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[Cys(O_2NH_2)^2]enkephalin analogues and dalargin: selectivity for δ-opioid receptors

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Abstract

To investigate the structure-activity relationships for potent and selective action of enkephalins at the δ-opioid receptors, two newly synthesized analogues, [Cys(O_2NH_2)^2,Leu^5]enkephalin and [Cys(O_2NH_2)^2,Met^5]enkephalin and the hexapeptide [D-Ala^2,Leu^5]enkephalyl-Arg (dalargin) were tested and compared with [Leu^5]enkephalin and [Met^5]enkephalin, for their effectiveness to inhibit 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 mouse vas deferens assays included evaluation of the effects of opioid agonists on the first, purinergic, and the second, adrenergic, components of electrically evoked biphasic responses (10 Hz and 20 Hz) and on ATP- or noradrenaline-evoked, tetrodotoxin-resistant responses. The opioids tested inhibited in a similar manner: (i) the purinergic and the adrenergic components of the electrically evoked contractions; and (ii) the ATP- and noradrenaline-induced postjunctional responses of the mouse vas deferens. Extremely low IC_{50} values (of 2–5 orders) were found for [Cys(O_2NH_2)^2,Leu^5]enkephalin, whose relative potency was between 239 and 1316 times higher than that of [Leu^5]enkephalin. The order of potency for the other peptides in this tissue was: [Cys(O_2NH_2)^2,Met^5]enkephalin > [Leu^5]enkephalin > dalargin > [Met^5]enkephalin. The highest IC_{50} values in the guinea pig ileum assays, indicating the lowest affinity for μ-/κ-opioid receptors, were obtained for the cysteine sulfonamide analogues, while dalargin showed a potency four times higher than that of [Met^5]enkephalin. The order of potency in this tissue was: dalargin > [Met^5]enkephalin > [Leu^5]enkephalin > [Cys(O_2NH_2)^2,Met^5]enkephalin > [Cys(O_2NH_2)^2,Leu^5]enkephalin. The ratio, IC_{50} in guinea pig ileum : IC_{50} in mouse vas deferens, indicating selectivity of the respective peptide for δ-opioid receptors, was extremely high for [Cys(O_2NH_2)^2,Leu^5]enkephalin and especially for the adrenergic component of the responses. This ratio for [Cys(O_2NH_2)^2,Met^5]enkephalin was higher than the ratios for dalargin, [Leu^5]enkephalin and [Met^5]enkephalin, which were about 3 orders of magnitude lower. The results suggest that incorporation of hydrophilic Cys(O_2NH_2) in the enkephalin molecule greatly increases the potency and selectivity of the analogues at δ-opioid receptors, while both D-Ala^2 substitution and lengthening of the peptide chain by Arg^6 in the molecule of [Leu^5]enkephalin decrease them.

Keywords: δ-Opioid receptor; Vas deferens, mouse; Cysteine sulfonamide enkephalin analog; Dalargin; Purinergic component; Adrenergic component; Electrical field stimulation

1. Introduction

After the discovery of endogenous opioid pentapeptides and of the heterogeneity of opiate receptors, thousands of enkephalin analogues have been investigated to answer questions related to opioid receptor functions and to design more potent and selective ligands. Hruby and Gehrig (1989), analyzing the structure-activity relations of numerous enkephalin analogues, summarized that the structure modifications having a substantial role in the affinity and selectivity of enkephalin ligands are the substitution at position 2 and the alterations in the C-terminal. It is well known that substitutions in position 2 and 5 by D- (or L-)cysteine or by D- (or L-)Pen (penicillamine = β-dimethylcysteine), yield δ-selective analogues with side-chain to side-chain intramolecular cyclization via a disulfide bridge (Schiller et al., 1981; Mosberg et al., 1983). However whether cysteine in position 2 with a protected SH group is of importance for obtaining δ-opioid

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receptor-selective linear enkephalin analogues is not understood. The present study was undertaken to investigate two newly synthesized (Stoeva et al., 1994) linear enkephalin analogues with $Cys(O_2NH_2)$ substitution in position 2: [$Cys(O_2NH_2)^2$,Leu^5]enkephalin and [$Cys(O_2NH_2)^2$, Met^5]enkephalin, whose δ-properties have not yet been studied. Also, to determine whether both the lengthening of the peptide chain with Arg in position 6 and substitution of D-Ala in position 2 would change the affinity and selectivity at δ-receptors, we studied [D-Ala^2,Leu^5]enkephalyl-Arg, dalargin a synthetic analogue of [Leu^5]enkephalin, which is used in clinical practice, but whose δ-opioid receptor action is unclear. [Leu^5]enkephalin and [Met^5]enkephalin were used for comparison with the native pentapeptide structures.

A part of the experiments was carried out with the guinea pig ileum longitudinal muscle preparation which has μ- and κ-opioid receptors but no δ-opioid receptors (Lord et al., 1977). Since the properties of the analogues tested in this tissue have been previously investigated (Pencheva et al., 1995), in this study we intended to obtain the IC_{50} values under the experimental conditions used and to compare them with the IC_{50} in mouse vas deferens. The majority of experiments were performed with isolated mouse vas deferens, containing μ-, δ- and κ-opioid receptors, but used as a standard model for studying the δ-properties of opioid ligands, because the enkephalin-selective δ-opioid receptors are predominant (Lord et al., 1977; Leslie, 1987). Sympathetic transmission in the mouse vas deferens is known to be due to at least two co-transmitters, noradrenaline and adenosine 5′-triphosphate (ATP). Both participate in the electrically evoked biphasic contractile responses consisting of a first, rapid, purinergic phase and a second, slow, adrenergic phase (Allcorn et al., 1986; Von Kügelgen et al., 1989). To more fully characterize the δ-properties of enkephalin analogues tested, we followed their effects separately on both purinergic and adrenergic components of electrically evoked motor responses. The effects of the peptides on the ATP- and noradrenaline-induced mechanical changes were also examined.

2. Materials and methods

2.1. Isolated tissue preparations

Male TO albino mice (23–29 g) were killed by cervical dislocation and the vasa deferentia were excised. The connective tissue-free preparations were mounted vertically in 10 ml siliconized organ baths with warm (37°C), oxygenated (95% O_2-5% CO_2), modified Krebs solution (according to Stjärne and Astrand, 1985, with lower content of Mg^{2+}), containing (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.6, $NaHCO_3$ 11.9, KH_2PO_4 0.5, glucose 11.5, pH 7.3–7.4 and peptidase inhibitor bacitracin 30 mg/l. The preparations were suspended under 1 g tension. The equilibration period was 60 min. The recorders of the contractile responses to electrical field stimulation and drugs were isometric, using a strain gauge (Microtechna 1101), connected to a line recorder (TZ 4620, Laboratorni Pristroje, Prague). Electrical field stimulation was applied with a pair of platinum electrodes situated 15 mm apart on diametrically opposite sides of the bath. Rectangular pulses at a frequency of 10 Hz or 20 Hz (train duration 10 s, pulse width 0.3 ms, submaximal voltage of 50 V at 60 s intervals), known to activate the neuronal structures in vas deferens (Stjärne and Astrand, 1985) were generated by a stimulator ST-02 (Experimetria, Hungary).

Segments of the ileum, isolated from male guinea pigs (Dunkin-Hartley, 250–300 g), approximately 2 cm long, containing intact myenteric plexus, were set up for field stimulation in a 10 ml siliconised organ bath (tension 1 g), as described elsewhere (Pencheva et al., 1995). The tissues were bathed in warm, oxygenated, Krebs solution, containing (mM): NaCl 120, KCl 5.9, $NaHCO_3$ 14.4, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 11.5 and 30 mg/l bacitracin and were stimulated electrically with three square pulses of 0.4 ms duration, frequency of 5 Hz and submaximal voltage of 40 V at 10 s intervals. Since opioids influence predominantly cholinergic neurotransmission in the intestine (Cox and Weinstock, 1966), we used stimulation with a relatively low frequency, known to elicit cholinergic responses mainly (Alberts and Stjärne, 1982).

In preliminary experiments, we found that in the guinea pig ileum and mouse vas deferens preparations the potencies of enkephalins and analogues were increased after bacitracin, but the changes were insignificant, except for [Met^5]enkephalin and [Leu^5]enkephalin in the mouse vas deferens assay, whose potencies were 1.5–2.5 times lower ($P < 0.05$).

2.2. Mouse vas deferens bioassay

2.2.1. General considerations

In the mouse vas deferens assay we used tetanic stimulation with a frequency of 10 Hz, because the biphasic responses elicited showed well pronounced components. The higher frequency of 20 Hz was applied as the potency of selective and non selective ligands during high frequency of stimulation would possibly be different, because the total number of excited nerve varicosities involved in the neurotransmitter release is larger (Alberts and Stjärne, 1982; Cunnane and Stjärne, 1984).

Since the participation of ATP and/or noradrenaline, alone or in combination, in biphasic contractile responses to single shocks or to low- and high-frequency stimulation depends on the stimulation parameters, animal species and experimental design (Amobi and Smith, 1987; Von Kügelgen and Starke, 1994), we characterized the phases of the electrically evoked responses in control experiments on separate groups of vas deferens preparations. In this preliminary series the postjunctional ATP- and noradrena-

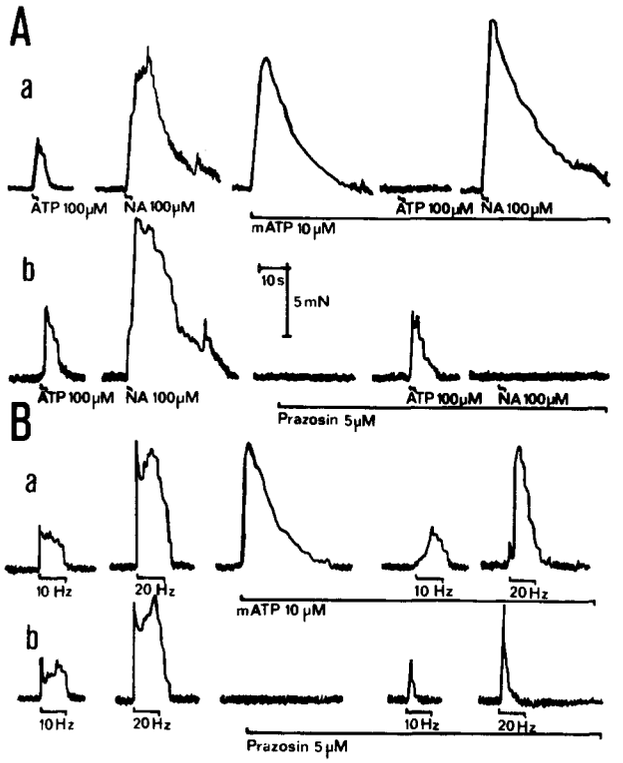

Fig. 1. Mouse vas deferens. Selective effects of α,β-methylene-adenosine-5'-triphosphate (mATP, 10 μM) and prazosin (5 μM) on contractile responses to exogenous ATP (100 μM) and noradrenaline (NA, 100 μM) (*A*, a, b) or on the purinergic and adrenergic components of the responses to electrical field stimulation (*B*, a, b) with the following parameters: 10 Hz or 20 Hz, train duration 10 s, pulse width 0.3 ms, submaximal voltage of 50 V, 60 s intervals. Upward and downward arrows indicate drug addition or stimulus application.

line-elicited responses were also determined. In order to isolate the adrenergic and purinergic components of the contractile responses, α,β-methylene-ATP (10 μM) or prazosin (5 μM) was added to the organ bath 15–20 min before repeated electrical (10 and 20 Hz) or drug stimulation. It was found that after desensitization of purinoceptors by α,β-methylene-ATP (the per se effect of α,β-methylene-ATP was a pronounced contraction), the tissue was selectively insensitive to doses of ATP (100 μM) which previously produced contractions, but not to noradrenaline (100 μM) (Fig. 1A,a). Pretreatment of intact preparations with prazosin blocked the contractile response to noradrenaline, but not that to ATP (Fig. 1A,b). On the other hand, electrical stimulation by 10 and 20 Hz (10 s, pulse width 0.3 ms, 50 V) elicited a tetanic response: i.e. a primary twitch component with a peak after 2–3 s, followed by a slow component with a peak after 5–7 s. After exposure of the tissue to α,β-methylene-ATP the primary component of the response was abolished at 10 Hz stimulation and was reduced by $88 \pm 9.5\%$ ($n = 4$) at 20 Hz stimulation (Fig. 1B,a), while the second component was unchanged. In the presence of prazosin the first component of the response to 10 and 20 Hz stimulation was unchanged but the second was completely abolished (Fig. 1B,b). This suggests that the purinergic and adrenergic component of the responses elicited by the stimulations used could be examined separately. The potency of enkephalins and analogues was compared with respect to the inhibitory effects on each component of the electrically or drug-evoked contractions.

2.2.2. Experimental procedure

To investigate the opioid agonistic activity of the peptides, two types of preparations were used: (i) electrically stimulated, and (ii) non-stimulated, where the inhibitory effects of opioids were tested on ATP- or noradrenaline-evoked responses. In the preparations of the first type, the effects of opioids were assessed on the individual components of the biphasic responses because their purinergic or adrenergic nature was proved in preliminary experiments. The cumulative concentration-response curves were obtained separately for purinergic and adrenergic components of the responses at two frequencies of stimulation (10 and 20 Hz), by the addition of increasing concentrations of opioids (1 pM–10 μM) at 2 min intervals. The changes in the amplitudes of the corresponding component, measured in linear units (mm) and recalculated in mN (according to the daily calibration of the transducer) were determined. The points of the concentration-response curves, expressed as percentages of the respective controls, were plotted for at least six points, each summarizing at least six effects obtained in different animals. The range between 16% and 84% was subjected to regression analysis and the IC_{50} values and the relative potency (according to the IC_{50} of [Leu^5]enkephalin considered to be 1) were estimated for each of the enkephalins or analogues tested with respect to purinergic and adrenergic components of the responses evoked by stimulation at frequencies of 10 and 20 Hz. To examine the opioid character of the effects exerted by enkephalins, we used the blocking agent, naloxone (1 μM; 10 min). Tetrodotoxin (1 μM; 10 min) was employed to block the neuronal input.

The preparations of the second type were subjected to the inhibitory action of the opioids as follows: ATP, applied non cumulatively to escape desensitization (intervals of 15 min with intervening washings were sufficient) and noradrenaline, administered cumulatively, at increasing concentrations from 1 pM to 1 mM, showed well expressed, concentration-dependent contractile responses; the submaximal contractile effects exerted by ATP and noradrenaline, reached at a concentration of 100 μM were taken to be 100% (controls); the effects of single concentrations of the enkephalins tested on ATP- and noradrenaline-evoked responses were followed over a range closely related to their IC_{50} values, obtained from the curves of electrically evoked responses. All effects of enkephalins on ATP- and noradrenaline-evoked responses were tested in the presence of tetrodotoxin (1 μM; 10 min) or naloxone (1 μM; 10 min). The maximal inhibitory effects of the

Table 1
Amino acid sequence of enkephalins and analogues

Enkephalins and analogues	Primary structure
[Leu^5]enkephalin	H-Tyr-Gly-Gly-Phe-Leu-OH
[Met^5]enkephalin	H-Tyr-Gly-Gly-Phe-Met-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-Ala^2,Leu^5]enkephalyl-Arg (dalargin)	H-Tyr-D-Ala-Gly-Phe-Leu-Arg-Oh

peptides on these responses, measured in linear units and expressed as percentages of the controls, were obtained for at least seven preparations.

All data are presented as means ± S.E.M. Differences between means were assessed for statistical significance using a t test for grouped data; $P < 0.05$ or lower was taken to be significant. The statistical procedures and the plotting of the curves were carried out with computer programs (Tallarida and Murray, 1981).

2.3. Guinea pig ileum bioassay

Cumulative concentration-response curves (1 pM–1 mM, at 2 min intervals) were constructed for each of the opioids. Potencies were expressed as IC_{50} values (nM). In order to test the selectivity of the opioids for δ-opioid receptors, the ratio, IC_{50} in guinea pig ileum : IC_{50} in mouse vas deferens, was calculated. This ratio is presented for adrenergic and purinergic components of the responses induced by stimulation at 10 Hz only, because the changes in the IC_{50} values in the mouse vas deferens assay upon stimulation at 20 Hz are connected with changes in neurotransmitter release rather than in the selectivity of opioid receptors.

2.4. Compounds

The peptides (described in Table 1) were: (i) [Leu^5]enkephalin (Sigma) and [Met^5]enkephalin (Sigma), used as test opioids; (ii) the newly synthesized cysteine sulfonamide analogues [Cys(O_2NH_2)^2,Leu^5]enkephalin and [Cys(O_2NH_2)^2,Met^5]enkephalin and the hexapeptide [D-

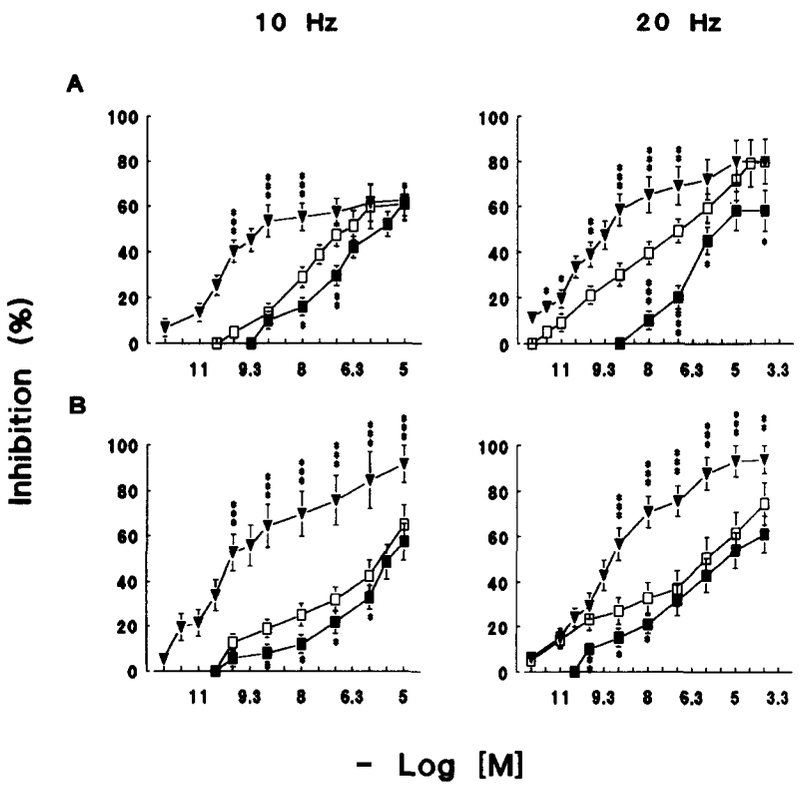

Fig. 2. Mouse vas deferens. Concentration-response curves for the inhibitory effects of [Leu^5]enkephalin (□), [Cys(O_2NH_2)^2,Leu^5]enkephalin (▼) and [D-Ala^2,Leu^5]enkephalyl-Arg (dalargin) (■) on the purinergic (*A*) and adrenergic (*B*) components of contractions evoked by 10 Hz and 20 Hz stimulation (train duration 10 s, pulse width 0.3 ms, submaximal voltage of 50 V at 60 s intervals). Means ± S.E.M. (vertical lines) of at least six preparations. Stars indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; t test for grouped data) compared to the effects of [Leu^5]enkephalin.

Ala[2],Leu[5]]enkephalyl-Arg (dalargin), were kindly supplied by the Group of Antimetabolites, headed by E. Golovinsky from the Institute of Molecular Biology, Bulgarian Academy of Sciences, Sofia. The drugs used were: α,β-methylene-adenosine-5′-triphosphate (mATP, Sigma), prazosin hydrochloride (Sigma), adenosine 5′-triphosphate (ATP, Sigma), noradrenaline hydrochloride (Sigma), naloxone (Sigma), bacitracin (Sigma) and tetrodotoxin (Sankyo).

The compounds were dissolved in distilled water and diluted to their final concentration in Krebs solution before use. They were administered in volumes not exceeding 0.5–1% of the bath volume.

3. Results

3.1. Mouse vas deferens assay

3.1.1. Effects of enkephalins and enkephalin analogues on responses to electrical stimulation

The electrically evoked biphasic responses of the mouse vas deferens preparations were not observed after treatment with tetrodotoxin (1 μM), indicating their neurogenic nature ($n = 4$; data not shown). The amplitude of the first, purinergic component of the response to 10 Hz stimulation was 3.92 ± 0.8 mN. This component of the response increased to 12.4 ± 1.7 mN ($n = 12$) upon 20 Hz stimulation, while the amplitude of the second, adrenergic component of the response was 4.34 ± 1.3 mN and 13.3 ± 2.1 mN ($n = 12$) upon stimulation at 10 Hz and 20 Hz, respectively.

The test opioids, [Leu[5]]enkephalin and [Met[5]]enkephalin, and their analogues, [Cys(O_2NH_2)[2],Leu[5]]enkephalin, [Cys(O_2NH_2)[2],Met[5]]enkephalin and dalargin, applied cumulatively at concentrations of 1 pM–10 μM, dose dependently inhibited the biphasic electrically induced contractile response to stimulation at 10 and 20 Hz frequency. Blockade of opiate receptors by naloxone (1 μM) antagonized the effects of all peptides on the neurogenic contractions, their purinergic and adrenergic components, at frequencies of 10 and 20 Hz. The concentration-response curves for [Cys(O_2NH_2)[2],Leu[5]]enkephalin with respect to its effects on the purinergic components of the responses to 10 and 20 Hz of stimulation were situated to the left of those for [Leu[5]]enkephalin, while those for dalargin were situated to the right (Fig. 2A). A similar effect of the enkephalin analogues was observed with respect to the adrenergic components of the responses to 10 and 20 Hz stimulation (Fig. 2B). The concentration-response curves for the other cysteine-containing analogue [Cys(O_2NH_2)[2], Met[5]]enkephalin were to the left of those for [Met[5]]en-

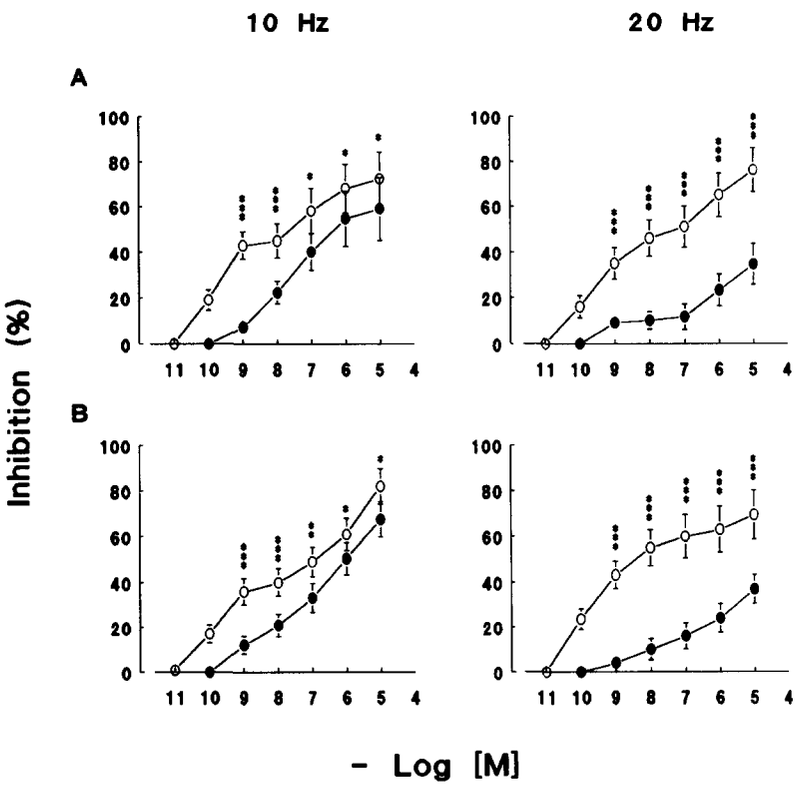

Fig. 3. Mouse vas deferens. Concentration-response curves for the inhibitory effects of [Met[5]] enkephalin (●) and [Cys(O_2NH_2)[2],Met[5]]enkephalin (○) on the purinergic (*A*) and adrenergic (*B*) components of contractions evoked by 10 Hz and 20 Hz stimulation (train duration 10 s, pulse width 0.3 ms, submaximal voltage of 50 V at 60 s intervals). Means ± S.E.M. (vertical lines) of at least six preparations. Stars indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; *t* test for grouped data) compared to the effects of [Met[5]]enkephalinon.

Table 2
The inhibitory effects of [Leu^5]enkephalin and [Met^5]enkephalin and of the enkephalin analogues [Cys$(O_2NH_2)^2$,Leu^5]enkephalin, [Cys$(O_2NH_2)^2$,Met^5]enkephalin and dalargin on electrically evoked contractions of the longitudinal muscle of the guinea pig ileum (3 pulses, 0.4 ms, 5 Hz, 40 V, at 10 s intervals) and of the mouse vas deferens (100 pulses, 0.3 ms, 10 Hz, 50 V at 60 s intervals) preparations

Enkephalins and analogues	Guinea pig ileum IC_{50} (nM)	Mouse vas deferens					
		Purinergic component			Adrenergic component		
		IC_{50} (nM)	Relative potency	Ratio IC_{50} in guinea pig ileum: IC_{50} in mouse vas deferens	IC_{50} (nM)	Relative potency	Ratio IC_{50} in guinea pig ileum: IC_{50} in mouse vas deferens
[Leu^5]enkephalin	138 ± 26	76.8 ± 84	1	1.79	158 ± 23 [a]	1	0.87
[Cys$(O_2NH_2)^2$,Leu^5]enkephalin	7980 ± 280	0.260 ± 0.038	295	30692	0.12 ± 0.02 [a]	1316	66500
Dalargin	9.5 ± 2.5	273 ± 35	0.28	0.035	332 ± 48	0.47	0.03
[Met^5]enkephalin	40.8 ± 6.35	531 ± 61	0.14	0.077	502 ± 71	0.31	0.08
[Cys$(O_2NH_2)^2$,Met^5]enkephalin	4200 ± 495	61 ± 4.6	1.26	68.96	83.4 ± 9.5 [a]	1.9	50.36

The mouse vas deferens assay comprised the biphasic responses of this tissue consisting of purinergic and adrenergic components. The values for IC_{50} are the means ± S.E.M.; the number of observations are at least 6. [a] Significant differences vs. the corresponding value for purinergic component at $P < 0.05$ (t test for grouped data).

kephalin with respect to both purinergic and adrenergic components of the responses to 10 and 20 Hz stimulation (Fig. 3A,B). The curves representing the effects of [Met^5]enkephalin were situated to the right for responses to 20 Hz stimulation, compared to those at 10 Hz, while the curves for effects of [Cys$(O_2NH_2)^2$,Met^5]enkephalin were not changed.

The values derived from the concentration-response curves for inhibition of both components of the responses to 10 Hz frequencies of stimulation, are summarized in Table 2. All enkephalins influenced the two components of the responses in a similar manner but to a different degree. The IC_{50} values of [Leu^5]enkephalin (considered to be 1) were lower for inhibition of the purinergic component of the responses ($P < 0.05$) than of the adrenergic one. [Cys$(O_2NH_2)^2$,Leu^5]enkephalin was the most potent inhibitor and the IC_{50} value was decreased to a very large extent compared to that of [Leu^5]enkephalin for both components of the responses. However, the IC_{50} value for inhibition of the adrenergic component by [Cys$(O_2NH_2)^2$,Leu^5]enkephalin was significantly lower ($P < 0.05$). The relative potency of [Cys$(O_2NH_2)^2$,Leu^5]-enkephalin was about 2 orders of magnitude higher with respect to the purinergic component and about 3 orders higher with respect to the adrenergic one. On the other hand, the IC_{50} values of dalargin were higher than those for [Leu^5]enkephalin and its relative potency was low. The highest IC_{50} values in the mouse vas deferens were found for [Met^5]enkephalin whose relative potency was the lowest. There were no significant differences between adrenergic and purinergic components for inhibition by [Met^5]enkephalin. Substitution of cysteine sulfonamide in the molecule of [Met^5]enkephalin led to a great increase in the relative potency of the analogue [Cys$(O_2NH_2)^2$,Met^5]enkephalin as compared to [Met^5]enkephalin and [Leu^5]enkephalin. The IC_{50} value of [Cys$(O_2NH_2)^2$,Met^5]enkephalin was lower for inhibition of the purinergic component than of the drenergic one. Comparison of the relative potency of [Cys$(O_2NH_2)^2$,Met^5]enkephalin with that of [Cys$(O_2NH_2)^2$,Leu^5]enkephalin showed it to be greatly decreased.

Summarizing the results derived from concentration-response curves at 20 Hz frequency of stimulation (Table 3), it could be concluded that the extent of inhibition of

Table 3
The inhibitory effects of [Leu^5]enkephalin and [Met^5]enkephalin and of the enkephalin analogues [Cys$(O_2NH_2)^2$,Leu^5]enkephalin, [Cys$(O_2NH_2)^2$,Met^5]enkephalin and dalargin on electrically evoked contractions of the mouse vas deferens preparations (100 pulses, 0.3 ms, 20 Hz, 50 V at 60 s intervals)

Enkephalins and analogues	Purinergic component		Adrenergic component	
	IC_{50} (nM)	Relative potency	IC_{50} (nM)	Relative potency
[Leu^5]enkephalin	129 ± 17	1	891 ± 75 [b]	1
[Cys$(O_2NH_2)^2$,Leu^5]enkephalin	0.54 ± 0.12	239	0.97 ± 0.12 [a]	918
Dalargin	4460 ± 438	0.03	3420 ± 480 [a]	0.26
[Met^5]enkephalin	94300 ± 10020	0.001	87600 ± 7600	0.01
[Cys$(O_2NH_2)^2$,Met^5]enkephalin	95.2 ± 11.3	1.35	98.5 ± 8.3	9.4

The purinergic and adrenergic components of the responses were evaluated. The values for IC_{50} are the means ± S.E.M.; the number of observations are at least 6. [a] Significant differences vs. the corresponding value for purinergic component at $P < 0.05$; [b] significant differences vs. the corresponding value for purinergic component at $P < 0.01$ (t test for grouped data).

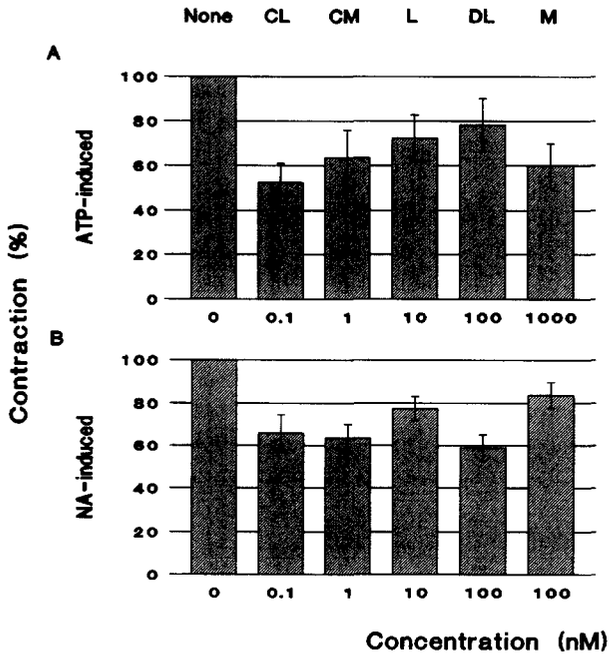

Fig. 4. Mouse vas deferens. The maximal inhibitory effects (%), of different concentrations (nM) of [Cys(O_2NH_2)^2,Leu^5]enkephalin (CL), [Cys(O_2NH_2)^2,Met^5]enkephalin (CM), [Leu^5]enkephalin (L), [D-Ala^2,Leu^5]enkephalyl-Arg (DL) and [Met^5]enkephalin (M), on ATP- (*A*) and noradrenaline-elicited (*B*) contractile responses; None: control responses of 100 μM ATP or noradrenaline. Columns: mean values of the maximum inhibition; vertical lines: S.E.M. of at least seven preparations.

electrically evoked contractions decreased when stimulation frequency was increased from 10 to 20 Hz for all enkephalins tested. However, the increase of the IC_{50} values was least pronounced for [Cys(O_2NH_2)^2,Leu^5]enkephalin and [Cys(O_2NH_2)^2,Met^5]enkephalin and was great for [Leu^5]enkephalin and especially so for dalargin and [Met^5]enkephalin. The IC_{50} values of the inhibition of the adrenergic component were significantly higher for [Cys(O_2NH_2)^2,Leu^5]enkephalin ($P < 0.05$) and [Leu^5]enkephalin ($P < 0.001$) and significantly lower for dalargin ($P < 0.05$).

3.1.2. Effects of enkephalins and enkephalin analogues on ATP- and noradrenaline-evoked responses

ATP applied non cumulatively at increasing concentrations, from 1 μM to 1 mM, produced transient concentration-dependent contractions, resistant to tetrodotoxin (1 μM). In the presence of enkephalins and enkephalin analogues the contractile effects of ATP, at a single submaximal concentration (100 μM), were decreased and the decrease was naloxone-sensitive. The maximal effects of the peptides on ATP-induced contractions were reached with different concentrations, revealing their different opioid potency. The maximal inhibitory effect was reached by [Cys(O_2NH_2)^2,Leu^5]enkephalin at a concentration of 0.1 nM (Fig. 4A). The order of inhibition produced by the other enkephalins was as follows: [Cys(O_2NH_2)^2,Met^5]enkephalin > [Leu^5]enkephalin > dalargin > [Met^5]enkephalin.

Noradrenaline applied cumulatively at concentrations of 1 μM to 1 mM elicited concentration-dependent contractile responses which were not sensitive to tetrodotoxin (1 μM). In the presence of enkephalins the effects of noradrenaline at a single submaximal concentration (100 μM), were inhibited and the inhibition was naloxone-dependent. The maximal inhibition was produced by [Cys(O_2NH_2)^2, Leu^5]enkephalin at a concentration of 0.1 nM and by [Cys(O_2NH_2)^2,Met^5]enkephalin at a concentration of 1 nM. The maximal inhibitory responses of the other peptides were reached with higher doses and the order of potency was: [Leu^5]enkephalin > dalargin > [Met^5]-enkephalin (Fig. 4B).

3.2. Guinea pig ileum assay

The IC_{50} values for enkephalins and enkephalin analogues in guinea pig ileum revealed significant differences in their effects and the order of opioid agonist potency was dalargin > [Met^5]enkephalin > [Leu^5]enkephalin > [Cys(O_2NH_2)^2,Met^5]enkephalin > [Cys(O_2NH_2)^2,Leu^5]-enkephalin (Table 2). The ratio of IC_{50} in guinea pig ileum : IC_{50} in mouse vas deferens was extremely high for [Cys(O_2NH_2)^2,Leu^5]enkephalin and especially for its effect on the adrenergic component. This ratio for the other cysteine sulfonamide analogues [Cys(O_2NH_2)^2,Met^5]enkephalin was higher than those for dalargin, [Met^5]enkephalin and [Leu^5]enkephalin, which were about 3 orders lower.

4. Discussion

The present study confirmed the biphasic pattern and the neurogenic nature of the electrically evoked motor responses of the mouse vas deferens (Von Kügelgen et al., 1989). The first and second phases of these responses corresponded to the purinergic and adrenergic components, as characterized in the present study and by other authors (Stjärne and Astrand, 1985; Burnstock, 1990; Von Kügelgen and Starke, 1991a).

In the mouse vas deferens the native opioids, [Leu^5]enkephalin and [Met^5]enkephalin, the hexapeptide, dalargin, and the newly synthesized analogues, [Cys(O_2NH_2)^2, Leu^5]enkephalin and [Cys(O_2NH_2)^2,Met^5]enkephalin, inhibited in a concentration-dependent manner: (i) the purinergic and adrenergic components of neurogenic contractions evoked by electrical stimulation at two frequencies (10 and 20 Hz); and (ii) the ATP- and noradrenaline-induced contractions. The effects were mediated by specific opioid receptors because of the naloxone-reversible manner of inhibition, similar to that observed in the guinea pig ileum and in the central nervous system (Bocheva et al., 1994). The purinergic and adrenergic components of

the responses to stimulation or ATP- and noradrenaline-induced changes were inhibited in a similar manner. Small, but statistically significant differences were observed in the inhibition of: the adrenergic component for the effect of [Cys(O_2NH_2)^2,Leu^5]enkephalin on the response evoked at 10 Hz and for the effect of dalargin on the response evoked at 20 Hz stimulation; and the purinergic component for the effect of [Cys(O_2NH_2)^2,Met^5]enkephalin on the response to 10 Hz and for effect of [Leu^5]enkephalin at 10 and 20 Hz stimulation. Prejunctionally mediated inhibition of the purinergic and adrenergic components in the responses of mouse vas deferens by selective opioid agonists with preference for inhibition of the adrenergic component, though with different parameters of stimulation, was described recently by Driessen et al. (1993). However they failed to report data concerning the opioid agonistic effects on postjunctional ATP- or noradrenaline-evoked responses. In the present study the inhibition of the ATP- and noradrenaline-elicited contractions at postjunctional level was manifested not only with respect to similarity but also to order of potency. The inhibition of the ATP- and noradrenaline-induced, tetrodotoxin-resistant responses by enkephalins could be considered as alternative parameter for estimation of opioid agonist potency. Thus, all these findings rule out the possibility of differential modulation by opioids of purinergic and adrenergic mechanisms in mouse vas deferens, which was proved for some other peptides (Trachte, 1988; Ellis and Burnstock, 1989), endogenous compounds (Ellis and Burnstock, 1990; Von Kügelgen and Starke, 1991b) and even for morphine (Forsyth and Pollock, 1988).

The quantitative correlations between the concentration-dependent inhibitory effects of enkephalins suggest that [Cys(O_2NH_2)^2,Leu^5]enkephalin is the most potent δ-opiate agonist. Obviously the [Cys(O_2NH_2)] substitution in position 2 in the chain of the pentapeptide structure of [Leu^5]enkephalin greatly increased the potency at the δ-opioid receptors. It is known that position 2 in the enkephalin molecule is important when preparing ligands conformationally restricted to the proper receptor type (Hruby and Gehrig, 1989), but the role of Cys(O_2NH_2) substitution in the δ-opioid receptor activity is unclear. It could be speculated that the less studied hydrophilic Cys(O_2NH_2) residue, substituted in position 2 probably meets the conformational and electronic requirements for high-affinity δ-opioid receptor binding, similar to other cysteine-containing, but cyclic opioid peptides, which have been thoroughly studied (Mosberg et al., 1983; Schiller et al., 1981; Keys et al., 1988). It is well known that the opioid peptides, especially the native enkephalins which possess a high degree of conformational flexibility, bind with high affinity to both μ- and δ-opioid receptors (Hruby, 1985; Goldstein, 1987). However, the different synthetic analogues of the opioid peptides exhibit varying affinity and selectivity for μ-, δ- and κ-opioid receptor types located on the sympathetic terminal axons of the mouse vas deferens (Illes, 1989). The extremely high potency of [Cys(O_2NH_2)^2,Leu^5]enkephalin in the mouse vas deferens, where enkephalin-selective δ-opioid receptors are predominant (Lord at all., 1977; Leslie, 1987), and the extremely low potency in the guinea pig ileum, which practically has only μ- and κ-receptors, suggest the high selectivity of this analogue at δ-opioid receptors. The incomplete blockade of endogenous peptidase activity by bacitracin only (McKnight et al., 1983) poses the question of a delayed enzymatic degradation of the analogues. However, the extremely large differences in the IC_{50} values of the enkephalin analogues between guinea pig ileum and mouse vas deferens preparations (of 1–5 orders of magnitude) and the presence of bacitracin in the Krebs solutions, give us ground to believe that the potency differences could be considered a result of their selectivity rather than of their differential enzymatic stability.

The properties of [Cys(O_2NH_2)^2,Met^5]enkephalin were further considered an argument in favour of the ability of Cys(O_2NH_2) in position 2 to increase the potency of enkephalins at δ-receptors. Comparison of the data about [Cys(O_2NH_2)^2,Met^5]enkephalin and [Met^5]enkephalin or [Leu^5]enkephalin, showed that this type of substitution in the molecule of the least potent [Met^5]enkephalin yielded the potent δ-analogue, [Cys(O_2NH_2)^2,Met^5]enkephalin, which proved to be: (i) more potent than [Met^5]enkephalin (from 6 to 1800 times higher relative potency); even at high frequency of stimulation, accompanied by an increased release of noradrenaline and ATP (Von Kügelgen and Starke, 1994), the relative potency of [Cys(O_2NH_2)^2, Met^5]enkephalin increased, while that of [Met^5]enkephalin decreased; (ii) more potent than [Leu^5]enkephalin (especially at high frequency of stimulation), which is a native agonist with preference for δ-receptors in this tissue, although in the molecule of [Cys(O_2NH_2)^2,Met^5]enkephalin contains methionine in position 5; and (iii) most selective among the enkephalins tested except for [Cys-(O_2NH_2)^2,Leu^5]enkephalin.

The importance of C-terminal leucine residue for the determination of the minimum active sequence necessary for interactions with δ-opioid receptors is still a matter of speculation (Hruby and Gehring, 1989). Comparing the characteristics of the following couples of enkephalins: [Leu^5]enkephalin/[Met^5]enkephalin, [Cys(O_2NH_2)^2, Leu^5]enkephalin/[Cys(O_2NH_2)^2,Met^5]enkephalin and dalargin/[Met^5]enkephalin, led us to believe that Leu^5 increases the potency of the enkephalin analogues at δ-receptors. This increase was especially pronounced with the higher frequencies of stimulation. After incorporation of cysteine sulfonamide into the pentapeptide structure of enkephalins (Aleksiev et al., 1971, 1972), analogues with cysteine sulfonamide substitution in position 2 and 5 and with a COOH-terminal in the form of amide derivates have been synthesized and investigated (Mancheva and Aleksiev, 1990). The poor δ-selectivity of these analogues and the properties of the cysteine sulfonamide-containing

enkephalins characterized in this study favoured the view that the carboxiamide terminal enkephalin analogues are less δ-opioid receptor-selective than the carboxylate terminal analogues. That the so-called COOH terminal changes are probably detrimental to δ-potency but not to μ-potency has been suggested by other authors (Hruby and Gehrig, 1989).

The observation on the effects of the enkephalin analogue, dalargin, demonstrated that another C-terminal change, i.e. lengthening of the peptide chain with Arg in position 6 decreased the potency of this peptide at δ-receptors in the mouse vas deferens but increased it in the guinea pig ileum, which has μ- and κ-receptors. However both the modification in position 5 and the substitution by D-Ala in position 2 greatly decreased the affinity for δ-receptors. Interdependence between residues 2 and 5 of enkephalins should be considered because: (i) analogues with D-Ala^2 substitution serve as prototypical δ opioid receptor ligands (Kosterlitz et al., 1980); (ii) the inhibitory effects of [Leu^5]enkephalyl-Arg^6 are decreased in both guinea pig ileum assay and in mouse vas deferens assay, as compared to the inhibitory effect of [Leu^5]enkephalin (Corbett et al., 1982). There are data about the therapeutic effects of dalargin on the gastrointestinal system (Timoshin et al., 1991), but very little is understood about the receptor preference of this enkephalin analogue. The poor δ-potency and selectivity of dalargin described here, could explain the pronounced effects of this analogue in the gut and its specific analgesic effects in the central nervous system (Bocheva et al., 1994), where analgesia is considered to be μ-dependent rather than δ-dependent (Chaillet et al., 1984).

In summary, the present results suggest that: (i) the native opioids, [Leu^5]enkephalin and [Met^5]enkephalin, the newly synthesized enkephalin analogues, [Cys(O_2NH_2)^2, Leu^5]enkephalin and [Cys(O_2NH_2)^2,Met^5]enkephalin, and the hexapeptide, dalargin, inhibit in a similar manner the electrically or drug-induced purinergic and adrenergic components of the evoked responses at both prejunctional and postjunctional levels in the mouse vas deferens; the postjunctionally mediated inhibition entirely mimics the typical prejunctional action of opioids as even the order of potency is preserved: [Cys(O_2NH_2)^2,Leu^5]enkephalin > [Cys(O_2NH_2)^2,Met^5]enkephalin > [Leu^5]enkephalin > dalargin > [Met^5]enkephalin; (ii) the opioids tested concentration dependently inhibit the electrically induced contractions of the guinea pig ileum, indicating the following order of potency: dalargin > [Met^5]enkephalin > [Leu^5]-enkephalin > [Cys(O_2NH_2)^2,Met^5]enkephalin > [Cys-(O_2NH_2)^2, Leu^5] enkephalin; (iii) incorporation of the hydrophilic Cys(O_2NH_2) into the molecule of [Leu^5]enkephalin in position 2 greatly increases the potency and selectivity of [Cys(O_2NH_2)^2,Leu^5]enkephalin for δ-opioid receptors; the same modification in the molecule of [Met^5]enkephalin also yields a selective and potent δ-agonist; and (iv) D-Ala^2 substitution and lengthening of the peptide chain by Arg^6 in the molecule of [Leu^5]enkephalin decrease both potency and selectivity of the analogue, dalargin, for δ-opioid receptors. All these findings reveal (i) structure-activity relationships for designing potent and selective enkephalin analogues at δ-receptors and (ii) alternative parameters for evaluation of opioid agonist potency with respect to purinergic and adrenergic components at the pre- and postjunctional levels.

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