Pharmacology Biochemistry & Behavior, Vol. 19, pp. 879–882, 1983.

Antinociceptive Activity of N-(4-Hydroxyphenacetyl)-4-aminoclonidine, a Novel Analog of Clonidine: Role of Opioid Receptors and *Alpha*-Adrenoceptors

MARTIN D. HYNES,* DAPHNE ATLAS† AND ROBERT R. RUFFOLO, JR.*

**The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285*
and †Department of Biological Chemistry, Institute of Life Sciences
The Hebrew University, Jerusalem, Israel

Received 6 April 1983

HYNES, M. D., D. ATLAS AND R. R. RUFFOLO, JR. *Antinociceptive activity of N-(4-hydroxyphenacetyl)-4-aminoclonidine, a novel analog of clonidine: Role of opioid receptors and* alpha-*adrenoceptors.* PHARMACOL BIOCHEM BEHAV **19**(5) 879–882, 1983.—N-(4-hydroxyphenacetyl)-4-aminoclonidine, a derivative of the *alpha*-adrenoceptor agonist p-aminoclonidine, was found to exhibit dose-dependent antinociceptive activity in the mouse writhing assay. In this measure of antinociceptive activity it was less potent than clonidine or xylazine. Naloxone, an opioid receptor antagonist, at a dose sufficient to abolish the antinociceptive activity of morphine, did not affect the antinociceptive activity of N-(4-hydroxyphenacetyl)-4-aminoclonidine, clonidine or xylazine. In contrast, yohimbine, a *alpha*-adrenoceptor antagonist, reduced the antinociceptive activity of N-(4-hydroxyphenacetyl)-4-aminoclonidine, clonidine and xylazine, but not morphine. The affinity of N-(4-hydroxyphenacetyl)-4-aminoclonidine, clonidine and xylazine for *alpha*-adrenoceptors in rat aorta was correlated highly with the relative potency for writhing inhibition. These results suggest that the antinociceptive activity of N-(4-hydroxyphenacetyl)-4-aminoclonidine is mediated by *alpha*-adrenoceptors.

Opioid receptors *Alpha*-adrenoceptors Clonidine Xylazine
N-(4-hydroxyphenacetyl)-4-aminoclonidine Morphine Naloxone Yohimbine

CLONIDINE is an imidazoline with potent antihypertensive activity resulting from stimulation of *alpha*-adrenoceptors in the central nervous system. In addition to its cardiovascular activity, clonidine has pronounced psychopharmacological activity (for reviews see [6, 9, 16]). One such psychopharmacological action of clonidine is antinociception. Clonidine has been found to exhibit antinociceptive activity in a wide variety of animal models such as writhing [3,7], hot plate [14], tail withdrawal [7,15], and the Randall Selitto test [7] (for a complete review of clonidine antinociceptive activity, see [6, 9, 16]). Xylazine, an *alpha*-adrenoceptor agonist closely resembling clonidine in structure, has been used in veterinary medicine as an analgesic [4]. The exact mechanism of clonidine- and xylazine-induced analgesia is not known. However, an interaction with opioid receptors has been ruled out as a possibility since naloxone, a potent narcotic antagonist, does not antagonize the antinociceptive activity of clonidine in a variety of tests [7,10]. Additionally, clonidine does not exhibit cross tolerance to morphine in dependent rodents [17]. Recently a new compound, N-(4-hydroxyphenacetyl)-4-aminoclonidine hydrochloride (HP-aminoclonidine), which is a derivative of the potent *alpha*-adrenoceptor agonist, p-aminoclonidine, was reported to inhibit the binding of tritiated D-Ala^2-Met^5-enkephalinamide and tritiated dihydromorphine to brain opioid receptors [1]. In view of HP-aminoclonidine's ability to inhibit opioid receptor binding, we undertook this investigation to ascertain if this compound would produce an opiate receptor-mediated antinociceptive activity in the mouse writhing assay.

METHOD

Mouse Writhing

The mouse writhing response was defined as a contraction of the abdominal musculature followed by the extension of the hind limbs. Acetic acid administration by the intraperitoneal route (10 ml/kg) at a concentration of 0.6 percent was used to induce this response. Five Cox Standard male albino mice (Laboratory Supply Co., Indianapolis, IN), weighing 20 to 22 grams after being fasted overnight, were observed simultaneously for the occurrence of the writhing response. Each mouse was used only once. The observation period was ten minutes in length and started five minutes after the administration of acetic acid. Inhibition of mouse writhing was calculated from the total number of writhes utilizing the control and drug-treated groups according to the following formula:

$$\text{Percent Inhibition} = 100 - \frac{\text{Experimental Group} \times 100}{\text{Control Group}}$$

TABLE 1

INHIBITION OF MOUSE WRITHING BY CLONIDINE, XYLAZINE AND HP-AMINOCLONIDINE

Treatment*	Inhibition of Mouse Writhing† ED_{50} (95 Percent Confidence Limits)	Relative Potency
Clonidine	0.012 (0.009–0.016)	82
Xylazine	0.292 (0.231–0.368)	3
HP-Aminoclonidine	0.985 (0.524–1.852)	1
HP-Aminoclonidine + Naloxone‡	0.958 (0.611–1.501)	—

*All drugs were administered by the subcutaneous route 30 minutes prior to testing.
†Mouse writhing ED_{50} values are expressed in mg/kg and were computed by the use of the regression line in reverse [5].
‡Naloxone was administered subcutaneously at a dose of 10 mg/kg.

On the average, control mice exhibit 225 to 250 writhes in the ten-minute observation period. The dose required to reduce the frequency of writhing by 50 percent was defined as the ED_{50}.

Determination of Alpha-Adrenoceptor Dissociation Constants

Male albino rats (Harlan Wistar, 220–350 g) were sacrificed by a sharp blow to the head. The thoracic aorta was removed and dissected free of fat and connective tissue in physiological salt solution (PSS, pH 7.40) at room temperature. Helically cut strips, approximately 2 mm wide and 30 mm long, were prepared as described by Furchgott and Bhadrakom [8] and were suspended in 10-ml organ baths containing PSS maintained at 37.5°C and aerated with a 5 percent carbon dioxide-95 percent oxygen mixture. The composition of PSS was (millimolar concentrations): NaCl, 118; KCl, 4.7; $MgCl_2$, 0.54; $CaCl_2$, 2.5; NaH_2PO_4, 1.0; $NaHCO_3$, 25; and glucose, 11; dissolved in demineralized water. The aortic strips were attached to Grass FT-03 isometric transducers connected to a Grass model 7 polygraph recorder. Dose-response curves were constructed by increasing bath concentrations of agonist approximately 3-fold [18]. Dissociation constants for *alpha*-adrenoceptors in rat aorta were determined by classical methodology described by us in detail previously [12,13].

All the drugs employed in these experiments were dissolved in water. Clonidine hydrochloride was a gift from Boehringer-Ingelheim, xylazine a gift of Bayer AG and the naloxone hydrochloride was donated by Endo Laboratories. The N-(4-hydroxyphenacetyl)-4-aminoclonidine was synthesized according to previously published procedures [1]. Yohimbine hydrochloride was purchased from the Sigma Chemical Company.

RESULTS

The ED_{50} values for the inhibition of mouse writhing by clonidine, xylazine and HP-aminoclonidine are summarized in Table 1. The data depicted in this table show that all of the compounds investigated are potent inhibitors of mouse writhing. The most active compound of those investigated was clonidine, which had an ED_{50} value of 0.012 mg/kg when administered by the subcutaneous route. HP Aminoclonidine was the least active of those compound investigated, being 82 times less potent than clonidine and times less potent than xylazine.

The inhibition of mouse writhing produced by singl doses of clonidine, xylazine and HP-aminoclonidine was no antagonized by administration of naloxone at a dose of 1 mg/kg (Fig. 1). However, this dose of naloxone was suffi cient to antagonize the inhibition of writhing produced by th opiate receptor agonist, morphine. Naloxone did not affec the writhing response by itself. Since HP-aminoclonidine i known to bind to opioid receptors [1], we examined i greater detail the possible interaction between HP aminoclonidine and naloxone by using a fixed dose of th narcotic antagonist in conjunction with increasing doses o HP-aminoclonidine. Naloxone, 10 mg/kg, did not shift th dose response curve for HP-aminoclonidine-induced writh ing inhibition. The HP-aminoclonidine ED_{50} value for writh ing inhibition in the presence and absence of naloxone i given in Table 1.

The effect of the *alpha*-adrenoceptor antagonist yohim bine on the analgesic activity of morphine, clonidine xylazine and HP-aminoclonidine was subsequently investi gated (Fig. 1). Yohimbine, at a dose of 8 mg/kg, did not affec the writhing response itself or the analgesia produced b morphine. Clonidine, xylazine and HP-aminoclonidine induced inhibition of writhing was significantly antagonize by yohimbine pretreatment.

Alpha-adrenoceptor affinity for clonidine, xylazine an HP-aminoclonidine was determined in rat aorta. The result of these determinations are shown in Table 2. All of thes compounds had high affinity for *alpha*-adrenoceptors, wit clonidine having the highest affinity of those compounds in vestigated in this study. HP-Aminoclonidine has the lowes affinity for *alpha*-adrenoceptors, being 100 times less poten than clonidine. The relative potencies of clonidine, xylazin and HP-aminoclonidine for inhibition of writhing and affinity for *alpha*-adrenoceptors are shown in Tables 1 and 2, re spectively. The rank order of activity on these tests were identical for these three *alpha*-adrenoceptor agonists.

DISCUSSION

HP-Aminoclonidine was found to inhibit, dose depend ently, the acetic acid-induced writhing response in th

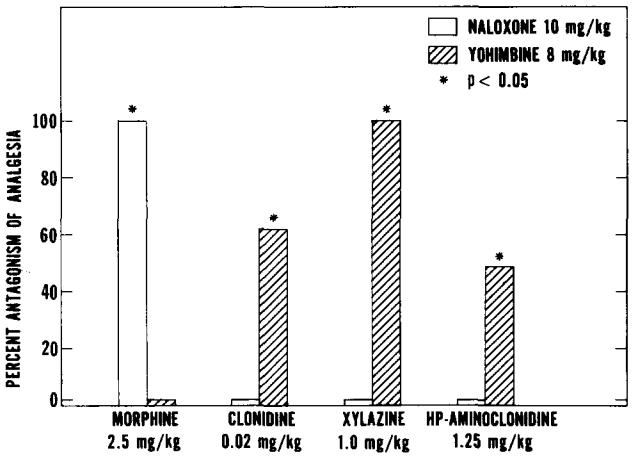

FIG. 1. Antagonism of the antinociceptive effects of morphine, clonidine, xylazine and HP-aminoclonidine by naloxone and yohimbine. The writhing response was not affected by the administration of either naloxone or yohimbine by themselves. Morphine, clonidine, xylazine and HP-aminoclonidine inhibited writhing by 86, 50, 45 and 43 percent, respectively, at the doses tested. A minimum of five mice were used in each treatment group. All drugs were administered subcutaneously.

TABLE 2

DISSOCIATION CONSTANTS OF CLONIDINE, XYLAZINE AND HP-AMINOCLONIDINE FOR THE *ALPHA*-ADRENOCEPTOR OF THE RAT AORTA

Treatment	K_B (nM)	−log KB	Relative Affinity
Clonidine	22	7.66 ± 0.06	102
Xylazine	339	6.47 ± 0.12	7
HP-Aminoclonidine	2239	5.65 ± 0.13	1

mouse. In this measure of antinociceptive activity, HP-aminoclonidine was found to be less active than clonidine and xylazine. The clonidine- and xylazine-induced inhibition of writhing was not antagonized by the narcotic antagonist, naloxone, at a dose which completely antagonized the antinociceptive effects of morphine. These results are in agreement with literature reports in which naloxone was found not to antagonize the antinociceptive activity of clonidine in a variety of analgesic tests [7, 10, 17]. Similarly, the results reported herein show naloxone ineffective in antagonizing the inhibition of mouse writhing produced by HP-aminoclonidine. HP-Aminoclonidine has been reported to produce a naloxone-reversible inhibition of adenylate cyclase, while also inhibiting the binding of tritiated D-Ala^2-D-Met^5-enkephalinamide and dihydromorphine to brain opiate receptors [1]. We conclude that these *in vitro* opioid-like effects of HP-aminoclonidine are not related to the *in vivo* antinociceptive activity of the compound.

HP-Aminoclonidine has previously been found to inhibit the binding of ^{3}H-clonidine to rat brain and NG108-15 neuroblastoma X glioma hybrid cell membranes with an affinity relative to clonidine of 0.3 [2]. The results reported here confirm the binding of HP-aminoclonidine to *alpha*-adrenoceptors of rat aorta. However, in this smooth muscle preparation, HP-aminoclonidine exhibited only 1/100 the activity of clonidine. The difference in the relative affinity values between these tests may be the result of several factors. HP-Aminoclonidine may, for example, diffuse poorly across tissue membrane, thereby limiting its activity in the rat aorta preparation. This discrepancy might also result from the fact that the rat aorta preparation assesses both affinity and efficacy, while the membrane system only provides affinity data. Thus HP-aminoclonidine may have a high affinity for the *alpha*-adrenoceptors, but low efficacy.

The antinociceptive activity of clonidine is now known to be mediated via *alpha*-adrenoceptors [6, 7, 9, 10, 11]. We therefore decided to assess the significance of *alpha*-adrenoceptors in the antinociceptive effects of HP-aminoclonidine. Yohimbine, an *alpha*-adrenoceptor antagonist, was found to reduce the antinociceptive activity of clonidine, xylazine and HP-aminoclonidine, but not that of morphine. The results implicate *alpha*-adrenoceptors in the writhing inhibition produced by clonidine, xylazine and HP-aminoclonidine. Additionally, there was an excellent correlation ($r=0.9995$) which was statistically significant ($p<0.05$) between the relative potency for inhibiting writhing and affinity for *alpha*-adrenoceptor of the rat aorta. Taken together, these results support the contention that HP-aminoclonidine exerts its antinociceptive activity via *alpha*-adrenoceptor stimulation and not through interactions with opioid receptors.

REFERENCES

1. Atlas, D. and S. L. Sabol. A novel analogue of clonidine with opiate activity. *Biochem Biophys Res Commun* **94:** 924–931, 1980.
2. Atlas, D. and S. L. Sabol. Interaction of clonidine and clonidine analogues with α-adrenergic receptors of neuroblastoma X glioma hybrid cells and rat brain. *Eur J Biochem* **113:** 521–529, 1981.
3. Bentley, G. A., I. W. Copeland and J. Starr. The actions of some alpha-adrenoceptor agonists and antagonists in an antinociceptive test in mice. *J Clin Exp Pharmacol Physiol* **4:** 405–419, 1977.
4. Booth, N. H. Nonnarcotic analgesics. In: *Veterinary Pharmacology and Therapeutics,* edited by L. M. Jones, N. H. Booth and L. E. McDonald. Iowa: The Iowa State University Press, 1977, pp. 351–372.
5. Brownlee, K. A. In: *Statistical Theory and Methodology in Science and Engineering,* 2nd edition. New York: John Wiley, 1965.
6. Fielding, S. and H. Lal. Clonidine: New research in psychotropic drug pharmacology. *Med Res Rev* **1:** 97–123, 1980.
7. Fielding, S., J. Walker, M. Hynes, M. Szewzak, W. Novick and H. Lal. A comparison of clonidine with morphine for antinociceptive and antiwithdrawal actions. *J Pharmacol Exp Ther* **207:** 899–905, 1978.
8. Furchgott, R. F. and S. Bhadrakom. Reactions of strips of rabbit aorta to epinephrine, isoproterenol, sodium nitrite and other drugs. *J Pharmacol Exp Ther* **142:** 39–58, 1953.
9. Lal, H. and G. T. Shearman. Psychotropic actions of clonidine. *Prog Clin Biol Res* **77:** 99–145, 1981.

10. Paalzow, G. and L. Paalzow. Clonidine antinociceptive activity: Effects of drugs influencing central monoaminergic and cholinergic mechanisms in the rat. *Naunyn Schmiederbergs Arch Pharmacol* **292:** 119–126, 1976.
11. Reddy, S. V. R., J. L. Maderdrut and T. L. Yaksh. Spinal cord pharmacology of adrenergic agonist-mediated antinociception. *J Pharmacol Exp Ther* **213:** 525–533, 1980.
12. Ruffolo, R. R., R. D. Dillard, E. L. Yaden and J. E. Waddell. Receptor interactions of imidazolines II. Affinities and efficacies of hydroxy-substituted tolazoline derivatives in rat aorta. *J Pharmacol Exp Ther* **211:** 74–79, 1979.
13. Ruffolo, R. R., E. L. Rosing and J. E. Waddell. Receptor interactions of imidazolines I. Affinity and efficacy for alpha adrenergic receptors in rat aorta. *J Pharmacol Exp Ther* **209:** 429–436, 1979.
14. Schmitt, H., J. C. LeDouasec and N. Petillot. Antinociceptive effects of some alpha-sympathomimetic agents. *Neuropharmacology* **13:** 289–294, 1974.
15. Sewell, R. D. E. and P. S. J. Spencer. Antinociceptive activity in mice after central injections of alpha- and beta-adrenoceptor antagonists. *Br J Pharmacol* **54:** 256–257, 1975.
16. Shearman, G. T. and H. Lal. Psychopharmacology of clonidine. In: *Neuropharmacology: Clinical Applications*, edited by W. Essuian and L. Valzilli. New York: Spectrum Publications, 1982, pp. 221–255.
17. Spaulding, T. C., S. Fielding, J. J. Venafro and H. Lal. Antinociceptive activity of clonidine and its potentiation of morphine analgesia. *Eur J Pharmacol* **58:** 19–25, 1979.
18. van Rossum, J. M. Cumulative dose response curves. II. Techniques for making dose-response curves in isolated organs and the evaluation of drug parameters. *Arch Int Pharmacodyn Ther* **143:** 299–300, 1963.