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A NOVEL ANALOGUE OF CLONIDINE WITH OPIATE-RECEPTOR AGONIST ACTIVITY

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SUMMARY

A new analogue of the α_2-adrenergic receptor ligand clonidine, N-(4-hydroxphenacetyl)-4-aminoclonidine, was synthesized. The analogue possesses opiate-receptor agonist activity in addition to α-adrenergic partial agonist activity. The analogue elicits inhibition of adenylate cyclase of NG108-15 neuroblastoma x glioma hybrid cells; most of the inhibition is reversed by the opiate-receptor antagonist naloxone. The analogue also inhibits the binding of [^{3}H]D-Ala^2-Met^5-enkephalinamide and [^{3}H]dihydromorphine to rat brain opiate receptors. The structure of the analogue suggests common elements in the ligand binding sites of α- and opiate receptors and may lead to a new class of opiate analgesics.

INTRODUCTION

Opiate receptors found in the nervous system, as well as in cultured cells of neuronal origin, have been well defined, characterized in detail, and widely discussed during the last decade (1-6). The major groups of compounds that interact specifically and with high affinity with opiate receptors include the morphine-like analgesics, related synthetic compounds, and the endogenous opioid peptides such as the enkephalins and β-endorphin (7,8). In this communication we present a new synthetic compound whose structure is not related to those of known opioid compounds but which behaves as an opiate-receptor agonist in in vitro systems. The new compound, N-(4-hydroxyphenacetyl)-4-aminoclonidine hydrochloride (HP-aminoclonidine[1]), is a derivative of p-aminoclonidine, a potent α-adrenergic receptor ligand (9-11). It was synthesized originally to be used as an α-receptor probe and indeed was found to bind with high affinity to α-receptors of rat brain and NG108-15 neuroblastoma x glioma hybrid cells (11). We demonstrate here that in addition to its high affinity for α-receptors, HP-aminoclonidine acts as an opiate agonist as indicated by its naloxone-reversed inhibition of adenylate cyclase (EC 4.6.1.1) of NG108-15 homogenates and by its binding affinity for rat brain opiate receptors.

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[1]Abbreviations: HP-aminoclonidine: N-(4-hydroxyphenacetyl)-4-aminoclonidine. PGE_1: prostaglandin E_1; IC_{50}: concentration eliciting half-maximal inhibition.

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MATERIALS AND METHODS

Synthesis of HP-aminoclonidine. p-Aminoclonidine (318 mg, 1.0 mmole) was partially dissolved in a solution of dimethylformamide (0.5 ml) and dry dioxane (2.0 ml). Triethylamine (0.14 ml, 2.0 mmole) was added to the stirred suspension. p-Hydroxyphenacetyl succinimide ester (302 mg, 1.2 mmole), prepared by reacting N-hydroxy-succinimide ester and p-hydroxyphenylacetic acid in the presence of dicyclohexylcarbodiimide, was added in 0.2 ml dioxane. The reaction mixture was stirred at room temperature (23°C) for 1 hr. The reaction mixture was evaporated under reduced pressure, and the residue was extracted with ethyl acetate and water. The aqueous phase was separated and adjusted to pH 8.5 with NaOH. The resultant white precipitate was collected by filtration on sintered glass, acidified with concentrated HCl, and precipitated from traces of methanol and ether. The precipitation procedure was repeated three times. The precipitate was dried over anhydrous $MgCl_2$. Net dry weight was 82 mg (20% yield). When chromatographed on thin layer plates in n-butanol: acetic acid: water 4:1:4, the compound migrated as a single spot (Rf 0.39) visualized by iodine vapor. A single peak absorbing at 290 nm was observed when the preparation was applied to an HPLC reverse-phase C_{18} column and eluted with a gradient of 1-30% acetonitrile in 1% acetic acid. The percent nitrogen was 13.1% (calculated 13.9%).

Cell culture. NG108-15 cells (passage 18-22) were cultured, harvested, and homogenized as described (13). Protein was determined by the method of Lowry (17).

Adenylate cyclase assay. Reaction mixtures (100 µl) contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 87 mM sucrose, 0.25 mM Ro20-1724 (a cyclic nucleotide phosphodiesterase inhibitor from Hoffmann-La Roche), 1 mM [α-^{32}P]ATP-(6 x 10^6 cpm, New England Nuclear), 1 µM GTP, 1 mM cAMP, 20 mM disodium creatine phosphate, 5 units (34-40 µg protein) creatine phosphokinase, 1 µM puromycin (to inhibit enkephalin aminopeptidase (18)), 10 µM PGE_1 where indicated, and 87-140 µg NG108-15 homogenate protein. Incubations were carried out for 20 min at 37° unless otherwise indicated. [^{32}P]Cyclic AMP was isolated by a modification (12) of the procedure of Salomon, et. al. (19). Results are expressed as means of duplicates, which were generally within 3% of the means.

Opiate receptor binding assay. Washed membranes (P_2 pellet) from rat brain (minus cerebella) were prepared by differential centrifugation as described (11). Binding mixtures contained, in a volume of 0.4 ml, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 247 µg membrane protein, radioactive opiate-receptor ligand as indicated and appropriate concentrations of unlabeled tested ligands Binding mixtures were incubated for 30 min at 25°C. Four ml ice-cold buffer (0.05 M Tris-HCl, pH 7.5, 0.01 $MgCl_2$) were then added, and the mixtures were immediately filtered onto Whatman GF/B filters (25 mm diameter) under reduced pressure. The filters were then immediately washed three times with 4 ml cold buffer and counted in 10 ml Aquasol (New England Nuclear) in a liquid scintillation counter. Specific binding of each labeled ligand to opiate receptors was defined as binding displaced by 10 µM naloxone.

The apparent dissociation constant $K_{D_{app}}$ for naloxone was calculated from the equation

$$K_{D_{app}} = \frac{EC_{50}}{1 + [S]/K_{S_{app}}}$$

where EC_{50} is the naloxone concentration eliciting half-maximal reversal, [S] is the agonist concentration, and $K_{S_{app}}$ is the apparent dissociation constant for agonist, here approximated by the half-maximally inhibitory concentration.

Chemicals. The following were kind gifts: p-aminoclonidine from Dr. G. Leclerc (Univ. of Strasbourg), clonidine from Boehringer-Ingelheim, phentolamine from Ciba-Geigy, etorphine from the National Institute of Drug Abuse, and naloxone from Endo. Sources of other chemicals are indicated elsewhere (13).

RESULTS

The structure of HP-aminoclonidine is shown in Fig. 1.

HP-aminoclonidine, morphine, and leucine-enkephalin inhibit basal adenylate cyclase of NG108-15 homogenates with half-maximally effective concentrations (IC_{50} values) of 700, 500, and 40 nM, respectively (Fig. 2). The maximum inhibition (59%) is similar for each compound and is of a magnitude normally found in this assay for full opiate or α-adrenergic agonists (12,13). The IC_{50} observed for morphine is somewhat lower than that (1500 nM) reported by Klee and Nirenberg (14), while the IC_{50} found for Leu-enkephalin is similar to their value. As shown in Fig. 3, HP-aminoclonidine also inhibits PGE_1 stimulated adenylate cyclase activity with an IC_{50} of 1000 nM and a maximum inhibition of at least 32%, which is similar to the maximum inhibition (38%) by etorphine, a highly potent opiate (IC_{50} = 2 nM).

The effects of opiate and α-adrenergic receptor antagonists at various concentrations on the inhibition of adenylate cyclase elicited by 3 μM HP-aminoclonidine are shown in Fig. 4. Naloxone, a specific competitive opiate-receptor antagonist reverses 70-90% of the inhibition, while dihydroergotamine, a

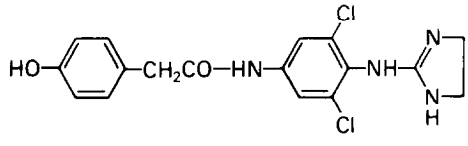

FIGURE 1: The structure of HP-aminoclonidine.

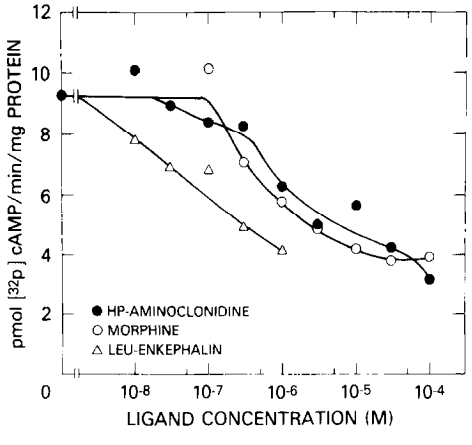

FIGURE 2: Inhibition of basal adenylate cyclase activity of NG108-15 neuroblastoma x glioma hybrid cells by HP-aminoclonidine (●), morphine sulfate (○), and leucine-enkephalin (△). Reaction mixtures contained 87 μg NG108-15 homogenate protein, indicated concentrations of tested ligands, and other components described in Materials and Methods.

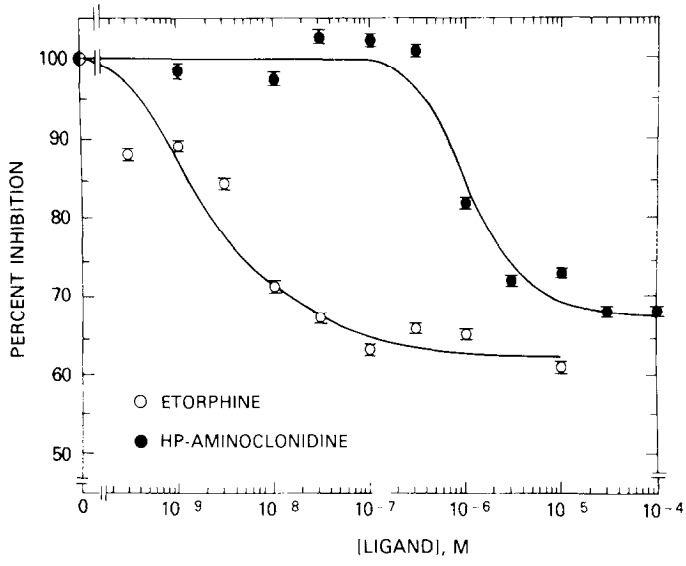

FIGURE 3: Inhibition of PGE_1-stimulated adenylate cyclase activity of NG108-15 cells by etorphine (○) and HP-aminoclonidine (●), added to reaction mixtures at indicated concentrations. Reaction mixtures contained 140 µg homogenate protein. The specific activity without added inhibitor was 66.4 pmoles cAMP/min/mg protein.

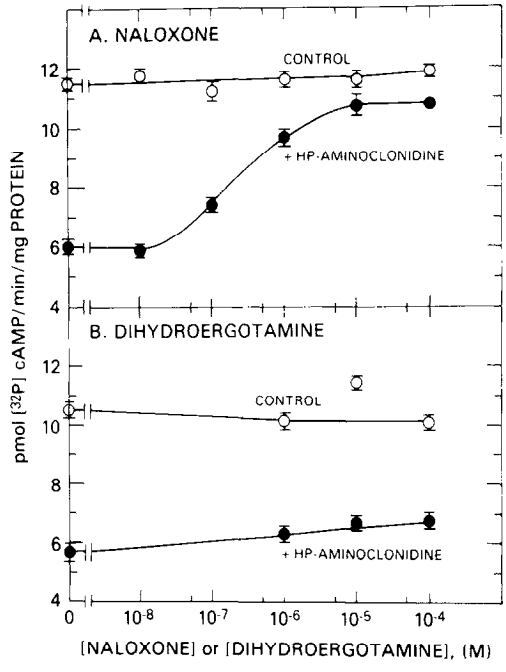

FIGURE 4: The effect of blockade of opiate or α-adrenergic receptors on the inhibition of NG108-15 adenylate cyclase activity by HP-aminoclonidine. Reaction mixtures containing 116 µg NG108-15 homogenate protein with (●) or without (○) 3 µM HP-aminoclonidine, were incubated for 10 min at 37°. Panel A: Naloxone was present at the concentrations indicated to block opiate receptors. Panel B: Dihydroergotamine hydrochloride was present at the concentrations indicated to block α-receptors.

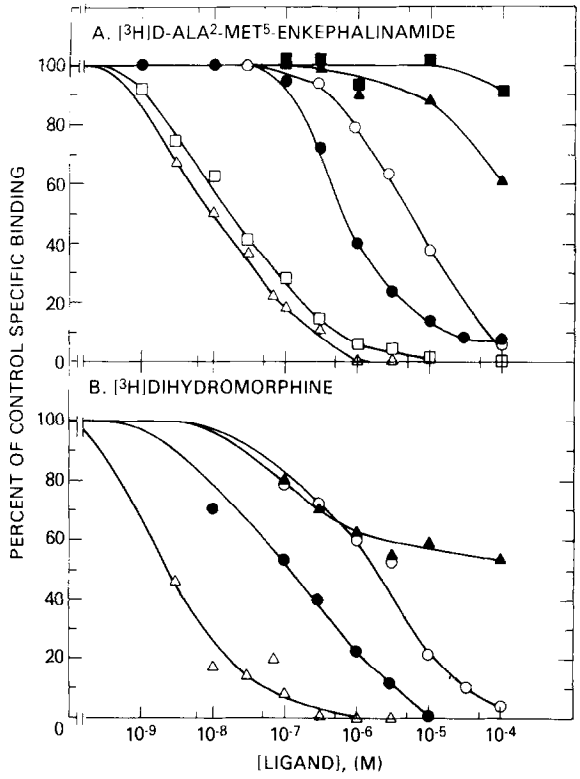

FIGURE 5: Displacement of labeled opiate-receptor ligands from rat brain opiate receptors by HP-aminoclonidine and other compounds. Panel A: Displacement of [^{3}H]-D-Ala^2-Met^5-enkephalinamide (39 Ci/mmol, Amersham) present in the binding mixture at 10 nM. Uncompeted specific binding was 72 fmol/mg protein, which was 60-70% of the total binding. Panel B: Displacement of [^{3}H]dihydromorphine (51.9 Ci/mmol, New England Nuclear) present in the binding mixture at 1 nM. The binding assay was conducted in dim light. Uncompeted specific binding was 14.2 fmol/mg protein, which was 50-60% of the total binding. Unlabeled compounds tested: naloxone hydrochloride (△), morphine sulfate (□), HP-aminoclonidine hydrochloride (●), phentolamine hydrochloride (○), p-aminoclonidine hydrochloride (▲), and clonidine hydrochloride (■).

competitive α-antagonist, reverses only 10-30% of the inhibition. Yohimbine, another α-antagonist, also fails to reverse most of the inhibition (not shown). The concentration of naloxone eliciting half-maximal reversal is 300 nM. The apparent dissociation constant of naloxone for the opiate receptor in the adenylate cyclase assay was calculated to be 55 nM. This value corresponds to the value of 20-30 nM reported for the reversal by naloxone of inhibition of adenylate cyclase by methionine-enkephalin (14) and supports the hypothesis that HP-aminoclonidine and naloxone compete for an opiate receptor.

As reported elsewhere (11) when NG108-15 opiate receptors are blocked by 50 μM naloxone, HP-aminoclonidine acts as a partial α-receptor agonist, inhibiting adenylate cyclase with an IC_{50} of 130 nM but with a maximum inhibition of only 10-30% of that of a full agonist. The naloxone-insensitive inhibition was reversed by α-receptor antagonists (not shown). Thus, HP-amino-

TABLE I: The opiate-receptor properties of clonidine, clonidine analogues, and related compounds.

Compound	Binding to rat brain opiate receptors		NG108-15 Adenylate Cyclase
	[^{3}H]D-Ala-Met-enkephalinamide	[^{3}H]Dihydromorphine	
	IC_{50} (nM)		IC_{50} (nM)
Clonidine-HCl	>100,000	n.d.[1]	>100,000
p-Aminoclonidine-HCl	>100,000	>100,000	>100,000
HP-aminoclonidine-HCl	600	120	700
Phentolamine-HCl	5,000	2,000	2,000[2]
Morphine sulfate	17	n.d.	500
Naloxone-HCl	10	2.5	none[3]

[1]n.d. Not determined.

[2]From reference 13.

[3]Pure opiate antagonist.

clonidine is a bifunctional ligand having a 6-fold greater potency for α-receptor activation than for opiate-receptor activation, and a 3-10 fold greater intrinsic efficacy at opiate receptors than at α-receptors.

In order to demonstrate a direct interaction of HP-aminoclonidine with opiate receptors, we examined its ability to displace radioactive opiate agonists from opiate receptors of rat brain membranes. We used [^{3}H]D-Ala^2-Met^5-enkephalinamide at 10 nM, which labels both "morphine" and "enkephalin" receptor subclasses, and [^{3}H]dihydromorphine at 1 nM, which labels predominantly the "morphine" receptors, as defined by Chang and Cuatrecasas (6). In the displacement of [^{3}H]-D-Ala-Met-enkephalinamide (Fig. 5A), the IC_{50} of HP-aminoclonidine (600 nM) is 1/35 of that of morphine (17 nM). Both p-aminoclonidine and clonidine, from which HP-aminoclonidine is derived, have little or no affinity for the D-Ala-Met-enkephalinamide receptors (IC_{50} >100 μM). Therefore, the hydroxyphenacetyl group is probably responsible, at least in part, for the opiate activity of the new analogue. Interestingly the α-antagonist phentolamine has a moderate affinity for opiate receptors (IC_{50} 2-5 μM), as originally reported by Cicero, et. al. (15). Phentolamine possesses some structural resemblance to HP-aminoclonidine by virtue of possessing phenolic and imidazoline moieties. In displacement of [^{3}H]dihydromorphine (Fig. 5B), HP-aminoclonidine is 1/48 as affine[2] as naloxone. The apparent dissociation constants for the inhibition of adenylate cyclase and the IC_{50} values for receptor binding are summarized in Table I.

[2]Affine: "having affinity." We suggest the use of this work in the biochemical literature because of its convenience. See A Supplement to the Oxford English Dictionary (1972) Oxford, Clarendon Press, p. 35.

DISCUSSION

In this paper we present a new clonidine analogue, HP-aminoclonidine, a high-affinity α-receptor partial agonist that behaves as a full opiate receptor agonist in the NG108-15 adenylate cyclase system. In the NG108-15 system, where opiate receptors have been reported to be mainly of the "enkephalin" subclass (6), its potency as an opiate agonist resembles that of morphine. However, in direct binding to rat brain opiate receptors (which include both subclasses), HP-aminoclonidine is considerably less affine than morphine (Table 1). When injected into mice, HP-aminoclonidine possesses analgesic activity, which is currently under investigation (R. Ruffolo and D. Atlas, in preparation).

The structure of HP-aminoclonidine (Fig. 1) bears little or no resemblance to known rigid opioid analgesics (such as the opium alkaloids, the morphinans and the benzomorphans) or to the flexible analgesics (such as the methadones and etonitazene) or to the opioid peptides. However, it has a phenolic group and a basic moiety (the imidazoline group) which are generally common requirements within the morphine-like compounds and the opioid peptides (16). The preparation of other analogues based on the structure of HP-aminoclonidine might lead to a new series of opioid analgesics, possibly with interesting clinical properties. Furthermore, findings presented in this study may open a new direction for the investigation of a possible common structural denominator between the ligand binding sites of α-adrenergic receptors and opiate receptors.

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