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Side Chain Modifications Change the Binding and Agonist Properties of Endomorphin 2

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Side chain modifications were introduced to endomorphin 2 (E2) to improve its binding properties and biological activity. A number of C-terminal modifications decreased the binding affinity to the mu-opioid receptor and the intrinsic activity in rat brain membranes. The exception was E2-ol, which showed increased binding affinity to MOR and higher potency in stimulating [^{35}S]GTPγS binding. N-methylation of Phe^3 (MePhe^3) attenuated the binding affinity and produced a rightward shift of [^{35}S]GTPγS binding curves. All derivatives had lower intrinsic activity than E2. Some of the modified peptides partially inhibited, while YPF-benzyl-allyl-amide fully inhibited, the E2 or [D-Ala^2,MePhe^4,Gly^{5}ol]enkephalin stimulated [^{35}S]-GTPγS binding. Marked differences were found between the results obtained using tritiated E2, tritiated naloxone, and [^{35}S]GTPγS binding, indicating the possible involvement of multiple binding sites. The data presented demonstrate that the C-terminal amide group has an essential role in the regulation of the binding and the agonist/antagonist properties of E2. © 2002 Elsevier Science

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Endomorphin 2 (E2:Tyr-Pro-Phe-Phe-NH_2) is a putative endogenous mu-opioid receptor (MOR) ligand (1) that displays strong analgesic activity (1, 2) and most of its physiological effects are similar to those of other MOR specific opioid ligands (for review see Vaccarino *et al.* (3)). Despite the similar physiological effects, the amino acid sequence of E2 is quite distinct from those of the traditional "typical" endogenous opioids (enkephalins, endorphins, dynorphins). Beside being the shortest (it is only four amino acids long), the second amino acid in E2 is proline instead of glycine and its C-terminal is amidated (1), which seems to be crucial for efficient binding to MOR (4). The presence of the cyclic amino acid Pro at position 2 not only influences the conformation of peptide chains, but also confers stability against most proteases (5). Its pharmacological characterisation, using different cell membrane preparations, has shown that E2 binds to the MOR with high affinity (1, 6, 7), activates G-proteins, and inhibits adenylyl cyclase (7, 8).

The binding of the radiolabelled non-hydrolyzable GTP analog [^{35}S]GTPγS has been previously used to provide a measure of G-protein activation by agonists (9, 10). Because G-protein activation is the first biochemical step after opioid receptor activation and is not limited by downstream effector systems, this assay provides a very direct measurement of efficacy. The correlation between the intrinsic activity of a drug in this assay and its potency *in vivo* makes it an appropriate system for measuring the relative efficacies of endogenous opioid peptides, which are not well characterised (11–13). [^{35}S]GTPγS binding stimulated by the MOR selective ligand DAMGO ([D-Ala^2, MePhe^4,Gly^{5}ol]enkephalin) has been shown to occur in several different membrane preparations (7, 12–15) and is absent in MOR knockout mice brain (8). DAMGO acts as a full agonist in the [^{35}S]GTPγS binding assay, producing a naloxone reversible stimulation (15). E2 is as effective in analgesic test as DAMGO (2, 16–18); however, its intrinsic activity in the [^{35}S]GTPγS binding assay is only about 65–70% of that induced by DAMGO (15, 19). Hence, E2 belongs to the

Abbreviations used: amino acids are abbreviated by their single-letter or 3-letter conventional codes; ACSF: artificial cerebrospinal fluid; MePhe: *N*-methylphenylalanine; DAMGO: [D-Ala^2,MePhe^4, Gly^{5}ol]enkephalin; E1: endomorphin 1; E2: endomorphin 2; Tyr-Pro-Phe-Phe-NH_2; E2-OH: Tyr-Pro-Phe-Phe-OH; E2-ol: Tyr-Pro-Phe-phenylalaninol; MOR: mu-opioid receptor; n_H: Hill coefficient; Nx: naloxone.

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same class of opioid agonists as morphine and fentanyl, which have been termed high-potency partial agonists (19, 20).

Comparison of the structures of DAMGO and E2 by NMR revealed similarities between the two peptides, but also highlighted that the structure of E2 is more restricted, which might explain the lower intrinsic activity in the [^{35}S]GTPγS binding assay. The similarity in the biological activity as well as in the predicted conformation population prompted us to study the influence of modifications of Phe^3 and Phe^4. Since in DAMGO the C-terminal residue does not contain any carboxylic acid related functionality, we applied a wide set of modifications to study the effect of the structural changes (elimination or replacement of the carboxamide group) on the binding affinity to MOR and the intrinsic activity of E2.

MATERIALS AND METHODS

Chemicals. All amino acid derivatives, piperidine, trifluoroacetic acid, Rink amide, and 2-chlorotritylchloride resins were obtained from Reanal, Hungary. Peptide amides were synthesised on Rink amide resin whereas peptide acids were prepared on 2-chlorotrityl resin with our standard Fmoc protocol (21). Substituted peptide amides were prepared by the combination of solid phase and solution phase peptide synthesis, and the peptide acids synthesised on solid phase were coupled with the corresponding amines in solution.

Tyr-Pro-Phe-Phe-ol (endomorphin 2-ol, E2-ol): Fmoc-Phe-ol was synthesized from Fmoc-Phe-OCH_3 following literature procedures (22) with the modification that acetoxyborohydride (23) was prepared separately. Fmoc-Phe-ol was coupled to 2-chlorotrityl resin in the presence of pyridine and dimethyl formamide (24). Peptide synthesis and the further steps followed the standard peptide synthetic protocol.

[^{3}H]Pro^2-E2 (sp act, 51 Ci/mmol (25)) and [^{3}H]naloxone (sp act, 35 Ci/mmol (26)) were synthesised at the Isotope Laboratory of the Biological Research Centre, Szeged. [^{35}S]GTPγS was purchased from Amersham. All other chemicals used in this study were of analytical grade and purchased from Sigma (St. Louis, MO).

Preparation of rat brain membranes. A crude membrane fraction was prepared from Wistar rat brains according to an earlier published method (27). The protein concentration was determined by the Bradford method (28), using bovine serum albumin as standard.

Receptor binding assay. The experiments were performed in glass tubes in 50 mM Tris–HCl buffer (pH 7.4) in a final volume of 1 ml. The protein concentration was 0.24 mg/ml. Incubation (45 min at 25°C for [^{3}H]Pro^2-E2 and 60 min at 0°C for [^{3}H]Nx) was started by addition of membrane suspension and terminated by rapid vacuum filtration through Whatman GF/C filters using a Brandel cell harvester. After three washes with 5 ml portions of ice-cold buffer, the filters were dried at 37°C. To determine the sodium index (Na^+ index) the displacement experiments were repeated in the presence of 100 mM NaCl and the resulting IC_{50} values were divided by the values obtained in the absence of sodium. Values below 1 indicate antagonist and above 10 full agonist properties. Partial agonists have Na^+ indexes between 1 and 10 (29). The radioactivity was measured in a toluene-based scintillation cocktail using a Wallac 1409 scintillation counter. The displacement curves were analysed with the software GraphPad Prism (30) using a nonlinear least-squares algorithm. All experiments were carried out in duplicate assays and repeated at least three times. The given values are means ± SEM.

[^{35}S]GTPγS binding. Tubes containing 10 μg of protein, 30 μM GDP, 10^{-9}–10^{-5} M opioid ligands, and 0.05 nM [^{35}S]GTPγS, all in 50 mM Tris–HCl (pH 7.4) buffer containing 1 mM EGTA, 100 mM NaCl, and 3 mM $MgCl_2$ in a final volume of 1 ml were incubated for 1 h at 30°C. Basal activity was determined by subtracting the non-specific binding (measured in the presence of 100 μM unlabelled GTPγS) from the total counts (measured in the absence of tested compounds). The incubation was started by the addition of the [^{35}S]GTPγS and was terminated by filtrating the samples through Whatman GF/B filters using a Brandel cell harvester. Filters were washed three times with ice-cold 50 mM Tris–HCl buffer (pH 7.4) and then dried. Bound radioactivity was measured in a Wallac 1409 scintillation counter using a toluene-based scintillation cocktail. Stimulation is given as percentage of basal activity. Data were calculated from at least three independent experiments performed in triplicates. Statistical analysis of the data was made by Student's t test or by ANOVA. A probability level of $P < 0.05$ was accepted as indicating significant differences.

RESULTS

Systematic investigation of the effect of C-terminal phenylalanine modification and N-methylation of Phe^3 on the E2 structure was performed in this study (Fig. 1). Besides the C-terminal acid derivative of E2 (Tyr-Pro-Phe-Phe-OH, E2-OH) complete elimination of Phe^4-NH_2 (Tyr-Pro-Phe-OH), several tripeptide amide and partially modified tetrapeptide derivatives were synthesized as described under Materials and Methods. The carboxamide group of E2 was substituted by hydroxymethyl (E2-ol), by allyl (Tyr-Pro-Phe-benzyl-allyl-amide), by hydrogen (Tyr-Pro-Phe-phenethyl-amide) to test the importance of hydrogen bonding and electron donor–acceptor properties at the C-terminal position. In addition, Phe^3 was substituted by *N*-methylphenylalanine in E2 (MePhe^3-E2) and E2-ol (MePhe^3-E2-ol).

The ability of these peptides to bind to MOR was assessed by using tritiated endomorphin 2 ([^{3}H]Pro^2-E2) (25) and tritiated naloxone ([^{3}H]Nx) (26) in competitive radioligand binding experiments in order to determine their equilibrium inhibition constants (IC_{50}). The MOR binding affinity of the different E2 derivatives was compared to DAMGO and E2. All peptides fully displaced tritiated E2 (Fig. 2) but their affinity to MOR was different (Table 1), reflecting the effect of structural modifications on the binding affinity. E2-ol had an IC_{50} similar to DAMGO (0.63 and 0.63 nM, respectively) while E2, the parent compound, was less potent in displacing [^{3}H]Pro^2-E2 (IC_{50} = 4 nM). Substitution of Phe^3 by *N*-methylphenylalanine in E2 (MePhe^3-E2) produced a small increase in the IC_{50} values compared to the parent compound (IC_{50} = 10.2 nM). Applying both the amide to alcohol conversion and the N-methylation of Phe^3 on E2 molecule the MOR affinity of MePhe^3-E2-ol decreased by two orders of magnitude (IC_{50} = 69 nM). Apart from E2-ol, the C-terminally modified peptides were considerably less potent in inhibiting the binding of [^{3}H]Pro^2-E2 (Table 1).

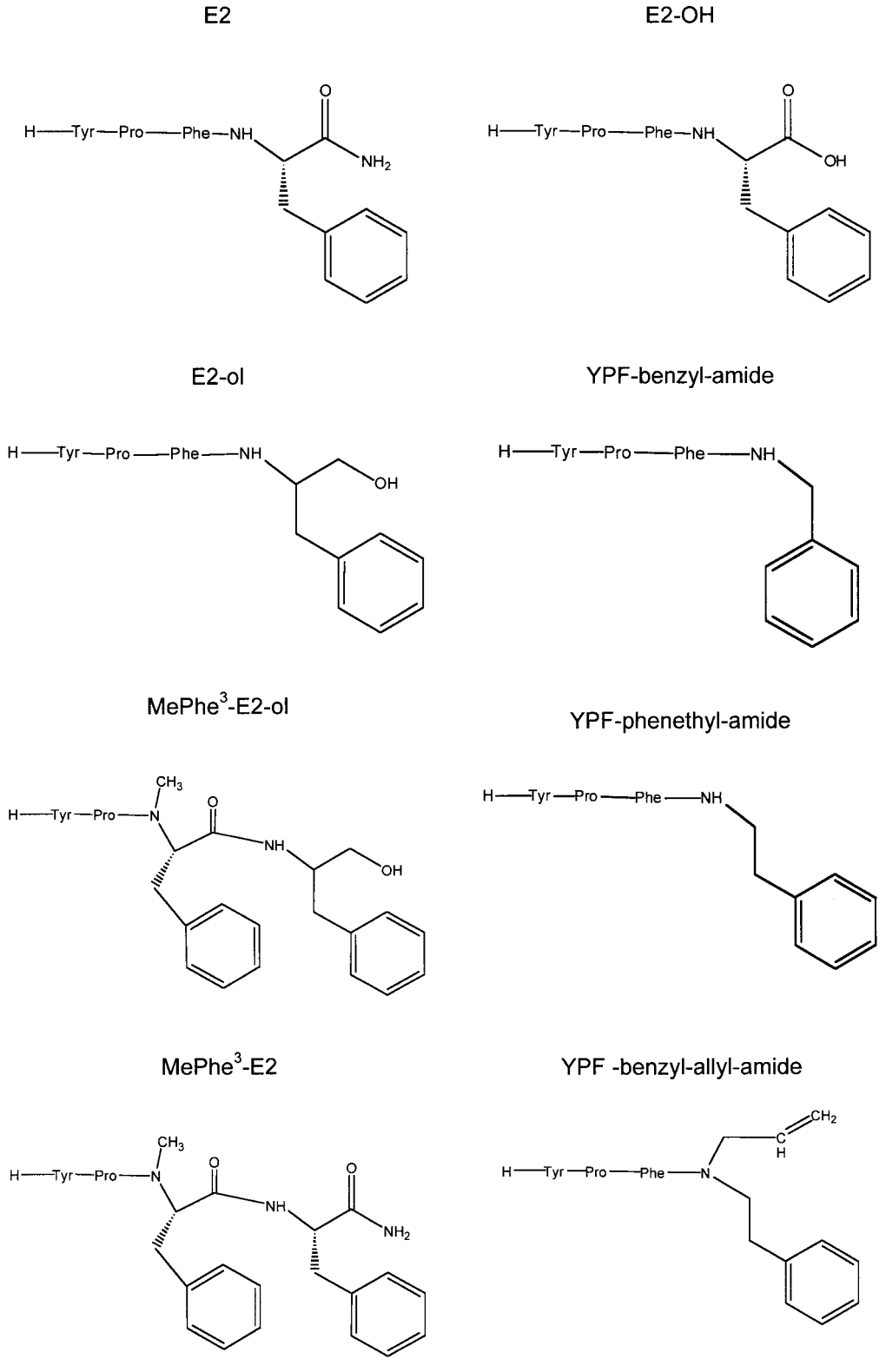

FIG. 1. Schematic diagram of E2 derivatives discussed in the current study.

[^{3}H]Nx binding was inhibited by E2 in the low nanomolar concentration range (IC_{50} = 7.2 nM) while E2-ol was less potent in displacing this ligand (IC_{50} = 51.3 nM). The rest of the derivatives had >100 nM affinities for the [^{3}H]Nx binding site (Table 1). The [^{3}H]Nx binding assay was repeated in the presence of 100 mM

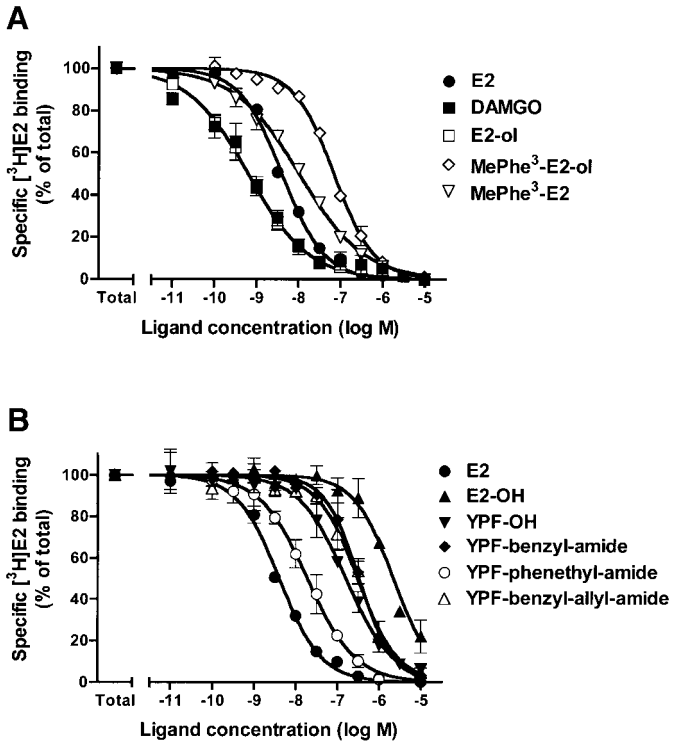

FIG. 2. Displacement of [^{3}H]Pro^2-E2 with DAMGO, E2, and its derivatives. Rat brain membranes were incubated with 0.4 nM radioligand as described under Materials and Methods in the presence of increasing concentrations (10^{-11}–10^{-5} M) of E2 derivatives that resemble DAMGO (A) or modified on the C-terminus only (B). Specific binding was determined by subtracting the values obtained in the presence of 10^{-5} M E2 from the total binding and used for the normalisation of the data as 100%. Data points are the averages of at least 3 independent experiments in duplicates ± SEM.

NaCl, a similar condition that was used to measure [^{35}S]GTPγS binding. Inclusion of 100 mM NaCl inhibited ligand binding to different extent (Table 1), producing a more than 10-fold IC_{50} increase in case of E2, DAMGO, MePhe^3-E2-ol and about 10-fold increase with YPF-benzyl-amide and YPF-phenethyl-amide. Less than 10-fold shift was observed in the case of E2-ol, MePhe^3-E2, and YPF-benzyl-allyl-amide, implying that these compounds are partial agonists.

The displacement curves were shallow for DAMGO, E2-ol, and Me-Phe^3-E2, as indicated by their Hill coefficients (n_H), (0.53, 0.6, and 0.56 respectively), while the rest of the peptides had an n_H close to unity (data not shown). These results suggest that the amide–alcohol substitution changed the way the ligand interacts with the receptor. It is interesting to note, however, that in the case of the N-methylated molecule (MePhe^3-E2-ol) the competitive binding curve follows again the mass action with serious loss of binding potency. In the presence of 100 mM NaCl the n_H for all ligands was around unity (data not shown).

To determine the effects of the chemical modifications on a more complex system involving not only binding but also G-protein activation, the stimulation of [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate ([^{35}S]GTPγS) binding to rat brain membranes by the structurally modified E2 derivatives was measured and compared to the effect exerted by E2 and DAMGO (Fig. 3). The E2 stimulated maximal [^{35}S]GTPγS binding (154%) was approximately 70% of the DAMGO stimulated maximal activity (181%) (Table 2). The maximal stimulations of [^{35}S]GTPγS binding by MePhe^3-E2, E2-ol, MePhe^3-E2-ol, YPF-phenethyl-amide, or YPF-benzyl-amide (142, 131, 130, 116, and 117%, respectively) were all below the E2 or DAMGO stimulated maximum (Table 1). Despite inhibiting both [^{3}H]Pro^2-E2 and [^{3}H]Nx binding, YPF-benzyl-allyl-amide, E2-OH, and YPF-OH had no agonist properties in this assay.

The stimulation of [^{35}S]GTPγS binding was concentration dependent. The most potent activator was E2-ol (EC_{50} = 27.2 nM), while the potencies of E2 or DAMGO (EC_{50}: 496 and 313 nM, respectively) were one order of magnitude lower (Table 1). N-methylation of E2-ol or E2 decreased the potencies by one order of magnitude compared to the corresponding non-methylated compounds (MePhe^3-E2-ol: 450 nM; MePhe^3-E2: 3600 nM). YPF-phenyl-ethyl-amide had an IC_{50} of 205 nM, comparable to potencies of E2 and DAMGO, while the IC_{50} for YPF-benzyl-amide was 20500 nM.

Because of the altered receptor affinity (determined from the [^{3}H]Nx binding) and the decreased intrinsic activity in the [^{35}S]GTPγS binding assays, we expected that several of the ligands will be able to at least partially inhibit the E2 or DAMGO stimulated [^{35}S]GTPγS binding. Indeed, the 10^{-6} M E2 or DAMGO stimulated [^{35}S]GTPγS binding was suppressed by 10^{-4} M E2-ol, YPF-phenethyl-amide, YPF-benzyl-amide, and MePhe^3-E2 (Fig. 4) to the levels produced by the derivatives themselves. Despite of its partial agonist nature, based on its Na^+ index, YPF-benzyl-allyl-amide fully inhibited both E2 or DAMGO stimulated [^{35}S]GTPγS binding (Fig. 4). This inhibition was concentration dependent (IC_{50} = 1019 nM), but weak compared to the inhibition produced by naloxone (IC_{50} = 8 nM) (Fig. 5). Simultaneous N-methylation of Phe^3 and the C-terminal amide to alcohol conversion (MePhe3-E2-ol) did not alter the E2 or DAMGO stimulated [^{35}S]GTPγS binding.

DISCUSSION

Because of the high selectivity for mu-opioid receptors (1, 6–8, 31) and the potent analgesic action at low concentrations, without the adverse effects of other opioids (32), synthesis and characterisation of new analogues of endomorphins are in the forefront of opioid research. Here we report how certain modifications introduced to the C-terminus and N-methylation of Phe^3 alter the binding affinity and the [^{35}S]GTPγS binding, used as a measure of biological activity, of E2.

TABLE 1

Receptor Constants and Functional Effects of DAMGO, E2, and Its Derivatives in Rat Brain Membranes

		[^{3}H]N$_x$IC$_{50}$ (nM)[a]			[^{35}S]GTPγS	
	[^{3}H]E2 IC$_{50}$ (nM)[a] (95% CI)	−Na$^+$	+Na$^+$/−Na$^+$[c]	+Na$^+$	EC$_{50}$ (nM)[b] (95% CI)	Maximal effect (95% CI)
DAMGO	0.63 (0.47 to 0.83)	3.1	*202.6*	628.0	323.9 (251 to 418)	184.4 (179.5 to 189.4)
E2	4.02 (3.59 to 4.49)	7.24	*84.5*	612.0	529.8 (331 to 817)	154.8 (149.4 to 160.2)
E2-ol	0.63 (0.52 to 0.77)	51.3	*7.6*	390.0	529.8 (16 to 47)	154.8 (149.4 to 160.2)
MePhe3-E2	10.25 (8.79 to 11.89)	229.0	*6.6*	1510.0	450.1 (2087 to 6211)	142.6 (136.8 to 148.3)
MePhe3-E2-ol	69.11 (60.81 to 78.25)	506.0	*28.1*	14200.0	3600.0 (234 to 867)	130.2 (126.4 to 134.0)
YPF-benzyl-amide	331.00 (287.20 to 383.45)	752.0	*10.6*	8007.0	22190.0 (7467 to 65980)	116.3 (111.6 to 121.0)
YPF-phenethyl-amide	19.10 (16.05 to 22.79)	310.0	*12.3*	3810.0	161.0 (52 to 500)	116.7 (113.4 to 120.0)
YPF-benzyl-allyl-amide	306.00 (254.90 to 366.65	768.0	*2.2*	1720.0	ND	100 (95.41 to 101.8)
E2-OH	2220.00 (1835.00 to 2678.00)	7470.0	*171.5*	1281000.0	ND	104.5 (99.68 to 109.4)
YPF-OH	160.50 (135.20 to 188.45)	22700.0	*122.9*	2791000.0	ND	102 (96.41 to 107.7)
Nx	1.4[d]	3.6	*0.9*	3.1	ND	ND

Note. All values are the averages of at least three independent experiments in duplicates.

[a] To determine the equilibrium inhibition constants (IC$_{50}$ in nM), rat brain membranes were incubated with 0.4 nM [^{3}H]Pro^2-E2 or 1 nM [^{3}H]Nx in the presence of the unlabelled peptides (see Fig. 2). The IC$_{50}$ values were calculated by fitting sigmoid dose–response curves with variable slope using the Prism program nonlinear least-squares algorithm. The 95% confidence intervals are listed in brackets.

[b] Half maximal stimulation (EC$_{50}$ in nM) and maximal effects (% stimulation over basal level) in the [^{35}S]GTPγS binding assay were calculated by fitting sigmoid dose–response curves with variable slope using the Prism program nonlinear least-squares algorithm. The 95% confidence intervals are listed in brackets.

[c] The Na$^+$ index (+Na/−Na) was calculated by dividing the IC$_{50}$ values measured in the presence and absence of 100 mM NaCl. Values below 1 indicate antagonist, between 1 and 0 partial antagonist, and above 10 agonist feature.

[d] Taken with permission from Spetea *et al.* (7).

In this paper we have shown that the only modification that improved the binding affinity and biological activity of E2 was the substitution of the phenylalanine-amide with phenylalaninol at position 4.

The structural features of a ligand determine its binding characteristics and biological effects; hence modifications of amino acids may have profound effects on receptor selectivity and physiological action (33–35). The five-amino-acid-long DAMGO has been derived from the original sequence of enkephalins (36) following the observations that N-methylation of the amino group of a phenylalanine[4] and alteration of the carboxyl group to an alcohol and replacement of the last amino acid (Met[5]) to ethanolamine (glycinol) dramatically increased the analgesic activity and receptor binding affinity to a naloxone sensitive opiate receptor (36–38) resulting in the most widely used MOR specific ligand with full agonist properties so far. Based on these previous successful modifications E2 was altered on its C-terminal and its Phe[3] in an attempt to increase its biological activity and to further our understanding of how structural modifications might influence the binding properties.

C-terminal residues play a critical role in the biological activity of MOR specific ligands (33). In fact, the C-terminal amino acid is essential for the high affinity binding of endomorphins to MOR (1, 39). For example, in Tyr-W-MIF-1 (1), the affinity significantly increases when Gly[4] is substituted by hydrophobic residues, and even more when it is substituted by Phe, an observation that actually led to the discovery of endomorphins. Because of the three aromatic side chains, endomorphins, in contrast to their small size, are relatively well structured (40). However, the last amino acid, Phe[4], is free to adopt a "bio-active" conformation that is independent of the correct orientation or the stereochemistry of this residue (41). The present study demonstrated that beside the conformational freedom, the spacing between the amide bond and the phenyl ring is also essential for obtaining reasonable ligand binding and [^{35}S]GTPγS stimulation (compare YPF-benzyl-amide and YPF-phenethyl-amide in Table 1). A simple

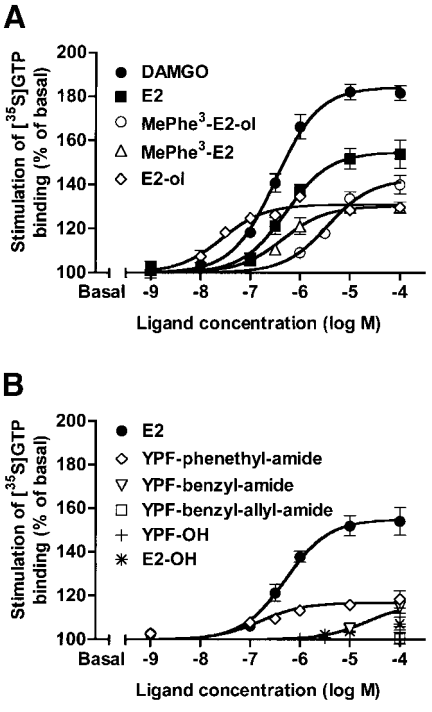

FIG. 3. Stimulation of [^{35}S]GTPγS binding to rat brain membranes by various concentrations of E2 derivatives. For comparison the stimulation induced by DAMGO was used. Incubations were carried out for 60 min at 30°C. Nonspecific binding was ~30%. Points represent the averages of 3–5 independent experiments in triplicates ± SEM.

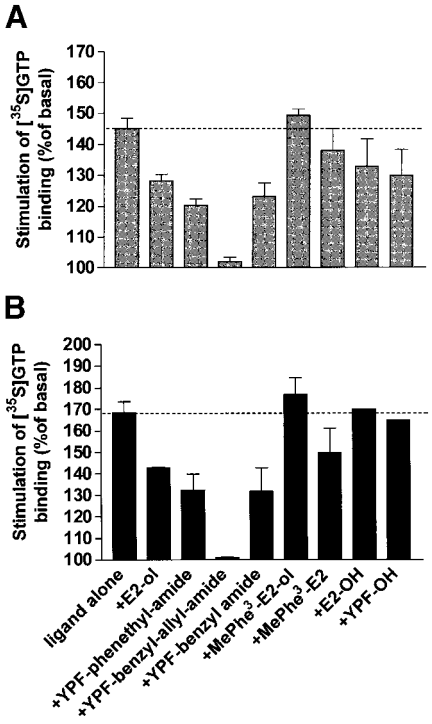

FIG. 4. Inhibition of submaximal stimulation of [^{35}S]GTPγS binding by 10^{-6} M E2 (A) or DAMGO (B) to rat brain membranes by 10^{-4} M E2 derivatives. Incubations were carried out for 60 min at 30°C. Nonspecific binding was ~30%. Points represent the averages of at least 3 independent experiments in triplicates ± SEM.

-CH_2 deletion resulted in one order of magnitude loss in receptor binding affinity and more than two orders of magnitude decrease in [^{35}S]GTPγS binding.

After the amide group was replaced with an alcohol, E2-ol became as potent in displacing [^{3}H]Pro^2-E2 from its binding site as DAMGO. Furthermore, it had higher affinity for the [^{3}H]Pro^2-E2 binding site than E2 itself (Table 1) (7). This enhanced binding could be brought about by the C-terminal hydroxyl group by improving the otherwise very similar solution structure of E2 and DAMGO (41). Despite the high affinity binding, the maximal biological activity induced by E2-ol was only about one third produced by DAMGO, and about half produced by E2. Based on the Na^+ index, the lower intrinsic activity could have been the result of E2-ol becoming a partial agonist in both E2 and DAMGO induced [^{35}S]GTPγS binding. This hypothesis is supported by experiments on guinea pig ileum and mouse vas deferens (42). Therefore, instead of producing a more potent agonist, replacement of -$CONH_2$ with -CH_2OH produced a high affinity partial agonist. It has been suggested that E2 itself is a partial agonist in the [^{35}S]GTPγS binding assay (15, 19). In our experiments, however, E2 was unable to antagonise the DAMGO induced GTP-binding (data not shown), it was a full agonist based on its Na^+ index, and it behaved like a full agonist in previous pharmacological studies (42). Differences in assay conditions (e.g., GDP concentration) have been shown to affect the intrinsic activity and antagonistic properties of a ligand (19, 20) and might be the cause of this discrepancy.

Similarly to DAMGO, N-methylation of Phe^3 enhanced the binding of morphiceptin to MOR (43), suggesting that a similar modification might improve the binding properties and biological activity of E2 as well. It has been shown in NMR studies that N-methyl

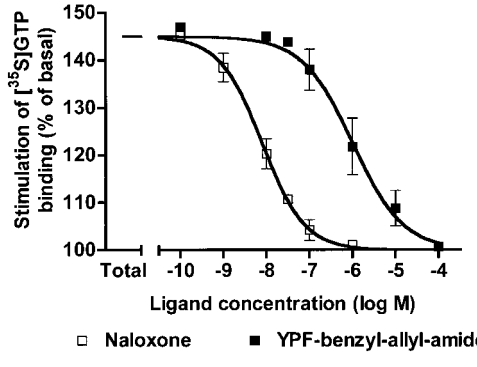

FIG. 5. Concentration-dependent inhibition of 10^{-6} M E2 stimulated [^{35}S]GTPγS binding by YPF-phenethyl-allyl-amide (■) or naloxone (□). Points represent the averages of 3–5 independent experiments in triplicates ± SEM.

amide conformationally restricts the peptide backbone and forces the ligand to adopt an extended conformation (44, 45) that was used to model the active conformation of E1 (40, 41, 45). In this study N-methylation only marginally decreased the binding affinity of E2, supporting the extended structure model for E2. However, *N*-MePhe^3-E2 became a low efficacy partial agonist, suggesting that this peptide does not possess the preferred conformation to induce the biological response. N-methylation of E2-ol yielded a 2 order of magnitude decrease in the potency of displacing [^{3}H]Pro^2]-E2 binding and turned the partial agonism of E2-ol into full agonism. The decreased biological activity of the N-methylated peptides showed that the restricted configuration mentioned above or the increased steric requirement of the methyl group is not favoured by the MOR. Interestingly, the inhibition of [^{3}H]Pro^2-E2 binding by the ligands that contained either N-methylated Phe^3 or an alcohol at their C-terminus was shallow ($n_H < 0.6$) compared to the other ligands ($n_H \sim 1$), suggesting that the interaction of these compounds with the MOR is different, presumably because of their different receptor occupancy and efficacy (42), but even involvement of multiple binding sites cannot be excluded. This possibility is even more pronounced considering the displacement values obtained with tritiated naloxone (Table 1).

While E2-ol became a high affinity, low efficacy analogue of E2, most C-terminal modifications decreased the MOR binding affinity and the intrinsic activity of E2, producing partial agonists with varying degrees of inhibitory potencies. Modification of E2 to E2-OH dramatically decreased the binding of the tetrapeptide to MOR especially in the presence of Na^+, which was accompanied by a complete loss of biological activity, a similar result we obtained earlier using E1-OH (46). Therefore, this derivative is unlikely to play a role in physiological events, despite the fact that E2-OH has been co-purified from brain with E2 (47). Similarly to our previous results on E1 (46), deletion of Phe^4 from E2 decreased the receptor binding affinity and biological activity of the remaining tripeptide, a result further emphasising the importance of Phe^4 in the binding of endomorphins to the MOR. In fact, proper orientation and the distance of ~12 Å from the aromatic ring of Tyr^1 is important for the interaction of Phe^4 with MOR (41). It is not surprising, therefore, that YPF-benzyl-amide was only a very weak partial agonist of MOR. However, both the aromatic ring and its position are important for biological activity since molecules like E2-OH and YPF-benzyl-allyl-amide were unable to stimulate [^{35}S]GTPγS binding. It is also important to note that YPF phenethyl-amide performs well in both receptor binding and [^{35}S]GTPγS assays, indicating that the C-terminal carboxamide or hydroxymethyl group is not essential for binding or G-protein activation. However, to achieve maximal stimulus such groups are essential. Replacement of phenylalanine amide to benzyl-allyl amide not only decreased the binding affinity by two orders of magnitude and transformed E2 into a partial agonist, but completely abolished its intrinsic activity. This new compound became a weak but full, probably allosteric, inhibitor of E2 or DAMGO induced [^{35}S]GTPγS binding.

Inhibition of [^{3}H]Nx binding was also affected by the modifications introduced earlier but the change in binding affinity followed a different pattern. The binding affinities to the [^{3}H]Nx binding site were all lower than that of the parent compound, suggesting that the interactions of E2 and Nx with MOR are different. This is not an unexpected result as it has been shown that the interactions of agonists and antagonists with opiate receptors are different (4, 48, 49).

In summary, we found that conformational restrictions between Pro^2 and Phe^3 or alteration of the C-terminal carboxamide group mostly decreased the binding affinity and biological activity of E2. We also showed that the distance between the C-terminal aromatic ring and the peptide backbone has a strong effect on receptor binding and G-protein activation. Although the C-terminal carboxamide group can be eliminated from the molecule without serious loss of binding activity, for efficient receptor stimulation and naloxone antagonism at least one polar group is necessary at the C-terminus.

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