MOLECULAR PHARMACOLOGY, 20:68–75

N-Demethylchlordimeform

A Potent Partial Agonist of Octopamine-Sensitive Adenylate Cyclase

JAMES A. NATHANSON AND EDWARD J. HUNNICUTT

Departments of Neurology and Pharmacology, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts 02114

Received December 9, 1980; Accepted February 5, 1981

SUMMARY

NATHANSON, J. A., AND E. J. HUNNICUTT. *N*-Demethylchlordimeform: a potent partial agonist of octopamine-sensitive adenylate cyclase. *Mol. Pharmacol.* **20**:68–75 (1981).

Physiological studies suggest that the formamidine pesticides, chlordimeform (CDM) and *N*-demethylchlordimeform (DCDM), may affect octopaminergic neurotransmission. To elucidate the possible mechanism of this interaction, the present study examined in detail the biochemical effects of these compounds on a very active octopamine-sensitive adenylate cyclase present in washed particulate preparations of the firefly light organ. DCDM was a partial agonist (70% of octopamine V_{max}) and was 6-fold more potent ($K_a = 2.2 \times 10^{-6}$ M) than octopamine ($K_a = 1.4 \times 10^{-5}$ M) in activating enzyme activity. At high concentrations ($IC_{50} = 3 \times 10^{-4}$ M), DCDM caused a 30% inhibition of octopamine stimulation. Activation by DCDM was reversible, nonadditive to that caused by octopamine, and could be competitively inhibited by several receptor antagonists, including cyproheptadine ($K_i = 2 \times 10^{-6}$ M), clozapine ($K_i = 4 \times 10^{-6}$ M), fluphenazine ($K_i = 6 \times 10^{-6}$ M), phentolamine ($K_i = 1.8 \times 10^{-5}$ M), and propranolol ($K_i = 4.7 \times 10^{-5}$ M). These inhibitory constants correlated well with those for inhibiting octopamine stimulation. The agonist activity of DCDM was specific for tissue containing an octopamine-activated adenylate cyclase; enzyme activity in the rat caudate nucleus (activated by dopamine) and in the heart and liver (activated by isoproterenol) was little affected by DCDM. In contrast to DCDM, CDM was a weak octopamine agonist ($K_a = 3 \times 10^{-5}$ M; 9% of octopamine V_{max}) in the light organ. At higher concentrations ($IC_{50} = 3$ to 10×10^{-4} M), CDM was an octopamine antagonist, causing nearly complete inhibition of octopamine stimulation at 1 to 3×10^{-3} M. This inhibitory effect of CDM was reversible, pH-dependent, and noncompetitive with octopamine. It was also nonselective, since CDM inhibited dopamine-sensitive, *beta*-adrenergic-sensitive, and non-hormone-dependent adenylate cyclases in mammalian brain and liver. The above results indicate that, at low concentrations, DCDM can bind specifically and reversibly to octopamine receptors with a resultant activation of adenylate cyclase. CDM, on the other hand, has little direct effect on the octopamine receptor; its octopamimetic actions *in vivo* are a probable result of its conversion to DCDM. At high, nonpharmacological doses, CDM exerts noncompetitive and non-receptor-specific inhibitory effects on adenylate cyclase. These data have relevance to understanding the structural requirements necessary for interaction with octopamine-sensitive adenylate cyclase.

INTRODUCTION

Considerable biochemical (1, reviewed in ref. 2) and physiological (3–8) evidence indicates that octopamine functions as a neurotransmitter or neuromodulator in a number of invertebrate species. Further evidence supports the presence of specific, membrane-bound octopamine receptors, some or all of which are associated with adenylate cyclase (9–12). In an effort to define the structural requirements necessary for activation of this enzyme, recent studies (13, 14) have examined the effects of a number of phenylethylamine derivatives on octopamine-sensitive adenylate cyclase in the firefly light organ, a tissue highly enriched in octopamine, but not dopamine, adrenergic, or serotonin receptors. With the possible exception of synephrine (*N*-methyloctopamine), which is about 20% more potent than octopamine, no

0026-895X/81/010068-08$02.00/0

other compound has been found with a potency greater than that of octopamine itself.

Recently, the formamidine pesticide CDM[1] and its *N*-monodemethyl derivative DCDM (Fig. 1) have been reported to mimic the effects of octopamine in stimulating light emission in the firefly lantern (15) and in affecting nerve-evoked muscle responses in the locust leg (16). Although these physiological experiments suggest that the formamidines may act directly on octopamine receptors, a prior biochemical study (17) has reported that CDM has no effect on activating the octopamine-sensitive adenylate cyclase known to be present in cockroach thoracic ganglia (9). This latter finding, coupled with the known action of the formamidines as monoamine oxidase inhibitors (18, 19), makes it difficult to determine with certainty, from intact tissue preparations, whether or not the formamidines are direct octopamine receptor agonists.

In order to clarify the above situation and determine whether the formamidines might be useful chemical tools for understanding the nature of the octopamine receptor, we have investigated, in detail, the effects of CDM and DCDM on the octopamine-sensitive adenylate cyclase present in particulate preparations of the firefly light organ. We find that DCDM (the probable *in vivo* metabolite of CDM) is about 6-fold more potent than octopamine itself as a partial agonist of light organ adenylate cyclase. Since this action of DCDM is reversible and can be competitively inhibited by antagonists known to inhibit the effects of octopamine, it appears that DCDM is the most potent octopaminergic compound yet described. In marked contrast, CDM is only a weak activator of light organ adenylate cyclase and, in the presence of octopamine, is a noncompetitive antagonist. These results have implications for understanding the structural characteristics necessary for binding to and activating octopamine-sensitive adenylate cyclase. Furthermore, because CDM and DCDM have little or no effect on activating adrenergic or dopaminergic-sensitive adenylate cyclases found in other tissues, these compounds have potential usefulness in biochemical and physiological studies of octopamine action.

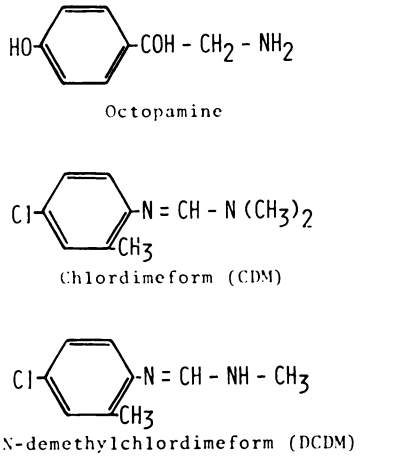

FIG. 1. *Structural formulae of octopamine and the formamidines, CDM and DCDM*

MATERIALS AND METHODS

Specimens of *Photinus pyralis* were collected in summer, frozen on Dry Ice, and stored at −90°. Under these conditions, octopamine-sensitive enzyme activity remains stable for 6 months or longer (14).[2] For each experiment, a number of insects were thawed and maintained at 4°. Tail sections were opened through a dorsal midline incision and the abdominal cavity was cleaned of all gut, fat, reproductive organs, and ganglia. The light organs were then 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). This homogenate was diluted to a volume of 30 ml in 6 mM Tris-maleate and centrifuged at 120,000 × *g* for 20 min. The supernatant was discarded, and the pellet was resuspended by homogenization in 30 ml of buffer and again centrifuged at 120,000 × *g* for 20 min. The resulting pellet (P_2 fraction) was resuspended in a volume of 6 mM Tris-maleate equivalent to the starting amount and maintained at 0° until it was used.

Adenylate cyclase activity 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 ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; 2 mM ATP; 0.06 ml of P_2 fraction; and various compounds to be tested. Prior experiments had determined that, under these conditions, octopamine-sensitive adenylate cyclase activity was optimized (14). CDM and DCDM were initially solubilized (prior to aqueous dilution) in 50% (v/v) methanol. Appropriate solvent controls were run in parallel. The enzyme reaction (5 min at 30°) was initiated by addition of ATP, 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 (20). Under the above assay conditions, enzyme activity was linear with respect to time and enzyme concentration, and phosphodiesterase activity was nearly completely inhibited. Previous experiments (13) have shown that the cyclic AMP produced in this reaction cochromatographs on Dowex AG-50X resin with authentic cyclic AMP. By using the above procedures, adenylate cyclase activity was also measured in homogenates and particulate preparations of the caudate nucleus, heart ventricle, and liver of adult male Sprague-Dawley rats. Starting tissue concentrations were 10 mg/ml for the caudate and 50 mg/ml for the heart and liver. Protein concentration was determined by the method of Lowry *et al.* (21).

K_i values for various receptor antagonists were calculated from two types of experiments. In the first, the concentration of agonist was held constant and the concentration of antagonist was varied. Assuming competitive inhibition, the K_i was calculated from the equation (22) $K_i = IC_{50}/(1 + S/K_a)$, where IC_{50} is the concentration of antagonist necessary to give 50% inhibition of agonist-stimulated activity, S is the concentration of agonist, and

[1] The abbreviations used are: CDM, chlordimeform; DCDM, *N*-demethylchlordimeform; DDCDM, *N,N*-didemethylchlordimeform.

[2] J. A. Nathanson, manuscript in preparation.

K_a is the concentration of agonist necessary for half-maximal activation of enzyme activity. In the second method, the concentration of antagonist was held constant and the concentration of agonist was varied. The K_i was calculated (22) from the equation $K_i = I/(K_a'/K_a - 1)$, where I is the concentration of antagonist, and K_a' and K_a are the concentrations of agonist necessary to cause half-maximal stimulation of enzyme activity in the presence and absence of antagonist, respectively.

All drugs and common reagents were obtained from Sigma Chemical Company (St. Louis, Mo.). CDM and DCDM (both as hydrochloride salts) were kindly supplied by Dr. H. M. LeBaron. Fluphenazine was supplied by E. R. Squibb and Sons, Inc. (Princeton, N. J.), clozapine by Sandoz Pharmaceuticals, Inc. (East Hanover, N. J.), phentolamine by CIBA-Geigy (Summit, N. J.), and cyproheptadine by Merck and Company (West Point, Pa.).

RESULTS

Activation of octopamine-sensitive adenylate cyclase. Figure 2 compares the effects of octopamine, CDM, and DCDM on adenylate cyclase activity present in the P_2 fraction of the firefly light organ. In confirmation of previous studies (13, 14), octopamine caused a marked stimulation of enzyme activity. In the experiment shown, the activation constant (K_a) for octopamine stimulation was 1.4×10^{-5} M and, at optimal concentrations, octopamine stimulated basal activity (13.3 ± 1 pmole/mg of protein per minute) more than 45-fold.

Although CDM also caused a significant stimulation of enzyme activity (approximately a 4-fold increase over basal levels), it was only 9% as active as octopamine. The K_a for enzyme stimulation by CDM was about 3×10^{-5} M. DCDM, on the other hand, was a potent activator of enzyme activity. This compound caused significant stimulation at 10^{-8} M, with a K_a of 2.2×10^{-6} M, and maximal stimulation occurred at 10^{-4} M. At optimal concentrations, DCDM caused over a 35-fold stimulation of basal adenylate cyclase activity. Maximal stimulation by DCDM was consistently about 25–30% less than that caused by octopamine. This partial agonistic effect is discussed in greater detail below.

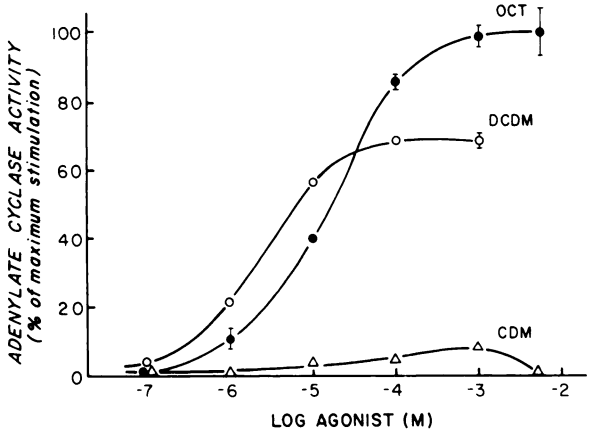

FIG. 2. *Activation of adenylate cyclase activity in firefly light organ particulate fractions by octopamine (OCT), DCDM, or CDM*

Activity is expressed as a percentage of the stimulation (674 ± 49 pmoles/mg of protein per minute) seen in the presence of a maximally effective concentration of octopamine. The values shown here and in subsequent figures represent the mean (± range) for replicate samples, each assayed for cyclic AMP content in duplicate. At those points which lack error bars, the range was within the size of the symbol. In the experiment shown, basal activity was 13.3 ± 1 pmoles/mg of protein per minute.

It seemed very unlikely that DCDM stimulation was a result of its known action as an monoamine oxidase inhibitor, since in previous studies (14) we found no stimulatory action of the monoamine oxidase inhibitor, pargyline, on either basal or octopamine-sensitive adenylate cyclase activity in light organ particulate fractions.

In additivity studies, the stimulation (1082 ± 10 pmoles/mg of protein per minute) above basal activity due to a combination of 10^{-3} M DCDM and 10^{-3} M octopamine was not significantly greater than the stimulation caused by 10^{-3} M octopamine (1398 ± 27) or 10^{-3} M DCDM (1053 ± 16) alone. This result supplies some evidence that DCDM and octopamine affect the same receptor. Additional evidence that DCDM and octopamine were competing for the same site came from experiments in which lantern adenylate cyclase activity was stimulated by low concentrations of octopamine in the presence or absence of a fixed, low concentration of DCDM. It was found that, in the presence of 10^{-6} M DCDM, the additional stimulation (70 ± 15 pmoles/mg of protein per minute) of enzyme activity by concentrations of octopamine from 0.1 to 3×10^{-6} M was less than that (230 ± 10) due to the same concentrations of octopamine in the absence of DCDM. When the octopamine concentration was increased further, from 3 to 10×10^{-6} M, the increment of octopamine stimulation in the presence of DCDM (240 ± 25) was at least as great as that in the absence of DCDM (185 ± 10). In other words, when the concentration of octopamine was approximately the same as that of DCDM, it was the DCDM (because of its probable greater affinity) that primarily determined receptor response; at higher concentrations, octopamine was able to compete effectively with 1×10^{-6} M DCDM for stimulation of the enzyme.

Reversibility of DCDM stimulation. Experiments were performed in which a light organ homogenate was first preincubated for 5 min at 30° with 10^{-4} M DCDM and then washed twice, as described under Materials and Methods, to remove soluble DCDM and produce a P_2 fraction. This fraction was then assayed for adenylate cyclase activity in the absence or presence of additional DCDM or octopamine. A similar procedure was followed with fractions preincubated with octopamine or with blank.

It was found that washing totally removed the stimulatory activity due to DCDM; in other words, basal activity after preincubation with DCDM and then washing (23 ± 1 pmole/mg of protein per minute) was similar to basal activity in a washed preparation not preincubated with DCDM (21 ± 1). Furthermore, DCDM was removed as effectively as octopamine itself. Following washing, the enzyme could be reactivated by either 10^{-3} M DCDM (727 ± 10) or 10^{-3} M octopamine (598 ± 19) to levels similar to those of enzyme which had not been preincubated with DCDM.

Inhibition of DCDM stimulation by receptor antagonists. It was of some interest to determine whether activation of adenylate cyclase by DCDM could be blocked by antagonists known to block the activation of enzyme activity by octopamine. Figure 3 compares the dose-dependent activation of light organ P_2 adenylate cyclase activity by DCDM in the absence and presence of 10^{-5} M cyproheptadine. [As determined by previous studies (10, 12, 14), cyproheptadine is a potent octopamine antagonist, with a K_i for octopamine of 5×10^{-6} M.] Cyproheptadine caused a shift in the DCDM dose-response curve to the right, resulting in an increase in the K_a for DCDM without causing any significant change in the maximal activation of the enzyme. A double reciprocal plot of these data (not shown) was consistent with competitive inhibition, and the calculated inhibitory constant of cyprohepatine for DCDM activation was 2.6×10^{-6} M.

In other inhibition experiments, the concentration of agonist (DCDM) was held constant, and the concentration of various antagonists was varied. As determined by previous experiments (14),[2] the particular antagonists chosen displayed a wide range of potency against octopamine-sensitive adenylate cyclase. Included were the serotonin and histamine antagonist, cyproheptadine; the dopamine antagonists, clozapine and fluphenazine; the *alpha*$_1$-adrenergic antagonist, phentolamine; the *beta*-adrenergic antagonist, propranolol; and the *alpha*$_2$-adrenergic antagonist, yohimbine. All compounds, save yohimbine, caused a dose-dependent decrease in adenylate cyclase stimulation by 10^{-5} M DCDM. Table 1 lists the IC_{50} values for the various antagonists to DCDM. Also shown are the IC_{50} values for these same compounds in blocking stimulation by 10^{-5} M octopamine (data from ref. 14). Comparison of these two sets of values reveals a substantial correspondence. The calculated correlation coefficient between the two sets of IC_{50} values (columns A and B) yields an r value of 0.997 ($p < 0.01$). Shown also in Table 1 (columns C and D) are the K_i values calculated from the data in columns A and B. Comparison of these two sets of values also reveals a significant correlation ($r = 0.997$; $p < 0.01$). These data, together with those above, support the possibility that the stimulatory action of DCDM on P_2 light organ adenylate cyclase may occur through a reversible activation of the octopamine-binding portion of the enzyme-receptor complex.

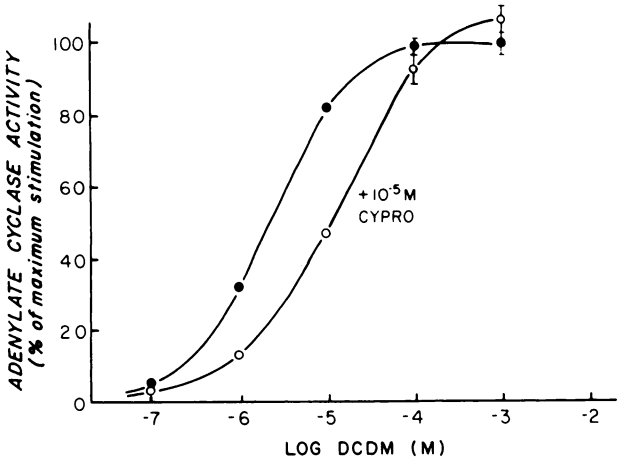

FIG. 3. *Activation of light organ adenylate cyclase activity by DCDM in the absence or presence of 10^{-5} M cyproheptadine (CYPRO)*

Activity is expressed as a percentage of the stimulation (472 ± 15 pmoles/mg of protein per minute) seen in the presence of a saturating concentration (10^{-4} M) of DCDM alone. Basal activity was 13.8 ± 0.5 pmoles/mg of protein per minute.

TABLE 1

IC_{50} values and calculated K_i values for various antagonists in inhibiting the stimulation of light organ adenylate cyclase activity by either DCDM (10^{-5} M) or octopamine (OCT) (10^{-5} M)

Compound	IC_{50}		K_i	
	DCDM (A)	OCT (B)	DCDM (C)	OCT (D)
	μM		μM	
Cyproheptadine	12	10	2	5
Clozapine	23	16	4	9
Fluphenazine	33	30	6	16
Phentolamine	100	85	18	46
Propranol	260	330	47	180
Yohimbine	>1000	>1000	>180	>550

Inhibitory action of DCDM. Although the predominant action of DCDM was stimulation, the compound also exhibited inhibitory effects on octopamine-sensitive adenylate cyclase activity; thus, DCDM was a partial agonist. Additivity studies showed that a maximally effective concentration of octopamine (10^{-3} M) was inhibited about 25–30% in the presence of 10^{-3} M DCDM. This result is consistent with the observation that DCDM alone, at optimal concentrations, stimulated enzyme activity only about 70–75% as much as octopamine (Fig. 2). (The apparent inhibitory effect of DCDM appeared to be due to an actual inhibition of adenylate cyclase rather than an activation of phosphodiesterase, because, under the standard incubation conditions, phosphodiesterase activity was nearly completely inhibited.)

The inhibitory action of DCDM is depicted in more detail in Fig. 4, which shows the effects on adenylate cyclase activity of varying concentrations of DCDM, alone and in the presence of a fixed, maximally effective concentration of octopamine. Increasing concentrations of DCDM caused a gradual decrease in octopamine stimulation to 68% of the starting value. The IC_{50} of DCDM for this inhibition was 2.2×10^{-4} M.

Inhibitory action of CDM. Figure 4 also shows the inhibitory effects of CDM on light organ adenylate cyclase activity. As noted before, CDM alone caused only a small stimulation of enzyme activity. In the presence of a fixed concentration of octopamine (10^{-3} M), increasing concentrations of CDM caused a progressive decrease in octopamine stimulation until, at 10^{-3} M CDM, there was complete inhibition of activity above that which was due to CDM alone. Further experiments showed that the concentration of CDM required for inhibition of octopamine stimulation was somewhat variable. For example, in the experiment shown in Fig. 4, as well as in others, the IC_{50} for inhibition of 10^{-3} M octopamine by CDM was 3×10^{-4} M, similar to the IC_{50} (2.2×10^{-4} M) for the partial antagonism of octopamine stimulation caused by

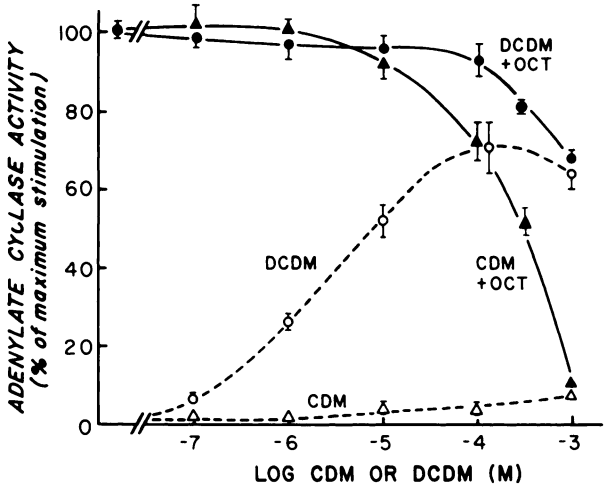

FIG. 4. *Stimulatory and inhibitory effects of DCDM and CDM on firefly light organ adenylate cyclase*

The effects of various concentrations of DCDM alone or CDM alone on enzyme activity (- - -) and the effects of various concentrations of DCDM or CDM on the stimulation of enzyme activity by a fixed (10^{-3} M) concentration of octopamine (——). Basal activity was 14.5 ± 1 pmoles/mg of protein per minute, and maximal stimulation in the presence of 10^{-3} M octopamine (OCT) alone was 1445 ± 33 pmoles/mg of protein per minute.

DCDM. In other cases, the IC_{50} of CDM for 10^{-3} M octopamine varied from 3×10^{-4} M to 3×10^{-3} M. Experiments investigating drug stability and solubility, order of addition (of agonist and antagonist) to enzyme, and time of preincubation (2–20 min) of CDM with the P_2 fraction prior to initiation of the enzyme reaction failed to reveal the cause for this variability. In another experiment, however, it was noted that inhibition of octopamine (10^{-3} M) stimulation by 10^{-3} M CDM varied according to the pH of the incubation buffer; inhibition was greater at pH 8.4 than at pH 7.4 and little inhibition occurred at pH 6.4. This effect of pH may have been due to the change in proportion of CDM (pK_a about 7.0) existing in solution as ion versus free base. [Similar effects of pH have previously been reported for the uncoupling action of CDM on liver mitochondria (23).]

Figure 5 describes an additional experiment in which the dose-dependent activation of lantern P_2 adenylate cyclase by octopamine was measured in the absence or presence of 10^{-4} M or 10^{-3} M CDM. As can be seen, both concentrations of CDM caused a decrease in the V_{max} of the octopamine dose-response curve with relatively little change in the apparent K_a for octopamine. This effect is distinctly different from the competitive inhibition of octopamine or DCDM stimulation shown by such antagonists as cyproheptadine (Fig. 3), and indicates that increasing concentrations of octopamine are unable to overcome the inhibition due to CDM.

These data suggest at least two possible explanations for the inhibitory action of CDM: (a) at higher concentrations, CDM bound irreversibly to the octopamine binding site; or (b) CDM affected enzyme activity at a separate site or possibly altered other factors necessary for enzyme activity (e.g., cofactor or substrate availability). To try to distinguish between these possibilities, experiments were performed to determine whether the inhibitory effects of CDM were reversible. A light organ homogenate was first preincubated for 5 min at 30° with either 10^{-3} or 3×10^{-3} M CDM, and then washed twice to remove CDM and produce a P_2 fraction. This fraction was then assayed for adenylate cyclase activity in the absence or presence of 10^{-4} M octopamine or in the presence of both octopamine and 10^{-3} M CDM, 3×10^{-3} M CDM, or 10^{-3} M cyproheptadine. A similar procedure was followed with fractions preincubated initially with 10^{-3} M cyproheptadine or with blank.

By using this protocol, it was found that washing removed nearly all of the inhibitory activity of CDM. In other words, octopamine-stimulated activity after preincubation with CDM and then washing was similar to octopamine-stimulated activity of enzyme not preincubated with CDM. Furthermore, CDM seemed to be removed as effectively by washing as the competitive antagonist, cyproheptadine. In addition, following preincubation with CDM and washing, octopamine-stimulated activity could be reinhibited either by CDM or by cyproheptadine. These data suggest that CDM inhibition of adenylate cyclase was not due to irreversible binding to the octopamine binding site of the enzyme-receptor complex. Further evidence for receptor-independent inhibition of adenylate cyclase by CDM was provided by experiments, described below, performed in other tissues.

Effect of DCDM and CDM on adrenergic- and dopaminergic-stimulated adenylate cyclase. To determine further the specificity of DCDM and CDM for activation and inhibition of octopamine-sensitive adenylate cyclase, the effects of these compounds were tested in tissues known to contain adenylate cyclases activated by other phenylethylamines but not by octopamine (11). These tissues included the rat caudate nucleus, known to contain a dopamine-sensitive adenylate cyclase (24); rat heart ventricle, known to contain a predominately *beta*$_1$-adrenergic-sensitive adenylate cyclase (25); and the rat liver, known to contain a *beta*$_2$-adrenergic-sensitive adenylate cyclase (25).

Figure 6 (B–D) shows the effects of DCDM on adenylate cyclase activity in each of these tissues compared

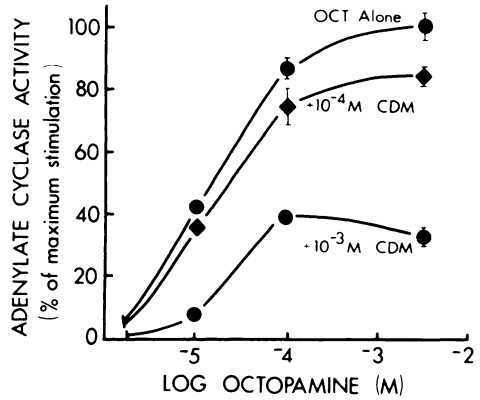

FIG. 5. *Stimulation of light organ adenylate cyclase activity by octopamine (OCT) alone or by octopamine in the presence of 10^{-4} M CDM or 10^{-3} M CDM*

Basal activity was 24 ± 2 pmoles/mg of protein per minute, and maximal stimulation in the presence of 5×10^{-3} M octopamine was 1770 ± 280 pmoles/mg of protein per minute.

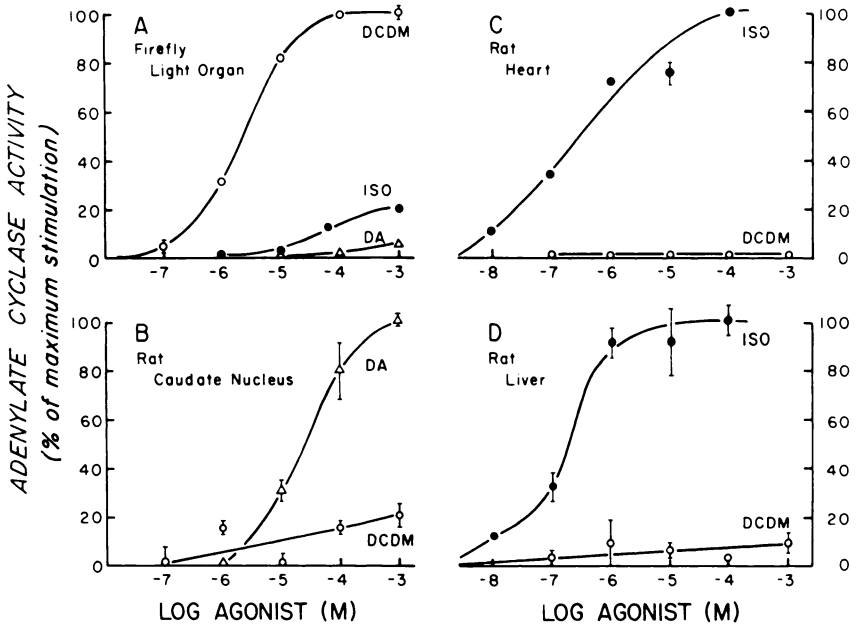

FIG. 6. *Comparison of the relative effects of DCDM, isoproterenol (ISO), or dopamine (DA) in activating adenylate cyclase activity in the firefly light organ (A), caudate nucleus (B), rat heart ventricle (C), or rat liver (D)*

In each graph, stimulation is expressed as a percentage of the maximal stimulation produced by the most active amine in that particular tissue. Basal and maximal activities (in picomoles per milligram of protein per minute) were as follows: 14.3 ± 0.5 and 472 ± 15 in A; 150 ± 9 and 245 ± 3 in B; 16.3 ± 0.5 and 35.1 ± 0.3 in C; 2.5 ± 0.1 and 6.0 ± 0.2 in D.

with the effects of dopamine or isoproterenol. Shown for comparison (Fig. 6A) are the effects of DCDM, dopamine, and isoproterenol on firefly light organ adenylate cyclase activity. In this latter tissue, both dopamine and isoproterenol were much less potent and less effective than DCDM; isoproterenol was 20% as active, with a K_a of $>6 \times 10^{-5}$ M; and dopamine showed only a small degree of stimulation (5% of DCDM) at 10^{-3} M. In contrast, in the rat caudate nucleus DCDM was much less effective than dopamine; at 10^{-3} M, the formamidine stimulated enzyme activity only about 20% as well as dopamine. In the rat heart, DCDM was totally without agonist activity, and in the rat liver DCDM was only 10% as active as isoproterenol. In additivity experiments in the heart and liver (data not shown), combinations of isoproterenol (10^{-4} M) with DCDM (10^{-3} M) resulted in no greater stimulation than isoproterenol alone. These studies in tissues containing nonoctopaminergic amine receptors, combined with the previous data, suggest that DCDM has a high degree of specificity for octopamine receptors.

Figure 7 shows the inhibitory effects of CDM on basal and hormone-stimulated adenylate cyclase activity in rat liver (Fig. 7A) or caudate nucleus (Fig. 7B), compared with the inhibitory effects of the *beta*-adrenergic antagonist, propranolol, and the dopamine antagonist, fluphenazine. These data can be compared with the effects of CDM on basal and octopamine-stimulated enzyme activity in the firefly light organ (Fig. 4).

In the rat caudate, CDM, at concentrations above 10^{-4} M, progressively inhibited both basal ($IC_{50} = 2 \times 10^{-4}$ M) and 10^{-4} M dopamine-stimulated ($IC_{50} = 4 \times 10^{-4}$ M) activity. Fluphenazine was a much more potent antagonist of dopamine stimulation ($IC_{50} = 2 \times 10^{-7}$ M), and, at these low concentrations, had little effect on basal activity. An analogous result was found in the rat liver, in which propranolol ($IC_{50} = 2 \times 10^{-7}$ M) displayed selective ability to inhibit 10^{-5} M isoproterenol-stimulated activity more than basal activity, whereas CDM, at higher concentrations, inhibited both basal activity ($IC_{50} = 2.5 \times 10^{-4}$ M) and isoproterenol-stimulated ($IC_{50} = 10^{-4}$ M) activity.

Thus, in contrast to the selective action of DCDM in stimulating adenylate cyclase with greater potency in the firefly light organ than in the rat caudate nucleus or liver, the inhibitory action of CDM on hormone-activated adenylate cyclase was nonselective. Furthermore, in the mammalian tissues, CDM inhibited both basal and hormone-stimulated activity to a similar degree. These results supply additional evidence that the stimulatory action of DCDM on light organ adenylate cyclase may be specific for the octopamine receptor, whereas inhibition by CDM may occur through an action, or at a site, separate from the octopamine binding portion of the receptor-adenylate cyclase complex.

DISCUSSION

At low concentrations ($<10^{-6}$ M), the formamidines cause marked behavorial abnormalities in susceptible arthropods, suggesting that part of their pesticidal activity is through a neurotoxic effect. Although a wide range of biochemical actions have been ascribed to these compounds, most reported effects [including inhibition of DNA, RNA, and protein synthesis (26); respiratory uncoupling (27); monamine oxidase inhibition (18, 19); local anesthesia (28); and blockade of cholinergic and glutamine synaptic transmission (29, 30)] occur at relatively high concentrations ($>10^{-5}$ M), and it is unclear whether these effects can explain the behavioral actions of the formamidines.

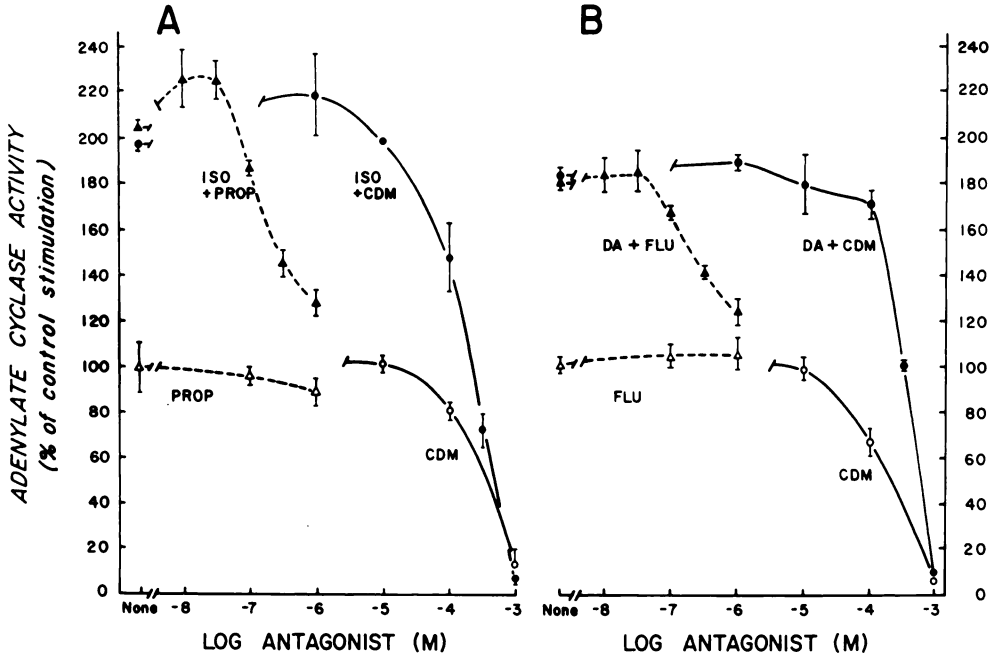

FIG. 7. *Comparison of the relative inhibitory effects of CDM, propranolol (PROP), or fluphenazine (FLU) on basal and 10^{-5} M isoproterenol (ISO)-stimulated adenylate cyclase activities in rat liver (A) and on basal and 10^{-4} m dopamine (DA)-stimulated adenylate cyclase activities in rat caudate nucleus (B)*

In each graph, stimulation or inhibition is expressed as a percentage of basal activity, which was 4.4 ± 0.2 pmoles/mg of protein per minute in A and 138 ± 7 pmoles/mg of protein per minute in B.

Evans and Gee (16) have reported that, in the superfused locust leg muscle, concentrations of DCDM as low as 10^{-7} M mimic the action of octopamine in potentiating twitch amplitude and muscle relaxation rate. Interestingly, the EC_{50} concentrations of DCDM for both actions was about 3×10^{-6} M, similar to the K_a of DCDM for activating light organ adenylate cyclase (Fig. 2). CDM, although nearly as effective as DCDM, was about 10-fold less potent.

Both CDM and DCDM have also been shown to mimic the action of octopamine in inducing glowing in the firefly lantern (15) but, whereas DCDM has a rapid onset of action (minutes), CDM has a delayed effect (4-hr lag). Because CDM is thought to be demethylated *in vivo* to form DCDM (31), these latter results suggest that the octopaminergic action of CDM seen in physiological studies likely results from its conversion to DCDM. This would be consistent with the results of the present study which show only a very weak effect of CDM, but a potent effect of DCDM, in stimulating the octopamine-sensitive adenylate cyclase of the light organ. (In fact, the possibility cannot be ruled out, in our experiments, that the small amount of agonist activity shown by CDM was due to some conversion of DCDM during the incubation *in vitro*.)

The present results demonstrate an inhibitory action of high concentrations of CDM on octopamine-stimulated adenylate cyclase. Although reversible, this inhibitory action of CDM is noncompetitive (Fig. 5). Furthermore (Fig. 7), because CDM also inhibits isoproterenol- and dopamine-stimulated adenylate cyclases in the rat liver and caudate nucleus [tissues which do not contain an octopamine-stimulated adenylate cyclase (11)], it seems unlikely that the inhibitory effect of CDM results from a selective interaction with octopamine receptors. This is further supported by the observation that CDM inhibits non-hormone-dependent adenylate cyclase activity (Fig. 7).

In contrast to CDM, the stimulatory action of DCDM on light organ adenylate cyclase shows considerable selectivity for the octopamine receptor site. DCDM activation is reversible and is competitively inhibited by antagonists known to inhibit octopamine activation of the enzyme. The significant correlation between the IC_{50} values of various antagonists for octopamine stimulation versus DCDM stimulation suggests that DCDM and octopamine are competing either for the same site or for two sites with very similar characteristics. Table 1 shows that the absolute values of the calculated K_i values of various antagonists for inhibitiong DCDM stimulation were less than the corresponding values for octopamine stimulation. Why the observed IC_{50} values were not larger for DCDM, however (given the greater affinity of DCDM for the receptor and the fact that the experiments were run with equal agonist concentrations of octopamine and DCDM), is not clear. Thus, we cannot rule out the possibility that the greater agonist potency of DCDM in stimulating enzyme activity might involve factor(s) in addition to greater receptor affinity alone.

In other experiments, both in the insect ganglia (11) and in the firefly light organ (14), it has been found that the presence of a hydroxyl group on the *beta*-position of the ethylamine side chain causes a considerable increase in agonist activity of phenylethylamines for octopamine-sensitive adenylate cyclase. It is of interest, then, that with DCDM the presence of an imino nitrogen (in place

of the hydroxylated carbon) also resulted in considerable agonist activity. As noted above, at the experimental pH employed, DCDM likely existed both as ion and free base.

When CDM is compared with DCDM, there is a marked decrease in agonist potency and efficacy associated with the presence of the second NH_2-terminal methyl group. This effect is similar to but somewhat more pronounced than that seen with addition of a second NH_2-terminal methyl group to *N*-methyloctopamine ($K_a = 1 \times 10^{-5}$ M; V_{max} 100%) to form *N,N*-dimethyloctopamine ($K_a = 2.1 \times 10^{-4}$ M; V_{max} 42%). The fact that the addition of still larger substituents to the NH_2 terminus further decreases the activity of phenylethylamines (14) suggests that similar larger NH_2-terminal substitutions on DCDM might also decrease activity. In the other direction, if substitutions of the NH_2-terminal of the formamidines do, in fact, follow the pattern that has been observed for phenylethylamines, then *N,N*-didemethylchlordimeform (DDCDM), the chlordimeform analogue of octopamine, should be almost as potent as DCDM as an octopamine agonist. Supporting this possibility are (a) the observation that DDCDM can mimic the action of DCDM in causing glowing in the firefly tail (15) and (b) preliminary data from our laboratory showing that DDCDM stimulated light organ adenylate cyclase with a K_a of 4×10^{-6} M.

In summary, these data support a direct interaction of certain formamidine pesticides with octopamine-sensitive adenylate cyclase. The specificity and potency of these compounds should make them useful tools for understanding the nature of the octopamine receptor as well as investigating the physiological actions of octopamine *in vivo*.

ACKNOWLEDGMENT

We wish to thank Professor J. E. Lloyd for his help in speciating the fireflies used in this study.

REFERENCES

1. Molinoff, P. B., and J. Axelrod. Distribution and turnover of octopamine in tissues. *J. Neurochem.* **19**:157–163 (1972).
2. Axelrod, J., and J. M. Saavedra. Octopamine. *Nature* (*Lond.*) **265**:501–504.
3. Barker, D. L., P. B. Molinoff, and E. A. Kravitz. Octopamine in the lobster nervous system. *Nature New Biol.* **236**:61–62 (1972).
4. Walker, R. J., A. G. Ramage, and G. N. Woodruff. The presence of octopamine in the brain of *Helix aspersa* and its action on specific snail neurons. *Experientia* (*Basel*) **28**:1173–1174 (1972).
5. Carpenter, D. O., and G. L. Gaubatz. Octopamine receptors on *Aplysia* neurones mediate hyperpolarization by increasing membrane conductance. *Nature* (*Lond.*) **252**:483–485 (1974).
6. Hoyle, G. Evidence that insect dorsal unpaired median (DUM) neurons are octopaminergic. *J. Exp. Zool.* **193**:425–431 (1975).
7. Evans, P. D., and M. O'Shea. An octopaminergic neurone modulates neuromuscular transmission in the locust. *Nature* (*Lond.*) **270**:257–259 (1977).
8. Robertson, H. A., and A. V. Juorio. Octopamine and some related noncatecholic amines in invertebrate nervous systems. *Int. Rev. Neurobiol.* **19**:173–224 (1976).
9. Nathanson, J. A., and P. Greengard. Octopamine-sensitive adenylate cyclase. Evidence for a biological role of octopamine in nervous tissue. *Science* (*Wash. D. C.*) **180**:308–310 (1973).
10. Nathanson, J. A., and P. Greengard. Serotonin-sensitive adenylate cyclase in neural tissue and its similarity to the serotonin receptor: a possible site of action of lysergic acid diethylamide. *Proc. Natl. Acad. Sci. U. S. A.* **71**:797–801 (1974).
11. Nathanson, J. A. Octopamine-sensitive adenylate cyclase and its similarity to the octopamine receptor, in *Trace Amines and the Brain* (E. Usdin and M. Sandler, eds. Marcel Dekker, New York, 161–190 (1976).
12. Harmar, A. J., and A. S. Horn. Octopamine-sensitive adenylate cyclase in cockroach brain: effects of agonists, antagonists and guanylyl nucleotides. *Mol. Pharmacol.* **13**:512–520 (1977).
13. Nathanson, J. A. Octopamine receptors, adenosine 3′,5′-monophosphate, and neural control of firefly flashing. *Science* (*Wash. D. C.*) **203**:65–68 (1979).
14. Nathanson, J. A., and E. J. Hunnicutt. Octopamine-sensitive adenylate cyclase: properties and pharmacological characterization. *Neurosci. Abstr.* **5**: 346 (1979).
15. Hollingworth, R. M., and L. L. Murdock. Formamidine pesticides: octopamine-like actions in a firefly. *Science* (*Wash. D. C.*) **208**:74–76 (1980).
16. Evans, P. D., and J. D. Gee. Action of formamidine pesticides on octopamine receptors. *Nature* (*Lond.*) **287**:60–62 (1980).
17. Matsumura, F., and R. W. Beeman. Biochemical and physiological effects of chlordimeform. *Environ. Health Perspect.* **14**:71–82 (1976).
18. Aziz, S. A., and C. O. Knowles. Inhibition of monamine oxidase by the pesticide chlordimeform and related compounds. *Nature* (*Lond.*) **242**:417–418 (1973).
19. Beeman, R. W., and F. Matsumura. Chlordimeform: a pesticide acting upon amine regulatory mechanism. *Nature* (*Lond.*) **242**:273–274 (1973).
20. Brown, B. L., R. P. Elkins, and J. D. M. Albano. Saturation assay for cyclic AMP using endogenous binding protein. *Adv. Cyclic Nucleotide Res.* **2**:25–40 (1972).
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
22. Chen, Y.-C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of an inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
23. Hollingworth, R. M. Chemistry, biological activity, and uses of formamidine pesticides. *Environ. Health Perspect.* **14**:57–69 (1976).
24. Kebabian, J. W., G. L. Petzold, and P. Greengard. Dopamine-sensitive adenylate cyclase in caudate nucleus of rat brain and its similarity to the dopamine receptor. *Proc. Natl. Acad. Sci. U. S. A.* **69**:2145–2150 (1972).
25. Minneman, K. P., A. Hedberg, and P. B. Molinoff. Comparison of beta-adrenergic receptor subtypes in mammalian tissues. *J. Pharmacol. Exp. Ther.* **211**:502–508 (1979).
26. Murakami, M., and J. Fukami. Effects of chlorphenamidine and its metabolites on HeLa cells. *Bull. Environ. Contam. Toxicol.* **11**:184 (1974).
27. Abo-Khatwa, N., and R. M. Hollingworth. Chlordimeform: the relation of mitochondrial uncoupling to toxicity in the German cockroach. *Life Sci.* **11**: 1181–1190 (1972).
28. Chinn, C., W. R. Pfister, and G. K. W. Yim. Local anesthetic-like actions of the pesticide chlordimeform. *Fed. Proc.* **35**:729 (1976).
29. Wang, C. M., T. Narahashi, and J. Fukami. Mechanism of neuromuscular block by chlordimeform. *Pestic. Biochem. Physiol.* **5**:119–125 (1975).
30. Yamamoto, D., and J. Fukami. Effect of chlordimeform on nerve-muscle system of the larva of the waxmoth. *J. Insect Physiol.* **22**:1511–1516 (1976).
31. Benezet, H. J., K.-M. Chang, and C. O. Knowles. Formamidine pesticides-metabolic aspects of neurotoxicity, in *Pesticide and Venum Neurotoxicity* (D. Shankland, R. Hollingworth, and T. Smyth., Jr., eds.) Plenum Press, New York, 189–206 (1978).

Send reprint requests to: Dr. James A. Nathanson, Departments of Neurology and Pharmacology, Massachusetts General Hospital, Boston, Mass. 02114.