amines in isolated firefly tails are due to the activation of adenylate cyclase-associated octopamine receptors located postsynaptically in the light organ (9, 15, 20). The dose-response curve in Fig. 2A shows that, for a group of animals, octopamine stimulated light emission with an EC_{50} (concentration of half-maximal effect) for octopamine of about 5 nmol per tail. NC5, in contrast, had an EC_{50} of about 0.3 nmol, and thus was >16 times as potent as octopamine. Relative to octopamine, NC5 was even more potent at low levels of stimulated light emission. At high doses, maximal stimulation by the two compounds was comparable and, at maximally effective doses, a combination of both compounds did not elicit more light production than either compound alone.

It is known that the formamidines, which have been reported to be partial agonists of octopamine-sensitive adenylate cyclase, can, in certain species of susceptible insects, cause motor-behavioral disruption (including tremors, hyperactivity, and inability to feed) (13, 21, 22, 23). To determine whether the phenyliminoimidazolidines have any similar behavioral actions, I examined the effects of certain of these compounds and of octopamine itself on the behavior of *Manduca sexta* (tobacco hornworm) larvae feeding on spray-treated tomato leaves. Both octopamine and the phenyliminoimidazolidines caused qualitatively similar effects: within 30 min, larvae began to manifest tremors,

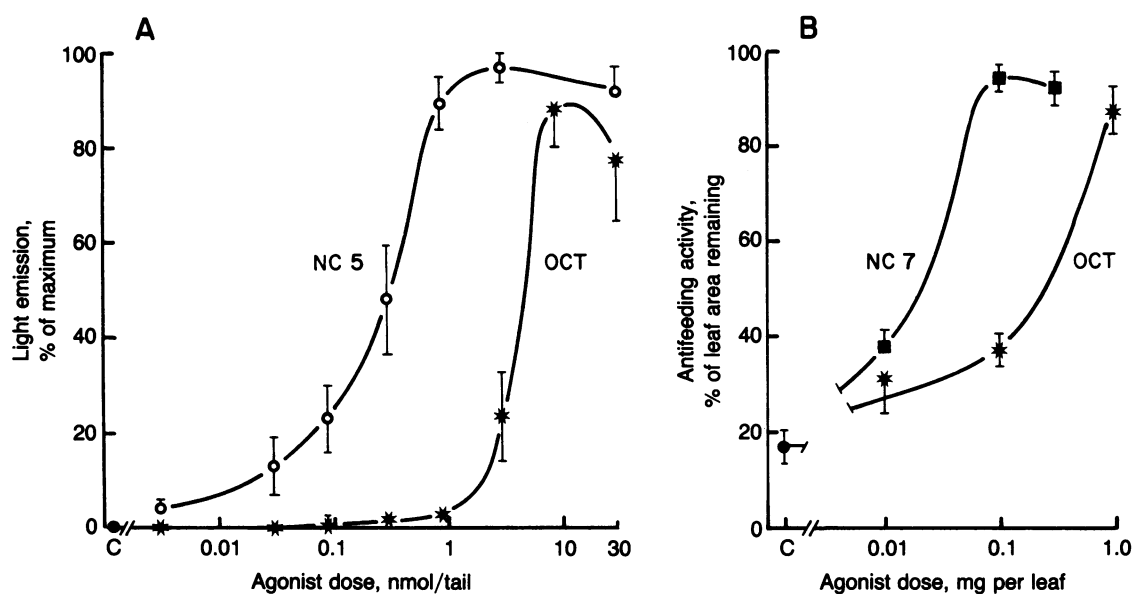


FIG. 2. Comparison of the relative effects of octopamine (OCT) or substituted phenyliminoimidazolidines (NC5 or NC7) on light emission in the isolated firefly tail (A) and on the ability of living tobacco hornworm larvae to feed (B). Values shown in A are expressed as a percent of maximal octopamine-stimulated illumination (mean \pm SEM, $n = 5$). Values shown in B are means (\pm mean deviation) of duplicate area measurements expressed as percent of total (starting) leaf area. Drugs in B were tested in the presence of *i*-BuMeXan (0.1 mg).

hyperactivity, and leaf walk-off behavior. At higher doses, larvae demonstrated particular difficulty during molting (a task demanding motor coordination), frequently being unable to shed their old cuticle and subsequently dying. These effects, which persisted as long as larvae continued to feed on the treated leaves, resulted in a marked decrease in the rate of leaf consumption. Fig. 2B quantitates this effect and shows that NC7, for example, was >15-fold more potent than octopamine. This value compares well with data obtained from biochemical experiments utilizing broken cell preparations of *Manduca* nerve cord, in which it was found that NC7 was a potent activator of nerve-cord adenylate cyclase activity, with a $K_{act} \approx 0.06$ that for octopamine. Together, the *in vivo* results in Fig. 2A and B support the above biochemical studies and indicate that selected phenyliminoimidazolidines are very potent agonists of octopamine-sensitive adenylate cyclase.

In addition to being potent, the phenyliminoimidazolidines were also highly selective for octopamine-activated adenylate cyclase as compared with adenylate cyclases activated by dopamine or adrenergic agents (24, 25). Fig. 1B and C show, respectively, the potent activation by isoproterenol of β_1 - and β_2 -adrenergic receptor-stimulated adenylate cyclase in rat heart and liver, tissues not known to contain any octopamine-activated adenylate cyclase (6). In the heart, octopamine caused a small stimulation of the β -adrenergic receptor whereas NC5 was almost completely inactive. In the liver, neither octopamine nor NC5 were active. Similarly, in the rat caudate nucleus, although dopamine stimulated the dopamine-sensitive adenylate cyclase known to be present, neither octopamine nor NC5 caused any significant enzyme stimulation. In none of the three rat tissues did NC5 cause any inhibition of basal adenylate cyclase activity.

Because the effects of NC5 and NC7 on octopamine-sensitive adenylate cyclase were blocked more effectively by α - than β -adrenergic antagonists (see above), the question arises whether the effectiveness of these phenyliminoimidazolidine derivatives as agonists might be due to their affecting a class of octopamine receptors that bear a close similarity to vertebrate α -adrenergic receptors. This is not likely to be the case for the following reasons: First, modulation of adenylate cyclase by vertebrate α -adrenergic agonists results in an inhibition of enzyme activity rather than the

marked activation observed with octopamine or the active phenyliminoimidazolidines (26).

Second, although certain phenyliminoimidazolidines are known to affect mammalian α_2 receptors (27, 28), the binding of various amines to vertebrate α_2 -adrenergic receptors does not correlate at all with the ability of these same amines to activate octopamine-sensitive adenylate cyclase. This is shown in Table 2, which lists the potency (relative to clonidine = 1) of a number of compounds in binding to the mammalian brain α_2 receptor (as measured by competition with [3 H]clonidine binding) and to the mammalian brain α_1 receptor [as measured by competition with WB-4101 [(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane] binding] and in activating firefly octopamine-sensitive adenylate cyclase. For example, norepinephrine has almost 100-fold greater affinity than octopamine for the α_2 -adrenergic receptor (and >50-fold greater affinity than octopamine for the α_1 -adrenergic receptor), whereas norepinephrine is only 0.12 as potent as octopamine in activating light organ adenylate cyclase (19). Also, whereas NC5 is 20-fold more potent than clonidine as an octopamine agonist, NC5 has been reported to be ≈ 0.1 as potent as clonidine in binding to rat brain. Other examples can also be seen.

There is also a poor correlation between the abilities of various antagonists to block octopamine-stimulated adenylate cyclase and to bind to vertebrate α -adrenergic receptors (data not shown). For example, yohimbine binds to α_2 receptors with about 4-fold the affinity of chlorpromazine (27), whereas the latter is >100-fold more potent than yohimbine in blocking octopamine-stimulated adenylate cyclase (19).

As mentioned above, recent studies have shown that the formamidines, which have been used as insecticides and acaricides for a number of years, are partial octopaminergic agonists. The present experiments, describing the effects of phenyliminoimidazolidines and octopamine, now provide evidence that three structurally distinct chemical groups [phenyliminoimidazolidines, phenylethanolamines (octopamine), and formamidines], with a shared ability to activate octopamine-sensitive adenylate cyclase, can exert adverse behavioral and physiological effects on certain species of insects. In other experiments, the toxicological effects of all three groups were markedly enhanced by phosphodiesterase

Table 2. Relative potency of compounds in binding to α_1 - and α_2 -adrenergic receptors and in activating octopamine-sensitive adenylate cyclase

Compound	α_2 binding	Octopamine-sensitive adenylate cyclase	α_1 binding
UK14304-18	4.5	0.12*	0.001 [†]
<i>p</i> -Aminoclonidine	4.5	0.70*	1.0
Dihydroergotamine	2.4	0.06 [‡]	120
Epinephrine	1.0	0.20	1.2
Clonidine	1.0	1.0*	1.0
NC9	0.48	5.4	—
Norepinephrine	0.33	0.13	2.2
NC7	0.28	10	—
NC5	0.15	20	—
Phenylethanolamine	0.005	0.11	0.018
Octopamine	0.0037	1.1	0.040
Tyramine	0.0009	0.15*	0.019

Values shown are ratios of potency relative to clonidine, calculated by dividing the K_d for clonidine binding or K_{act} for adenylate cyclase activation by the K_d or K_{act} of the compound indicated; thus, values >1 indicate a greater potency (and a lower absolute K_d or K_{act}). Data on octopamine-sensitive adenylate cyclase were derived from activation of firefly light organ adenylate cyclase (see *Materials and Methods*). K_d s of compounds for α_2 and α_1 binding (as measured by competition with [³H]clonidine and WB-4101 binding, respectively) were averaged values from studies in mammalian tissues, usually brain (27–31).

*Partial agonist.

[†]Measured by competition with prazosin binding.

[‡]Antagonist activity only, compared with antagonist activity of clonidine.

inhibitors (32) and mimicked by certain cyclic AMP analogs, evidence further supporting a mechanism of action related to stimulation of octopamine-sensitive adenylate cyclase. (Indeed, in the absence of a phosphodiesterase inhibitor, octopamine shows little toxic effect.) Additionally, the rank-order ability of octopamine, NC7, and the formamidine DDCDM to cause toxic effects in *Manduca* is the same as the rank-order ability of these three compounds to cause increases of cyclic AMP content in isolated intact *Manduca* nerve cord *in vitro*.

Interestingly, I have found that the ability of the phenyliminoimidazolidines and the formamidines to activate adenylate cyclase varies somewhat among different species. In the firefly, as noted above, NC5 and NC7 are, respectively, 20- and 10-fold more potent than octopamine, and DDCDM is a partial agonist with ≈ 4 times the potency of octopamine. In the cockroach (*Periplaneta americana*), NC5 and NC7 are about equipotent, with 10–12 times the potency of octopamine, but DDCDM has almost no activity. In *Manduca*, DDCDM (20-fold more potent than octopamine) is more potent than either NC7 (16-fold) or NC5 (8-fold). Reported experience from the use of formamidines as pesticides (23) has indicated that *Manduca* is quite sensitive to formamidine effects whereas cockroaches are almost completely resistant; this observation is consistent with the relative potency of the formamidines as agonists of adenylate cyclase in these two species. Also consistent with these biochemical findings is the observation that, in *Manduca*, NC7 is more potent than NC5 in disrupting motor activity. Such species differences could indicate genetic variability in the octopamine-2 receptor or the existence of more than a single receptor subtype associated with adenylate cyclase. These species differences may also help explain why it has previously been reported that octopamine and the formamidines can actually stimulate feeding in the blowfly (20). [However, the doses of compounds injected resulted in

such high levels (>1 mM) that it is difficult to compare such results with those in the present study, where both *in vitro* and *in vivo* experiments used concentrations in the low micromolar range.]

Tissue levels of octopamine are 10- to 50-fold higher in invertebrates than in vertebrates (1, 33), and attempts to identify octopamine-sensitive adenylate cyclase in vertebrates have failed (6). This and other evidence suggests that octopamine may function as a neurohormone and neurotransmitter primarily in invertebrates. For this reason, certain of the substituted phenyliminoimidazolidines described above, as well as other novel and selective agonists of octopamine-sensitive adenylate cyclase (alone or together with substances that augment cyclic AMP increases), could have potential as pestistatic or pesticidal agents with low toxicity for vertebrates.

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