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INCREASED BIOLOGICAL ACTIVITY OF DIMERS OF OXYMORPHONE AND ENKEPHALIN: POSSIBLE ROLE OF RECEPTOR CROSSLINKING

Eli Hazum, Kwen-Jen Chang, H.J. Leighton, O. William Lever, Jr. and Pedro Cuatrecasas

The Wellcome Research Laboratories, Research Triangle Park, NC 27709

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SUMMARY. Four analogs of oxymorphone, oxymorphaminoethylthiol, oxymorphaminoethyldisulfide, oxymorphaminoethyl-nitrobenzoic acid disulfide and oxymorphone thiazolidine, as well as the enkephalin analogs, enkephalin-thiol, Tyr-D-Ala-Gly-Phe-Leu-Lys(ε-NH)$COCH_2CH_2SH$ and the enkephalin-dimer, [Tyr-D-Ala-Gly-Phe-Leu-Lys(ε-NH)$COCH_2CH_2S$-]$_2$, were examined for binding to enkephalin and morphine receptors. The analogs gained substantial affinity for enkephalin and lost affinity for morphine receptors. The affinity of the dimers of both opiates and enkephalins was slightly greater than that achieved by the corresponding thiol monomers. However, in the guinea pig ileum the dimeric analogs were much more active than the monomers. Receptor dimerization or cross-linking may be involved in the biological activity of opiates and opioid peptides.

INTRODUCTION. It is now recognized that receptor microaggregation or cross-linking, perhaps independent of internalization, may be important for certain biological responses in some systems such as insulin, epidermal growth factor and immunoglobulins [1-6; for review, 7]. Opiate receptors in neuroblastoma cells can form large, visual clusters which do not internalize [8], and both sulfhydryl and disulfide groups are involved in cluster formation [9]. The inhibitory effects of opioids on adenylate cyclase occur before, and are independent of, the formation of gross, visual clusters [10].

Because of the possible importance of receptor-receptor and dimerization reactions, we have prepared monomers and dimers of oxymorphone and enkephalin which contain sulfhydryl and disulfide groups. Such analogs might undergo sulfhydryl-disulfide exchange reactions with reactive receptor moieties, or the dimers might passively act to cross-link receptor structures by non-covalent binding. The analogs were tested for binding to morphine (μ) and enkephalin (δ) receptors [11-12; for review, 13], as well as for activity in the guinea pig ileum assay. While the dimers of both opiates and enkephalins exhibit slightly

The abbreviations used are OMET, oxymorphaminoethylthiol; OMED, oxymorphaminoethyldisulfide; OM-NBD, oxymorphaminoethyl-nitrobenzoic acid disulfide; OMTZ, oxymorphone thiazolidine; enkephalin-thiol, Tyr-D-Ala-Gly-Phe-Leu-Lys (ε-NH)$COCH_2CH_2SH$; enkephalin-dimer, [Tyr-D-Ala-Gly-Phe-Leu-Lys(ε-NH)$COCH_2CH_2S$-]$_2$

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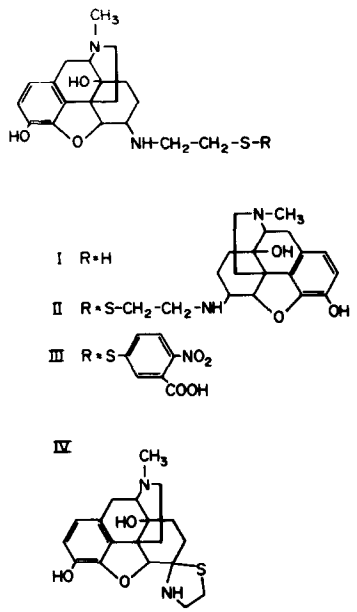

Fig 1. The structures of oxymorphaminoethylthiol (I, OMET), oxymorphaminoethyl-disulfide (II, OMED), oxymorphaminoethyl-nitrobenzoic acid disulfide (III, OM-NBD) and oxymorphone thiazolidine (IV, OMTZ).

greater affinity than the monomers for receptors in membranes, their potencies in the guinea pig ileum are much greater (7-times and 2-times, respectively), suggesting the possible importance of receptor aggregation or cross-linking.

MATERIALS AND METHODS. Materials. The protected hexapeptide, Boc-Tyr(OtBu)-D-Ala-Gly-Phe-Leu-Lys, was provided by Dr. S. Wilkinson, The Wellcome Research Laboratories, Beckenham, England. Oxymorphone was kindly donated by Endo, Garden City, N.Y. ^{125}I-[D-Ala^2, D-Leu^5]enkephalin (11,12) had a specific activity of 1 to 2 Ci per μmol. [^{3}H]Naloxone (23 Ci per mmol) was from NEN. We wish to thank B. Soltmann for the mass spectral data.
Synthesis. Reaction of oxymorphone hydrochloride with cysteamine (10 equivalents) in absolute methanol in the presence of triethylamine (1 equivalent) led to thiazolidine (OMTZ, IV in Fig. 1) as the major product. When the reaction was quenched after 2 hr with 5% sodium borohydride (4°C), chloroform extraction and recrystallization from chloroform-petroleum ether provided a mixture of IV and oxymorphaminoethylthiol (OMET, I in Fig. 1), which had Rf values of 0.40 and 0.29, respectively, on silica gel with methanol:ammonia (97:3, v/v; the R_f value for oxymorphone was 0.46 in this system). Preparative TLC under these conditions followed by recrystallization from chloroform-hexane provided oxymorphaminoethylthiol (OMET, I in Fig. 1); molecular ion m/e 362 by field desorption mass spectrometry: dipping method, B.A.T. 20 mA, containing a small amount of the corresponding disulfide (OMED, II; m/e 722). The stereochemistry at C-6 was not determined.

Thiazolidine (IV in Fig. 1) was also obtained by refluxing a well-stirred suspension of oxymorphone hydrochloride and cysteamine (1.4 equivalents) in degassed toluene under nitrogen for 1.5 hr. An additional 0.6 equivalent of cysteamine was added and refluxing continued 1.5 hr longer. After removal of solvent under reduced pressure, the residue was treated with water and the pH was adjusted to 8 with sodium bicarbonate. Chloroform extraction followed by preparative TLC on silica gel with chloroform:methanol:ammonia (50:49.9:0.10, v/v) and recrystallization from chloroform-hexane gave OMTZ (IV in Fig. 1) as a mixture of stereoisomers (nmr in $CDCl_3$:H_5 protons at

δ4.98 and δ4.54) which migrated as a single spot on silica gel in two solvent systems (Rf 0.40 in methanol:ammonia, 97:3; Rf 0.24 in chloroform:methanol, 1:1). The mass spectrum (EI, 70eV, 245°C) showed a molecular ion at m/e 360 (100%). Oxymorphaminoethyl-nitrobenzoic acid disulfide (OM-NBD, III in Fig. 1) was prepared by reacting compound I with 5,5'-dithiobis(2-nitrobenzoic acid). Enkephalin-dimer, [Tyr-D-Ala-Gly-Phe-Leu-Lys(ε-NH)$COCH_2CH_2S$-]$_2$, was prepared by reaction of 2 equivalents of Boc-Tyr(OtBu)-D-Ala-Gly-Phe-Leu-Lys with one equivalent of dithiobis(succinimidyl propionate) in absolute methanol (10% dimethylformamide) in the presence of 2.2 equivalents of triethylamine. After standing at 24°C for 24 hr, the reaction was evaporated to dryness *in vacuo* and treated for 10 min with anhydrous trifluoroacetic acid. The deprotected dimer was crystallized from dimethylformamide-ethyl acetate, and finally purified on TLC (silica); R_f = 0.78 in chloroform:methanol (1:2, v/v). Enkephalin-thiol, Tyr-D-Ala-Gly-Phe-Leu-Lys(ε-NH)$COCH_2CH_2SH$, was obtained by reducing the enkephalin-dimer with DTT; R_f = 0.68 in chloroform:methanol (1:2, v/v).

Binding Assay. Brain membranes (11) were suspended in 2 volumes (original wet weight) of 50 mM Tris·HCl and stored at -20°C. Binding assays were performed for 60 min at 24°C [11,12] using a filtration method (GF/C filters). Final volumes were 0.25 ml (containing 2 mM Mg^{2+}) for ^{125}I-[D-Ala^2,D-Leu^5]-enkephalin (0.2 nM) and 2 ml for the ^{3}H-naloxone (0.38 nM) narcotics. The variability of the duplicates was usually less than 10% of the mean.

Guinea Pig Ileum Assays. Ilea were prepared according to Paton and Vizi [14] except that the longitudinal muscle was not prepared from the circular muscle. Following equilibration (isometric conditions, 37°C, 1.0 g resting tension) in Krebs-Henseleit buffer (composition in mmol per liter: NaCl, 118.1; KCl, 4.15; $NaHCO_3$, 25.5; $MgSO_4$, 1.2; $CaCl_2$, 2.5; KH_2PO_4, 1.23; and D-glucose, 11.1), the tissues were field-stimulated (square wave pulse, 0.1 Hz, 0.5 ms duration, supramaximal voltage). After the twitch responses to electrical stimulation had stabilized, non-cumulative concentration-response curves were run. Biological assays of OMET (I in Fig. 1) were done in DTT to ensure complete reduction of disulfide; conversion of OMET to the dimeric OMED (II in Fig. 1) prior to assay was done by *in situ* oxidation with hydrogen peroxide. Hydrogen peroxide and DTT alone did not affect the binding or ileum assays.

RESULTS. *Synthesis of monomers and dimers*. Four analogs of oxymorphone were prepared by modification of the C-6 position as shown in Fig. 1. Oxymorphaminoethylthiol (OMET, I) and oxymorphone thiazolidine (OMTZ, IV) were synthesized by coupling cysteamine to oxymorphone, and oxymorphaminoethyl-disulfide (OMED, II) was prepared by oxidizing OMET with hydrogen peroxide. Oxymorphaminoethyl-nitrobenzoic acid disulfide (OM-NBD, III) was prepared by reacting OMET with 5,5'-dithiobis(2-nitrobenzoic acid) [15]. The coupling was done by substitution of the oxygen at the C-6 position of oxymorphone since modification at this position does not alter the agonist-antagonist properties [16,17]. The two enkephalin analogs were prepared by addition of a lysine residue at the carboxyl terminal and subsequent chemical modification with dithiobis(succinimidyl propionate) to give the dimer, [Tyr-D-Ala-Gly-Phe-Leu-Lys(ε-NH)$COCH_2CH_2S$-]$_2$. The thiol, Tyr-D-Ala-Gly-Phe-Leu-Lys(ε-NH)$COCH_2CH_2SH$, was obtained by reducing the dimer. The protected hexapeptide, Boc-Tyr(OtBu)-D-Ala-Gly-Phe-Leu-Lys, was used as the starting material since a) substitution of D-Ala in the second position of enkephalin results in a more potent and metabolically stable derivative [18] and b) the ε-amino group of lysine

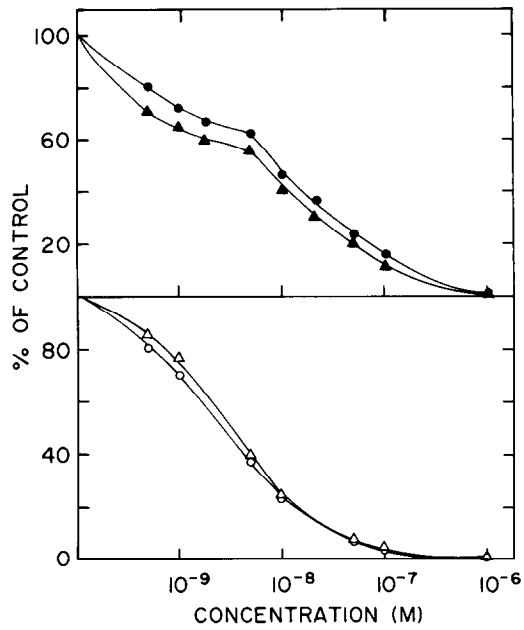

Fig. 2. Lower panel, inhibition of binding of [^{3}H]naloxone to brain membranes by OMED (O——O) and OMET (Δ——Δ). Upper panel, competition of binding of ^{125}I-[D-Ala^2,D-Leu^5] enkephalin to brain membranes by OMET (O——O) and OMED (Δ——Δ).

serves as a spacer for the substitution reaction. The two peptides were homogeneous by TLC and stained with ninhydrin.

Binding properties. Using ^{125}I-[D-Ala^2, D-Leu^5]enkephalin OMET (I) and OMED (II) display apparent IC_{50} values of about 10 nM, 6 nM, 15 nM and 8 nM, for compounds I to IV, respectively (Fig. 1 and 2, upper; Table 1). (The affinities of morphine and oxymorphone are 35 nM and 21 nM, respectively [12]). Thus, the analogs gain substantial affinity for enkephalin receptors. With [^{3}H]naloxone the apparent IC_{50} values were 3.0 nM, 2.8 nM, 3.0 nM and 1.6 nM (Fig. 1

TABLE 1. Comparison of the potency of oxymorphone and enkephalin derivatives for enkephalin and morphine receptors in brain membrane preparations and their biological activity in the guinea pig ileum assay

Compound	IC_{50}[a], nM ± S.E.		ID_{50}[d], μM ± S.E.
	Enkephalin (δ) receptors[b]	Morphine (μ) receptors[c]	
Oxymorphone	21.0 ± 1.8	0.3 ± 0.1	0.02 ± 0.01
OMET (I)	10.0 ± 1.2	3.0 ± 0.3	0.40 ± 0.05
OMED (II)	6.1 ± 0.6	2.8 ± 0.2	0.06 ± 0.01
OM-NBD (III)	15.0 ± 2.2	3.0 ± 0.4	0.50 ± 0.22
OMTZ (IV)	8.0 ± 0.7	1.6 ± 0.1	0.24 ± 0.16
[D-Ala^2,Leu^5] enkephalin	1.2 ± 0.2	5.0 ± 2	0.20 ± 0.05
Enkephalin-thiol	1.2 ± 0.1	50 ± 4	0.86 ± 0.08
Enkephalin-dimer	1.0 ± 0.1	40 ± 3	0.48 ± 0.20

[a] IC_{50}, concentration causing 50% inhibition of specific binding.
[b] Binding assays with ^{125}I-[D-Ala^2,D-Leu^5]enkephalin.
[c] Binding assays with [^{3}H]naloxone.
[d] ID_{50}, concentration causing 50% depression of smooth muscle contractions.

and 2, lower; Table 1) for compounds I to IV, respectively, suggesting the loss of affinity for morphine receptors. (The affinities of morphine and oxymorphone are 0.4 nM and 0.3 nM, respectively [12]). The enkephalin-thiol and dimer have normal binding affinities for enkephalin receptors (1.2 nM and 1.0 nM, respectively) but are lower for morphine receptors (50 nM and 40 nM, respectively) (Table 1).

Guinea pig ileum assay. The ID_{50} values of OMET (I) and OMED (II) were 400 nM and 60 nM, respectively (Fig. 1, Table 1); for oxymorphone, morphine, OM-NBD (III) and OMTZ (IV) they were 20, 77, 500 and 240 nM, respectively. The ID_{50} values for enkephalin-thiol and enkephalin-dimer were 860 nM and 480 nM, respectively. The potencies of the dimers are thus greater than those of the monomers.

DISCUSSION. Several recent studies suggest that receptor microaggregation or cross-linking may be important for certain biological responses in some systems [1-7]. Bivalent antibodies to the insulin receptor bind to insulin receptors and trigger many of the same biological responses caused by insulin [1,2]. Monovalent Fab' fragments compete for binding but do not initiate biological responses. However, addition of anti-Fab' antibodies to crosslink the Fab'-receptor complexes restores the insulin-like activity [2]. Furthermore, under certain conditions bivalent but not univalent antibodies directed to insulin and EGF can dramatically enhance the activity of very low concentrations of these hormones in fibroblasts [3]. An inactive analog of EGF (cyanogen bromide-cleaved EGF) retains its ability to bind but does not induce aggregation (patch formation) of receptors [4]. Addition of bivalent anti-EGF antibody restores both the bioactivity and the morphological cross-linking (patch formation). There is evidence that basophil and mast cell systems require cross-linking of their Fc receptors for induction of histamine release [5,6].

We have shown previously [11,13] the existence of two types of opiate receptors in brain membranes: enkephalin (δ) receptors, which bind enkephalins and their analogs with high affinity and narcotics with low affinity, and morphine (μ) receptors, which bind enkephalins and narcotics with reversed affinities. The oxymorphone analogs studied here gained substantial affinity for enkephalin receptors and lost some affinity for morphine receptors, while the two enkephalin analogs have higher affinity for enkephalin receptors. The affinities of the dimers (OMED and enkephalin-dimer) for both receptors are slightly higher than those of the monomers (OMET and enkephalin-thiol), suggesting that dimerization of receptors may enhance slightly the binding affinity (at 24°) in isolated brain membranes.

Opiates are more potent than enkephalins in suppressing electrically stimulated muscle contractions in the isolated guinea pig ileum, indicating

that this tissue contains predominantly morphine (μ) receptors [19], or that the intrinsic efficacy of the μ-receptors is greater. The potency of OMED (II) was 7-times greater than that achieved with OMET (I). This is probably due to the dimerization of OMET and not simply to the presence of a disulfide bond or decrease in activity of the thiol compound since OM-NBD (III), which also contains a disulfide bond, is less active than OMED (II) but equally active to OMET (I) (Fig. 1, Table 1). It is also not the result of a bulky substituent on the C-6 position since OM-NBD (III) and OMTZ (IV) are less active (Fig. 1, Table 1). Finally, the increased activity is not due to the formation of covalent bonds with sulfhydryl groups in the receptor [20-23] since in both the binding and ileum assays the effects of all the analogs are readily reversible. The enkephalin-dimer was 2-times more active than its monomer in this assay. Another type of dimer, N^{α}, N^{ε}-bis(D-Ala^2, Met^5) enkephalin, is 4-times more potent than [D-Ala^2, Met^5]enkephalinamide in the rat after central injection [24].

Dimerization of opiates and enkephalins may thus dramatically increase their biological activity. Although little effect was shown on binding affinities, these studies were done in isolated membrane systems, where receptor rearrangements (such as aggregation) may not occur readily, and the assays were performed at 24°, also possibly decreasing microaggregation. However, the data suggest that aggregation or cross-linking of opiate receptors, or simultaneous occupation of two binding sites on the same receptor by the dimers, may be important for the immediate biological responses of opiates and opioid peptides. Other possible explanations could of course exist. The acute inhibitory effects of opiates and enkephalins on adenylate cyclase activity can occur when the receptors are in a diffuse state rather than in clustered patterns. Therefore, if microaggregation is involved in these effects it must be submicroscopic, perhaps involving only dimerization or limited cross-linking reactions.

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