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Structure–activity relationships of naturally occurring and synthetic opioid tetrapeptides acting on locus coeruleus neurons

Yea-Ru Yang [a], Tsai-Hsien Chiu [b,*], Chien-Liang Chen [b]

[a] *Faculty of Physical Therapy, National Yang-Ming University, Shih-Pai, Taipei, Taiwan*
[b] *Department of Physiology, National Yang-Ming University, Shih-Pai, Taipei, Taiwan*

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Abstract

Intracellular recording was used to study the effects of eight opioid tetrapeptides with similar amino acid sequences, namely endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2), endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2), morphiceptin (Tyr-Pro-Phe-Pro-NH_2), hemorphin-4 (Tyr-Pro-Trp-Thr), Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2), Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2), TAPS (Tyr-D-Arg-Phe-Sar) and DALDA (Tyr-D-Arg-Phe-Lys-NH_2), on neurons of the rat locus coeruleus, using a submerged brain slice preparation. All the tetrapeptides inhibited the spontaneous firing of all neurons of the locus coeruleus tested. Higher concentrations also caused hyperpolarization of the neurons and a reduction in input resistance. These inhibitory effects were rapidly and completely reversed by CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2, a selective μ-opioid receptor antagonist). The IC_{50} of the opioid tetrapeptides, in terms of inhibition of spontaneous firing of locus coeruleus neurons, as compared to the concentrations which produced a 5-mV hyperpolarization ($HC_{5\ mV}$) were calculated, giving the same rank order of potency: TAPS (IC_{50} = 1.9 nM, $HC_{5\ mV}$ = 3.4 nM) > endomorphin-1 (IC_{50} = 8.8 nM, $HC_{5\ mV}$ = 22.1 nM) and endomorphin-2 (IC_{50} = 5.3 nM, $HC_{5\ mV}$ = 16.1 nM) > DALDA (IC_{50} = 20 nM, $HC_{5\ mV}$ = 143 nM) > morphiceptin (IC_{50} = 65 nM, $HC_{5\ mV}$ = 335 nM) > Tyr-W-MIF-1 (IC_{50} = 3.8 μM, $HC_{5\ mV}$ = 6.7 μM) > hemorphin-4 (IC_{50} = 6.7 μM, $HC_{5\ mV}$ = 36.9 μM) > Tyr-MIF-1 (IC_{50} = 37.5 μM, $HC_{5\ mV}$ = 76.2 μM). Comparison of the ability of endomorphin-1 and endomorphin-2 to inhibit spontaneous firing based on single-cell recordings (n = 5) showed these two peptides to be equipotent. Based on these results, the structure–activity relationships of these opioid tetrapeptides are discussed herein.

Keywords: Opioid tetrapeptide; μ-Opioid receptor agonist; Structure–activity relationship; Locus coeruleus

1. Introduction

Many opioid peptides occur naturally and are divided into 'typical' and 'atypical' types (Teschemacher, 1993). Since the N-terminal tetrapeptide of most atypical opioid peptides represents the minimum sequence for full opioid activity (Montecucchi et al., 1981; Chang et al., 1983; Brantl et al., 1986), it was of interest to compare the actions (potency) of those opioid tetrapeptides which have similar amino acid sequences. Firstly, a group of naturally occurring opioid tetrapeptides, listed below, consists of peptides with the N-terminal dipeptide sequence Tyr-Pro. Morphiceptin (Tyr-Pro-Phe-Pro-NH_2) and hemorphin-4 (Tyr-Pro-Trp-Thr) are formed by enzymatic digestion of milk protein (casein) and hemoglobin, respectively (Chang et al., 1981; Brantl et al., 1986). Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2, MIF = melanocyte-stimulating hormone release inhibiting factor = Pro-Leu-Gly-NH_2) and Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2) have been isolated from bovine hypothalamus and human brain cortex (Horvath and Kastin, 1989, 1990; Erchegyi et al., 1992; Hackler et al., 1993). More recently, two novel opioid tetrapeptides, Tyr-Pro-Trp-Phe-NH_2 and Tyr-Pro-Phe-Phe-NH_2, have been isolated from both bovine and human brain (Hackler et al., 1997; Zadina et al., 1997); since these two new tetrapeptides have a high affinity and a clear specificity for the opioid receptor preferred by morphine, they were named endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2) (Zadina et al., 1997). Secondly, another group of opioid tetrapeptides, shown below, belongs to the dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) family. Dermorphin, isolated from both frog skin and rat brain, contains a D-amino acid (Montecucchi et al.,

* Corresponding author. Tel.: +886-2-28267084; Fax: +886-2-28264049; E-mail: thchiu@ym.edu.tw

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1981; Buffa et al., 1982) and interacts with opioid receptors through its N-terminal sequence Tyr-D-Ala-Phe-Gly (Montecucchi et al., 1981). This N-terminal tetrapeptide of dermorphin has been the subject of a variety of modifications that have resulted in many potent receptor-specific analogs, such as Tyr-D-Arg-Phe-Sar (TAPS, Sar = *N*-methylglycine) and Tyr-D-Arg-Phe-Lys-NH_2 (DALDA) (Sato et al., 1987; Schiller et al., 1989).

Both the above Tyr-Pro and D-amino acid (dermorphin) groups of tetrapeptides appear to act as specific agonists of μ-opioid receptors and have both central and peripheral opioid activity (Chang et al., 1981; Brantl et al., 1986; Sato et al., 1987; Schiller et al., 1989; Erchegyi et al., 1992; Zadina et al., 1997). All of the atypical opioid tetrapeptides, except Tyr-MIF-1, mimic the typical opioid peptides (β-endorphin, enkephalins and dynorphins) in terms of their analgesic properties. For example, i.c.v. administration of morphiceptin, hemorphin-4, Tyr-W-MIF-1, endomorphin-1, endomorphin-2 or DALDA induces analgesia in the tail-flick assay (Chang et al., 1982; Davis et al., 1989; Bickel et al., 1994; Zadina et al., 1993, 1997), while TAPS elicits antinociceptive effects following i.c.v., i.v. or p.o. administration in the rat (Paakkari et al., 1993). In the cardiovascular system, morphiceptin produces transient bradycardia following i.v. administration (Wei et al., 1980). With hemorphin-4, endomorphin-1 or endomorphin-2, a significant decrease in arterial blood pressure is detected after i.v. injection in the rat (Liebmann et al., 1989; Champion et al., 1997b; Czapla et al., 1998). These results provide evidence that these endogenous or synthetic tetrapeptides exert opioid actions in vivo and, therefore, may play an important physiological role.

The rat locus coeruleus has served as a useful model for studying opioid action for many years (Nestler et al., 1994). It consists of a compact group of norepinephrine-containing cell bodies close to the floor of the fourth ventricle at the upper border of the pons, and projections from this small pontine nucleus give rise to more than half the noradrenergic neurons in the brain (Amaral and Sinnamon, 1977). The noradrenergic neurons of the locus coeruleus show both a high opioid receptor density (Mansour et al., 1986) and high opioid receptor mRNA expression (Mansour et al., 1995); both electrophysiological studies (Williams and North, 1984; Aghajanian and Wang, 1987) and microscopic autoradiography (Moyse et al., 1997) indicate that the opioid receptors on the somatic and dendritic membrane of the locus coeruleus neurons are of the μ-opioid receptor type. Moreover, immunocytochemical and immunofluorescent studies show that opioid peptide-containing nerve terminals innervate neurons of the locus coeruleus (Finley et al., 1981; Léger et al., 1983). The locus coeruleus is involved in many physiological functions, including the central neural control of the cardiovascular system (see the Discussion section of Yang et al., 1997) and, more important, it is the primary origin of descending noradrenergic analgesic fibers (for reviews, see Proudfit, 1988; Jones, 1991; Lipp, 1991). Thus, in this study, firstly we have chosen to investigate whether the above-mentioned eight opioid tetrapeptides are able, like other opioids, to influence neuronal activity in the locus coeruleus; then finally, to determine their rank order of potency and to discuss their structure–activity relationships.

2. Materials and methods

2.1. Preparation and maintenance of slices of locus coeruleus

The methods used to prepare and maintain rat locus coeruleus slices were similar to those described previously (Chiu et al., 1990, 1993, 1995). Male Sprague–Dawley rats (120–200 g) were killed and their brains were rapidly removed. A block of tissue containing the pons was excised and attached to a small Plexiglass stage with cyanoacrylate glue; an agar block, placed next to the tissue, acted as support during sectioning. The tissue was then submerged in oxygenated artificial cerebrospinal fluid (artificial CSF), maintained at 3–5 C, in the well of a Lancer 1000 vibratome. Several 300- to 350-μm-thick coronal sections of the pons were cut, then, a slice containing a cross-section through the caudal end of the locus coeruleus was mounted in the recording chamber and allowed to equilibrate for 1 h. The slice was completely submerged in a heated (33–34 C) flowing (2.3 ml/min) solution of the following millimolar composition: NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 26.2, $MgCl_2$ 1.3, $CaCl_2$ 2.4, glucose 11.1, gassed with 95% O_2/5% CO_2, and was viewed from above using a dissection microscope. In the trans-illuminated slice, the locus coeruleus was seen as a translucent area lying on the lateral aspect of the periventricular gray, below the fourth ventricle.

2.2. Intracellular recording

Intracellular recording from the neurons of the locus coeruleus was performed using sharp microelectrodes, filled with 2 M KCl, with a d.c. tip resistance of 40–70 MΩ. The recording microelectrodes were inserted into the locus coeruleus under visual control. Intracellular potentials were recorded using an amplifier with an active bridge circuit, permitting current injection through the recording electrode (WPI M707). Current and voltage traces were displayed on a storage oscilloscope (Textronix 5113) and a rectilinear pen recorder (Gould 2400). Input resistance was measured by passing hyperpolarizing constant current pulses of sufficient duration to fully charge the membrane capacitance and reach a steady state voltage deflection. In order to minimize possible errors from inward rectification, only weak constant current pulses were used to create a voltage deflection within 10–15 mV.

2.3. Perfusion of solutions and drugs

A valve system was used to switch the perfusion solution between control artificial CSF and drug-containing CSF. The period required for test solutions to reach the chamber was known, and ranged from 25–35 s. The drugs used were endomorphin-1 and endomorphin-2 (Tocris Cookson, Bristol, UK), TAPS, DALDA, morphiceptin, Tyr-MIF-1, Tyr-W-MIF-1 and CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2) (Bachem, CA, USA) and hemorphin-4 (Peninsula Laboratories, CA, USA).

2.4. Data analysis

The IC_{50} is defined as the concentration which gives a 50% inhibition of neuronal firing and is obtained by interpolating from the data points in the dose–response curves. Numerical data are expressed as the means ± the standard error of the mean (S.E.M.). Paired or unpaired Student's *t*-tests were used to analyze the differences between individual means. *P* values equal to, or less than, 0.05 were judged to be statistically significant.

3. Results

3.1. Membrane properties of locus coeruleus neurons

Electrophysiological properties were examined in a total of 96 neurons of the locus coeruleus with stable intracellular impalement. All neurons of the locus coeruleus included in this study showed spontaneous activity, the frequency of spontaneous firing ranging from 0.3 to 4.4 Hz (2.1 ± 0.1 Hz, $n = 96$). The pattern of locus coeruleus neuron spontaneous firing recorded in each slice was very regular, i.e., the interspike interval was remarkably uniform. The neurons had resting membrane potentials of -48 to -74 mV (-55.5 ± 0.8 mV, $n = 96$) and apparent input resistances of 106–370 MΩ (178 ± 7 MΩ, $n = 96$).

Table 1
IC_{50} and $HC_{5\ mV}$ for different opioid tetrapeptides on the inhibition of locus coeruleus neuronal activities

Tetrapeptide	Amino acid sequence	IC_{50} (nM)	$HC_{5\ mV}$ (nM)
Endomorphin-1	Tyr-Pro-Trp-Phe-NH_2	8.8	22.1
Endomorphin-2	Tyr-Pro-Phe-Phe-NH_2	5.3	16.1
Morphiceptin	Tyr-Pro-Phe-Pro-NH_2	65	335
Hemorphin-4	Tyr-Pro-Trp-Thr	6.7×10^{3}[a]	36.9×10^{3}
Tyr-MIF-1	Tyr-Pro-Leu-Gly-NH_2	37.5×10^{3}[a]	76.2×10^{3}
Tyr-W-MIF-1	Tyr-Pro-Trp-Gly-NH_2	3.8×10^{3}[a]	6.7×10^{3}
TAPS	Tyr-D-Arg-Phe-Sar	1.9[a]	3.4
DALDA	Tyr-D-Arg-Phe-Lys-NH_2	20	143

[a] These figures were taken from the previous studies of Yang and Chiu (1997) and Yang et al. (1998).
The rank order of potency was the same whether based on inhibition of the spontaneous firing rate or hyperpolarization of the membrane potential.

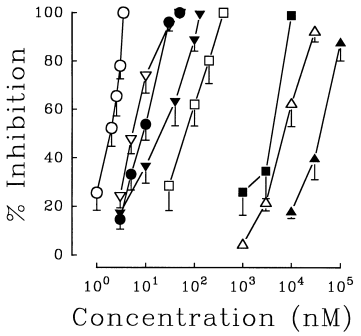

Fig. 1. Dose–response curves for different opioid tetrapeptides for inhibition of neuronal firing in the locus coeruleus. The points represent the mean ± S.E.M. ($n = 5$–9 per point) decrease in firing rate during superfusion with morphiceptin (□), hemorphin-4 (△), Tyr-MIF-1 (▲), Tyr-W-MIF-1 (■), endomorphin-1 (●), endomorphin-2 (▽), TAPS (○) and DALDA (▼). The IC_{50} is defined as the concentration which gave 50% inhibition of neuronal firing. The calculated IC_{50} for the opioid tetrapeptides are listed in Table 1.

These data are similar to those we obtained in earlier studies (Chiu et al., 1990, 1993, 1995; Yang et al., 1998).

3.2. Rank order of potency

Superfusion of all eight tetrapeptides tested resulted in inhibition of the spontaneous firing rate, caused hyperpolarization of the membrane potential and reduced the input resistance of the locus coeruleus neurons. Inhibition of the firing rate was the most sensitive parameter and showed a marked change even at low concentrations. TAPS (1–3.5 nM), endomorphin-1 (3–50 nM), endomorphin-2 (3–50 nM), DALDA (3–130 nM), morphiceptin (30–400 nM), Tyr-W-MIF-1 (1–10 μM), hemorphin-4 (1–30 μM) and Tyr-MIF-1 (10–100 μM) produced dose-related inhibition of spontaneous firing in all locus coeruleus neurons tested; these data were used to construct dose–response curves. Please note that, as shown in Table 1, some of the data were taken from our previous studies for the sake of comparison (see Yang and Chiu, 1997; Yang et al., 1998). Fig. 1 shows the inhibitory effects of different opioid tetrapeptides on the firing rate of many neurons ($n = 5$–9 per point). The rank order of potency of these opioid tetrapeptides was determined by measuring the IC_{50} for inhibition of spontaneous firing of locus coeruleus neurons from the dose–response curves. Most opioid tetrapeptides produced a decrease in firing rate with IC_{50} values in the nM range, whereas the IC_{50} values of hemorphin-4, Tyr-MIF-1 and Tyr-W-MIF-1 were in the μM range (Table 1). Since the IC_{50} for endomorphin-1 and endomorphin-2 were very close, dose–response curves for endomorphin-1 and endomorphin-2 on inhibition of firing rate were further compared for single neurons ($n = 5$). The neurons were perfused individually with different concentrations of one drug, then of another. No significant difference was seen between the IC_{50} for endomorphin-1 and endomorphin-2 (endomorphin-1: 8.4 ± 1.5 nM, endo-

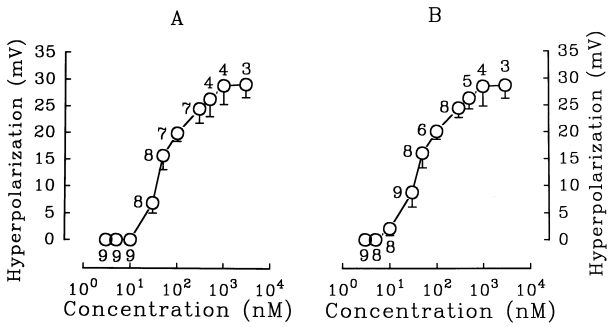

Fig. 2. Dose-dependent effects of endomorphin-1 (A) and endomorphin-2 (B) on the membrane potential of locus coeruleus neurons. The vertical bars represent the S.E.M. for the number of neurons indicated.

morphin-2: 6.6 ± 0.9 nM, $n = 5$, $P > 0.05$). The rank order of potency of the above 8 tetrapeptides was also determined by measuring the concentration which produced 5-mV hyperpolarization ($HC_{5\,mV}$) from the dose–response curves. These $HC_{5\,mV}$ values are summarized in Table 1. Based on either inhibition of the spontaneous firing rate or hyperpolarization of the membrane potential, the rank order of potency was: TAPS > endomorphin-1 = endomorphin-2 > DALDA > morphiceptin > Tyr-W-MIF-1 > hemorphin-4 > Tyr-MIF-1.

3.3. Effects of endomorphins

Since the endomorphins (endomorphin-1 and endomorphin-2) are very potent newly discovered endogenous opioid tetrapeptides with several important bioactivities in vivo, we then examined their electrophysiological actions on neurons of the locus coeruleus. Although the neurons varied in their sensitivity to endomorphins, both the inhibition of firing rate and hyperpolarization of membrane potential were concentration-dependent (Figs. 1 and 2). For instance, endomorphin-2 at a concentration of 3 or 5 nM caused a decrease of 24.3% ($n = 9$) or 47.8% ($n = 8$), respectively, in neuronal firing rate, but produced no hyperpolarization while, at higher concentrations (10 or 30 nM), greater inhibition of firing was seen together with hyperpolarization of the membrane potential. At a given concentration of endomorphin-2, there was considerable inter-neuron variation in the size of the hyperpolarization. For example, the amplitude of the hyperpolarization in response to 10 or 30 nM endomorphin-2 ranged from 0 to 9 mV (2.1 ± 1.3 mV, $n = 8$) or from 0 to 23 mV (8.8 ± 2.7 mV, $n = 9$), respectively. At 50 nM, endomorphin-2 produced complete inhibition of firing of all neurons tested ($n = 8$), which was associated with a 16.1-mV hyperpolar-

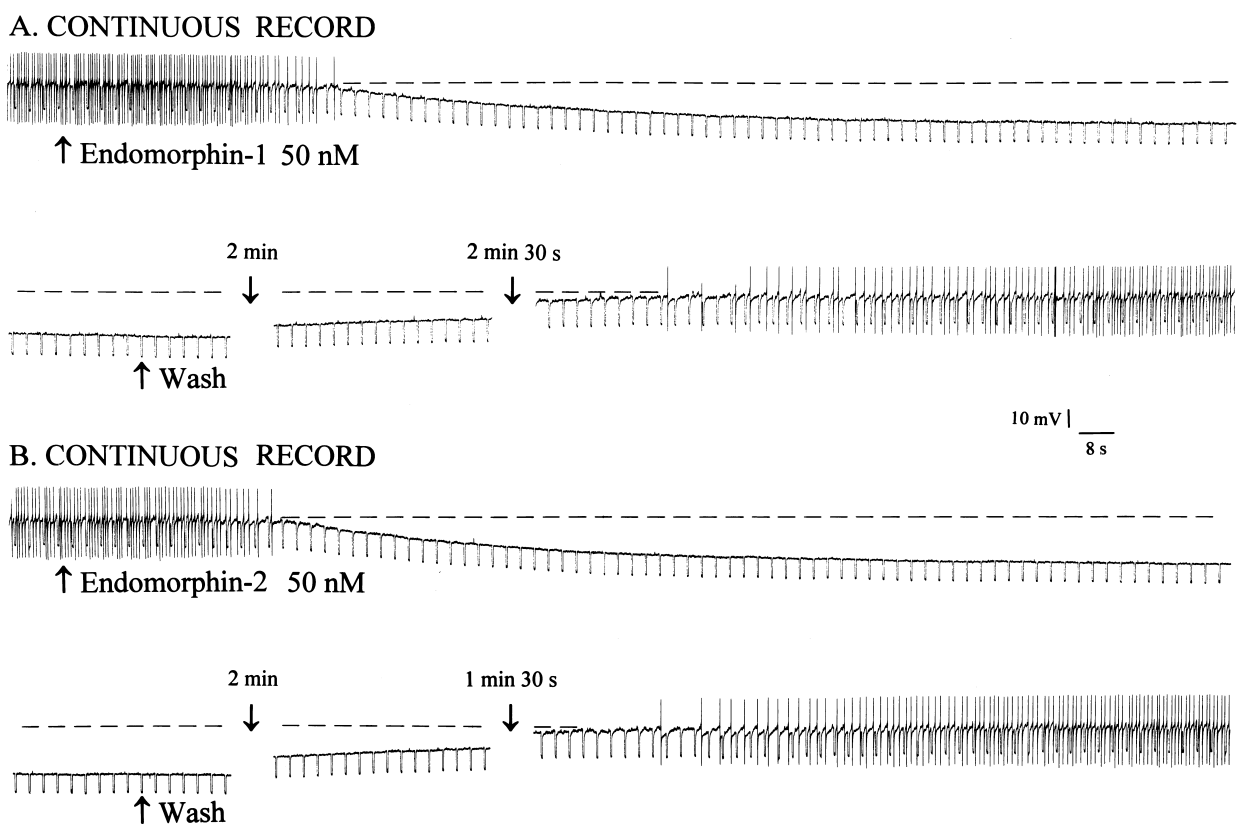

Fig. 3. Effects of endomorphin-1 and endomorphin-2 on firing rate, membrane potential and input resistance of a typical locus coeruleus neuron. Superfusion with 50 nM endomorphin-1 (A) or endomorphin-2 (B) for 5 min caused complete inhibition of the firing rate, hyperpolarization of the membrane potential (26 and 29 mV for endomorphin-1 and endomorphin-2, respectively) and a decrease in input resistance. The broken horizontal lines indicate −65 mV.

ization (range 6–29 mV, $n = 8$) (Fig. 3). The amplitude and time course of the electrophysiological effects caused by the same concentration (50 nM) of endomorphin-1 and endomorphin-2 were very similar (Fig. 3). The endomorphin-induced hyperpolarization reached a plateau between 1 and 3 μM (Fig. 2). The maximum hyperpolarization observed was about 30 mV. This is similar to the hyperpolarizing effect of other opiates or opioids on the locus coeruleus neurons (Williams and North, 1984).

3.4. Effect of CTAP on reversing the inhibitory actions caused by tetrapeptides

Superfusion of CTAP (300 nM), a selective antagonist of μ-opioid receptors, altered neither the membrane potential nor the firing rate in neurons of the locus coeruleus. Superfusion of endomorphin-1 (30 nM), endomorphin-2 (30 nM), DALDA (300 nM), morphiceptin (400 nM), Tyr-W-MIF-1 (10 μM), hemorphin-4 (50 μM) or Tyr-MIF-1 (100 μM) resulted in both complete inhibition of the spontaneous firing and hyperpolarization of the membrane potential (2–16 mV). These inhibitory effects were antagonized by CTAP (300 nM). Neither firing rate nor membrane potential was altered, compared with the control state ($n = 4$ for each tetrapeptide).

4. Discussion

We have, for the first time, assessed the actions of six endogenous and two synthetic opioid tetrapeptides on neurons of the locus coeruleus using intracellular recording analysis techniques and have described the effects and structure–activity relationships of both the Tyr–Pro group and D-amino acid group of opioid tetrapeptide on brain neurons. Our investigation demonstrated that all opioid tetrapeptides tested in this study inhibit spontaneous firing, cause hyperpolarization of the membrane potential and reduce the input resistance of the locus coeruleus neurons. In an earlier study, we demonstrated that TAPS binds to μ-opioid receptors on the cell membrane of neurons of the locus coeruleus, resulting in hyperpolarization of the membrane and reduction of the input resistance of the neurons (Yang et al., 1998). The present study also demonstrated that the effects of the other seven tetrapeptides on neurons of the locus coeruleus appear to be mediated through the same receptors (μ-opioid receptors), since CTAP was effective to reverse these tetrapeptide-induced inhibitory effects. This inference is further supported by the following findings. Firstly, all opioid tetrapeptides tested in this study exhibited affinity for μ-opioid receptors. The binding affinity of hemorphin-4 for μ-opioid receptors is almost in the same range as that for δ-binding sites (Liebmann et al., 1989), whereas the other seven opioid tetrapeptides have a much higher affinity for μ-opioid receptors than for δ- or κ-opioid receptors (Chang et al., 1981; Schiller et al., 1989; Zadina et al., 1994, 1997). Secondly, the opioid receptors on the plasma membranes of noradrenergic perikarya and dendrites of the locus coeruleus are of the μ type (Moyse et al., 1997). Thirdly, quantitative pharmacological techniques have demonstrated that the inhibitory effects of opioids on the locus coeruleus neurons are due to activation of μ-opioid receptors (Williams and North, 1984; Aghajanian and Wang, 1987). Moreover, our present findings are consistent with those of previous studies which indicated that opiates or opioids, acting via μ-opioid receptors, reduce neuronal excitability by opening inward-going rectification potassium channels and producing hyperpolarization (Williams and North, 1984; Aghajanian and Wang, 1987; Chiu et al., 1990, 1993).

Although TAPS (a synthetic compound) was the most potent opioid tetrapeptide tested in this study, the most potent endogenous opioid tetrapeptides were endomorphin-1 and endomorphin-2. Moreover, these two newly discovered tetrapeptides are also more potent than other opiates or opioids in terms of hyperpolarizing effects on the locus coeruleus neurons, since the concentrations required to produce 15-mV hyperpolarization of membrane potential were 48 and 46.4 nM for endomorphin-1 and endomorphin-2, respectively (Fig. 2), much lower than those for other opiates or opioids reported to produce the same effect, such as dermorphin, morphine, normorphine, β-endorphin, met-enkephalin and [D-Ala^2-NMePhe^4-Gly-ol^5]enkephalin (DAGO) (see Chiu et al., 1990). The present results are compatible with recent findings that endomorphin-1 and endomorphin-2 have higher affinities and greater selectivity for the μ-opioid receptor than do the three typical endogenous mammalian peptides with opiate activity, namely β-endorphin, met-enkephalin and dynorphin (Zadina et al., 1997). As these two new tetrapeptides have been isolated from both bovine and human brain and are found in human brain in much higher amounts than in the bovine frontal cortex (Hackler et al., 1997; Zadina et al., 1997), the potent μ-selective bioactivity of these two endomorphins, including analgesia and vasodepressor activity (Champion et al., 1997a,b; Zadina et al., 1997; Czapla et al., 1998), suggests that they may be of physiological importance not only in animals, but also in humans.

From the structural point of view, it has been established that naturally occurring opioid peptides consist of two components, a biologically important N-terminal tri- or tetrapeptide fragment (message sequence) and the remaining C-terminal fragment (address sequence) (Yamazaki et al., 1993). The N-terminal message sequence shows a strict requirement for the amine and phenolic groups of Tyr in position 1, an appropriate spacer (Pro or D-Ala in position 2 or Gly in positions 2 and 3) and an aromatic group (Trp in position 3 or Phe in positions 3 or 4), the amine and phenolic groups of Tyr and the aromatic group of Trp or Phe being required for opioid receptor recognition (Yamazaki et al., 1993; Zadina et al., 1997).

We therefore speculate that endomorphin-1 and endomorphin-2 are the most potent tetrapeptides in the Tyr–Pro group due to their possession of an aromatic group (Trp or Phe) in both positions 3 and 4, resulting in increased μ-opioid receptor binding and potent bioactivity. Similarly, the fact that there is no Trp or Phe residue in positions 3 or 4 within Tyr-MIF-1 may account for its having the lowest potency in our tests. The above explanation is also supported by our finding that, when the leucine residue in position 3 in Tyr-MIF-1 is replaced by tryptophan, the ability of the peptide to hyperpolarize neuronal membrane potential is significantly improved, i.e., the concentrations which produced 5-mV hyperpolarization for Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2) and Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2) are 76.2 and 6.7 μM, respectively. Our present results also demonstrate that endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2) are almost equally potent, i.e., their electrophysiological effects on locus coeruleus neurons show a very similar amplitude and time course (Fig. 3), indicating that the replacement of tryptophan by phenylalanine in position 3 scarcely affects the ability to reduce neuronal excitability. It can therefore be suggested that the potency of opioid tetrapeptides containing the Tyr-Pro-Trp-X-NH_2 or Tyr-Pro-Phe-X-NH_2 sequence is determined by the nature of the fourth residue. In support of this inference, the present results show that the order of potency on neurons of the locus coeruleus is: Phe-4 tetrapeptide (endomorphin-1 or endomorphin-2) > Pro-4 tetrapeptide (morphiceptin) > Gly-4 tetrapeptide (Tyr-W-MIF-1). Turning now to the C-terminus of these 6 Tyr-Pro group tetrapeptides, interestingly, in this series, hemorphin-4 was the only tetrapeptide without an amine group at the C-terminus. Zadina et al. (1997) synthesized peptides containing all possible natural amino acid substitutions in position 4 of Tyr-W-MIF-1 and found that hemorphin-4-amide (Tyr-Pro-Trp-Thr-NH_2) had an increased affinity for μ-opioid receptors compared to Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2). Moreover, the absence of amidation at the C-terminus of morphiceptin and Tyr-W-MIF-1 results in a substantial decrease in the affinity for μ-opioid receptors (Erchegyi et al., 1992; Sartania et al., 1996). Accordingly, amidation of the C-terminus seems to be essential for μ-opioid receptor specificity and might explain the low potency of hemorphin-4.

The present results are consistent with those of other studies using different methods to investigate the potency of some tetrapeptides of the Tyr–Pro group; however, previous reports did not thoroughly discuss the issue of structure–activity relationships. It has been shown that, in the receptor binding assay, the order of affinities for μ-opioid receptors is: morphiceptin > Tyr-W-MIF-1 > hemorphin-4 > Tyr-MIF-1 (Erchegyi et al., 1992; Zadina et al., 1996). Using the guinea pig ileum assay, Erchegyi et al. (1992) also demonstrated that the IC_{50} for inhibition of electrically induced contractions are 2.4, 13.7 and 21.2 μM for Tyr-W-MIF-1, hemorphin-4 and Tyr-MIF-1, respectively. Comparison of the IC_{50} for hemorphin-4, Tyr-MIF-1 and Tyr-W-MIF-1 obtained in the guinea pig ileum assay study and those from our study (Table 1) shows the IC_{50} values to be essentially in the same μM range. Additionally, our recent results suggest that these three tetrapeptides act on the μ-opioid receptor as partial agonists (Yang and Chiu, 1997).

The present study also showed that dermorphin analogs have potent effects on neurons of the locus coeruleus (see also Chiu et al., 1990). The high potency of dermorphin analogs (TAPS and DALDA) may be explained as follows. Firstly, these peptides are very protease-resistant, owing to the presence of a D-amino acid residue in the molecule and, consequently, are only slowly degraded or inactivated (Pert et al., 1976; Roemer et al., 1977; Sato et al., 1987). Secondly, it has been proposed that opioid peptides carrying a net positive charge would accumulate in the vicinity of the μ-opioid receptor and, therefore, would show μ-opioid receptor preference (Schwyzer, 1986; Schiller et al., 1989). This may also contribute to the high potency of TAPS and DALDA, both of which contain a positively charged D-arginine residue in position 2. The present study also showed that TAPS (Tyr-D-Arg-Phe-Sar, Sar = *N*-methylglycine) is 10 times more potent than DALDA (Tyr-D-Arg-Phe-Lys-NH_2) to inhibit neuronal firing; this can be explained by the observations of both Zadina et al. (1997), who demonstrated that Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2) has a higher affinity and selectivity for the μ-opioid receptor than does Lys^4-substituted Tyr-W-MIF-1 (Tyr-Pro-Trp-Lys-NH_2) and of Sasaki et al. (1984), who argued that the methyl group of sarcosine stabilizes the C-terminus against carboxypeptidases.

In summary, our results showed that all these opioid tetrapeptides have inhibitory effects on neurons of the locus coeruleus and provide an insight into their structure–activity relationships. We speculate that the six endogenous opioid tetrapeptides may exert an opioid action under normal physiological conditions. Consequently, the results of this study might contribute to the development of more potent, yet smaller opioid peptides acting on the μ-opioid receptors of brain neurons.

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