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Opioid profiles of Cys^2-containing enkephalin analogues

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Abstract

To elucidate the structural features determining δ-opioid receptor properties of enkephalin analogues containing $Cys(O_2NH_2)$ in position 2, a series of Cys^2-containing derivatives were synthesized and tested for their effectiveness in depressing electrically evoked contractions of the mouse vas deferens (predominantly enkephalin-selective δ-opioid receptors) and the guinea-pig ileum (μ- and κ-opioid receptors). The peptidase resistance of the compounds was also tested. The ratio IC_{50} in the guinea-pig ileum/IC_{50} in the mouse vas deferens, indicating selectivity for δ-opioid receptors, was high for $Cys(O_2NH_2)^2$-containing analogues and especially for [$Cys(O_2NH_2)^2$, Leu^5]enkephalin, which was about seven times more selective than δ-opioid receptor selective ligand cyclic [D-Pen^2, D-Pen^5]enkephalin (DPDPE). The dissociation constant (K_A) and relative efficacy (e_{rel}) of the compounds in the mouse-isolated vas deferens were determined using explicit formulae derived by fitting of the data points with two-parametric hyperbolic function. The obtained values for K_A and e_{rel} suggest that: (i) incorporation of $Cys(O_2NH_2)^2$ in the molecule of [Leu^5]enkephalin highly increases the efficacy and does not change significantly the affinity of the respective analogues to δ-opioid receptors; [$Cys(O_2NH_2)^2$, Leu^5]enkephalin has higher affinity than DPDPE, but is less resistant to enzyme degradation; the effect of this modification on the efficacy is decreased when methionine is in position 5; (ii) D-configuration of $Cys(O_2NH_2)^2$-containing analogues increases their peptidase resistance, but reduces efficacy and affinity of the peptides towards δ-opioid receptors; (iii) the substitution of $Cys(O_2NH_2)$ with $Hcy(O_2NH_2)$ reduces the efficacy, affinity and potency of the respective analogues and maintains their sensitivity to endogenous peptidases; (iv) the substitution of the sulfonamide group with benzyl group in the molecule of Cys in position 2 decreases their efficacy and affinity toward δ-opioid receptors, but attaches resistance to enzyme degradation. The results obtained in this study allow: (i) to involve the receptor affinity and agonist efficacy as drug-design consideration for δ-opioid receptor properties of newly synthesized compounds and (ii) to characterize some of the structural features, which set the pattern for their opioid profiles.

Keywords: Enkephalin; Cysteine; δ-Opioid receptor; Affinity; Efficacy

1. Introduction

Early investigations suggested that the μ rather than the δ subtype of opioid receptors appears to be involved in enkephalin-induced analgesia (Chaillet et al., 1984). But with the discovery of compounds with improved selectivity for δ-opioid receptors, it became clear that these receptors also mediate analgesia (Quock et al., 1999). Moreover, antinociception studies in recombinant mice with disrupted expression of the μ-opioid receptor revealed that δ-opioid receptor selective agonists do not require functional μ-opioid receptors to mediate antinociception (Matthes et al., 1998). At the same time, δ–opioid receptor selective drugs

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share morphine's positive therapeutic effects (particularly analgesia) and exert greater relief of neuropathic pain (Dickenson, 1997), but reduce negative properties such as respiratory depression (Cheng et al., 1993) and constipation (Burks et al., 1988; Sheldon et al., 1990) and reveal a minimal potential for the development of physical dependence (Cowan et al., 1988). However, these advantages are closely related not only with the selectivity of δ-opioid compounds which concerns specific recognition between receptor and drug, but with their efficacy which depends on the maximal biological response of the drug.

There is evidence for increased δ-opioid receptor recognition and potency of linear or cyclic enkephalin analogues with Cys in position 2, which is due in part to increased efficacy (Guis et al., 1993; Kramer et al., 1993, 1997). Our earlier studies have shown that incorporation of Cys(O_2NH_2) in the enkephalin molecule greatly increases the potency and selectivity of the analogues at δ-opioid receptors (Pencheva et al., 1995, 1996, 2003). To further elucidate the structural features important for selective action of these compounds and to characterize their opioid profiles, a series of linear enkephalin derivatives containing modified Cys in position 2 were synthesized. Since the animal models of nociception are unable to determine the maximal effect of the compounds, we tested the peptides by in vitro bioassays, which provide more precise quantitative information. During the course of our investigation, it also became necessary to: (i) examine the potencies of peptides in the presence and in the absence of peptidase inhibitors; and (ii) differentiate receptor affinity and agonist efficacy of the peptides using explicit formulae derived by modeling of concentration-response relations with two-parametric hyperbolic function.

2. Methods

2.1. Bioassays in isolated tissues

The myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum (hereafter referred to as guinea-pig ileum) and the vas deferens of the mouse were set up for field stimulation in 3 or 5 ml siliconised organ baths as described previously (Pencheva et al., 1999). Male Dunkin–Hartley (250–350 g) guinea pigs and TO albino mice (23–29 g) were used. When the vas deferens preparation was mounted, modified Krebs solution (according to Stjärne and Astrand, 1985, with lower content of Mg^{2+}) was used. Electrically evoked responses were measured under isometric conditions after standard calibration of mechanoelectrical transducer (Experimetria, Hungary) connected to a recording device TZ 4620 (Laboratorni Pristroje, Praha) at a tension equivalent to a load of 0.5 g for ileum and 0.1 g for vas deferens. Electrical field stimulation with single, bipolar rectangular pulses of supramaximal voltage, 75-mA current, 0.5-ms duration and 0.1-Hz frequency was applied to guinea-pig ileum preparations. Mouse vasa deferentia were stimulated using trains, consisting of three pulses of supramaximal voltage, 25-mA current and 1-ms duration at intervals of 250 ms (4 Hz); the trains were repeated at 0.1 Hz. The experimental protocols were carried out in accordance with the ethical guidelines of the European Community about Care and Use of Laboratory Animals for scientific purposes and approved by local, institutional ethics committee.

2.2. Experimental procedure

After an equilibration period (60 min), control agonist concentration–response curves were constructed by cumulative addition of opioid peptide at concentrations increasing from 0.1 pM to 1 mM at 2–3 min intervals. The tissues were then exposed to a cocktail of the peptidase inhibitors, bestatin 10 μM, captopril 10 μM, thiorphan 3 μM and Leu–Leu 2 mM (McKnight et al., 1983), for at least 20–25 min and concentration–response curves for the same compounds were obtained again. A standard agonist was used to monitor changes in tissue sensitivity during experimentation. The δ-opioid receptor agonist cyclic [D-Pen^2, D-Pen^5]enkephalin (DPDPE) (Mosberg et al., 1983) was the standard for the vas deferens and the μ-opioid receptor agonist [D-Ala^2, *N*-Me-Phe^4, Leu^5]enkephalyl-Arg-NH_2 (Pencheva et al., 1999) for the myenteric plexus. Tissues from the same animal were exposed to various concentrations of either naloxone or *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864), and 20 min later, the effect of cumulatively increasing or single concentrations of the opioid peptides was determined. This procedure was repeated until at least five replicates from different animals were obtained.

2.3. Analysis of data and curve fitting

The following generally accepted notations are used: A–opioid agonist; $[A]$–concentration of the agonist; E–the measured inhibitory effect; E_m^T–potential maximum response of the tissue; E_m^A–maximum response of A; IC_{50}–concentration of an agonist, which produces 0.5 E_m^T; $[A_{50}]$–concentration of an agonist, which produces 0.5 E_m^A; K_A–dissociation constant with units of $[A]$. Further, we bring into use e_{rel}–relative efficacy of the agonist, which is unitless; $e_{rel}=e_A/C$, where e_A is Stephenson's efficacy (Stephenson, 1956), while C is a constant, which depends only from the tissue, but not from the drug.

Each individual set of $E/[A]$ curve data were calculated as fractional inhibition of electrically evoked twitch by the following relationship:

$$\text{fractional inhinbition of twitch} = \frac{\text{height}_{[0]} - \text{height}_{[A]}}{\text{height}_{[0]}},$$

where $\text{height}_{[0]}$ is contraction height (in mm) in the absence of agonist and $\text{height}_{[A]}$ is the contraction height (in mm) associated with a given agonist concentration; operationally, $\text{height}_{[0]}$ defines E_m^T. Mean concentration response data

Table 1
Amino acid sequence of Cys^2-containing enkephalin fragments and analogues used in this investigation

Peptide	Primary structure
Tyr-Cys(O_2NH_2)	H–Tyr-Cys(O_2NH_2)–OH
Tyr-D-Cys(O_2NH_2)	H–Tyr-D-Cys(O_2NH_2)–OH
Tyr-Cys(Bzl)	H–Tyr-Cys(Bzl)–OH
[Cys(O_2NH_2)^2, Leu^5]enkephalin	H–Tyr-Cys(O_2NH_2)-Gly-Phe-Leu–OH
[Cys(O_2NH_2)^2, Met^5]enkephalin	H–Tyr-Cys(O_2NH_2)-Gly-Phe-Met–OH
[D-Cys(O_2NH_2)^2, Leu^5]enkephalin	H–Tyr-D-Cys(O_2NH_2)-Gly-Phe-Leu–OH
[D-Cys(O_2NH_2)^2, Met^5]enkephalin	H–Tyr-D-Cys(O_2NH_2)-Gly-Phe-Met–OH
[Hcy(O_2NH_2)^2, Leu^5]enkephalin	H–Tyr-Hcy(O_2NH_2)-Gly-Phe-Leu–OH
[H-Cys(O_2NH_2)^2, Met^5]enkephalin	H–Tyr-Hcy(O_2NH_2)-Gly-Phe-Met–OH
[Cys(Bzl)^2, Leu^5]enkephalin	H–Tyr-Cys(Bzl)-Gly-Phe-Leu–OH
[Cys(Bzl)^2, Met^5]enkephalin	H–Tyr-Cys(Bzl)-Gly-Phe-Met–OH

from at least six tissues were fitted by nonlinear least-squares regression to a two-parametric or three-parametric hyperbolic function: $E=(a_0[A])/([A_{50}]+[A])$ and $E=(a_0[A]^n)/([A_{50}]^n+[A]^n)$, respectively, in which a_0, $[A_{50}]$ and n are the asymptote, location or slope parameters, respectively. Location parameter was actually estimated as logarithms and recalculated in nM.

Potencies of agonists were evaluated by calculation of IC_{50} values (nM); the data are presented as means±S.E.M. Differences between means as logarithms were assessed for statistical significance using t-test for grouped data; $p<0.05$ was taken to be significant.

Using nonlinear modeling (Milanov et al., 2003) of the two-parametric hyperbolic function, we obtained the following explicit formulas for computation of K_A and e_{rel} of the opioid peptides: $K_A=[A_{50}]/(1-\lambda_A)$ and $e_{rel}=\lambda_A/(1-\lambda_A)$, where $\lambda_A=E_m^A/E_m^T$ ($E_m^A=a_0$).

The statistical procedures, the plotting of the graphs and the calculations of the numerical results were carried out using software package Slide Write Plus and computer programs prepared according to Tallarida and Murray (1981).

2.4. Peptides, drugs and solutions

Cys^2-containing analogues (Table 1) were synthesized by classical procedure in solution using (2+3) strategy with a combination of chemical (azide) and enzymatic methods as described previously (Pencheva et al., 2001). The final products were purified by column chromatography on Sephadex G-15, solvent system of 0.2N NaOH and reversed phase column liquid chromatography-middle pressure (RP-C_{18}). Pure peptides were characterized by amino acid analysis, analytical reversed phase-high performance liquid chromatography, optical rotation and mass spectrometry.

Inhibitors of peptidases: L-Leu–L-Leu, bestatin, thiorphan and captopril, were from Sigma. Other peptides and drugs: [Leu^5]enkephalin, [Met^5]enkephalin, DPDPE and naloxone, were from Sigma; [D-Ala^2, *N*-Me-Phe^4, Leu^5]enkephalyl-Arg-NH_2 used as μ-opioid receptor selective enkephalin analogue (Pencheva et al., 1999) was kindly supplied by Prof. Tomislav Barth from the Academy of Sciences of the Czech Republic (Institute of Organic Chemistry and Biochemistry); ICI 174864, a gift from Research Biochemicals International (Natick, MA, USA) as a part of the NIMN Chemical Synthesis Program, Contract N01MH30003, was sent by Linda Brady, PhD.

The Krebs solution contained (mM): NaCl 120, KCl 5.9, $NaHCO_3$ 14.4, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5 and glucose 11.5. The composition of modified Krebs solution was (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.6, $NaHCO_3$ 11.9, KH_2PO_4 0.5 and glucose 11.5, pH 7.3–7.4.

3. Results

3.1. Effects of Cys^2-containing enkephalin fragments

Cys^2-containing dipeptides had no effects in the guinea-pig ileum with exception of Tyr-Cys(Bzl), which had very low agonist activity in this tissue with $E_m^A=0.29\pm0.01$ and $[A_{50}]=18.0\pm0.4$ nM. However, these compounds were more active in the mouse vas deferens (Fig. 1), with IC_{50} (nM) values as follows: Tyr-Cys(O_2NH_2)-302±58, Tyr-D-Cys(O_2NH_2)→10 000 ($[A_{50}]$=98±13 nM) and Tyr-Cys(Bzl)-144±29. The differences (at $p<0.05$) toward IC_{50} values obtained in the presence of peptidase inhibitors were not significant. As shown in Fig. 1, the maximum inhibitory effect of Tyr-Cys(O_2NH_2) ($E_m^A=0.82\pm0.02$) was higher than that of Tyr-Cys(Bzl) ($E_m^A=0.72\pm0.02$), although its IC_{50}

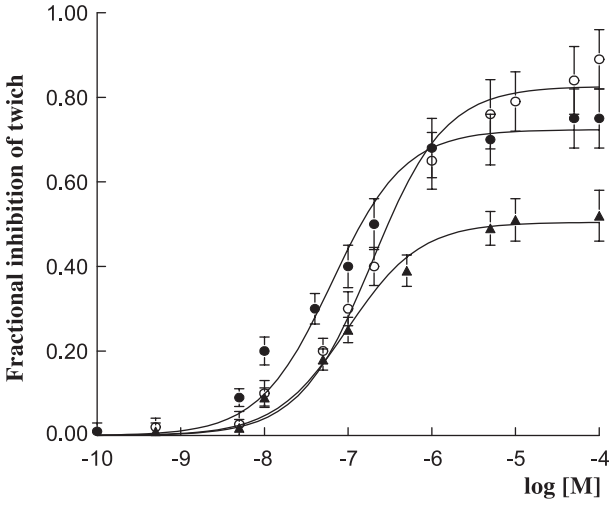

Fig. 1. Mouse vas deferens. Concentration–response curves of Tyr-Cys(O_2NH_2) (○), Tyr-Cys(Bzl) (●) and Tyr-D-Cys (O_2NH_2) (▲). Each point is mean±S.E.M. of five to seven replicants.

Table 2
The inhibitory effects (IC_{50}, nM) of Cys^2-containing and related analogues on electrically evoked contractions of the guinea-pig ileum and the vas deferens of the mouse

Peptide	Guinea-pig ileum IC_{50} (nM)	Mouse vas deferens IC_{50} (nM)	Ratio IC_{50} in g.p. ileum/ IC_{50} in mouse vas deference
[$Cys(O_2NH_2)^2$, Leu^5]enkephalin	>20 000[a]	1.29±0.31[a]	>15 504
[$Cys(O_2NH_2)^2$, Met^5]enkephalin	>20 000[a]	2.22±0.45[a]	>9009
[D-$Cys(O_2NH_2)^2$, Leu^5]enkephalin	1496±302	11.40±2.01	131
[D-$Cys(O_2NH_2)^2$, Met^5]enkephalin	1367±210	75.96±11.67	18
[$Hcy(O_2NH_2)^2$, Leu^5]enkephalin	ND	31.92±5.10[a]	ND
[$Hcy(O_2NH_2)^2$, Met^5]enkephalin	ND	16.09±1.90[a]	ND
[$Cys(Bzl)^2$, Leu^5]enkephalin	280±38	8.30±1.40	34
[$Cys(Bzl)^2$, Met^5]enkephalin	1266±208	9.53±1.20	131
DPDPE	13 273±2296	6.18±1 17	2148
[D-Ala^2, *N*-Me-Phe^4, Leu^5]enkephalyl-Arg-NH_2	1.63±0.24	1371±294	0.0012
[Leu^5]enkephalin	236±34[a]	11.45±2.06[a]	21
[Met^5]enkephalin	162±21[a]	18.91±2.15[a]	9

Each value (except ratio) represents mean±S.E.M. of at least six experiments. ND—not determined (because the values of their [A_{50}] were higher than 30 000 nM and E_m^A<0.4); g.p.—guinea pig.

[a] Significant differences at p<0.05 vs. IC_{50} values, obtained with peptidase inhibitors.

value was lower. The inhibitory effects of the fragments in mouse vas deferens were blocked by 100 nM naloxone.

3.2. Effects of Cys^2-containing analogues

[$Cys(O_2NH_2)^2$, Leu^5]enkephalin and [$Cys(O_2NH_2)^2$, Met^5]enkephalin had a very low activity in the guinea-pig ileum (Table 2) where their E_m^A were 0.52±0.06 and 0.56±0.06, respectively (for [$Cys(O_2NH_2)^2$, Leu^5]enkephalin, see Fig. 2). Combination of peptidase inhibitors bestatin (10 μM), thiorphan (3 μM), captopril (10 μM) and Leu–Leu (2 mM) increased their potency in this tissue about two times. The same analogues were much more active in the mouse vas deferens (Table 2) where [$Cys(O_2NH_2)^2$, Leu^5]enkephalin revealed the highest potency as compared with all peptides investigated, including DPDPE. The inhibitory effects of $Cys(O_2NH_2)^2$-containing analogues were blocked by either the selective δ-opioid receptor selective antagonist ICI 174 864 (100 nM) or by 100 nM naloxone, while 10 nM naloxone did not antagonize their action (data not shown). The potency of [$Cys(O_2NH_2)^2$, Leu^5]enkephalin and [$Cys(O_2NH_2)^2$, Met^5]enkephalin increased 10 times in the presence of peptidase inhibitors.

$Hcy(O_2NH_2)^2$-containing analogues revealed a poor activity in guinea-pig ileum (Table 2) with E_m^A<0.4 and relatively low potency in mouse vas deferens (for [$Hcy(O_2NH_2)^2$, Leu^5]enkephalin, see Fig. 2). The potency of these compounds was sensitive to the peptidase inhibitors, which increased their IC_{50} values about five times in guinea-pig ileum and mouse vas deferens. In contrast, D-$Cys(O_2NH_2)^2$- and $Cys(Bzl)^2$-containing peptides were resistant to inhibition of endogenous peptidases in both tissues. [D-$Cys(O_2NH_2)^2$, Leu^5]enkephalin, [D-$Cys(O_2NH_2)^2$, Met^5]enkephalin and [$Cys(Bzl)^2$, Met^5]enkephalin were equipotent in guinea-pig ileum (with IC_{50} values higher than 1 μM), while [$Cys(Bzl)^2$, Leu^5]enkephalin shows a higher potency, similar to that of the endogenous [Leu^5]enkephalin in this tissue. $Cys(Bzl)^2$-containing peptides and [D-$Cys(O_2NH_2)^2$, Leu^5]enkephalin revealed relatively high potency in mouse vas deferens, while IC_{50} value of [D-$Cys(O_2NH_2)^2$, Met^5]enkephalin was about 7–10 times higher (Table 2). The inhibitory effects of Hcy^2- and D-Cys^2-containing analogues and [$Cys(Bzl)^2$, Met^5]enkephalin were antagonized by 100 nM naloxone, while opioid effect of [$Cys(Bzl)^2$, Leu^5]enkephalin was blocked by 50 nM naloxone. As shown in Fig. 2, the concentration–response data for the Cys^2-containing analogues are much closer

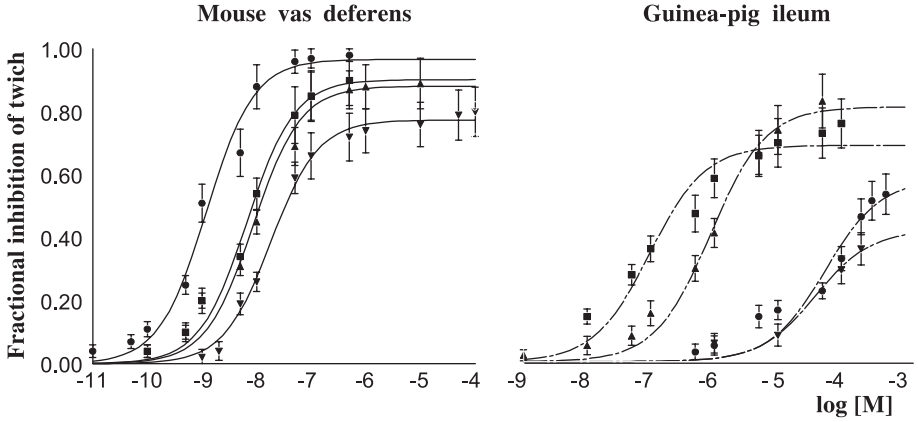

Fig. 2. Concentration–response curves of [$Cys(O_2NH_2)^2$, Leu^5]enkephalin (●), [$Cys(Bzl)^2$, Leu^5]enkephalin (■), [D-$Cys(O_2NH_2)^2$, Leu^5]enkephalin (▲) and [$Hcy(O_2NH_2)^2$, Leu^5]enkephalin (▼) in mouse vas deferens and guinea-pig ileum. Each point is mean±S.E.M. of at least six preparations.

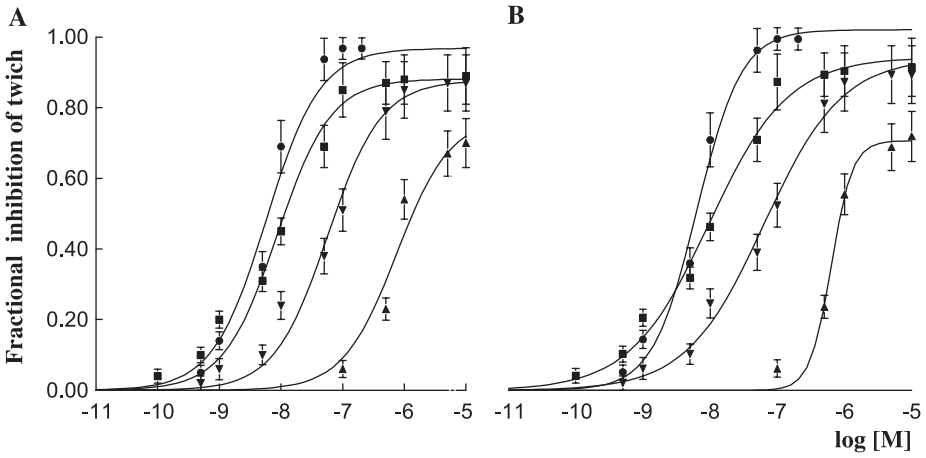

Fig. 3. Mouse vas deferens. Concentration–response curves of DPDPE (●), [D-Cys(O_2NH_2)^2, Leu^5]enkephalin (■), [D-Cys(O_2NH_2)^2, Met^5]enkephalin (▼) and [D-Ala^2, *N*-Me-Phe^4, Leu^5]enkephalyl-Arg-NH_2 (▲), approximated with two-parametric (A) or three-parametric (B) hyperbolic function. Each point is mean±S.E.M. of at least six preparations.

together in the mouse vas deferens than in the guinea-pig ileum. Moreover, their E_m^A values in the mouse vas deferens are between 0.74 and 0.98, whereas in the guinea-pig ileum, the values are between 0.35 and 0.75.

The ratio of the IC_{50} value in the guinea-pig ileum to the IC_{50} value in the mouse vas deferens as a tentative measure of the degree of selectivity of the peptides for the δ- or μ-opioid receptors show that selectivity of Cys(O_2NH_2)^2-containing analogues towards the δ-opioid receptors was higher than that of DPDPE. The selectivity of Hcy(O_2NH_2)^2-containing analogues is probably also high because their potency in guinea-pig ileum, which contains μ- and κ-opioid receptors, is extremely low.

3.3. Determination of K_A and e_{rel} of Cys^2-containing analogues

Since the validity of the explicit formulae for calculation of K_A and e_{rel} concerns the two-parametric hyperbolic function, which is not commonly used, we compare the fitting of $E/[A]$ curve data obtained by both two- and three-parametric hyperbola. Curves of the dose–response relations for some peptides in mouse vas deferens and guinea-pig ileum are presented in Figs. 3 and 4, respectively. Obviously, the additional slope parameter included in the three-parametric hyperbolic function gives more flexible approximation, especially concerning DPDPE and [D-Ala^2, *N*-Me-Phe^4, Leu^5]enkephalyl-Arg-NH_2 in mouse vas deferens and [Cys(Bzl)^2, Leu^5]enkephalin and [Cys(O_2NH_2)^2, Leu^5]enkephalin in guinea-pig ileum. However, the comparison in respect of asymptote and location ($[A_{50}]$) parameters shows that there are no statistically significant differences between the extrapolations with both types of hyperbolic functions, including the cases with no optimal curve fitting mentioned above (Table 3).

Using the explicit formulae derived from the two-hyperbolic function, we calculate K_A and e_{rel} for all peptides in mouse vas deferens. As shown in Table 4, the e_{rel} of

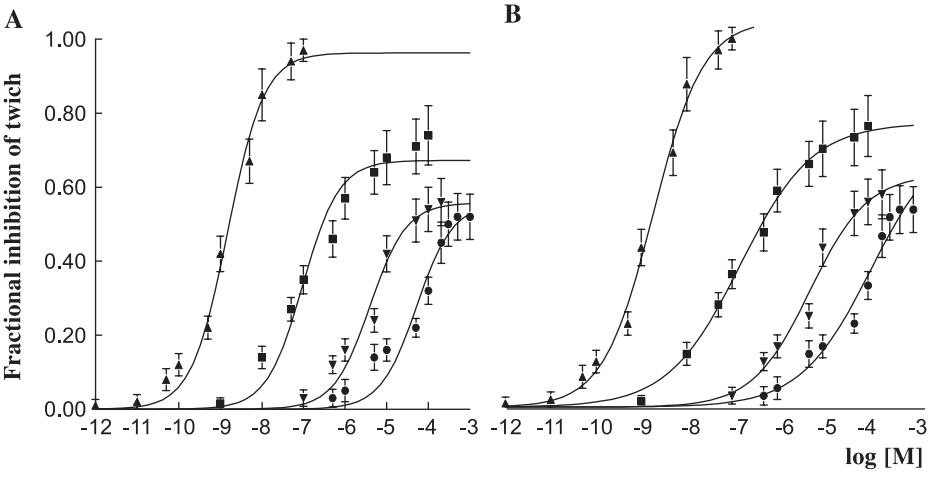

Fig. 4. Guinea-pig ileum. Concentration–response curves of [D-Ala^2, *N*-Me-Phe^4, Leu^5]enkephalyl-Arg-NH_2 (▲), [Cys(Bzl)^2, Leu^5]enkephalin (■), [Cys(O_2NH_2)^2, Met^5]enkephalin (▼) and [Cys(O_2NH_2)^2, Leu^5]enkephalin (●), approximated with two-parametric (A) or three-parametric (B) hyperbolic function. Each point is mean±S.E.M. of six preparations.

Table 3
Comparison between asymptote and location ([A_{50}], nM) parameters obtained by fitting of $E/[A]$ data of Cys^2-containing and related analogues to both two-parametric or three-parametric hyperbolic functions in mouse vas deferens and guinea-pig ileum

Peptide	E_m^A	Asymptote		[A_{50}] (nM)	
		Two-parametric hyperbola	Three-parametric hyperbola	Two-parametric hyperbola	Three-parametric hyperbola
Mouse vas deferens					
[$Cys(O_2NH_2)^2$, Leu^5]enkephalin	0.98±0.02	0.97±0.03	1.00±0.03	1.21±0.20	1.35±0.27
[$Cys(O_2NH_2)^2$, Met^5]enkephalin	0.92±0.07	0.89±0.03	0.91±0.03	1.70±0.34	1.91±0.43
[D-$Cys(O_2NH_2)^2$, Leu^5]enkephalin	0.89±0.08	0.89±0.03	0.92±0.03	8.68±1.53	9.61±1.76
[D-$Cys(O_2NH_2)^2$, Met^5]enkephalin	0.87±0.08	0.88±0.03	0.91±0.02	57.21±9.74	63.68±11.78
[$Hcy(O_2NH_2)^2$, Leu^5]enkephalin	0.80±0.07	0.77±0.01	0.77±0.01	17.38±1.23	17.43±1.35
[$Hcy(O_2NH_2)^2$, Met^5]enkephalin	0.84±0.07	0.82±0.01	0.83±0.01	10.20±0.71	11.07±0.66
[$Cys(Bzl)^2$, Leu^5]enkephalin	0.90±0.08	0.90±0.03	0.95±0.04	6.67±1.05	7.84±1.54
[$Cys(Bzl)^2$, Met^5]enkephalin	0.73±0.08	0.78±0.01	0.79±0.02	5.01±0.36	5.64±0.52
DPDPE	0.97±0.03	0.97±0.03	1.01±0.07	5.78±1.07	6.56±1.30
[D-Ala^2, *N*-Me-Phe^4, Leu^5]enkephalyl-Arg-NH_2	0.70±0.07	0.77±0.07	0.69±0.03	749±143	635±56
[Leu^5]enkephalin	0.85±0.08	0.85±0.02	0.84±0.03	8.07±1.00	7.76±1.06
[Met^5]enkephalin	0.80±0.08	0.78±0.01	0.79±0.01	10.62±1.18	11.07±1.14
Guinea-pig ileum					
[$Cys(O_2NH_2)^2$, Leu^5]enkephalin	0.52±0.06	0.56±0.04	0.72±0.02	>30000	>30000
[$Cys(O_2NH_2)^2$, Met^5]enkephalin	0.64±0.05	0.56±0.03	0.61±0.06	3951±1035	5267±2261
[$Cys(Bzl)^2$, Leu^5]enkephalin	0.74±0.08	0.67±0.03	0.74±0.02	96.52±24.72	151±25
[D-Ala^2, *N*-Me-Phe^4, Leu^5]enkephalyl-Arg-NH_2	0.97±0.03	0.96±0.03	1.02±0.04	1.54±0.21	1.80±0.31

The values represent means±S.E.M. of at least six experiments.

[$Cys(O_2NH_2)^2$, Leu^5]enkephalin is equal to that of the selective δ-opioid receptor ligand DPDPE, but its affinity is about five times higher. D-Cys^2-containing analogues were 4 times less effective with 2–13 times lower affinity in this tissue. $Hcy(O_2NH_2)^2$-containing peptides and [$Cys(Bzl)^2$, Met^5]enkephalin possess relatively high affinity, but their efficacy was similar to that of endogenous enkephalins. [$Cys(Bzl)^2$, Leu^5]-enkephalin was about three times less effective than DPDPE, but its affinity was three times higher. Concerning C-terminal part, Leu in position 5 gives improvement of the efficacy of the analogues [with exception of the $Hcy(O_2NH_2)^2$-containing], while is harmful for the affinity (with exception of D-Cys^2-containing), including endogenous enkephalins.

Table 4
Dissociation constant K_A (nM) and relative efficacy e_{rel} (unitless) of Cys^2-containing and related enkephalin analogues in mouse vas deferens

Peptide	K_A (nM)	e_{rel}
[$Cys(O_2NH_2)^2$, Leu^5]enkephalin	36.4±16.4	29.2±9.5
[$Cys(O_2NH_2)^2$, Met^5]enkephalin	14.1±5.4	7.3±2.0
[D-$Cys(O_2NH_2)^2$, Leu^5]enkephalin	73.4±12.7	7.4±1.9
[D-$Cys(O_2NH_2)^2$, Met^5]enkephalin	463±161	7.1±1.8
[$Hcy(O_2NH_2)^2$, Leu^5]enkephalin	76.4±7.1	3.4±0.2
[$Hcy(O_2NH_2)^2$, Met^5]enkephalin	55.7±6.1	4.5±0.3
[$Cys(Bzl)^2$, Leu^5]enkephalin	68.5±29.7	9.3±3.2
[$Cys(Bzl)^2$, Met^5]enkephalin	23.8±3.0	3.5±0.3
DPDPE	180±35	30.2±10.0
[D-Ala^2, *N*-Me-Phe^4, Leu^5]enkephalyl-Arg-NH_2	3302±1106	3.4±1.5
[Leu^5]enkephalin	54.9±13.1	5.8±1.0
[Met^5]enkephalin	48.4±7.5	3.6±0.3

Each value represents mean±S.E.M. of six experiments.

4. Discussion

The present investigation provides more detailed data for the potency, selectivity, affinity and efficacy of Cys^2-containing linear enkephalin analogues toward opioid receptors (Pencheva et al., 1995, 1996, 2003). The results reveal a specific involvement of δ-opioid receptor activation in the biological activities of these peptides (with exception of [$Cys(Bzl)^2$, Leu^5]enkephalin) because: (i) they have poor activity in guinea-pig ileum (the E_m^A values are between 0.35 and 0.75), which has μ- and κ-opioid receptors, but not δ-opioid receptors, and well pronounced effects in mouse vas deferens, which contains μ-, δ- and κ-opioid receptors, but is considered to be a standard model for studying the δ-properties of opioid ligands (Leslie, 1987); (ii) their inhibitory effects were blocked by either the selective δ-opioid receptor antagonist ICI 174864 (Cotton et al., 1984) or by 100 nM naloxone, while 10 nM naloxone did not antagonize their action. So it is permissible to compare IC_{50} values in the guinea-pig ileum with IC_{50} values in the mouse vas deferens as a direct correlation of μ-potency with δ-potency. By the way, we compare the IC_{50} values and generated by them selectivity ratio in the absence of peptidase inhibitors because: (i) the biological response is less manipulated; (ii) the combination of endogenous peptidases existing in the guinea-pig ileum or mouse vas deferens preparations and especially in the vicinity of the opioid receptors is still unanswered; (iii) the effects of degradation are different between both tissues guinea-pig ileum and mouse vas deferens; (iv) the enzymatic stability of the analogues is different. According to selectivity ratio, incorporation of $Cys(O_2NH_2)^2$ in the

molecule of [Leu^5]enkephalin and [Met^5]enkephalin increases the selectivity of the peptides from five to seven times as compared with that of δ-opioid receptor selective ligand DPDPE. However, this selectivity ratio has only a tentative character and, at some extent, is a confused measure because it depends on both: (i) the content and reactivity of the opioid receptors in two tissues, containing different receptor populations and thus reflects the stage of recognition between the compound and the respective opioid receptors; (ii) the potency of the peptides to evoke biological response, which concerns the post-recognition stage and stimulus response mechanisms. Basing only on selectivity ratio, the two different stages of drug-receptor action such as agonist-receptor and receptor–response interaction remain ill-resolved. That is why we need additional parameter to characterize the effects of peptides investigated. To differentiate receptor affinity and agonist efficacy of the analogues toward the δ-opioid receptors in mouse vas deferens, we determined their K_A and e_{rel} values. The explicit formulae used (Milanov et al., 2003) for evaluation of these parameters are a result of: (i) fitting by a two-parametric hyperbolic function; and (ii) an additional suggestion in the theory of drug-receptor modeling; we suppose that the unknown function of Stephenson $E=f(s)$ (Stephenson, 1956; s—stimulus) is also two-parametric hyperbola. Many other approaches for determination of K_A and e_{rel}, including the so-called operational model (Black and Leff, 1983; Quock et al., 1999; Burkey et al., 1998), failed to obtain explicit formulae. Generally, the theoretical modeling of the drug–receptor interaction begins with two parametric hyperbola, but the behavior of the concentration–response curves is described with at least three parameters (Leff and Dougall, 1989; Kramer et al., 1993, 1997). Moreover, both couples E_m^A/E_m^T and IC_{50}/A_{50} are not used in a proper way. Thus, the commonly used procedure to calculate K_A and e_{rel} is the technique of partial irreversible receptor inactivation. However, using the explicit formula for K_A, we obtained for the selective δ-opioid receptor agonist DPDPE the value K_A=180±35 nM, which is comparable with that of Kramer et al. (1997; K_A=230±45 nM). Other evidence for the relevancy of the formulae for K_A and e_{rel} was the comparison between the fitting of the data points with two- and three-parametric hyperbola. It was established that there are no statistical differences of the asymptote and location parameter ([A_{50}]) between two- and three parametric hyperbolic functions.

All these give us ground to suggest the following structure–affinity and structure–efficacy relationships: (i) incorporation of Cys(O_2NH_2)^2 in the molecule of [Leu^5]enkephalin highly increases the efficacy and does not change significantly the affinity of the respective analogues to δ-opioid receptors; [Cys(O_2NH_2)^2, Leu^5]enkephalin has higher affinity than DPDPE, but is less resistant to enzyme degradation similar to the endogenous enkephalins; the effect of this modification on the efficacy is decreased when methionine is in position 5: (ii) D-configuration of Cys(O_2NH_2)^2-containing analogues increases their peptidase resistance, but reduces efficacy and affinity of the peptides towards δ-opioid receptors; (iii) the substitution of Cys(O_2NH_2) with Hcy(O_2NH_2) decreases the efficacy, affinity and potency of the respective analogues and maintains their sensitivity to endogenous peptidases; (iv) the substitution of the sulfonamide group with benzyl group in the molecule of Cys in position 2 decreases their efficacy and affinity toward δ-opioid receptors, but attaches resistance to enzyme degradation.

Evidence for the role of Cys(O_2NH_2) in position 2 in the improvement of the δ-opioid properties of the respective analogues is the opioid profiles of Cys^2-containing fragments as a minimum structure with opioid-like activity (Vavrek et al., 1981). While some dipeptides containing Gly-Phe sequence exert antagonist properties (Radomirov et al., 1994), di- or tripeptides revealed to the Tyr–Gly–Gly sequence could mimic the opioid agonist effect (Morgan et al., 1976). Thus, we established that Tyr-Cys(O_2NH_2) was potent and selective towards δ-opioid receptors, while Tyr-D-Cys(O_2NH_2) was less potent and Tyr-Cys (Bzl) was less selective. At the same time, this enkephalin fragment exerts more effective action because its E_m^A was highest and similar to that of the pentapeptide sequences, although reached at higher doses.

In conclusion, these findings are consistent with a high potency and selectivity of the linear, Cys(O_2NH_2)^2-containing enkephalin analogues for δ-opioid receptors. Furthermore, the present results allow: (i) to involve the receptor affinity and agonist efficacy as drug-design consideration for δ-opioid properties of newly synthesized compounds; and (ii) to characterize some of the structural features, which set the pattern for their opioid profiles. Thus, Cys^2-containing linear enkephalin analogues may provide a starting point for the design of δ-selective agonist in the development of new therapeutic compounds with analgesic properties.

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