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Stimulation of Adenosine 3′,5′-Monophosphate Formation by *Alpha* and *Beta* Adrenergic Agonists in Rat Cerebral Cortical Slices: Effects of Clonidine

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SUMMARY

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Clonidine has no significant effect on basal levels of cyclic 3′,5′-AMP in incubated slices of rat cerebral cortex, but this drug and oxymetazoline antagonize norepinerphrine-elicited accumulation of cyclic AMP to a degree which corresponds to the *alpha* adrenergic component of the norepinephrine response. Phenylephrine has virtually no agonist or antagonist activity in the brain slice system. Clonidine does not antagonize the accumulation of cyclic AMP elicited by maximal concentrations of a *beta* adrenergic agonist, isoproterenol, and markedly potentiates the stimulatory effects of submaximal concentrations of this agonist. Clonidine completely antagonizes the formation of cyclic AMP elicited by methoxamine, an *alpha* adrenergic agonist. The results indicate that clonidine and oxymetazoline function as potent antagonists with respect to norepinephrine- and methoxamine-stimulated formation of cyclic AMP in brain tissue, presumably at an *alpha* adrenergic locus. In addition, clonidine, oxymetazoline, and phenylephrine appear to interact synergistically with the *beta* adrenergic component of cyclic AMP-generating systems, thus functioning as partial agonists in brain slices under certain conditions.

INTRODUCTION

Clonidine (Catapresan, 2-[(2,6-dichlorophenyl)amino]-2-imidazoline HCl) is a potent antihypertensive agent whose action is believed to be mediated through stimulation of *alpha* adrenergic receptors in vasomotor centers of the central nervous system, resulting in a feedback-linked depression of vasomotor function (1–4). Although the exact locus of action of clonidine in the central nervous system is unclear, the drug has been shown to be a potent *alpha* adrenergic agonist in both central (2, 3) and peripheral (1, 5, 6) systems. At high dosages a modest *alpha* antagonist activity was manifest in certain peripheral systems (7). Activation of presynaptic *alpha* adrenergic receptors by clonidine and certain other *alpha* agonists, such as oxymetazoline, reduces the stimulus-evoked release of norepinephrine in brain (8–10) and heart (1, 11). It has been presumed that activation of such presynaptic *alpha* adrenergic receptors is involved in the central hypotensive action of clonidine. Activation of central postsynaptic *alpha* receptors by clonidine, however, appears to be involved

in the clonidine-evoked increases in flexor responses in reserpine-treated rats (3). A recent report further implicated the activation of postsynaptic *alpha* adrenergic receptors by clonidine in the genesis of central effects by this drug (12). Our interest in *alpha* adrenergic receptors of the central nervous system and their relationship to the adenylate cyclase system (13, 14) prompted us to examine the effects of clonidine, alone and in combination with other adrenergic agonists, on the accumulation of cyclic 3′,5′-AMP in rat cerebral cortical slices.

MATERIALS AND METHODS

Adult, male F-344 rats (Microbiological Associates, Bethesda, Md.) were used in these experiments. This strain of rats was selected for use because of the relatively high accumulation of cyclic AMP obtained in cerebral cortical slices following stimulation by norepinephrine (13). Clonidine HCl was provided by Boehringer-Ingleheim; methoxamine HCl, by Schering; oxymetazoline, by Merck; phenoxybenzamine, by Smith Kline & French; and phenylephrine HCl, by Sterling-Winthrop Laboratories. *l*-Norepinephrine bitartrate and *l*-isoproterenol HCl were purchased from Sigma Chemical Company.

Animals were killed by decapitation, and chopped cerebral cortical slices were prepared as previously described (15). Experiments usually consisted of the pooled cortical tissue from two rats. The time from decapitation to initial incubation of slices was less than 10 min. Chopped tissue was incubated for 15 min at 37° in Krebs-Ringer-bicarbonate–glucose medium (16) gassed with 95% O_2–5% CO_2. Tissue was transferred to 17 μM adenine in Krebs-Ringer medium for an additional 40 min. Slices were then washed twice with medium for an additional 10 min, collected on nylon mesh, divided into portions, and transferred to 30-ml beakers containing the appropriate test agents in 10 ml of medium. Tissue was incubated for 9–15 min. During this period accumulations of cyclic AMP elicited by catecholamines remain at nearly constant maximal levels (14, 17). In experiments with combinations of agonist and antagonist, a 3-min incubation with antagonist preceded addition of agonist. Slices were finally collected on nylon mesh and transferred to homogenizers containing 1 ml of cold 8% trichloracetic acid. Tissues were homogenized, and 5 pmoles of cyclic [^{14}C]AMP (1 nCi) were added to monitor recovery. Extraction and determination of cyclic AMP were carried out using the method of Gilman (18) as modified by Schultz and Daly (19). Protein was determined by the method of Lowry *et al.* (20) as modified by Miller (21).

RESULTS

Clondine at concentrations from 1 to 100 μM did not alter levels of cyclic AMP in rat cerebral cortical slices compared with nonstimulated controls (Table 1), although the increase in cyclic AMP with 100 μM clonidine approached statistical significance at the $p < 0.05$ level. The stimulatory effect of norepinephrine, an agent which has been shown to possess both *alpha* and *beta* adrenergic agonist properties in the adenylate cyclase system of rat brain (14, 22, 23), was significantly inhibited by the presence of clonidine, the latter in concentrations as low as 0.1 μM (Figs. 1 and 2). At each concentration utilized, clonidine reduced the accumulation of cyclic AMP elicited by maximal concentrations of norepinephrine to levels comparable to the maximal accumulation elicited by isoproterenol (Fig. 2). A combination of clonidine and the *alpha* adrenergic antagonist phenoxybenzamine resulted in a reduction in the norepinephrine-stimulated accumulation of cyclic AMP no greater than the reduction seen with either phenoxybenzamine or clonidine alone, indicative of an *alpha* adrenergic blockage by these two agents at an identical locus. At the concentration of phenoxybenzamine utilized (15 μM), no effects were observed on basal levels of cyclic AMP. This agent has been shown to effectively block the *alpha* adrenergic component of norepinephrine-elicited accumulation of cyclic AMP in rat cortical slices (17) as well as the accumulation of cyclic AMP elicited by methoxamine, an *alpha* adrenergic agonist (14). Combinations of clonidine and the *beta* adrenergic antagonist

propranolol reduced the norepinephrine-stimulated accumulations of cyclic AMP to control levels (Fig. 2). Clonidine also completely antagonized the methoxamine-stimulated accumulation of cyclic AMP (Table 2). The later compound is a potent *alpha* adrenergic agonist in many peripheral systems and elicits at least a 2-fold accumulation of cyclic AMP in cortical slices from the F-344 rat when used at maximal concentrations of 100 μM (Table 2; cf. ref. 14).

TABLE 1

Effect of alpha adrenergic agonists on accumulation of cyclic AMP in rat cerebral cortical slices

Values represent means ± standard errors. Each experiment was performed on the pooled cortices of two rats.

Agent (μM)	No. of experiments	Cyclic AMP
		pmoles/mg protein
None	16	47 ± 4
Clonidine (1)	5	49 ± 7
Clonidine (10)	8	50 ± 7
Clonidine (100)	8	62 ± 7
Oxymetazoline (5)	4	52 ± 12
Oxymetazoline (100)	3	59 ± 18
Phenylephrine (100)	4	57 ± 11

At maximal or nearly maximal stimulatory concentrations of isoproterenol (greater than 1 μM) clonidine at a concentration of 100 μM had no significant effect on the stimulated formation of cyclic AMP (Fig. 3). However, at submaximal concentrations of isoproterenol, a marked potentiation of the stimulatory effects of the *beta* agonist was observed. Indeed, at the lowest concentration of isoproterenol tested (0.1 μM), the presence of clonidine caused a 2-fold increase in the isoproterenol-stimulated accumulation of cyclic AMP. The levels of cyclic AMP attained under this condition were not significantly different from levels elicited with maximal stimulatory concentrations of isoproterenol. The potentiative effect of clonidine with submaximal concentrations of isoproterenol was concentration-dependent between 1 and 100 μM clonidine (Fig. 4).

Two classical *alpha* adrenergic agonists, phenylephrine and oxymetazoline (see refs. 1 and 11), were also investigated for their effects on the cyclic AMP-generating system in rat cortical slices. Although neither agent elicited a significant accumulation of cyclic AMP (Table 1), oxymetazoline at both 5 and 100 μM completely antagonized what would appear to be the *alpha* adrenergic component for norepinephrine-elicited accumulations of cyclic AMP (Ta-

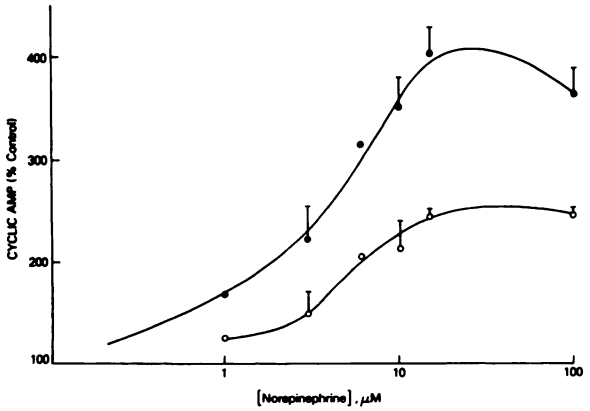

FIG. 1. *Effect of clonidine on norepinephrine-stimulated formation of cyclic AMP in rat cerebral cortical slices*

●——●, norepinephrine; ○——○, norepinephrine plus 100 μM clonidine. Values represent means ± standard errors of at least three experiments or the averages of duplicate determinations (1 and 6 μM), each experiment consisting of the pooled cortices from two rats. Nonstimulated values for these series of experiments were 50 ± 8 pmoles/mg of protein.

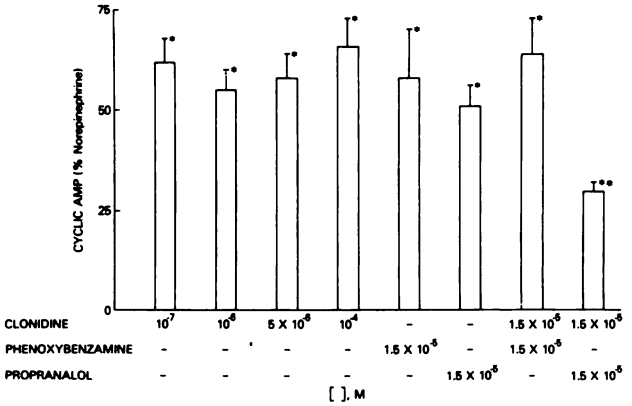

FIG. 2. *Effects of clonidine, phenoxybenzamine, and propranolol on norepinephrine-stimulated formation of cyclic AMP in rat cerebral cortical slices*

Values represent means ± standard errors of at least four experiments, each experiment consisting of the pooled cortices of two rats. Nonstimulated values for cyclic AMP were 58 ± 4 pmoles/mg of protein, while values for 100 μM norepinephrine were 208 ± 22 pmoles/mg of protein for these series of experiments.

*p <0.05 compared with 100 μM norepinephrine.

**Not significantly different from nonstimulated values.

TABLE 2

Effect of clonidine on methoxamine-stimulated formation of cyclic AMP in rat cerebral cortical slices

Values represent means ± standard errors of at least four experiments, each experiment consisting of the pooled cortices of two rats.

Agent (μM)	Cyclic AMP
	pmoles/mg protein
None	58 ± 4
Methoxamine (100)	121 ± 12
Methoxamine (100) + clonidine (100)	61 ± 11[a]

[a] $p < 0.01$ compared with methoxamine alone.

ble 3). Levels of cyclic AMP elicited by combinations of oxymetazoline and norepinephrine were not significantly different from levels obtained with maximal concentrations of isoproterenol. Phenylephrine, another *alpha* agonist in peripheral systems, was relatively ineffective either as an agonist by itself or as an antagonist in combination with norepinephrine (Tables 1 and 3). Oxymetazoline and phenylephrine were also tested for their ability to potentiate the effects of submaximal concentrations of isoproterenol, analogous to the potentiation observed with clonidine. At a 100 μM concentration both oxymetazoline and phenylephrine potentiated the effects of 0.1 μM isoproterenol to an extent such that the elicited accumulation of cyclic AMP was not significantly different from that obtained with the maximal concentration of isoproterenol (Fig. 4).

DISCUSSION

Clonidine in both central and peripheral systems appears to be a potent *alpha* adrenergic agonist. Activation of presynaptic *alpha* receptors by this drug or by oxymetazoline reduces the rate of efflux of norepinephrine from adrenergic neurons (1, 8–11). Another *alpha* adrenergic agonist, phenylephrine, is only weakly active in depressing the stimulus-induced efflux of norepinephrine in heart preparations (1). *Alpha* adrenergic antagonists such as phenoxybenzamine potentiate the stimulus-evoked release of norepinephrine (9). Evidence for activation of central and peripheral postsynaptic adrenergic receptors by clonidine has also been obtained (3, 12). Thus the present finding that clonidine is a potent *alpha* adrenergic antagonist with regard to norepinephrine-elicited accumulation of cyclic AMP in rat cortical slices (Figs. 1 and 3) was entirely unexpected. The evidence for this conclusion, however, is very strong and includes (a) the antago-

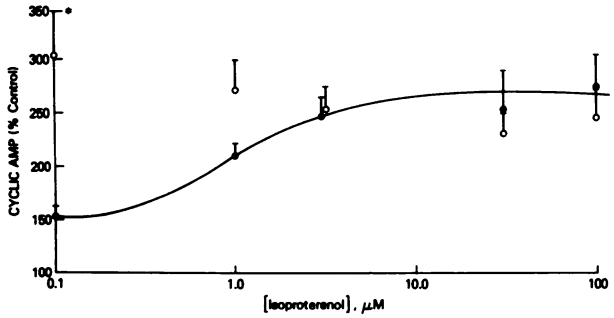

FIG. 3. *Effect of clonidine on isoproterenol-stimulated formation of cyclic AMP in rat cerebral cortical slices*

●——●, isoproterenol; ○——○, isoproterenol plus 100 μM clonidine. Values represent means ± standard errors of at least three experiments, each experiment consisting of the pooled cortices from two rats. Nonstimulated values for these series of experiments were 38 ± 6 pmoles/mg of protein.

*p <0.05 comparing isoproterenol with isoproternol plus clonidine, using a paired t-test.

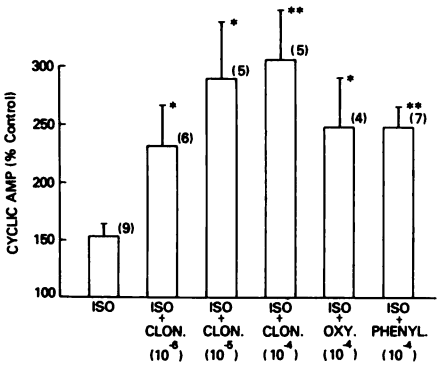

FIG. 4. *Potentiation of isoproterenol-stimulated formation of cyclic AMP by adrenergic agents*

Isoproterenol (ISO) was present at 0.1 μM. Values represent means ± standard errors of the number of experiments in parentheses. Nonstimulated levels of cyclic AMP in these series of experiment were 35 ± 5 pmoles/mg of protein. CLON., clonidine; OXY., oxymetazoline; PHENYL., phenylephrine.

*p <0.05 as determined by a paired t-test comparing values obtained with isoproterenol alone with values obtained with isoproterenol plus the adrenergic agent in the same experiment.

**p <0.02.

nism of norepinephrine responses to a similar extent by clonidine and phenoxybenzamine (Fig. 2); (b) the lack of additivity of the effects of clonidine and phenoxybenzamine on norepinephrine responses (Fig. 2); (c) the lack of antagonism of the response to isoproterenol (Fig. 3); (d) complete antagonism of the norepinephrine response by a combination of clonidine and propranolol (Fig. 2); and (e) complete blockade of the methoxamine response by clonidine (Table 2). Oxymetazoline would appear, like clonidine, to be an *alpha* antagonist with respect to norepinephrine-elicited accumulations of cyclic AMP in rat cortical slices (Table 3), while phenylephrine would appear to have little activity as either agonist or antagonist in rat cortical slices (Tables 1 and 3). In rat cerebellar slices, where the *beta* antagonist sotalol

TABLE 3

Effect of oxymetazoline and phenylephrine on norepinephrine-stimulated formation of cyclic AMP in rat cerebral cortical slices

Values represent means ± standard errors of at least four experiments, each experiment consisting of the pooled cortices of two rats.

Agent (μM)	Cyclic AMP
	pmoles/mg protein
None	65 ± 9
Norepinephrine (15)	247 ± 22
Norepinephrine (100)	258 ± 34
Norepinephrine (15) + oxymetazoline (5)	159 ± 19[a]
Norepinephrine (100) + oxymetazoline (100)	150 ± 19[a]
Norepinephrine (100) + phenylephrine (100)	247 ± 23
Isoproterenol (100)	148 ± 8

[a] p < 0.02 compared with norepinephrine alone at 15 and 100 μM, respectively.

completely blocks responses to norepinephrine, clonidine has no effect on norepinephrine-elicited accumulation of cyclic AMP (24).

The lack of significant agonist activity in brain slices for clonidine and oxymetazoline and their potent *alpha* antagonist activity are clearly difficult to reconcile with the proposal that the presynaptic *alpha* adrenergic receptors which regulate the rate of neurotransmitter release do so via a cyclic AMP-dependent mechanism. Instead, it would appear that the large accumulations of cyclic AMP elicited in rat cortical slices by interaction of norepinephrine with what appear to be *alpha* receptors, and antagonized by clonidine and oxymetazoline, are not related to the *alpha* receptor-mediated feedback control of norepinephrine release. It is possible, however, that clonidine, oxymetazoline, and phenylephrine do elicit large increases in cyclic AMP in a minor compartment represented by presynaptic elements of the noradrenergic nerve ending and that this small accumulation is not detectable in the heterogeneous brain slice preparation. Indeed, small but not statistically significant increases in levels of cyclic AMP did occur on exposure of rat cortical slices to these agents (Table 1). Alternatively, clonidine and oxymetazoline might have only very weak partial agonist activity at a presynaptic *alpha* receptor sufficient to "trigger" cyclic AMP-mediated inhibition of norepinephrine release, but because of their high affinity for this site they would block the stimulatory effects of a complete agonist, norepinephrine. It is also possible that accumulations of cyclic AMP may be elicited by these *alpha* adrenergic agents in minor postsynaptic elements and that cyclic AMP might therefore be involved in central postsynaptic effects of these drugs (see refs. 3 and 12). These observations emphasize the complexity of adrenergically regulated mechanisms and their interrelationship with accumulation of cyclic AMP in the central nervous system. Thus, although there is evidence which indicates that postsynaptic *alpha* and *beta* adrenergic receptors mediate the accumulation of cyclic AMP in brain tissue (22, 25), definitive evidence for a catecholamine-mediated accumulation of cyclic AMP at presynaptic sites or for a role of cyclic AMP in the presynaptic modulation of neurotransmitter turnover and release has not been obtained (see ref. 26). Further studies are clearly necessary to delineate both the morphological location and physiological significance of the norepinephrine-sensitive *alpha* receptors controlling adenylate cyclase activity in cerebral cortex which are sensitive to blockade by clonidine and oxymetazoline.

A further unexpected observation with rat cerebral cortical slices is the potentiation by clonidine, oxymetazoline, and phenylephrine of the stimulatory effects of submaximal concentrations of the *beta* agonist isoproterenol. The mechanism of this apparent synergism between *alpha* and *beta* adrenergic agents is unknown. Synergism with respect to stimuated formation of cyclic AMP has previously been observed in brain slices between histamine and norepinephrine (27) and between adenosine and biogenic amines (17, 27, 28). One interpretation of the present results is that potentiation of isoproterenol responses by clonidine is due to an alteration in the affinity of the *beta* adrenergic receptor for isoproterenol. Indeed, the accumulations of cyclic AMP elicited by combinations of very low concentrations of isoproterenol with clonidine are not significantly different from the accumulations elicited by maximal stimulatory concentrations of the *beta* agonist alone (Fig. 3).

Altogether, both biochemical and physiological studies indicate that clonidine and other *alpha* adrenergic agonists have multiple loci of action within noradrenergic synapses of the central nervous system. With regard to cyclic AMP mechanisms, clonidine effectively blocks the *alpha* component of norepinephrine-elicited accumulations of cyclic AMP and potentiates the *beta* response to low concentrations of isoproterenol in rat cortical slices. In addition, clonidine may have some intrinsic *alpha* agonist activity with respect to cyclic AMP generation in rat cortical slices. The relationship of these effects to the pharmacological activity of clonidine

at presynaptic and postsynaptic loci cannot, however, be profitably discussed without further definition of the loci at which catecholamine-elicited accumulations of cyclic AMP occur in the brain slice system.

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