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PROTECTION BY OPIOID LIGANDS AGAINST MODIFICATION OF THE OPIOID RECEPTOR BY A CARBODIIMIDE

ALAN RICHARDSON,*† JOSEPH SIMON‡ and ERIC A. BARNARD
MRC Molecular Neurobiology Unit, MRC Centre, Hills Road, Cambridge CB2 2QH, U.K.

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Abstract—Opioid receptors in membranes prepared from guinea-pig cerebellum were modified irreversibly by treatment with a water soluble carbodiimide, 1-ethyl,3-(3-dimethylaminoethyl)carbodiimide (EDAC). This decreased the number of [^{3}H]bremazocine binding sites (B_{max} reduced from 140 to 100 fmol/mg by 1 mM EDAC) without changing their affinity. When the EDAC concentration used was sufficient (500 mM) to inactivate almost all of the opioid receptors, the modification was partly prevented by inclusion of high concentrations (100 μM) of opioid agonists ([D-Ala^2, MePhe^4, Glyol^5]-enkephalin, [D-Ala^2, D-Leu^5]-enkephalin,(+)-*trans*-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzo[*b*]thiophene-4-acetamide hydrochloride), although they exhibited equal efficacy irrespective of their μ, δ or κ type selectivity. However, almost all of the opioid binding sites were protected when a guanine nucleotide analogue (GppNHp, 100 μM) was also included with the agonists during carbodiimide treatment.

The receptors for the endogenous opioid peptides are generally accepted as being divided into three types, namely μ, δ and κ. These can be measured in radioligand binding assays by the agonists [D-Ala^2, MePhe^4, Glyol^5]-enkephalin (DAGO§), [D-Ala^2, D-Leu^5]-enkephalin (DADLE) and (+)-*trans*-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl] benzo[*b*]thiophene-4-acetamide hydrochloride (PD117302), respectively [1–3], although DADLE also binds to the μ site [4]. Ligands such as bremazocine bind to each type with similar affinities [5].

The opioid receptors are believed to be members of the G-protein-linked superfamily of seven transmembrane segment receptors. Members of this family, which include the β_2-adrenergic receptor, show a decrease in the affinity of agonist binding in the presence of GTP. A series of deletion studies using the latter receptor have suggested that the ligand binding site may be within a pocket delineated by the transmembrane segments [6] and that a conserved aspartate residue may act as the counterion to the cationic amine on the ligand [7–9]. This suggests that a similar residue may be present in the opioid receptors, acting as a counterion for the N-terminal amine of the endogenous peptides or the tertiary amine present in many opiates. Thus, carboxyl-modifying reagents would be predicted to decrease ligand binding by reducing the receptor number, and a submaximal concentration of such an agent would do so without altering the affinity at the remaining sites. If the modification occurs at the ligand binding site it should be possible to prevent the modification by inclusion of opioid ligands.

In order to test whether the opioid receptor does bear such a carboxyl group, which is involved in ligand binding, we have measured whether the carboxyl-modifying reagent 1-ethyl,3-(3-dimethylaminoethyl) carbodiimide (EDAC) can irreversibly block [^{3}H]bremazocine binding to membranes prepared from guinea-pig cerebellum. This tissue contains opioid receptors of the μ, δ and κ types [10]. We have estimated previously that these are present in the relative proportions 20, 30 and 50% by comparing the relative B_{max} values obtained for [^{3}H]DAGO, [^{3}H][D-Ser^2, Leu^5]-enkephalin and [^{3}H]5-α,7-α,8-β-(−)-*N*-methyl,*N*-[(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)-dec-8-yl)]-benzeneacetamide (U69593), agonists selective for these sites, respectively (A.R. and E.A.B., submitted). The ability of μ, δ and κ-selective agonists to protect against such modification was also tested. EDAC has been used previously to modify carboxyl groups on many proteins [11–13] including bacteriorhordopsin [14]. The latter is of particular significance as it is also a member of the seven transmembrane superfamily of proteins.

MATERIALS AND METHODS

[^{3}H]Bremazocine (35 Ci/mmol) was obtained from DuPont-NEN (Stevenage, U.K.), GppNHp was obtained from Boehringer-Mannheim (Mannheim, Germany) and PD117302 was a kind gift from Dr

* Present address: Dept. of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QJ, U.K.

† Corresponding author. Tel. (0223) 334062; FAX (0223) 334040.

‡ J.S. is on leave from The Institute of Biochemistry, Biological Research Centre of the Hungarian Academy of Sciences, 6701 Szeged, Hungary.

§ Abbreviations: EDAC, 1-ethyl,3-(3-dimethylaminoethyl)carbodiimide; DADLE, [D-Ala^2, D-Leu^5]-enkephalin; DAGO, [D-Ala^2, MePhe^4, Glyol^5]-enkephalin; GppNHp, guanyl-5′-(β, γ)-imido-triphosphate; MOPS, 3-[*N*-morphilino]-propane-sulphonic acid; PD117302 (+)-*trans*-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzo[*b*] thiophene-4-acetamide hydrochloride.

John Hunter, Parke-Davis, Cambridge. All other chemicals were obtained from either the Sigma Chemical Co. or BDH (both Poole, U.K.) laboratory supplies.

Guinea-pig cerebellar membranes were prepared according to a previously published method, used for preparing membranes from rat forebrain, [15] and stored at −70° prior to use. For carbodiimide modification, the membranes were washed with 40 volumes of 10 mM 3-[*N*-morphilino]-propanesulphonic acid (MOPS) buffer, pH 7.4. The membranes were resuspended to a final protein concentration of 1.2 mg/mL in buffer A (10 mM MOPS, 6 mM $MgSO_4$, 1 mM ethyleneglycolbis (aminoethylether) tetraacetate 0.04 mg/mL soybean trypsin inhibitor, 0.32 mg/mL benzamidine hydrochloride, 0.40 mg/mL bacitracin, 0.1 mM dithiothreitol. The reaction was initiated by the addition of a freshly prepared solution of EDAC to the desired final concentration. The membranes were agitated on an orbital shaker for 30 min at room temperature. In the case of reactions performed in the presence of DAGO, DADLE or PD117302, the membranes were incubated with the protecting agents for 30 min at room temperature prior to the addition of the carbodiimide. In the case of experiments performed in the presence of GppNHp, 100 mM NaCl were also included and both agents were added with the protecting ligands. The reaction was stopped by centrifugation (15,000 *g*, 10 min at 4°) and the membranes were washed by centrifugation six times with approximately 20 volumes of MOPS buffer. The membranes were resuspended in buffer A, but with *N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid replacing MOPS. [^{3}H] (TES) Bremazocine binding was measured in triplicate by incubating the membranes in a final volume of 1 mL and at a final protein concentration of approximately 0.15 mg/mL in the presence of the radioligand for 60 min at 30°. Non-specific binding was measured in the presence of 10 μM naloxone. The reaction was terminated by filtration on a Brandell cell harvester through GF/B filters and washing with 3 × 4 mL of Tris–HCl, pH 7.4, 4°. Protein concentrations were determined by a modification of the Lowry method [16].

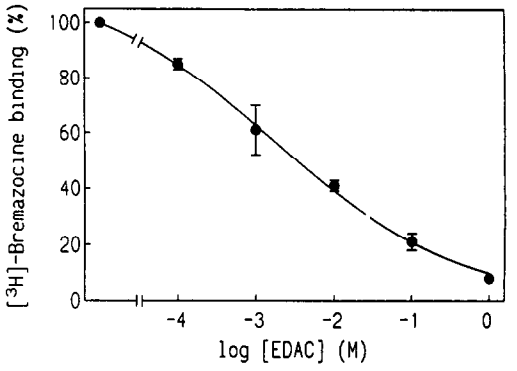

Fig. 1. Inhibition of [^{3}H]bremazocine (5 nM) binding to guinea-pig cerebellar membranes after exposure to EDAC. The results are expressed as a fraction of the binding in the absence of EDAC and are the means of three experiments, ± SEM.

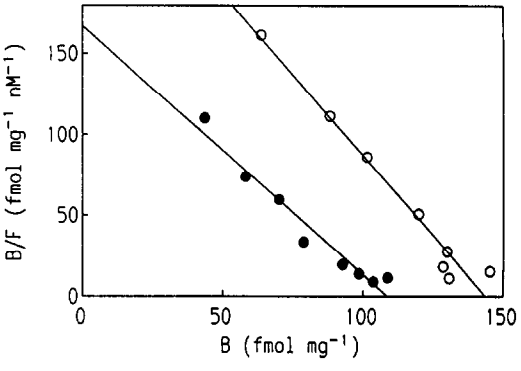

Fig. 2. Inhibition of [^{3}H]bremazocine binding to guinea-pig cerebellar by EDAC. Guinea-pig cerebellar membranes were treated with EDAC (1 mM) for 30 min (●) and the [^{3}H]bremazocine binding recovered compared to control membranes treated in parallel but in the absence of EDAC (○). In the experiment shown, the B_{max} was reduced by 21% compared to the control value and in two comparable experiments similar results were obtained. The mean reduction in B_{max} was 28 ± 5% but the affinity for [^{3}H]-bremazocine was not altered significantly.

RESULTS

The pre-treatment of guinea-pig cerebellar membranes with EDAC caused a concentration-dependent inhibition of [^{3}H]bremazocine binding (Fig. 1). EDAC had no significant effect on the non-specific binding of [^{3}H]bremazocine, which represented approximately 25% of the total binding measured. Almost complete inhibition was observed with 1 M EDAC, while half maximal inhibition was observed with approximately 5 mM EDAC. This inhibition was not reversed by washing the membranes up to seven times by centrifugation.

Saturation radioligand binding indicated that the modifying reagent (1.0 mM) decreased the number of binding sites by 28 ± 5% (N = 3) without altering the affinity of the remaining sites [B_{max} = 140 ± 17 fmol/mg, K_d = 0.55 ± 0.11 nM in control experiments and B_{max} = 100 ± 7 fmol/mg, K_d = 0.77 ± 0.09 after EDAC treatment (Fig. 2)]. In order to determine whether this inhibition could be protected against by the inclusion of DAGO, DADLE and PD117302, it was necessary to remove these ligands from the membranes after the EDAC treatment and prior to radioligand binding studies. This was achieved by centrifugation six successive times in a volume of buffer approximately equal to 20 times the volume of the membrane pellet. In experiments where the membranes were treated with 100 μM of each of the protecting agents in the absence of EDAC, 90–95% (N = 2) of the [^{3}H]-bremazocine (5 nM) binding was recovered when compared to parallel samples not exposed to the protecting agents.

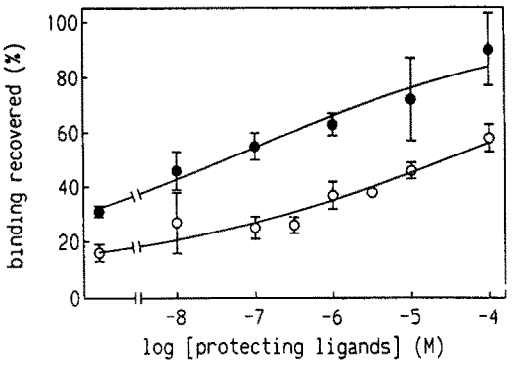

Fig. 3. Protection from EDAC inhibition of [^{3}H]-bremazocine binding by opioid ligands. Guinea-pig cerebellar membranes were treated with EDAC (500 mM) and the ability of several concentrations of DAGO, DADLE and PD117302 to protect [^{3}H]bremazocine binding (5 nM) was investigated. The protection against EDAC treatment was performed in the presence (●) or absence (○) of 100 μM GppNHp. In the experiments shown, the binding recovered was measured when all three protecting ligands were used at the same time and at equal concentrations, but indistinguishable results were obtained if the ligands were used individually. The results are expressed as a fraction of the binding in the absence of EDAC and are the means of 3–7 independent experiments ± SEM.

Membranes were treated with an almost maximal inhibitory concentration of EDAC (500 mM) in the presence of a range of concentrations of the protecting agents DAGO, DADLE and PD117302. These were unable to afford complete protection at concentrations up to 100 μM when used either individually or when all three were used at the same time and at equal concentrations, each up to 100 μM. However, in all cases a substantial proportion of the binding sites were protected from inactivation in a concentration-dependent manner (Table 1 and Fig. 3).

The protection experiments were repeated in the presence of 100 μM of a guanine nucleotide analogue, guanyl-5′-(β,γ)-imido-triphosphate (GppNHp). The optimal effects of this compound at the opioid receptors are measured in the presence of NaCl [15] and the latter was included with GppNHp in the experiments reported here for this reason. In the absence of GppNHp, 100 mM NaCl caused no significant change in the protection afforded by the ligands used above. In the absence of protecting ligands, GppNHp gave approximately a 20% protection against the carbodiimide modification. In combination with the protecting ligands, almost all the [^{3}H]bremazocine binding sites were protected by GppNHp [Fig. 3]. This was significantly greater than the protection observed in the absence of added nucleotide ($P = 0.05$). In these experiments, the protecting ligands DAGO, DADLE and PD117302 were again used either individually or all three together with GppNHp, indistinguishable results being obtained (data not shown).

DISCUSSION

EDAC caused a concentration-dependent decrease in the number of [^{3}H]bremazocine binding sites in guinea-pig cerebellum. This was not reversed by several successive washes, an observation which supports the expectation that this inhibition is irreversible. Three agonists, which at low concentrations are selective for the μ, δ and κ opioid sites, were used to protect the ligand binding site from EDAC. [A non-selective ligand, such as ethylketocyclazocine or bremazocine, could not be used on its own to protect the μ, δ and κ sites as these have slow dissociation rates (A.R. and E.A.B., unpublished observations), so complicating the subsequent recovery of [^{3}H]ligand binding.] This protection was incomplete even at a concentration of the protecting ligands of 100 μM and was not due to failure to remove the latter, as all the [^{3}H]-Bremazocine binding was recovered if the protection experiments were performed in the absence of

Table 1. Protection of [^{3}H]bremazocine binding from inactivation by EDAC by various opioid ligands

Protecting ligand	Binding recovered (%)
No ligand	19 ± 2
PD117302	55 ± 6
DAGO	47 ± 7
DADLE	50 ± 8
PD117302 + DAGO + DADLE	57 ± 8
Naloxone	16 ± 6

Guinea-pig cerebellar membranes were treated with EDAC (500 mM) in the presence of various opioid agonists (100 μM). The results are expressed as the [^{3}H]bremazocine (5 nM) binding recovered as a fraction of that obtained in the absence of added EDAC. Indistinguishable results were obtained irrespective of which agonist was used. When all three agonist were used together (each 100 μM), no further protection was achieved. The antagonist naloxone (100 μM) was unable to provide any measurable protection of [^{3}H]bremazocine binding.

EDAC. The high concentration of agonists needed to protect the ligand binding site probably reflects the need to ensure almost continual occupation of the binding site in the presence of a high concentration of EDAC. At the high ligand concentrations employed, the ligands cease to be selective for the respective opioid receptor subtypes and are able to protect the other subtypes. This explains why the same degree of protection is obtained by using the μ, δ and κ-preferring ligands either individually or in combination.

These results implicate the involvement of a carboxyl group(s) in ligand binding. However, the type of residue modified cannot be identified unambiguously as both tyrosine and cysteine can sometimes react with carbodiimides, although more slowly [17]. The modified group(s) may lie within the transmembrane region of the opioid receptor and in a position analogous to the counterion in β-adrenergic receptors [7–9]. Alternatively, the ligand binding site may lie in a large N-terminal region analogous to that found in the receptors for the polypeptide hormones lutropin-choriogonadotropia thyroid-stimulating hormone and follicle-stimulating hormone [18–20] as well as the metabotropic glutamate receptor [21]. The carboxyl group could also lie in a position outside the ligand binding site which is masked by a conformational change induced by agonist binding. The observation that the antagonist, naloxone, was unable to protect against modification supports this notion, assuming that the antagonist is unable to induce the necessary conformational change required to mask the carboxyl group. Alternatively, the agonist and antagonist binding sites may not fully overlap, as has been suggested for the muscarinic acetylcholine receptor [22], perhaps preventing the antagonist from protecting the carboxyl group.

The (small) protection obtained with GppNHp (100 μM) alone suggests that it causes some conformational change in the receptor which leads to partial protection of the ligand binding site. It is unlikely that GppNHp binds directly to the opioid ligand binding site and the degree of protection achieved was not increased with higher GppNHp concentrations (500 μM, data not shown). When GppNHp were used in combination with the opioid agonists, almost complete protection of the opioid binding site was achieved. The simplest interpretation is that the protection obtained with GppNHp is merely additive with that obtained with opioid agonists alone. An alternative and more attractive explanation is that the agonists are able to protect the ligand binding site better when it is in its low affinity state, which is induced by GppNHp and which corresponds to the receptor being uncoupled from its G-protein. Indeed, the low affinity state of the receptor may merely be one in which the agonist counterion is partially occluded by some conformational change in the protein and less available for ligand binding or EDAC modification.

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