ELSEVIER

European Journal of Pharmacology 314 (1996) 61–67

ejp

Inhibition of dynorphin-converting enzymes prolongs the antinociceptive effect of intrathecally administered dynorphin in the mouse formalin test

Koichi Tan-No [a,*], Aki Taira [a], Tsukasa Sakurada [b], Makoto Inoue [a], Shinobu Sakurada [a], Takeshi Tadano [a], Takumi Sato [a], Chikai Sakurada [b], Ingrid Nylander [c], Jerzy Silberring [d], Lars Terenius [d], Kensuke Kisara [a]

[a] *Department of Pharmacology, Tohoku College of Pharmacy, 4-4-1 Komatsushima, Aoba-ku, Sendai 981, Japan*
[b] *Department of Biochemistry, Daiichi College of Pharmaceutical Sciences, 22-1 Tamagawa-cho, Minami-ku, Fukuoka 815, Japan*
[c] *Department of Pharmaceutical Biosciences, Division of Pharmacology, University of Uppsala, Uppsala S-751 24, Sweden*
[d] *Department of Clinical Neuroscience, Drug Dependence Research Section, Karolinska Institute, Stockholm S-171 76, Sweden*

Received 14 March 1996; revised 19 June 1996; accepted 2 July 1996

Abstract

The effects of peptidase inhibitors on the antinociception induced by intrathecally (i.t.) administered dynorphin A and dynorphin B in the mouse formalin test were examined. When administered i.t. 5 min before the injection of 0.5% formalin solution into the dorsal surface of a hindpaw, dynorphin A (0.5–2 nmol) and dynorphin B (2–8 nmol) produced a dose-dependent and significant reduction of the paw-licking response. Dynorphin A (2 nmol) and dynorphin B (8 nmol)-induced antinociception disappeared completely within 90 min and 60 min, respectively. *p*-Hydroxymercuribenzoate, a cysteine proteinase inhibitor, and phosphoramidon, an endopeptidase 24.11 inhibitor simultaneously administered with dynorphin A or dynorphin B, significantly prolonged antinociception induced by both dynorphins. However, captopril, an angiotensin-converting enzyme inhibitor, bestatin (a general aminopeptidase inhibitor) and a serine proteinase inhibitor phenylmethanesulfonyl fluoride, were inactive. Dynorphin-converting enzyme(s) transform dynorphin-related peptides to [Leu^5]enkephalin and [Leu^5]enkephalin-Arg^6. Neither [Leu^5]enkephalin nor [Leu^5]enkephalin-Arg^6, even at high dose (10 nmol), produced any antinociceptive effect. However, [Leu^5]enkephalin-Arg^6, but not [Leu^5]enkephalin, produced a significant antinociceptive effect when co-administered with phosphoramidon. Therefore, the prolongation of the antinociception induced by both dynorphins in the presence of phosphoramidon, may be due to the inhibition of [Leu^5]enkephalin-Arg^6 degradation. The present results indicate that dynorphin-converting enzyme(s) may be important enzyme(s) responsible for terminating dynorphin-A- and dynorphin-B-induced antinociception at the spinal cord level in mice.

Keywords: Dynorphin A; Dynorphin B; Dynorphin-converting enzyme; Formalin test; Intrathecal administration; Peptidohydrolase; (Mouse)

1. Introduction

Dynorphin A and dynorphin B which are opioid peptides derived from pre-prodynorphin, have high affinity for κ-receptors (for review, see Höllt, 1986). These peptides are present in high concentrations in the dorsal horn of the spinal cord where processing of primary afferent nociceptive information occurs (for review, see Basbaum and Fields, 1984; Millan, 1986). It is known that intrathecally (i.t.) administered dynorphin-related peptides such as dynorphin A, dynorphin A-(1–13) and dynorphin B produce antinociceptive effects in rat and mice, measured by the tail-flick test (Han and Xie, 1982; Han et al., 1984; Spampinato and Candeletti, 1985; Ren et al., 1985; Herman and Goldstein, 1985; Sakurada et al., 1988).

Evidence indicates that several peptidases are involved in the metabolism of enkephalins. Endopeptidase 24.11 cleaves the Gly^3-Phe^4 bond in both [Met^5]enkephalin and [Leu^5]enkephalin (Malfroy et al., 1978; Sullivan et al., 1978; Almenoff et al., 1981; Fulcher et al., 1982). Inhibitors of this enzyme such as thiorphan (Roques et al., 1980), acetorphan (Lecomte et al., 1986), SCH 34826 (Chipkin et al., 1988) and *N*-[1-(*RS*)-carboxy-3-phenylpropyl]-Phe-*p*-aminobenzoate (Pozsgay et al., 1986) slow the degradation of enkephalins in vivo and possess antinociceptive properties (Roques et al., 1980; Chipkin et

* Corresponding author. Fax: (81-22) 275-2013.

PII S0014-2999(96)00518-3

al., 1982, 1988; De La Baume et al., 1983; Lecomte et al., 1986; Kest et al., 1991). Other studies using specific enzyme inhibitors indicate that aminopeptidase and angiotensin-converting enzyme also contribute to the degradation of enkephalins (Turner et al., 1985). Thus, administration of the aminopeptidase inhibitors, bestatin and amastatin alone or in combination with an angiotensin-converting enzyme inhibitor captopril, increase basal nociceptive thresholds and potentiate enkephalin-induced antinociception (Chaillet et al., 1983; Chou et al., 1984; Fournié-Zaluski et al., 1984). With regard to the enzyme cleaving dynorphin-related peptides, it has been found that dynorphin-converting enzyme(s) in bovine and human spinal cord (Silberring and Nyberg, 1989; Silberring et al., 1992, 1993), which are essentially cysteine proteinases, cleave dynorphin A and dynorphin B between Arg^6-Arg^7 and, to a lesser degree, Leu^5-Arg^6, generating the δ-receptor active fragments. It has also been reported that the antinociceptive effect produced by intracerebroventricularly (i.c.v.) administered dynorphin B is potentiated and prolonged by bestatin (Nakazawa et al., 1989), and that the inhibitory effect of dynorphin A-(1–8) on the electrically evoked contractions of guinea-pig ileum, mouse vas deferens and rabbit vas deferens is potentiated by amastatin, captopril and/or phosphoramidon (Numata et al., 1988). These reports suggest that these inhibitor-sensitive peptidases may be involved in the degradation of dynorphin-related peptides and enkephalins. However, the degradation of dynorphin-related peptides in the spinal cord in vivo is not fully understood.

The present study aims at clarifying the role of peptidase activity in modulating the antinociception induced by dynorphin A and dynorphin B, using the combined administration of several peptidase inhibitors.

2. Materials and methods

Male mice of ddY strain weighing 20–22 g were purchased from Shizuoka Laboratory Center (Shizuoka, Japan). They were housed in cages of 15–20 animals matched for weight and placed in a colony room. Animals were given standard food (Clea, Osaka, Japan) and tap water ad libitum in an air-conditioned room at 22–24°C and 50–60% relative humidity under a constant 12-h light: 12-h dark cycle (light on at 8:00 a.m.).

Approximately 1 h before the formalin test, the mice were placed individually in transparent observation chambers (22.0 × 15.0 × 12.5 cm). A mirror was placed behind the chamber to allow unobstructed observation of the hindpaws. After a period of adaptation, the mouse was taken out of the chamber and 20 μl of 0.5% formalin solution (0.185% formaldehyde in saline) was injected subcutaneously (s.c.) into the dorsal surface of the right hindpaw, using a microsyringe with a 26 gauge needle. Each animal was immediately returned to the observation

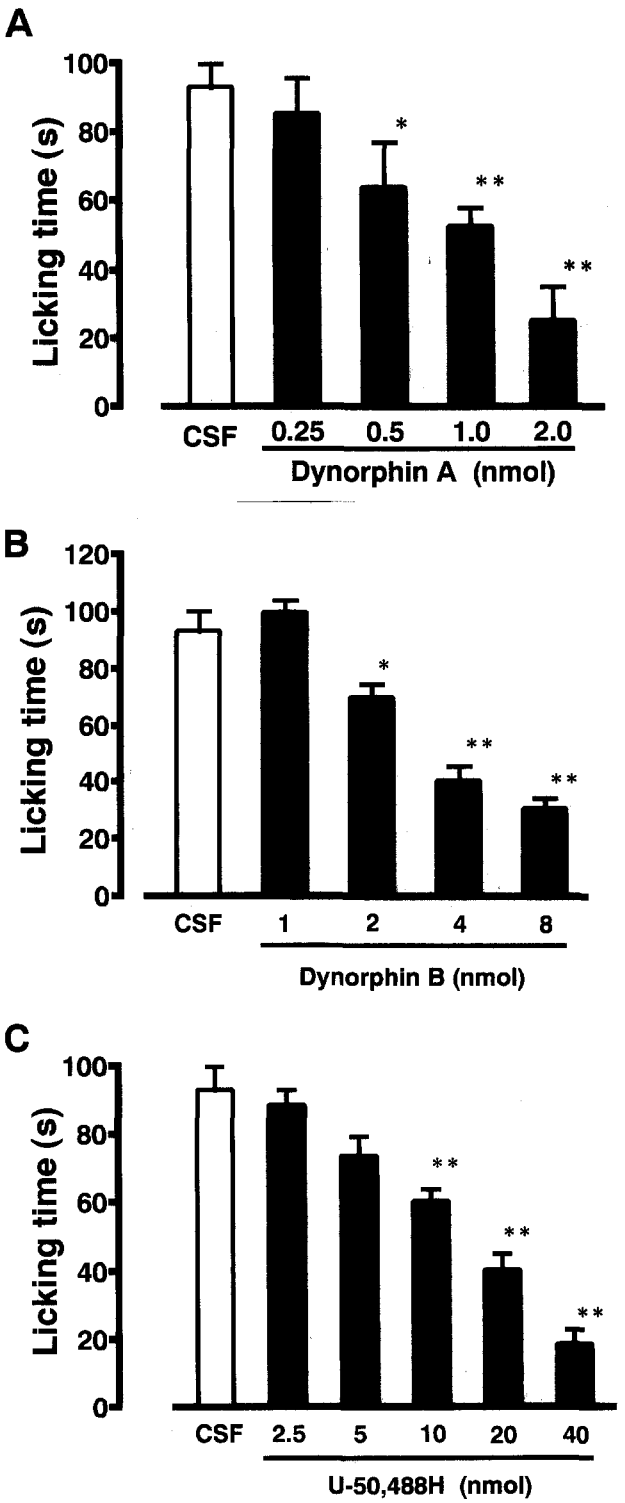

Fig. 1. Antinociceptive effects of intrathecally administered dynorphin A (A), dynorphin B (B) and U-50,488H (C) in the mouse formalin test. The duration of licking induced by 0.5% formalin solution was determined over a 5 min period, starting immediately after injection of formalin. Dynorphin A, dynorphin B and U-50,488H were administered i.t. 5 min before injection of 0.5% formalin. These data are given as the means ± S.E.M. for groups of 10 mice. * $P < 0.05$, ** $P < 0.01$ when compared to CSF-treated controls.

Table 1
Time-course of antinociceptive effects produced by i.t. administration of dynorphin A, dynorphin B and U-50, 488H in the mouse formalin test

Pretreatment time	CSF controls	Dyn A (2 nmol)	Dyn B (8 nmol)	U-50,488H (40 nmol)
5 min	92.6 ± 7.1	24.7 ± 10.0 [b]	30.3 ± 3.6 [b]	18.1 ± 4.6 [b]
15 min	104.2 ± 5.2	49.9 ± 9.0 [b]	53.0 ± 3.7 [b]	37.6 ± 4.2 [b]
30 min	113.9 ± 4.7	76.3 ± 4.8 [b]	84.2 ± 3.9 [b]	77.3 ± 3.5 [b]
60 min	100.7 ± 4.2	80.0 ± 7.8 [a]	95.1 ± 6.0	86.4 ± 4.9
90 min	92.2 ± 4.2	89.5 ± 4.8	N.D.	N.D.

Each mouse was i.t. administered with dynophin A (Dyn A), dynorphin B (Dyn B) and U-50,488H at indicated times before injection of formalin. These data are given as the means ± S.E.M. for groups of 10 mice. [a] $P < 0.05$, [b] $P < 0.01$ when compared to CSF-treated controls. N.D., not done.

chamber. Licking or biting of the injected hindpaw was defined as a nociceptive behavioural response and the total duration of the response was measured with a hand-held stop-watch during the test period. It has previously been found that a nociceptive behavioural response induced by 1 or 2% formalin solution has two peaks, 0–5 min (first phase) and 10–30 min (second phase), but when induced by 0.5% formalin solution has only the first phase (Sakurada et al., 1992). In the preliminary experiment, dynorphin A and dynorphin B produced only the slight antinociceptive effects during the first phase as measured in the 2% formalin test (data not shown). Thus, each animal was observed individually for 5 min, immediately after the s.c. injection of 0.5% formalin solution.

Chemicals were purchased from the following sources: dynorphin A, dynorphin B, [Leu^5]enkephalin-Arg^6 (Bachem, Bubendorf, Switzerland), U-50,488H, *p*-hydroxymercuribenzoate, phenylmethanesulfonyl fluoride, bestatin, captopril (Sigma, St. Louis, MO, USA), [Leu^5]enkephalin, phosphoramidon (Peptide Institute, Osaka, Japan) and formalin (Nacalai Tesque, Osaka, Japan). The reagents, except phenylmethanesulfonyl fluoride were dissolved in artificial cerebrospinal fluid (CSF), containing NaCl 7.4 g, KCl 0.19 g, $MgCl_2$ 0.19 g, $CaCl_2$ 0.14 g/1000 ml of distilled and sterilized water. Phenylmethanesulfonyl fluoride was dissolved in artificial CSF containing 3% ethanol. All these substances or vehicle were administered directly i.t. in a volume of 5 μl in unanesthetized mice, essentially

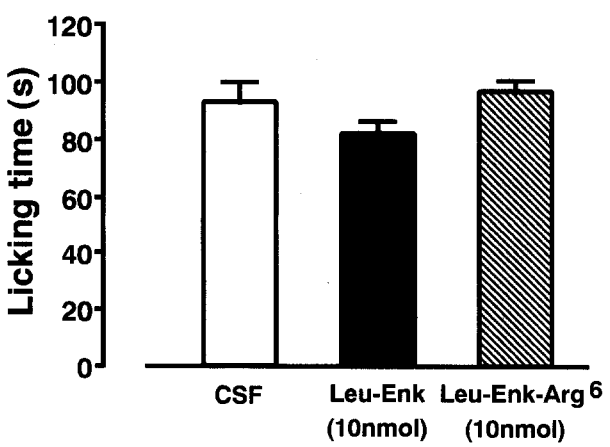

Fig. 2. Effects of intrathecally administered [Leu^5]enkephalin (Leu-Enk) and [Leu^5]enkephalin-Arg^6 (Leu-Enk-Arg^6) in the mouse formalin test. Each peptide was administered i.t. 5 min before injection of 0.5% formalin. These data are given as the means ± S.E.M. for groups of 10 mice.

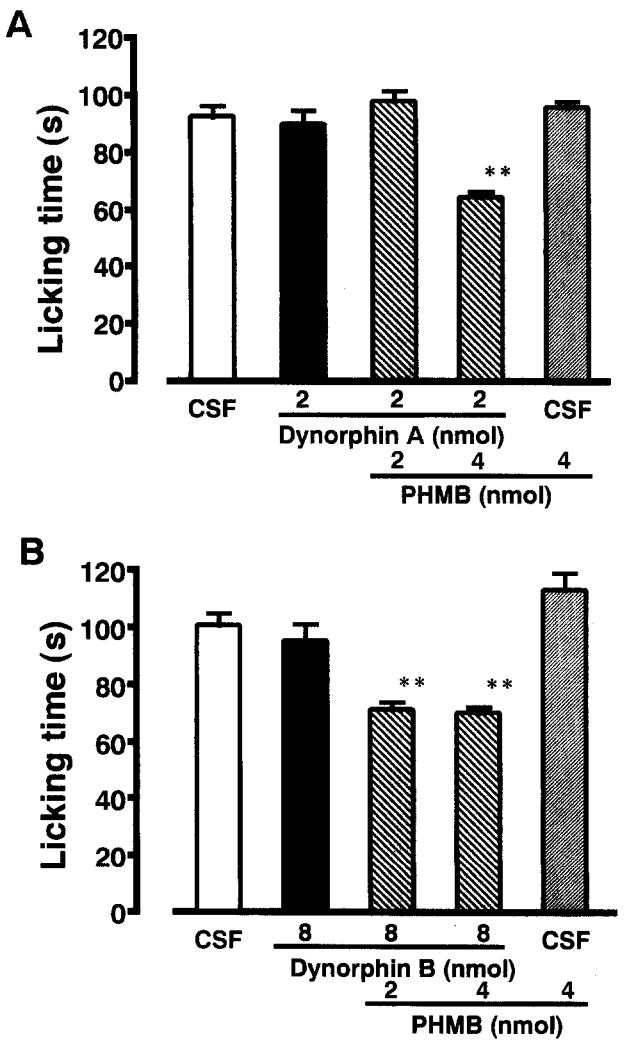

Fig. 3. Effects of *p*-hydroxymercuribenzoate (PHMB) on (A) dynorphin-A- and (B) dynorphin-B-induced antinociception in the mouse formalin test. PHMB was co-administered i.t. with dynorphin A (2 nmol) and dynorphin B (8 nmol) 90 min and 60 min before injection of 0.5% formalin, respectively. These data are given as the means ± S.E.M. for groups of 10 mice. ** $P < 0.01$ when compared to dynorphin A or dynorphin B alone.

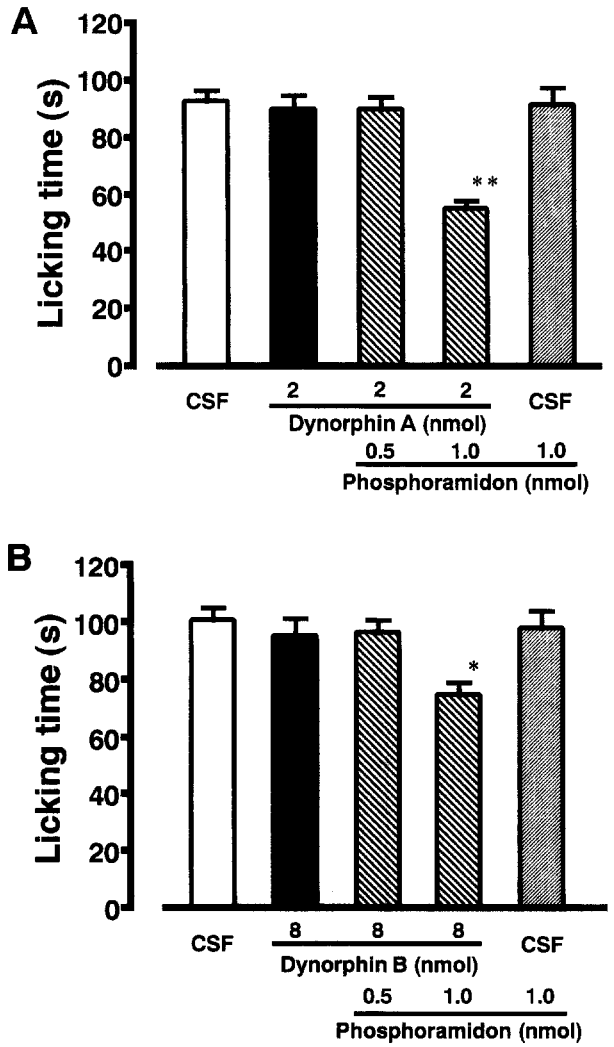

Fig. 4. Effects of phosphoramidon on (A) dynorphin-A- and (B) dynorphin-B-induced antinociception in the mouse formalin test. Phosphoramidon was co-administered i.t. with dynorphin A (2 nmol) and dynorphin B (8 nmol) 90 min and 60 min before injection of 0.5% formalin, respectively. These data are given as the means ± S.E.M. for groups of 10 mice. ** $P < 0.01$ when compared to dynorphin A or dynorphin B alone.

as described by Hylden and Wilcox (1980). Briefly, a lumbar puncture was performed using a 29 gauge needle connected to a Hamilton microsyringe. The needle was inserted between the L5 and L6 vertebrae and a rapid i.t. administration of 5 μl was made. The accurate placement of the administrations was indicated by a quick flick of the mouse's tail. The effect of peptidase inhibitors was determined by co-administration with a peptide, dynorphin A, dynorphin B, [Leu^5]enkephalin and [Leu^5]enkephalin-Arg^6.

Statistical analysis of the results included determination of ID_{50} values by the method of Litchfield and Wilcoxon (1949) and Tukey's test for multiple comparisons after analysis of variance (ANOVA). The criterion of significance was set at $P < 0.05$. All results are given as means ± S.E.M.

3. Results

3.1. Effects of peptides and U-50,488H on 0.5% formalin-induced nociceptive behavioural response

The injection of 0.5% formalin solution into the dorsal surface of a hindpaw caused an acute nociceptive behavioural response that lasted about 5 min. When administered i.t. 5 min before the injection of formalin, dynorphin A (0.5–2 nmol), dynorphin B (2–8 nmol) and U-50,488H (10–40 nmol) produced a dose-dependent and significant reduction of the nociceptive behavioural response (Fig. 1). The ID_{50} values for dynorphin A, dynorphin B and U-50,488H were 0.96 (0.58–1.59) nmol, 4 (2.16–7.41) nmol and 15 (9.02–24.95) nmol, respectively. The significant antinociceptive effects of dynorphin A (2 nmol), dynorphin B (8 nmol) and U-50,488H (40 nmol) lasted for 60 min, 30 min and 30 min, respectively (Table 1). The substances applied caused no motor impairment or hindlimb paralysis even at the highest doses used.

Neither [Leu^5]enkephalin nor [Leu^5]enkephalin-Arg^6 produced any antinociceptive effect even at the high dose of 10 nmol when administered 5 min before formalin injection (Fig. 2).

3.2. Effects of p-hydroxymercuribenzoate on dynorphin-A- and dynorphin-B-induced antinociception

Dynorphin-A (2 nmol)- and dynorphin B (8 nmol)-induced antinociception was no longer present at 90 min and 60 min after i.t. administration, respectively. When simultaneously administered with *p*-hydroxymercuribenzoate (4 nmol), a cysteine proteinase inhibitor, dynorphin A-induced antinociception was observed even 90 min after i.t.

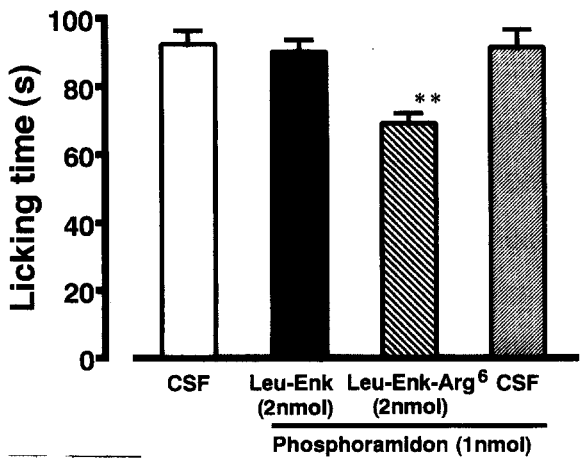

Fig. 5. Effects of phosphoramidon in combination with [Leu^5]enkephalin (Leu-Enk) and [Leu^5]enkephalin-Arg^6 (Leu-Enk-Arg^6) in the mouse formalin test. Phosphoramidon was co-administered i.t. with Leu-Enk (2 nmol) and Leu-Enk-Arg^6 (2 nmol) 90 min before injection of 0.5% formalin, respectively. These data are given as the means ± S.E.M. for groups of 10 mice. ** $P < 0.01$ when compared to CSF-treated control.

Table 2
Effects of phenylmethanesulfonyl fluoride, bestatin and captopril on dynorphin A- and dynorphin B-induced antinociception in the mouse formalin test

Inhibitor	60 min		90 min	
	CSF controls	Dyn B (8 nmol)	CSF controls	Dyn A (2 nmol)
CSF	100.7 ± 4.2	95.1 ± 6.0	92.2 ± 4.2	89.5 ± 4.8
PMSF (4 nmol)	99.0 ± 5.3	98.5 ± 4.6	91.6 ± 4.0	99.9 ± 5.2
Bestatin (4 nmol)	98.4 ± 3.6	108.2 ± 3.4	99.2 ± 5.5	80.7 ± 4.1
Captopril (4 nmol)	94.0 ± 3.8	90.9 ± 2.8	91.6 ± 2.1	82.0 ± 3.4

Each mouse was co-administered the various peptidase inhibitors in combination with dynorphin A (Dyn A) 90 min and dynophin B (Dyn B) 60 min before injection of formalin, respectively. These data are given as the means ± S.E.M. for groups 10 mice. PMSF, phenylmethanesulfonyl fluoride.

administration (Fig. 3A). A similar result was obtained for dynorphin B since a significant antinociception was observed 60 min after co-administration with *p*-hydroxymercuribenzoate (2 and 4 nmol) as shown in Fig. 3B. A single administration of *p*-hydroxymercuribenzoate (4 nmol) produced no antinociceptive effect in the assay.

3.3. Effects of phosphoramidon on dynorphin A-, dynorphin B-, [Leu^5]enkephalin- and [Leu^5]enkephalin-Arg^6-induced antinociception

A single administration of 2 nmol, but not 0.5–1 nmol phosphoramidon alone, caused a significant antinociceptive effect by itself, under conditions tested. Therefore, lower doses of this inhibitor, 0.5 and 1 nmol, were applied in combination with peptide administrations. Co-administration of dynorphin A or dynorphin B with phosphoramidon prolonged antinociceptive effects to 90 min and 60 min after administration, respectively (Fig. 4). It was also noted that co-administration of phosphoramidon (1 nmol) together with 2 nmol [Leu^5]enkephalin-Arg^6, but not [Leu^5]enkephalin, produced a significant antinociception 90 min after i.t. administration (Fig. 5).

3.4. Effects of phenylmethanesulfonyl fluoride, bestatin and captopril on dynorphin A- and dynorphin B-induced antinociception

As shown in Table 2, phenylmethanesulfonyl fluoride (4 nmol), bestatin (4 nmol) and captopril (4 nmol) simultaneously administered with dynorphin A or dynorphin B, did not prolong dynorphin A- or dynorphin B-induced antinociception. These substances alone caused no behavioural effect.

4. Discussion

The dynorphin converting enzyme(s) purified from the bovine and human spinal cords, which seem to be cysteine proteinases, cleave dynorphin A and dynorphin B between Arg^6-Arg^7 and Leu^5-Arg^6 thus generating [Leu^5]enkephalin-Arg^6 as a major product and small amounts of [Leu^5]enkephalin (Silberring and Nyberg, 1989; Silberring et al., 1992, 1993). Inhibition of dynorphin converting enzyme(s) by low doses of *p*-hydroxymercuribenzoate, a cysteine proteinase inhibitor, has also been observed in extracts from rat striatum (Nylander et al., 1995). Another dynorphin converting enzyme isolated from human CSF is essentially a serine proteinase (Nyberg et al., 1985). The present study shows that the antinociception induced by dynorphin A (2 nmol) and dynorphin B (8 nmol) completely disappeared at 90 min and 60 min after i.t. administration, respectively. Co-administration with *p*-hydroxymercuribenzoate, but not phenylmethanesulfonyl fluoride, a serine proteinase inhibitor, prolonged the antinociceptive effects produced by dynorphin A and dynorphin B. Neither of the products generated by dynorphin-converting enzyme(s), [Leu^5]enkephalin nor [Leu^5]enkephalin-Arg^6 given alone, produced the antinociceptive effect at a high dose (up till 10 nmol). These results agree with previous reports that enkephalins are rapidly degraded by several peptidases including neutral endopeptidase ('endopeptidase 24.11') in the CNS and peripheral tissues (Turner et al., 1985; Bourne et al., 1989). It has also been shown that the formation of [^{3}H][Leu^5]enkephalin from [^{3}H]dynorphin A-(1–8) by slices of rat lumbosacral spinal cord is reduced by *p*-hydroxymercuribenzoate and *N*-ethylmaleimide, another cysteine proteinase inhibitor (Dixon and Traynor, 1990).

Phosphoramidon, an 'endopeptidase 24.11' inhibitor, also prolonged both dynorphin A- and dynorphin B-induced antinociception when simultaneously administered with dynorphin A or dynorphin B. On the other hand, *N*-[1-(*RS*)-carboxy-3-phenylpropyl]-Phe-*p*-aminobenzoate, another inhibitor of this enzyme, simultaneously administered i.c.v. with dynorphin A-(1–8), has previously been shown ineffective in influencing the nociceptive stimuli in the rat tail-flick and electric footshock-induced jump tests (Kest et al., 1992). In addition to increasing the dynorphin A and dynorphin B effects, phosphoramidon also produced a significant antinociceptive effect when simultaneously administered with a relatively low dose of [Leu^5]enkephalin-Arg^6 (2 nmol) even at 90 min after i.t. administration. This may suggest that prolongation of both dynorphin A- and dynorphin B-induced antinociception by phosphoramidon is due to the inhibition of the degradation of [Leu^5]enkephalin-Arg^6 which is formed from dynorphin A and

dynorphin B by dynorphin-converting enzyme(s) and not due to direct inhibition of degradation of dynorphin A or dynorphin B by 'endopeptidase 24.11' which are poor substrates for this enzyme. Our previous studies indicate that phosphoramidon does not affect conversion of dynorphins to enkephalins by dynorphin-converting enzyme(s) (Silberring and Nyberg, 1989; Silberring et al., 1992). It is worth noting that this inhibitor shows no detectable influence on the action of [Leu^5]enkephalin, but potentiated analgesic response produced by [Leu^5]enkephalin-Arg^6. It is not, therefore unlikely that other proteinases take part in the degradation process (Oka et al., 1992; Roques et al., 1993) and an 'inhibitory cocktail' should be considered in order to slow down proteolysis (Roques et al., 1993). Phosporamidon administered alone at higher doses possessed intrinsic opioidergic activity in our experiments and, therefore, it has been applied at lower doses when simultaneously administered with the peptides.

Nakazawa et al. (1989) have reported that the antinociceptive effect produced by i.c.v. administration of dynorphin B is potentiated and prolonged by bestatin. Moreover, the inhibitory effect of dynorphin A-(1–8) on the electrically evoked contractions of guinea-pig ileum, mouse vas deferens and rabbit vas deferens was potentiated by amastatin, captopril and/or phosphoramidon (Numata et al., 1988). These reports suggested that aminopeptidase and angiotensin-converting enzyme may also be involved in the degradation of dynorphin-related shorter peptides. However, it appears that aminopeptidase and angiotensin-converting enzyme do not play an important role in terminating dynorphin A- and dynorphin B-induced antinociception in the mouse spinal cord, since the antinociceptive effects produced by these peptides were not significantly prolonged by 4 nmol of bestatin or captopril. This finding is supported by the earlier reports that kinetic constants obtained for angiotensin-converting enzyme and enkephalins do not favor the role of this enzyme in enkephalin metabolism (Benuck and Marks, 1979; Schwartz et al., 1981). However, in certain brain regions, captopril potentiated the antinociceptive effect of i.c.v. administered [Met^5]enkephalin (Stine et al., 1980). Our studies on dynorphin metabolism by dynorphin converting enzyme(s) also indicate that angiotensin-converting enzyme is not involved in this process.

There are several reports that i.t. administration of dynorphin-related peptides causes long-lasting hindlimb paralysis in the same dose range which produced the antinociceptive effect in the rat tail-flick test (Spampinato and Candeletti, 1985; Ren et al., 1985; Herman and Goldstein, 1985). This constitutes a problem for evaluating the antinociceptive effects produced by dynorphin-related peptides. In the present study using the 0.5% formalin test, i.t. administration of dynorphin A (0.5–2 nmol) and dynorphin B (2–8 nmol) caused no hindlimb paralysis, and produced a dose-dependent and significant antinociceptive effect. Therefore, the mouse formalin test using 0.5% formalin solution may be more useful to evaluate the antinociceptive effects of dynorphin-related peptides than for instance, the rat tail-flick test. I.t. administration of U-50,488H (10–40 nmol), a non-peptide κ-receptor agonist as a reference for comparison, also produced a dose-dependent and significant antinociceptive effect. Judging from the ID_{50} values, dynorphin A and dynorphin B were approximately 15 and 4 times more potent, respectively, in producing antinociceptive effects than U-50,488H.

In conclusion, the present results indicate that dynorphin-converting enzyme(s) may be important peptidases responsible for terminating both dynorphin A- and dynorphin B-induced antinociception at the spinal cord level in mice, and that the prolongation of both dynorphin A- and dynorphin B-induced antinociception by phosphoramidon may be due to inhibition of the degradation of [Leu^5]enkephalin-Arg^6.

Acknowledgements

This work was supported by a research grant, No. 07672374, from the Japanese Ministry of Education, Science and Culture, the Swedish Medical Research Council, the National Institute of Drug Abuse (Rockville, MD, USA) and the Swedish Alcohol Research Fund. We thank Mrs Delphi Post for excellent help in the preparation of the manuscript.

References

Almenoff, J., S. Wilk and M. Orlowski, 1981, Membrane-bound pituitary metalloendopeptidase: Apparent identity to enkephaliase, Biochem. Biophys. Res. Commun. 102, 206.

Basbaum, A.I. and H.L. Fields, 1984, Endogeneous pain control systems: brainstem spinal pathways and endorphin circuitry, Annu. Rev. Neurosci. 7, 309.

Benuck, M. and N. Marks, 1979, Co-identity of brain angiotensin converting enzyme with a membrane bound dipeptidyl carboxypeptidase inactivating Met-enkephalin, Biochem. Biophys. Res. Commun. 88, 215.

Bourne, A., K. Barnes, B.A. Taylor, A.J. Turner and A.J. Kenny, 1989, Membrane peptidases in the pig choroid plexus and other cell surfaces in contact with the cerebrospinal fluid, Biochem. J. 259, 69.

Chaillet, P.H., H. Marcais-Collado, J. Costetin, C.C. Yi, S. De La Baume and J.C. Schwartz, 1983, Inhibition of enkephalin metabolism by, and antinociceptive activity of, bestatin, an aminopeptidase inhibitor, Eur. J. Pharmacol. 86, 329.

Chipkin, R.E., M.B. Latranyi, L.C. Iorio and A. Barnett, 1982, Potentiation of *d*-Ala^2-enkephalinamide analgesia by thiorphan, Eur. J. Pharmacol. 83, 283.

Chipkin, R.E., J.G. Berger, W. Billard, L.C. Iorio, R. Chapman and A. Barnett, 1988, Pharmacology of SCH34826, an orally active enkephalinase inhibitor analgesic, J. Pharmacol. Exp. Ther. 245, 829.

Chou, J., J. Tang, J. Del Rio, H.-Y. T. Yang and E. Costa, 1984, Action of peptidase inhibitors on met-enkephalin-Arg-Phe and met-enkephalin metabolism and on electroacupuncture antinociception, J. Pharmacol. Exp. Ther. 230, 349.

De La Baume, S., C.C. Yi, J.C. Schwartz, P. Chaillet, H. Marçais-Col-

lado and J. Constentin, 1983, Participation of both enkephalinase and aminopeptidase activities in the metabolism of endogenous enkephalins, Neuroscience 8, 143.

Dixon, D.M. and J.R. Traynor, 1990, Formation of [Leu^5]enkephalin from dynorphin A (1–8) by rat central nervous tissue in vitro, J. Neurochem. 54, 1379.

Fournié-Zaluski, M.C., P. Chaillet, P. Bouboutou, A. Couland, P. Cherot, G. Waksman, J. Costentin and B.P. Roques, 1984, Analgesic effects of kelatorphan, a new highly potent inhibitor of multiple enkephalin degrading enzymes, Eur. J. Pharmacol. 102, 525.

Fulcher, I.S., R. Matsas, A.J. Turner and A.J. Kenny, 1982, Kidney neutral endopeptidase and the hydrolysis of enkephalin by synaptic membranes show similar sensitivity to inhibitors, Biochem. J. 203, 519.

Han, J.-S. and C.-W. Xie, 1982, Dynorphin: potent analgesic effect in spinal cord of the rat, Life Sci. 31, 1781.

Han, J.-S., G.-X. Xie and A. Goldstein, 1984, Analgesia induced by intrathecal injection of dynorphon B in the rat, Life Sci. 34, 1573.

Herman, B.H. and A. Goldstein, 1985, Antinociception and paralysis induced by intrathecal dynorphin A, J. Pharmacol. Exp. Ther. 232, 27.

Höllt, V., 1986, Opioid peptide processing and receptor selectivity, Ann. Rev. Pharmacol. Toxicol. 26, 59.

Hylden, J.L.K. and G.L. Wilcox, 1980, Intrathecal substance P elicits a caudally-directed biting and scratching behaviour in mice, Brain Res. 217, 212.

Kest, B., M. Orlowski, C.J. Molineaux and R.J. Bodnar, 1991, Antinociceptive properties of inhibitors of endopeptidase 24.15, Int. J. Neurosci. 56, 141.

Kest, B., M. Orlowski and R.J. Bodnar, 1992, Endopeptidase 24.15 inhibition and opioid antinociception, Psychopharmacology 106, 408.

Lecomte, J.M., J. Constentin, A. Vlaiculescu, P. Chaillet, H. Marcais-Collado, C. Llorens-Cortes, M. Leboyer and J.C. Schwartz, 1986, Pharmacological properties of acetorphan, a parentally active "enkephalinase" inhibitor, J. Pharmacol. Exp. Ther. 237, 937.

Litchfield, J.T. and F. Wilcoxon, 1949, A simplified method of evaluating dose-effect experiments, J. Pharmacol. Exp. Ther. 96, 99.

Malfroy, B., J.P. Swerts, A. Guyon, B.P. Roques and J.C. Schwartz, 1978, High affinity enkephalin-degrading peptidase in brain is increased after morphine, Nature 276, 523.

Millan, M.J., 1986, Multiple opioid systems and pain, Pain 27, 303.

Nakazawa, T., M. Ikeda, T. Kaneko, K. Yamatsu, K. Kitagawa and Y. Kiso, 1989, Bestatin potentiates the antinociception but not the motor dysfunction induced by intracerebrally administered dynorphin-B in mice, Neuropeptides 13, 277.

Numata, H., T. Hiranuma and T. Oka, 1988, Inactivation of dynorphin-(1–8) in isolated preparations by three peptidases, Jpn. J. Pharmacol. 47, 417.

Nylander, I., K. Tan-No, A. Winter and J. Silberring, 1995, Processing of prodynorphin-derived peptides in striatal extracts. Identification by electrospray ionization mass spectrometry linked to size-exclusion chromatography, Life Sci. 57, 123.

Nyberg, F., K. Nordstrom and L. Terenius, 1985, Endopeptidase in human cerebrospinal fluid which cleaves proenkephalin B opioid peptides at consecutive basic amino acids, Biochem. Biophys. Res. Commun. 131, 1069.

Oka, T., X. F. Liu, T. Kajita, N. Ohgiya, K. Ghoda, T. Taniguchi, Y. Arai and T. Matsumiya, 1992, Effects of the subcutaneous administration of enkephalins on tail-flick response and righting reflex of developing rats, Brain Res. 69, 271.

Pozsgay, M., C. Michaud, M. Liebman and M. Orlowski, 1986, Substrate and inhibitor studies of thermolysine-like neutral metalloendopeptidase from kidney membrane fractions: comparison with bacterial thermolysine, Biochemistry 25, 1292.

Ren, M. F., C. H. Lu and J. S. Han, 1985, Dynorphin-A-(1–13) antagonizes morphine analgesia in the brain and potentiates morphine analgesia in the spinal cord, Peptides 6, 1015.

Roques, B.P., M.C. Fournié-Zaluski, E. Soroca, J.M. Lecomte, B. Malfroy, C. Llorens and J.C. Schwartz, 1980, The enkephalinase inhibitor thiorphan shows antinociceptive activity in mice, Nature 288, 286.

Roques, B.P., F. Noble, V. Dauge, M.C. Fournié-Zaluski and A. Beaumont, 1993, Neutral endopeptidase 24.11: structure, inhibition, and experimental and clinical pharmacology, Pharmacol. Rev. 45, 87.

Sakurada, T., K. Takahashi, S. Sakurada, K. Kisara, R. Folkesson and L. Terenius, 1988, Enkephalins interact with substance P-induced aversive behaviour in mice, Brain Res. 442, 191.

Sakurada, T., K. Katsumata, H. Uchiumi, Y. Manome, K. Tan-No, S. Sakurada, S. Kawamura, R. Ando and K. Kisara, 1992, Spantide-induced antinociception in the mouse formalin test, in: International Symposium on Processing and Inhibition of Nociceptive Information, eds. R. Inoki, Y. Shigenaga and M. Tohyama (Excerpta Medica, Amsterdam) p. 227.

Schwartz, J.C., B. Malfroy and S. De La Baume, 1981, Biological inactivation of enkephalins and the role of enkephalin-dipeptidyl-carboxypeptidase ("enkephalinase") as neuropeptidase, Life Sci. 29, 1715.

Spampinato, S. and S. Candeletti, 1985, Characterization of dynorphin A-induced antinociception at spinal level, Eur. J. Pharmacol. 110, 21.

Stine, S.M., H.-Y. T. Yang and E. Costa, 1980, Inhibition of in situ metabolism of [^{3}H](Met^5)-enkephalin and potentiation of (Met^5)-enkephalin analgesia by captopril, Brain Res. 188, 295.

Sullivan, S., H. Akil and J.D. Barchas, 1978, In vitro degradation of enkephalin: evidence for cleavage at the Gly-Phe bond, Commun. Psychopharmacol. 2, 525.

Silberring, J. and F. Nyberg, 1989, A novel bovine spinal cord endoprotease with high specificity for dynorphin B, J. Biol. Chem. 264, 11082.

Silberring, J., M.E. Castello and F. Nyberg, 1992, Characterization of dynorphin A-converting enzyme in human spinal cord. An endoprotease related to a distinct conversion pathway for the opioid heptadecapeptide?, J. Biol. Chem. 267, 21324.

Silberring, J., H.-U. Demuth, P. Brostedt and F. Nyberg, 1993, Inhibition of dynorphin converting enzymes from human spinal cord by *N*-peptidyl-*O*-acyl hydroxylamines, J. Biochem. 114, 648.

Turner, A.J., R. Matsas and J. Kenny, 1985, Are there neuropeptide-specific peptidases?, Biochem. Pharmacol. 34, 1347.