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Reversible Inhibition of Cholinesterases by Opioids: Possible Pharmacological Consequences

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Abstract

The inhibitory potency of opioids belonging to different structural categories on electric eel and rat brain acetylcholinesterase (AChE) and horse serum butyrylcholinesterase (BuChE) was investigated.

The phenylazepine meptazinol, the pyrrolo-[2,3-*b*]-indole derivative eseroline and the benzomorphan normetazocine were the most potent inhibitors of AChE among the compounds tested. These were followed by (−)-metazocine, *N*-allylnorcyclazocine, 3-(1,3-dimethyl-3-pyrrodinyl)-phenol, levallorphan, levorphanol and pentazocine. The opioids which inhibited horse serum BuChE were in order of potency: meptazinol, methadone, profadol, levallorphan and 1,2,3-trimethyl-3-(3-hydroxyphenyl)-piperidine. The results of this work appear consistent with the fact that the anticholinesterase activity of the opioids is not confined to specific structural categories, although conformationally constrained molecules, like those of morphinans, benzomorphans or pyrrolo-[2,3-*b*]-indoles, appear to favour affinity for AChE, whereas highly flexible molecules, like those of acyclic opioids, inhibit BuChE in a rather selective way. In all cases, the inhibitory action of opioids markedly differed from that of carbamates or organophosphorous compounds, in that it was time-independent and immediately reversible on dilution.

In general the anticholinesterase action of opioids does not seem to influence appreciably the pharmacological properties of the drugs since it is evidenced at drug doses higher than those which are analgesic. However, in the case of mixed agonist/antagonist opioids with rather weak analgesic activity, the enzyme inhibition caused by the levels of circulating drugs can be so marked as to exert also a cholinergic component of action.

Anticholinesterase agents may potentiate the analgesic activity of opioids (see Green & Kitchen 1986, for review), but at the same time they may reverse other opioid responses such as respiratory depression, somnolence and intestinal motility inhibition.

We have evaluated for anti-AChE and anti-BuChE activity a number of opioids, many of which, to our knowledge, had not been tested before, with the aim of ascertaining whether this property was peculiar to a particular structural category of these drugs and if it might influence their pharmacological profile.

Materials and Methods

Materials

Acetylcholinesterase (AChE) (EC 3.1.1.7) from *Electrophorus electricus* and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) were purchased from Boehringer Mannheim GmbH, Germany. Butyrylcholinesterase (BuChE) (EC 3.1.1.8) from horse serum, acetylthiocholine iodide (ATCh), butyrylthiocholine iodide (BuTCh), ethopropazine hydrochloride, physostigmine sulphate, naloxone hydrochloride and naltrexone hydrochloride were from Sigma Chemical Co., St. Louis, MO, USA. Morphine hydrochloride was from Carlo Erba, Milan, Italy. The following drugs were gifts: levorphanol tartrate and levallorphan tartrate (F. Hoffman-La Roche & Co., Basel, Switzerland); normetazocine hydrochloride and (−)-metazocine fumarate (National Institute on Drug Abuse, Rockville, MD, USA); pentazocine hydrochloride, ketocyclazocine and ethylketocyclazocine methanesulphonate (Sterling-Winthrop Research Institute, Rensselaer, NY, USA); *N*-allylnormetazocine (Prof. J.H. Woods, University of Michigan, Ann Arbor, USA); eseroline salicylate (Prof. G. Renzi, University of Florence, Italy); (−)-5-(3-hydroxyphenyl)-2-methylmorphan (Dr E.L. May, Virginia Commonwealth University, Richmond, USA); 1,2,3-trimethyl-3-(3-hydroxyphenyl)-piperidine hydrobromide (Tanabe Seiyaku Co. Ltd., Osaka, Japan); ketobemidone hydrochloride (Ciba-Geigy, Basel, Switzerland); pethidine hydrochloride (Hoechst Italia s.p.a., Milan, Italy); 3-(1,3-dimethyl-3-pyrrodinyl)-phenol hydrochloride (Warner Lambert Co., Ann Arbor, USA); profadol (Parke, Davis & Co., Ann Arbor, USA); meptazinol (Wyeth Laboratories, Bucks, U.K.); methadone hydrochloride (Wellcome Italia s.p.a., Pomezia, Italy); (+)-propoxyphene hydrochloride (Lisapharma s.p.a., Erba, Italy).

A 20% w/v homogenate (glass/Teflon 0.13-0.18 mm clearance) of whole rat (250–300 g, Wistar) brain in 0.05 M sodium phosphate buffer, pH 7.2, was used as a source of brain AChE activity. The final dilution of wet tissue in the assay system was 1:300. This preparation hydrolysed on average 6.4 ± 0.8 μmol acetylthiocholine g^{-1} min^{-1}.

Methods

The measurements of cholinesterase activity were carried out according to the photometric method of Ellman et al (1961) using ATCh (eel and rat brain AChE) and BuTCh (horse serum BuChE) as substrates in a total 3 mL volume. Ethopropazine (10 μM) was added to brain homogenates to selectively inhibit BuChE.

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The inhibitory action of the test compounds towards cholinesterases was assessed in saturation experiments by using three different concentrations of the compounds and varying the substrate concentration between 0.031 and 0.5 mM (ATCh) or between 0.031 and 1 mM (BuTCh). The competitive, K_i, and the noncompetitive, K_i', inhibition constants were calculated from double reciprocal plots by replotting the slope and the vertical axis intercept values, respectively, vs drug concentrations (Dixon & Webb 1979).

The IC50 values on rat brain AChE of the opioids with the highest inhibitory activities were calculated from inhibition curves based on 4 or 5 different concentrations of the compounds in the presence of 0.031 mM ATCh, using the ALLFIT computer program (De Lean et al 1978).

All experiments were performed at room temperature (21–22°C) and the enzymic hydrolysis was started immediately after addition of inhibitor to the enzyme, since inhibition was seen to be time-independent.

Results

The inhibitory action of the opioids was tested on commercial preparations of electric eel AChE and horse serum BuChE and crude AChE activity from rat brain.

In all the cases examined, the inhibition was time-independent and immediately reversible upon dilution. This was ascertained by preincubating aliquots of eel AChE (0.2 U) and horse serum BuChE (0.28 U) with a fixed concentration of morphine (100 μM, AChE; 500 μM, BuChE), levorphanol (10 μM, AChE; 25 μM, BuChE) and (−)-metazocine (2 μM, AChE, 50 μM, BuChE) for time periods varying from 0 to 30 min before adding the substrate (0.5 mM ATCh or 1 mM BuChE) to start enzymic hydrolysis. It was seen that the initial (0 min preincubation) enzymic inhibition (44 ± 5% and 33 ± 4%, respectively, for morphine; 33 ± 3% and 30 ± 2%, respectively, for levorphanol; 21 ± 2% and 30 ± 4%, respectively, for (−)-metazocine) remained practically unmodified independently of preincubation duration. Besides, when aliquots of eel AChE (2.2 U), pretreated with highly inhibitory concentrations of morphine (1 mM) and levorphanol (0.3 mM), were rapidly diluted 1000 times with the chromogen solution and assayed at different times after dilution, the enzymes were seen to recover immediately (that is, at the first assay) their maximal theoretical activity (96 ± 3% and 100 ± 2%, respectively).

The double reciprocal plots obtained in saturation experiments were linear and generally indicated mixed inhibition, although sometimes this appeared prevalently competitive or, less often, non-competitive. Fig. 1 shows as an example the plots obtained using levorphanol as inhibitor of the different enzyme preparations.

The slope, K_i, and the intercept, K_i', inhibition constants (Dixon & Webb 1979) of all the opioids tested are given in Table 1. Since K_i represents the equilibrium dissociation constant of the complex between the inhibitor and the active centre of the enzyme and K_i' is thought to represent the dissociation constant between the inhibitor and a peripheral regulatory site of the enzyme (Berman et al 1981) or the acetyl-enzyme intermediate (Krupka 1963), they provide also an indication of the inhibition mechanism. As it appears from these results, all the opioids investigated possessed a certain degree of anticholinesterase activity. However, some of them, namely the morphinan antagonists naloxone and naltrexone and the phenylpiperidine pethidine, were extremely weak both as AChE or BuChE inhibitors since their competitive inhibition constant, K_i, was higher than 100 μM. By this standard, ketobemidone, methadone and (+)-propoxyphene possessed negligible affinity for AChE, but were not that weak toward BuChE. The reverse was true for morphine and eseroline. The phenylazepine meptazinol, the pyrrolo-[2,3-*b*]-indole derivative eseroline and the benzomorphan normetazocine were the most potent inhibitors of AChE of the compounds tested. These were followed by (−)-metazocine, *N*-allylnorcyclazocine, 3-(1,3-dimethyl-3-pyrrodinyl)-phenol, levallorphan, levorphanol and pentazocine. The opioids which inhibited horse serum BuChE were in order of potency meptazinol,

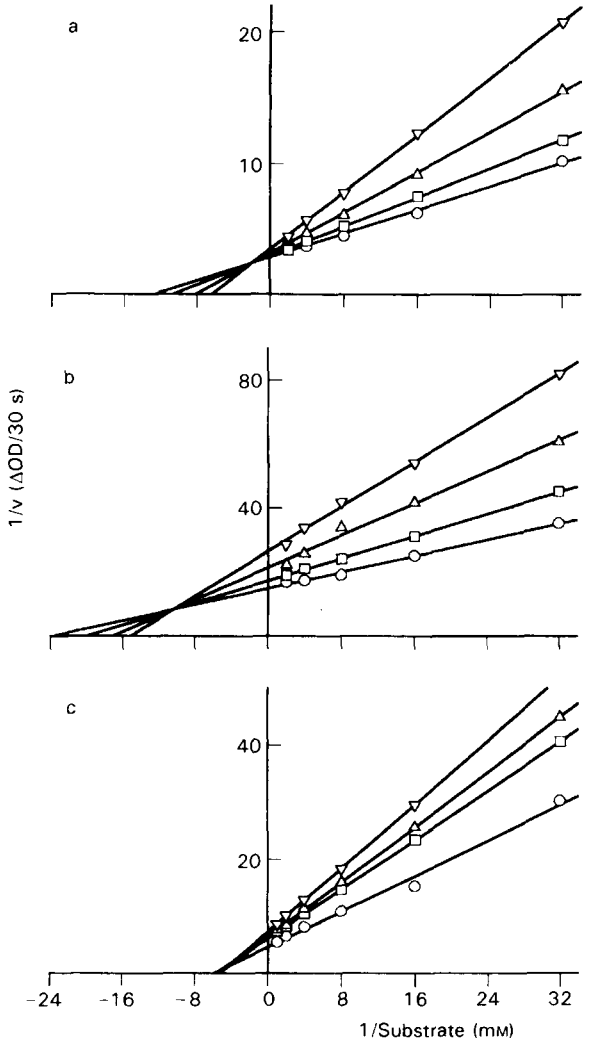

FIG. 1. Double-reciprocal plots for the inhibition of different cholinesterases by levorphanol. a. Eel AChE : ○ = buffer; □, △ and ▽ = 2.3, 4.6 and 9.2 μM levorphanol, respectively; substrate = ATCh in the range 0.031–0.5 mM. b. brain AChE: ○ = buffer; □, △ and ▽ = 2.5, 5 and 10 μM levorphanol, respectively; substrate = ATCh in the range 0.031–0.5 mM. c. serum BuChE: ○ = buffer; □, △ and ▽ = 12.5, 25 and 50 μM levorphanol, respectively; substrate = BuTCh in the range 0.031–1 mM. The points on the graph represent the means of three separate experiments.

Table 1. K_i and K_i' constants of opioids for different cholinesterases.

Compound	Electric eel AChE		Rat brain AChE		Horse serum BuChE	
	K_i (μM)	K_i' (μM)	K_i (μM)	K_i' (μM)	K_i (μM)	K_i' (μM)
Morphinans						
Levorphanol	6.0 ± 0.6	42 ± 16	5.5 ± 0.4	12.6 ± 2.0	72 ± 20	99 ± 17
Morphine	22 ± 4.0	1600 ± 120	61 ± 7.0	1370 ± 280	532 ± 60	2010 ± 240
Levallorphan	3.5 ± 0.2	13 ± 1.5	4.0 ± 0.3	7.0 ± 0.9	2.5 ± 0.2	5.5 ± 0.6
Naloxone	350 ± 42	1800 ± 210	500 ± 65	2900 ± 315	300 ± 35	1277 ± 115
Naltrexone	400 ± 51	> 10 000	280 ± 26	5430 ± 570	250 ± 21	620 ± 70
Benzomorphans						
Normetazocine	0.16 ± 0.02	10.7 ± 1.0	0.17 ± 0.02	5.5 ± 0.8	74 ± 6	44 ± 4
(−)-Metazocine	0.6 ± 0.04	55 ± 3	1.5 ± 0.1	11 ± 1.5	33 ± 3.5	80 ± 3
Pentazocine	5.5 ± 0.7	295 ± 34	9.0 ± 0.5	36 ± 3.2	16 ± 1.7	78 ± 8
N-Allylnorcyclazocine	0.9 ± 0.1	20 ± 1	2.5 ± 0.2	70 ± 7	22 ± 1	26 ± 2
Ketocyclazocine	50 ± 5	656 ± 62	55 ± 4	587 ± 50	40 ± 2	82 ± 7.5
Ethylketocyclazocine	69 ± 7	690 ± 52	40 ± 2	750 ± 57	35 ± 4	116 ± 15
Hexahydropyrrolo[2,3-*b*]-indoles						
Eseroline	0.1 ± 0.08	23 ± 4	0.6 ± 0.12	19 ± 2.1	200 ± 25	8300 ± 900
Phenylmorphans						
(−)-5-(3-hydroxyphenyl)-2-methylmorphan	43 ± 6	400 ± 55	95 ± 2	185 ± 8	30 ± 1.5	22 ± 3
Phenylpiperidines;						
1,2,3-Trimethyl-3-(3-hydroxyphenyl)piperidine	5.2 ± 0.5	360 ± 38	17 ± 2	410 ± 35	6.5 ± 0.7	2137 ± 190
Ketobemidone	250 ± 20	2260 ± 300	100 ± 8	2400 ± 185	40 ± 4.5	2100 ± 225
Pethidine	1500 ± 95	> 10 000	2400 ± 285	8200 ± 885	175 ± 15	9230 ± 735
Phenylpyrrolidines						
3-(1,3-Dimethyl-3-pyrrodinyl)-phenol	2.0 ± 0.3	30 ± 2.5	3.0 ± 0.3	345 ± 36	28 ± 2	53 ± 4.7
Profadol	11 ± 1.2	2000 ± 117	49 ± 4.1	267 ± 25	2.0 ± 0.15	9 ± 1
Phenylazepines						
Meptazinol	0.08 ± 0.01	2.0 ± 0.3	0.5 ± 0.07	2.0 ± 0.2	1.0 ± 0.15	4.9 ± 0.5
Acyclic opioids						
Methadone	132 ± 15	1900 ± 150	100 ± 8	1100 ± 105	1.5 ± 0.2	2.6 ± 0.2
(+)-Propoxyphene	2230 ± 250	5820 ± 605	1000 ± 75	5900 ± 610	18 ± 1.5	24 ± 2.5

The equilibrium dissociation constants K_i and K_i' were calculated from double reciprocal plots by replotting the slopes and the intercepts on 1/V axis versus three different concentrations of the compounds: the intercepts on base line gave K_i and K_i', respectively (Dixon & Webb 1979). The conditions of the activity assay were those reported in the legend of Fig. 1. The values are the means ± s.e.m. of 2-3 separate determinations performed in duplicate.

methadone, profadol, levallorphan and 1,2,3-trimethyl-3-(3-hydroxyphenyl)-piperidine. The ratio between the K_i values for BuChE and AChE provides a measure of selective affinity.

Table 2. IC50 values of various opioid agonists on rat brain AChE.

Compound	IC50	
	(μM)	(mg L^{-1})*
Meptazinol	0.3 ± 0.02	0.07 ± 0.005
Normetazocine	0.8 ± 0.07	0.17 ± 0.015
Eseroline	1.0 ± 0.12	0.22 ± 0.03
(−)-Metazocine	4.4 ± 0.3	1.02 ± 0.07
Pentazocine	5.0 ± 0.6	1.3 ± 0.16
Levorphanol	6.0 ± 0.5	1.56 ± 0.13
Profadol	16.0 ± 0.9	3.5 ± 0.20
Methadone	80 ± 9.2	24.8 ± 2.8
Morphine	86 ± 7.6	24.6 ± 2.2
Ketobemidone	560 ± 43	138 ± 11
(+)-Propoxyphene	1000 ± 140	333 ± 47
Pethidine	> 1000	> 247

IC50 values were calculated from 4-5 point inhibition curves, using the ALLFIT computer program (De Lean et al 1978) and are the means ± s.e.m. of at least three independent experiments performed in duplicate. ATCh (31.2 μM) was used as substrate. * Expressed as free base.

According to this criterion, the inhibitory actions of eseroline (ratio = 2000) and normetazocine (ratio = 462) were particularly selective towards AChE, whereas (+)-propoxyphene and methadone showed a preference for BuChE (ratio = 0.008 and 0.011, respectively). Meptazinol, being the most potent compound toward both AChE and BuChE, was scarcely selective (ratio = 12.5).

To have a more comprehensive measure of the overall anti-AChE potency of the opioid agonists in the central nervous system, the IC50 values of some of these drugs were measured on rat brain AChE (Table 2). In these experiments, a concentration of substrate (ATCh) close to that of the enzyme K_m (37.7 μM) was used. Under the same experimental conditions, but after a 15-min preincubation period, physostigmine IC50 was 0.02 ± 0.002 μM, corresponding to 5.5 ± 0.55 μg L^{-1}.

Discussion

These experiments confirm, and extend previous reports, indicating that opioids possess varying degrees of anticholinesterase activity.

The time-course of the inhibitory action of these drugs, however, differs markedly from that of traditional anticholinesterase agents. The opioids, lacking a reactive acylating

group, cannot covalently block the enzyme. Therefore, their inhibitory action resides exclusively on the formation of the reversible inhibitor-enzyme complex and accordingly is time-independent and instantaneously reversible upon dilution. In this respect, the action of opioids more closely resembles that of the ammonium phenols, such as edrophonium, or the tricyclic amine tacrine, which inhibit cholinesterases in a truly reversible way (Taylor 1990).

The results of this work appear consistent with the fact that the anticholinesterase activity of opioids is not confined to a specific structural category. Thus, the presence in a hydrophobic surrounding of a secondary or tertiary amino group approximately 7.4 Å away from a phenolic group, as occurs in most opioids, appears to be sufficient to confer considerable anticholinesterase activity. Also the fact that the molecule of the opioid analgesic eseroline exactly represents the polycyclic moiety of physostigmine (Galli et al 1979; Bartolini et al 1981), supports the concept that there is a certain overlap between the chemical features responsible for interaction with opioid receptors and cholinesterases. Coleman & Oswald (1993) have presented evidence that the benzomorphan *N*-allylnorcyclazocine interacts directly with the catalytic site of AChE. According to these authors, the phenol ring of the opioid binds to the catalytic residues of the enzyme and the quaternarized amine group, with its lipophilic surroundings, to an anionic subsite, consisting primarily of aromatic amino acids, located at the opening of the gorge containing the catalytic site (Sussman et al 1991). This inhibitory mechanism would explain the prevailing competitive character of the action of opioids on AChE. The non-competitive component of the inhibition, particularly present in the action of the opioids on BuChE, might underlie interaction of the drugs also with the acyl-enzyme intermediate as hypothesized for tacrine (Berman & Leonard 1992) and other reversible inhibitors (Krupka 1963). As to selectivity, a conformationally constrained structure, like that of morphinans, benzomorphans or pyrrolo-[2,3-*b*]-indoles, appears to favour affinity for AChE as compared with BuChE. The reverse seems to be true for molecules endowed with a high degree of flexibility, such as, for instance, the acyclic opioids.

In our experiments, the selective κ agonists ketocyclazocine and ethylketocyclazocine showed rather feeble anticholinesterase activity in comparison with benzomorphans interacting with μ (metazocine and normetazocine) or σ (*N*-allylnorcyclazocine) receptors. However, the inhibitory potencies of these compounds are not lower than those of several other μ opioids. Although these data are too scarce to draw definite conclusions, it seems unlikely that the anticholinesterase activity of opioids is correlated with the affinity for a specific receptor. Also the fact that among μ receptor agonists and antagonists there is no apparent correlation between anticholinesterase potency and receptor affinity as measured in binding assays (Pert & Snyder 1974), appears in line with this reasoning.

Although it is difficult to generalize about the in-vivo situation from the in-vitro one, the results of this work suggest that the opioids with the highest inhibitory potency on brain AChE, namely meptazinol, normetazocine, eseroline and (−)-metazocine, may present at high doses a component of cholinergic stimulation. The doses of these drugs used in analgesia experiments in small animals are generally high enough to inhibit supposedly a substantial fraction of the enzyme. Stephens et al (1978) have reported that in rats treated with analgesic doses of the opioid agonist/antagonist meptazinol, the drug's plasma levels were in the range 0.6–1.2 mg L^{-1}, that is considerably higher than its IC50 for brain AChE (0.07 ± 0.005 mg L^{-1}), consistent with the pharmacology of this analgesic. To our knowledge, data on the plasma levels of eseroline, normetazocine and (−)-metazocine which can be attained during analgesic treatment, are not available. However, at least in the case of eseroline (Bartolini et al 1981) and (−)-metazocine (Jacobson & May 1965), they are expected to be very low because these opioids are potent analgesics and accordingly are effective at low dosages. The analgesia induced by eseroline is reported to be unaffected by the anticholinergic atropine (Bartolini et al 1981). This finding appears to rule out the presence of a cholinergic component in eseroline analgesic action. However, we have previously shown that this opioid exerts a dual action on electrically-evoked contractions of guinea-pig ileum: inhibitory at low and excitatory at high concentrations (Fürst et al 1982). This behaviour can be explained by the assumption that the inhibitory action of the drug towards AChE prevails over the action on opioid receptors at high concentrations, whereas at low concentrations the opposite is true. As for the other opioids examined in this work, their anti-AChE activity is probably too feeble to be evident during analgesic treatment. In a clinical study, Dixon (1986) found that plasma levels of levorphanol as low as 10 μg L^{-1} are sufficient to induce analgesia. This concentration is considerably lower than its IC50 value for AChE (1.56 ± 0.13 mg L^{-1}), therefore, it is unlikely that analgesic doses of levorphanol, or of other opioids less potent than levorphanol itself, are able to affect brain AChE to such an extent as to cause a detectable cholinergic activation or to contribute to their pharmacological action.

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