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EFFECTS OF SYNTHETIC ANALOGUES OF HUMAN OPIORPHIN ON RAT BRAIN OPIOID RECEPTORS

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Human opiorphin (Gln-Arg-Phe-Ser-Arg; QRFSR-peptide) is a physiological inhibitor of enkephalin-inactivating peptidases. We previously demonstrated that opiorphin can substitute for the classic mixture of peptidase inhibitors and greatly improves the specific binding and affinity of the enkephalin-related peptide [^{3}H]MERF (Tyr-Gly-Gly-Phe-Met-Arg-Phe; YGGFMRF) for rat brain opioid receptors. To extend the metabolic stability of opiorphin in human plasma two functional derivatives were designed, i.e., Cys-[$(CH_2)_6$]-QRF-[Ser-O-octanoyl]-R peptide (monomeric CC_6-opiorphin) and its cystine-dipeptide (dimeric CC_6-opiorphin) derivative. We found that, in homologous competition experiments, the affinity of [^{3}H]MERF for rat brain opioid receptors was significantly increased in the presence of monomeric and dimeric CC_6-opiorphin, compared to control-Tris buffer. In addition ten times lower concentrations (5 μM) than those required for native opiorphin (50 μM) were sufficient. In heterologous competition experiments, using unlabeled dynorphin$_{(1-10)}$, affinity increases were also observed: increases in binding were similar with either monomeric or dimeric CC_6-opiorphin. Surprisingly, these opiorphin analogues displayed weak competitive effects on [^{3}H]MERF binding to rat brain opioid receptors in the absence of unlabeled MERF, effects never observed for the native opiorphin. In conclusion, CC_6-opiorphin compounds are certainly more potent than the native opiorphin in increasing the binding and the affinity of homologous and heterologous competition, but the binding enhancement occurs only at temperatures much higher than 0°C, specifically at 24°C.

Key words: *opioid receptors, radioligand binding, Met-enkephalin-Arg-Phe, rat brain membranes, peptidase inhibition*

INTRODUCTION

Opiorphin (Gln-Arg-Phe-Ser-Arg; QRFSR-peptide) is the first characterized human physiological inhibitor of amino-peptidase N and neutral endopeptidase (1). We previously investigated the effect of opiorphin on the binding of enkephalin containing peptides such as [^{3}H]MERF (Tyr-Gly-Gly-Phe-Met-Arg-Phe; YGGFMRF) on rat brain opioid receptors (2, 3). We demonstrated that opiorphin increases the specific binding and affinity of MERF to the rat brain membrane fraction (2).

Met-enkephalin or Leu-enkephalin containing peptides as MERF and dynorphin$_{(1-10)}$ are rapidly cleaved *in vivo* and *in vitro* by several ectopeptidases, such as neutral endopeptidase (NEP; EC 3.4.24.11), amino-peptidase N (AP-N; EC 3.4.11.2) and dipeptidyl carboxypeptidase I (DPCP; EC.3.4.15.1) (1, 4). It was necessary, up until now, to add at least three inhibitors - thiorphan or phosphoramidon for NEP, amastatin or bestatin for AP-N, and captopril for DPCP inhibition - to be able to measure the *in vitro* concentration of the peptides and their binding to opioid receptors (5-7). We found that opiorphin can substitute for this classic mixture of peptidase inhibitors (2).

A major limitation to the use of the opiorphin peptide, however, is its rapid degradation by circulating peptidases. Indeed, native QRFSR-peptide disappears from human plasma with a metabolic half-life evaluated at 5 min (8). Consequently, new peptide analogues of opiorphin were designed and synthesized to further improve its metabolic stability in human plasma for its potential use in pharmaceutics (4, 8). Among these designed opiorphin analogues, the best performing Cys-[$(CH_2)_6$]-Gln-Arg-Phe-[Ser-O-Octanoyl]-Arg peptidomimetic product (monomeric CC_6-opiorphin) and its cystine-dimeric derivative (dimeric CC_6-opiorphin) were found to be more stable with respect to the human plasmatic peptidases while showing reinforced *in vitro* inhibitory potency toward human NEP (more than 40-fold increase) and AP-N (more than 10-fold increase) activities relative to the QRFSR native peptide (4, 8). The importance of opiorphin is not only reflected by its analgesic activity, but also by its anti depressive-like activity in standard rat model (9). Here, we compared these compounds with native opiorphin and determined their effects on MERF binding to opioid receptors in rat brain.

MATERIALS AND METHODS

Peptides

Met-enkephalin-Arg^6-Phe^7 (MERF) (3), dynorphin$_{(1–10)}$ (decapeptide fragment of Dynorphin A 1-17) and [3`5`-I]-Tyr^1-

Met-enkephalin-Arg^6-Phe^7 were synthesized in the BRC Radiolab using Boc chemistry. MERF was tritiated by catalytic dehalogenation of [3`5`-I]-Tyr^1-Met^5-enkephalin-Arg^6-Phe^7 using tritium gas. Specific radioactivity of the tritiated MERF was 40 Ci/mmole (10). Human opiorphin native peptide and derivatives were synthesized by Genosphere Laboratories (France).

Membrane preparation

A crude membrane fraction from albino Wistar rat brain was prepared according to Pasternak *et al*. (11).

Radioligand binding studies

Radioligand binding studies were performed in 50 mM Tris-HCl buffer (pH 7.4) supplemented with 50 µM opiorphin or with the following peptidase inhibitors: 30 µM bestatin, 30 µM captopril, 0.3 µM thiorphan, 10 µg/ml bacitracin, 1 mM PMSF, 1 mM benzamidine, 4 µg/ml soybean trypsin inhibitor, 1 mM EDTA and 1 mM EGTA. This buffer is referred to as "buffer D". Opiorphin derivatives were dissolved in tri-distilled water at 25 mM final concentration, aliquoted and stored at –80°C until extemporaneously diluted, at desired concentrations in Tris buffer and used in binding experiments. The final volume of the reaction mixture was 1 ml and contained about 300 mg of membrane proteins. Incubations were performed either for 40 min at 0°C or 30 min at 24°C. Reactions were terminated by rapid filtration under vacuum followed by washing with 3 × 5 ml ice-cold 50 mM Tris-HCl buffer through Whatman GF/C glass fiber filters using a Brandel M24R cell harvester. Radioactivity was measured in UltimaGoldTM, using a Packard Tricarb 2300TR liquid scintillation analyzer. Nonspecific binding was calculated in the presence of excess MERF (10 µM). All assays were performed in duplicate and repeated several times.

Data analysis

Experimental displacement curves were fitted by non-linear regression using the one-site competition fitting option of the programme GraphPad Prism, version 5.0. Statistical analysis was done by ANOVA tests. A P value inferior or equal to 0.05 was considered as significant.

RESULTS

In homologous competition binding experiments, in the presence of 50 µM opiorphin, the [^{3}H]MERF binding at 24°C was increased by 140% compared to the classic peptidase inhibitor cocktail, buffer D (c.f. Materials and Methods). Affinity was also increased when opiorphin replaced the peptidase inhibitors in buffer D. IC_{50} at 24°C was 59 nM using buffer D and was 5.8 nM in the presence of 50 µM opiorphin (2).

Table 1. IC_{50} values of [^{3}H]MERF binding to rat brain membrane preparations in homologous and heterologous competition experiments.

Binding conditions / buffer	IC_{50} (nM)	±S.E.M.	No. Expts.
MERF / [^{3}H]MERF in Tris buffer	413	30	6
MERF / [^{3}H]MERF in Tris containing 10 µM opiorphin monomeric derivative	9	2	2
MERF / [^{3}H]MERF in Tris containing 5 µM opiorphin monomeric derivative	17***	6	5
MERF / [^{3}H]MERF in Tris containing 1 µM opiorphin monomeric derivative	33	11	2
MERF / [^{3}H]MERF in buffer D	10.9***	3.2	18
MERF / [^{3}H]MERF in buffer D + 1 mM DTT	81	9	6
MERF / [^{3}H]MERF in buffer D + 1 mM DTT containing 5 uM opiorphin monomeric derivative	27	7	3
MERF / [^{3}H]MERF in buffer D + 1 mM DTT containing 1 uM opiorphin monomeric derivative	62	9	3
Dynorphin$_{(1–10)}$ / [^{3}H]MERF in Tris buffer	383^{NS}	47	2
Dynorphin$_{(1–10)}$ / [^{3}H]MERF in Tris containing 5 µM opiorphin monomeric derivative	14.5	-	1
Dynorphin$_{(1–10)}$ / [^{3}H]MERF in buffer D	69	11	3
Dynorphin$_{(1–10)}$ / [^{3}H]MERF in Tris containing 5 µM opiorphin dimeric derivative	15.7	4.3	4
MERF / [^{3}H]MERF in Tris containing 5 µM opiorphin dimeric derivative	4.1***	0.4	10
MERF / [^{3}H]MERF in Tris containing 2.5 µM opiorphin dimeric derivative	4.8	0.7	3
MERF / [^{3}H]MERF in Tris containing 0.5 µM opiorphin dimeric derivative	6.0	1.4	5
MERF / [^{3}H]MERF in Tris containing 0.25 µM opiorphin dimeric derivative	6.8	1.9	3

*** P<0.0001; one-way ANOVA with Tukey's multiple comparison test. F=257.7; NS: not significant.

Here we demonstrated that monomeric and dimeric CC_6-opiorphin analogues also substantially increased the maximal binding and affinity of MERF to rat brain membrane preparations at 24°C (*Fig*. 1*a* and 1*b*). Similarly to opiorphin native peptide, they showed an improved protective effect on maximal [^{3}H]MERF binding compared to the classic peptidase inhibitor cocktail (buffer D) (*Fig*. *2*). Strikingly, both CC_6-opiorphin analogues significantly increased the ligand affinity of MERF at ten times lower concentrations (5 μM or less; $p<0.0001$, *Table 1*) than native opiorphin, (50 μM).

In heterologous competition experiments of [^{3}H]MERF, with unlabeled dynorphin$_{(1–10)}$ decapeptide as competitor, the affinity and maximal binding values at 24°C were also increased in the presence of both the monomeric and dimeric CC6-opiorphin peptides (IC_{50} at 14.5 nM and 15.7 nM, respectively, *Table 1*), compared with values obtained using buffer D (IC_{50} at 69 nM, *Table 1*, *Fig*. *4a* and *4b*).

However, monomeric and dimeric CC_6-opiorphin peptides did not increase [^{3}H]MERF binding at 0°C in homologous binding experiments. Moreover, the dimeric CC6-opiorphin inhibited binding of unlabeled MERF (data not shown), a phenomenon never observed with native opiorphin (2). Thus, when opiorphin was added at different concentrations to the reaction mixture, as a direct competitor-ligand without unlabeled MERF, no displacement occurred. However, in contrast, both CC_6-opiorphin analogues showed displacement potential over 1 μM concentration (*Fig*. *3*).

In order to investigate whether it is necessary to maintain the cysteine residue of the monomeric CC_6-opiorphin in the reduced form, we added 1 mM DTT (dithiothreitol) to the reaction mixture. Under these experimental conditions, the affinity of the CC_6-opiorphin peptide increased compared to the control: however, the control IC_{50} values were higher than those in non-reducing conditions (*Table 1*).

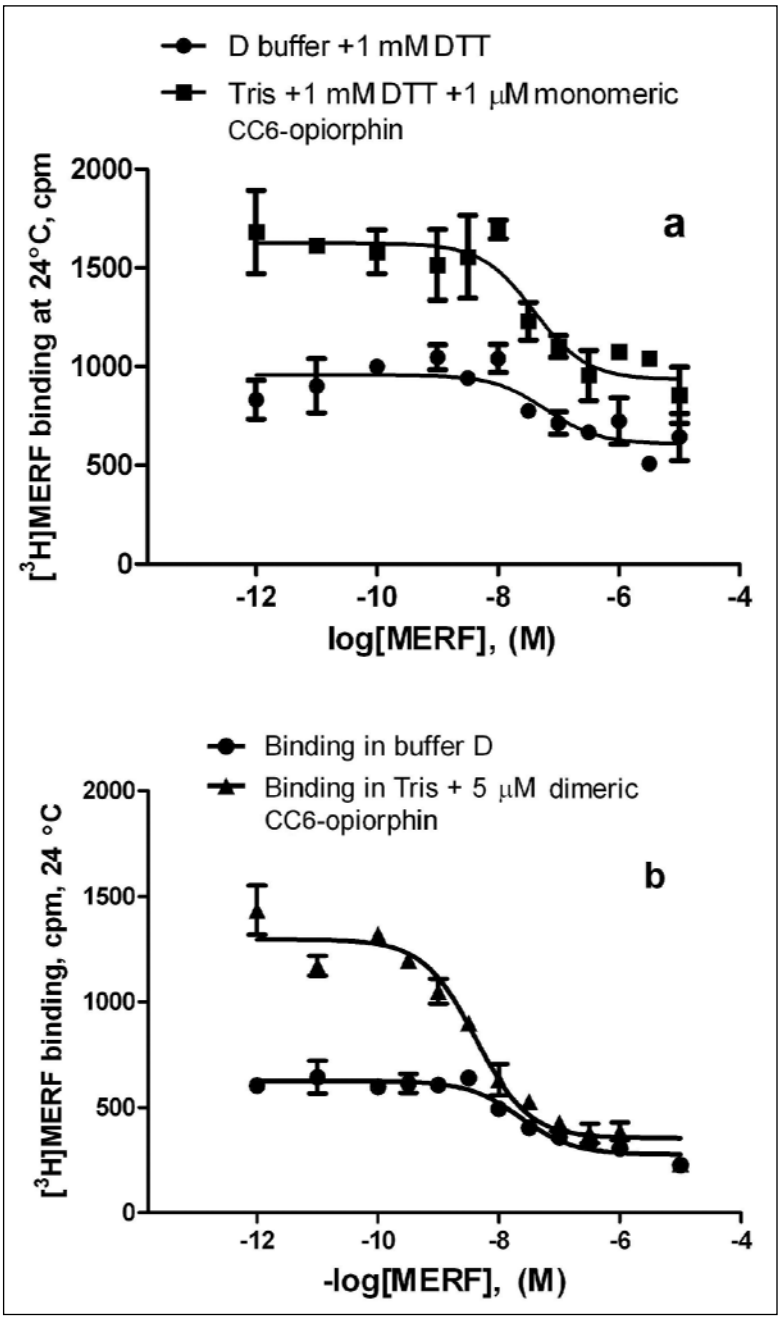

Fig. *1a*. Homologous competition binding of [^{3}H]MERF in rat brain membranes at 24°C. Points represent means ± S.E.M. of three independent experiments, each performed in duplicates. Square = competition by the monomeric CC_6-opiorphin in buffer D.

Fig. *1b*. Homologous displacement curves in the presence of buffer D or 5 μM dimeric CC_6-opiorphin analogue.

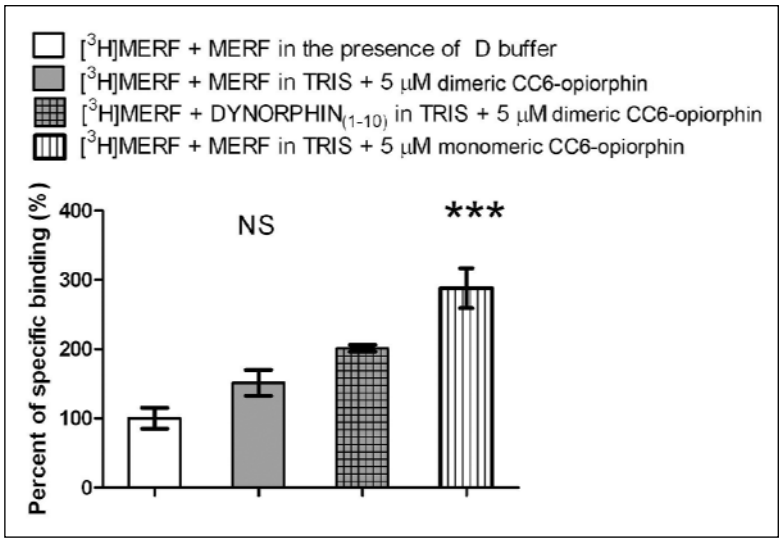

Fig. 2. Percentage of [^{3}H]MERF binding capacity to rat brain membrane preparation in homologous and heterologous competition studies. NS: non-significant; one-way analysis of variance, ANOVA with Tukey's multiple comparison post hoc test. P value summary 0.0003; F=18.64; R square 0.8614. * P<0.05; *** P<0.0001.

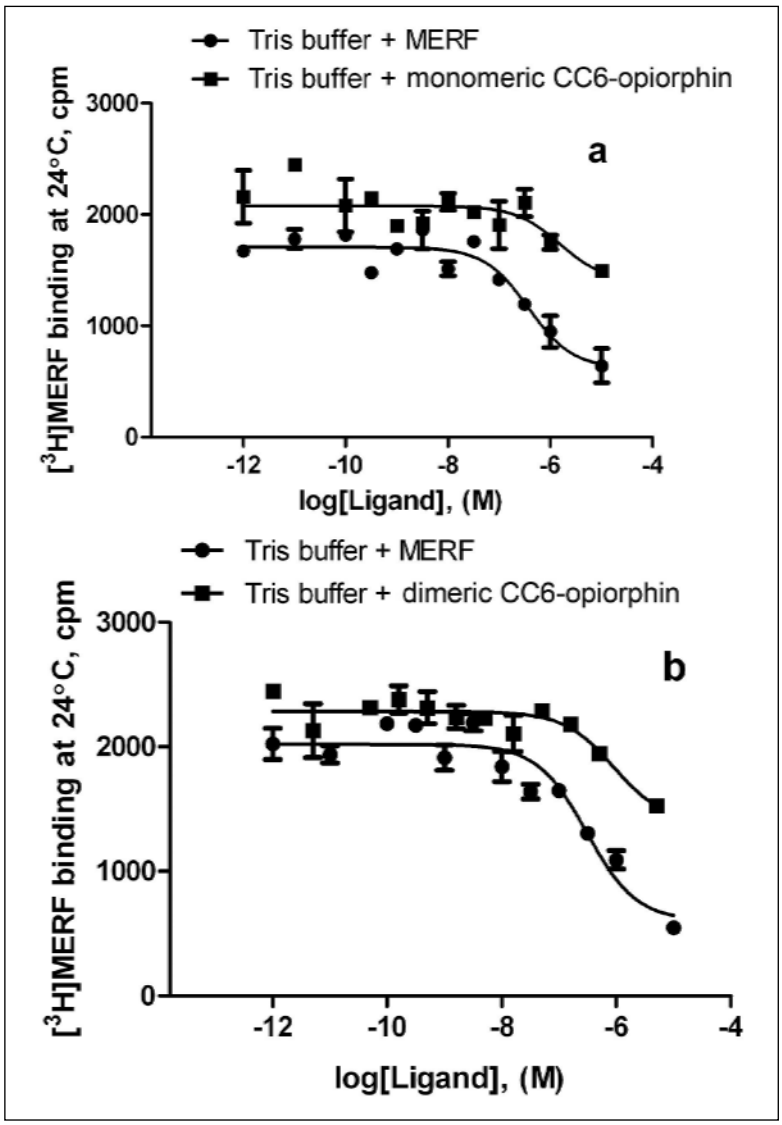

Fig. 3. Heterologous competition binding experiments by the monomeric CC_6-opiorphin analogue (*a*) and by the dimeric CC_6-opiorphin analogue (*b*).
Lower curves are the homologous displacement by unlabeled MERF.

DISCUSSION

Opiorphin is a potent natural inhibitor of peptidase enzymes (1). By blocking enkephalin degradation (2, 3), longer term actions of the opioid peptides are expected. In addition to the analgesic (12) and anti depressive activity (9), other endogenous agents act also through the opioid system as melatonin (13), whereas corticotrophin-releasing factor

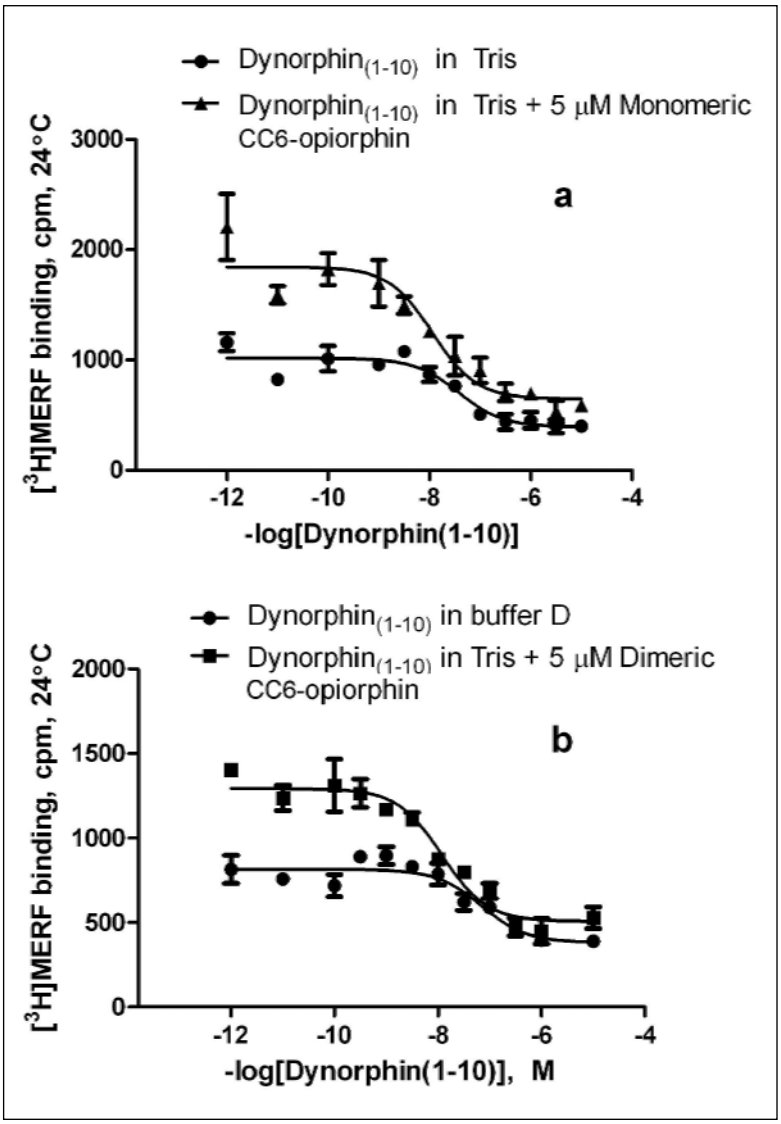

Fig. 4. Heterologous competition binding of [^{3}H]MERF and dynorphin$_{(1–10)}$ in the presence of 5 μM monomeric CC_6-opiorphin analogue (*a*) or 5 μM dimeric CC_6-opiorphin analogue (*b*).

(CRF) induced analgesia is mediated by non-opioid mechanisms (14).

Opiorphin peptidomimetics were designed and synthesized in a successful attempt to increase the stability of this pharmacologically important peptide against circulating peptidases (4, 8). In an *in vitro* kinetic model, native opiorphin, when added to human plasma, is rapidly metabolized with a metabolic half-life of about 5 min. After 15–60 min incubation only 13% of opiorphin remains free and native. In contrast, under similar experimental conditions, at least 42% of the synthesized monomeric CC_6-opiorphin peptide remains free and in its native conformation (4, 8). Previous data also demonstrated that CC_6-opiorphin peptide is at least ten times more potent than the opiorphin natural peptide in its ability to inhibit human NEP and AP-N *in vitro* (4, 8). Here, we demonstrate that both binding and affinity of MERF to rat brain membrane preparations at 24°C increases using lower concentrations of monomeric and dimeric CC_6-opiorphin analogues (5 μM or lower) compared to the parent opiorphin compound (50 μM). Monomeric and dimeric CC_6-opiorphin analogues also displayed a slight intrinsic affinity for rat brain opioid receptors at 5 μM (EC_{50}=1.1 μM) and were able to induce weak displacement of [^{3}H]MERF binding in the absence of MERF. However, this action of the analogues on the low affinity opioid binding site of [^{3}H]MERF is not inhibited by naloxone, hence it cannot be considered as a classical opioid ligand (3). We also observed that, whereas opiorphin increased binding and affinity at 0°C, the dimeric CC_6-opiorphin fully inhibited the competition of MERF at this temperature while the monomeric compound did not influence the reaction at all. The reason for these differences might be the presence of cysteine or cystine residues in the monomeric and dimeric CC_6-opiorphin peptides, respectively, and/or the presence of inserted alkyl groups, which increase the size and the lipophilic character of the opiorphin analogues. An other advantage of opiorphin peptides is their non-addictive analgesic action without drug tolerance effects (12). The effect of dynorphin$_{(1–10)}$ was investigated in heterologous competition experiments because it contains Leu-enkephalin unlike to MERF, therefore it was of interest to see whether opiorphin can increase the binding of C-terminally extended Leu-enkephalin as well (*Fig. 4*). In fact the new CC_6-opiorphin analogues were more effective than the native opiorphin peptide (*Figs. 2* and *4*).

Summarizing the results in terms of dose-efficacy, monomeric and dimeric CC_6-opiorphin analogues are certainly more potent than endogenous opiorphin, in raising the binding

and increasing the affinity of MERF homologous and dynorphin$_{(1–10)}$ heterologous competition. However, binding enhancement occurs only at temperatures much higher than 0°C (at 24°C). Interestingly, the affinity increase is more pronounced using Tris buffer as control, whereas the increase in maximal binding is more evident using classic peptidase inhibitor buffer D as control. Taken together, these data are a step forward in determining the pharmacological parameters necessary for optimal opiorphin drug-candidate activity.

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