Biochemical Pharmacology, Vol. 30, No. 20, pp. 2847–2850, 1981.
Printed in Great Britain.

0006-2952/81/202847-04 $02.00/0

IN VITRO BIOCHEMICAL EFFECTS OF NEFOPAM HYDROCHLORIDE, A NEW ANALGESIC AGENT*

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(*Received* 14 *July* 1980; *accepted* 25 *March* 1981)

Abstract—Nefopam hydrochloride (Acupan), an analgesic in laboratory animals and man, was a very weak inhibitor of [^{3}H]naloxone binding (IC_{50} 25 μM) to brain homogenates, in comparison to other analgesic agents. On the other hand, nefopam was a potent inhibitor of synaptosomal uptake of dopamine, norepinephrine, and serotonin, with IC_{50} values of 0.47, 0.89, and 0.34 μM respectively. The mechanism of analgesic action by nefopam probably is not related to direct actions on opiate receptors but may be related to an enhancement of monoaminergic function by uptake inhibition.

A new analgesic agent, nefopam hydrochloride (Fig. 1), is chemically and pharmacologically unique in comparison to known agents [1]. Its biochemical effects have not been examined previously. Because potent activity in an *in vitro* opiate receptor binding assay correlates with *in vivo* narcotic agonist and mixed narcotic agonist–antagonist analgesia, with most known analgesic drugs [2], demonstration of such binding affinity could indicate a mechanism of analgesic action as well as of narcotic liability.

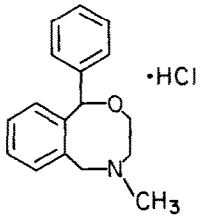

Fig. 1. Nefopam hydrochloride (5-methyl-1-phenyl-3,4,5,6-tetrahydro-1*H*-2,5-benzoxazocine hydrochloride).

Additionally, the preclinical observation of transient stimulatory and "antidepressant" effects in laboratory species [3, 4], as well as some clinical instances of a weak " stimulant-like" effect of short duration [5], suggested examination of a potential mechanism, inhibition of neuronal reuptake of biogenic amines.

In the present study the activity of nefopam hydrochloride in an opiate receptor binding assay is compared to known analgesics and to its structural predecessors, orphenadrine and diphenhydramine, which lack a rigid ring structure. Since the latter compounds and nefopam hydrochloride possess local anesthetic activity, procaine was included in these comparisons. The present study also evaluated the effects of these agents as well as tricyclic antidepressants on synaptosomal uptake of [^{3}H]dopamine, [^{3}H]norepinephrine, and [^{14}C]serotonin. Studies on amine uptake inhibition seem particularly appropriate since nefopam hydrochloride was originally studied preclinically as an antidepressant [6] prior to discovery of its analgesic properties.

METHODS

Radiolabeled materials. [^{3}H]Naloxone (10.84 Ci/mmole), 3,4-dihydroxyphenylethylamine[ethyl-1-^{3}H(N)] ([^{3}H]-DA, 15 Ci/mmole), and levo[7-^{3}H(N)]norepinephrine ([^{3}H]-NE, 5.85 Ci/mmole), were purchased from the New England Nuclear Corp. (Boston, MA). 5-Hydroxy[side chain-2-^{14}C]tryptamine creatinine sulfate ([^{14}C]-5-HT, 56 mCi/mmole) was purchased from Amersham/Searle (Arlington Heights, IL).

Opiate receptor binding assay. Male Charles River CD rats (200–250 g) were decapitated, their brains removed over ice, and the cerebella excised. After homogenization in 100 vol. of cold 50 mM Tris–HCl buffer (pH 7.7) using a Polytron PT-10, the homogenate was centrifuged at 14,500 *g* for 15 min at 0–4°, the pellet washed in Tris–HCl, and receptor binding activity determined as described by Pert and Snyder [7] and Pasternak *et al.* [8]. Following purification by thin-layer chromatography, [^{3}H]naloxone was routinely used at a final concentration of 2 nM. Drugs were dissolved in distilled water, and seven to ten concentrations were run in triplicate in the absence and presence of added sodium ion (100 mM).

Stereospecific binding of [^{3}H]naloxone was determined by subtraction of nonspecific binding, that which occurred in the presence of 100 nM levallorphan tartrate. Percent inhibition of stereospecific binding was based on comparison to control stereospecific binding (no drug) and a Litchfield–Wilcoxon ED_{50} program [9] provided IC_{50} values, the concentration required to produce 50 per cent inhibition. Statistical comparison of IC_{50} values was by one-way analysis of variance followed by LSD comparison.

Synaptosomal uptake. After brains were removed from four to five male Charles River CD rats per experiment and homogenized in ice-cold 0.32 M sucrose using Potter–Elvehjem homogenizers, they

* A preliminary report was presented at the 62nd Annual FASEB Meeting, Atlantic City, NJ, U.S.A. (1978).

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were pooled and centrifuged at 1000 *g* for 25 min at 0–4°. Synaptosomes (0.2 ml supernatant fraction) were preincubated for 2 min with Krebs–Henseleit buffer (pH 7.4) containing added glucose (11 mM), ascorbate (0.114 mM), nialamide (12.5 μM), Na_2-EDTA (27 μM), and drug. Duplicates of each reaction mixture were carried through the procedure at 37° and at 0°. Samples incubated at 37° were agitated (60 cycles/min), while those at 0° were not. At the end of the preincubation, a labeled neurotransmitter was added and the incubation continued for the time indicated: [^{3}H]DA, 0.025 μM, for 2 min; [^{3}H]NE, 0.05 μM, for 2 min; and [^{14}C]-5-HT, 0.05 μM, for 3 min. The linearity of preliminary time and concentration studies determined the incubation time and concentration for each neurotransmitter. Samples incubated at 37° were agitated under 95% O_2/5% CO_2, while 0° samples were agitated occasionally under air. Following termination of the reaction by transfer of 37° samples to a salted ice bath, synaptosomes were collected by ultrafiltration according to Baldessarini and Vogt [10] and White and Paton [11].

Active uptake was determined by subtraction of nonspecific uptake (0° sample) from its duplicate at 37°. Percent inhibition was based on comparison with controls (no drug). IC_{50} Values and statistical analyses were determined as in the binding assay.

Protein determination. Receptor and synaptosomal protein concentrations were monitored by the method of Lowry *et al.* [12], using crystalline bovine serum albumin as a standard and correcting for the presence of Tris buffer where necessary.

RESULTS

Opiate receptor binding activity. Based on a comparison of the IC_{50} values (Table 1), nefopam was 2500, 430, or 22 times less potent than morphine, pentazocine, or D-propoxyphene, respectively, in inhibiting stereospecific binding of [^{3}H]naloxone in the absence of added sodium ion (the "agonist" receptor configuration). In the presence of added sodium ("antagonist" receptor configuration), nefopam was 400, 260, or 6 times less potent, respectively. The effect of methampyrone was too weak for IC_{50} determination in either case.

Although nefopam appeared to have greater binding affinity than either of its parent compounds, orphenadrine or diphenhydramine, all three, as well as procaine, can at best be considered weakly effective in light of their high IC_{50} values.

Synaptosomal uptake. Nefopam was a potent inhibitor of the synaptosomal uptake of each of the neurotransmitters examined (Table 2). Based on comparison of the IC_{50} values in this assay nefopam was one-half to one-third as potent as *d*-amphetamine and 27 times as potent as imipramine in inhibiting [^{3}H]DA and [^{3}H]NE uptake. Although reproducible, the imipramine effect on [^{3}H]NE uptake was less potent than expected when compared to a previous study performed in this laboratory using Holtzman rats. Because of this, amitriptyline was added to the study. The amitriptyline IC_{50} correlated well with previous determinations in our laboratory, being about 4.4 times less potent than nefopam.

[^{14}C]-5-HT uptake was inhibited equally by nefopam and imipramine. Morphine, procaine, or methampyrone did not produce inhibition substantial enough to allow IC_{50} determination in any of the neurotransmitter systems, whereas orphenadrine and diphenhydramine were significantly less active then nefopam in all systems.

DISCUSSION

A positive correlation between opiate receptor binding affinity in the presence and absence of added sodium ion *in vitro* and narcotic agonist, antagonist, and agonist–antagonist potency *in vivo* has been well documented [2, 13, 14]. Excluding agents in which metabolism was involved *in vivo,* potent affinity for the agonist receptor configuration was also indicative of narcotic liability. The lack of significant interaction of nefopam with the opiate receptor, in either configuration, implies a mechanism of analgesic action different from that of the opiates, as well as a freedom from narcotic liability.

Table 1. Effects of drugs on stereospecific [^{3}H]naloxone binding to rat brain membrane preparations*

Drug	Mean IC_{50} (μM) ± S.E. Without Na^+ (−)		100 mM Na^+ (+)	
Nefopam HCl	25.83 ± 3.72	(3)	143.67 ± 36.67	(3)
Morphine sulfate	0.01 ± 0.001†	(4)	0.36 ± 0.05†	(4)
Pentazocine	0.06 ± 0.01†	(3)	0.55 ± 0.13†	(3)
D-Propoxyphene HCl	1.20 ± 0.01†	(3)	23.47 ± 2.15†	(3)
Methampyrone	‡	(3)	‡	(3)
Orphenadrine citrate	48.67 ± 0.64†	(3)	120.0 ± 20.8†	(3)
Diphenhydramine HCl	46.33 ± 0.84†	(3)	372.3 ± 54.5†	(3)
Procaine HCl	539.3 ± 47.8†	(3)	‡	(3)

* The concentration of drug producing 50 per cent inhibition of control stereospecific binding was determined by Litchfield–Wilcoxon analysis. Numbers in parentheses denote the number of IC_{50} determinations for that particular drug.

† Significantly different from nefopam hydrochloride in that category (+ or − Na^+) by one-way analysis of variance followed by LSD comparison, $\alpha = 0.05$.

‡ Effect at 10^{-3} M was too weak for IC_{50} determination.

Table 2. Effects of drugs on synaptosomal uptake*

Drug	Mean IC_{50} (μM) ± S.E.		
	[^{3}H]Dopamine	[^{14}C]Serotonin	[^{3}H]Norepinephrine
Nefopam HCl	0.47 ± 0.05	0.34 ± 0.01	0.89 ± 0.02
d-Amphetamine sulfate	0.21 ± 0.03†	6.05 ± 0.85†	0.28 ± 0.05†
Imipramine HCl	12.7 ± 1.0†	0.29 ± 0.04	24.12 ± 2.21†
Orphenadrine citrate	11.7 ± 3.1†	10.04 ± 0.90†	24.45 ± 2.38†
Diphenhydramine HCl	4.4 ± 0.80†	26.55 ± 11.9†	1.95 ± 0.46†
Morphine sulfate	‡	‡	‡
Procaine HCl	‡	‡	‡
Methampyrone		‡	
Amitriptyline HCl			3.92 ± 1.08†

* The concentration of drug producing 50 per cent inhibition of active uptake by synaptosomes was determined by Litchfield–Wilcoxon analysis; N = 5 for each group.

† Significantly different from nefopam hydrochloride by one-way analysis of variance followed by LSD comparison, $\alpha = 0.05$.

‡ Effect at 10^{-4} M was too weak for IC_{50} determination.

The endogenous ligand for the opiate receptor, enkephalin, produces a potent analgesia fully reversed by naloxone [15, 16]. If nefopam acted on the ligand rather than on the receptor site, by stimulating its production or its release, or by inhibiting its degradation, *in vivo* analgesia would be affected. Naloxone, however, would reverse such analgesia and that produced by nefopam was not reversed [1].

Recent reports have discussed the possibility of multiple opiate receptors in the brain [17, 18]. Although differential potencies between enkephalins and opiates in competition for the binding of labeled opiates and labeled enkephalins have been confirmed, the IC_{50} values in both instances are on the nanomolar level, several orders of magnitude lower than the results observed in these experiments with nefopam. Since these differential ligand affinities were subtle, our conclusions concerning nefopam would not be affected unless the relative ratio of opiate to enkephalin receptor sites in the whole brain preparation was disproportionate. This was not the case in the study by Chang *et al.* [17], which examined relative distribution of the two receptor sites.

The metabolites of nefopam are not active *in vivo* as analgesics (A. C. Conway, personal communication), suggesting that activity resides in the parent molecule. Nefopam also differs from the anti-inflammatory class of analgesics [1].

Although other investigators have reported the interaction of local anesthetics [19] and tricyclic antidepressants [20] at the opiate receptor site, the concentrations needed to produce inhibition of labeled ligand binding were high (greater than 10^{-5} M) as were results with nefopam. Such low affinity for the receptor can hardly be considered meaningful, in view of the basic concept that pharmacologic action of an opiate requires very low fractional occupancy of the receptors and, thus, high specific affinity [21]. Moreover, such results could be produced indirectly by nonspecific membrane effects which result in a conformational change in the receptor, decreasing its affinity for the labeled ligand. These considerations are particularly appropriate in view of the clinical analgesic potency of nefopam, which is one-third to one-half the potency of morphine [22].

Synaptosomal uptake studies in all three neurotransmitter systems differentiated nefopam from its structural predecessors, orphenadrine and diphenhydramine. Although the potency of nefopam in catecholaminergic systems may explain the stimulation mentioned earlier, the psychogenic properties of nefopam in monkeys resemble those of procaine more closely than *d*-amphetamine (D. M. Hammerbeck, personal communication).

Like imipramine, nefopam produced potent inhibition of the synaptosomal uptake of serotonin, which would increase serotonin availability at the post-synaptic receptor site. The literature concerned with the role of serotonin in pain sensitivity and narcotic analgesia, based on pharmacological, surgical, electrophysiological, and dietary manipulations of brain and spinal cord serotonin has been reviewed by Messing and Lytle [23]. They concluded that serotoninergic neurons have a role in the nociceptive response and in the effect of analgesic agents. Manipulations which increase serotonin neurotransmission decrease pain sensitivity or reactivity and enhance opiate analgesia, and the converse. The assumption cannot be made, however, that this is the only mechanism involved in pain sensitivity and analgesia. For example, β-endorphin has been reported to affect the metabolism of both dopamine and serotonin [24, 25].

In conclusion, nefopam is a clinically potent analgesic that appears free from narcotic liability. Although inhibition of the neuronal uptake of serotonin may be involved, further clarification of the biochemistry of central nociceptive systems and analgesia is required before the precise mechanism of analgesic action can be elucidated.

Acknowledgements—The technical assistance of D. L. Schwab is gratefully acknowledged.

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