Roles of Endogenous Opioid Peptides in Modulation of Nocifensive Response to Formalin

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ABSTRACT

Roles of endogenous opioid peptides and their receptors in modulation of the nocifensive responses to formalin in mice were studied. Mice were pretreated i.c.v. or intrathecally (i.t.) with selective opioid receptor antagonists or intrathecally with antisera against endogenous opioid peptides and the nocifensive licking responses to intraplantar injection of formalin (0.5%, 25 μl) were then observed. Pretreatment with the ε-opioid receptor antagonist β-endorphin(1–27) or the selective μ-opioid receptor antagonist d-Phe-Cys-Tyr-Orn-Thr-Pen-Thr-NH₂ (CTOP) given i.c.v. dose dependently enhanced the second, but not the first phase of the nocifensive response. However, i.c.v. pretreatment with the selective δ-receptor antagonist naltrindole or κ-receptor antagonist nor-binaltrophimine did not affect the nocifensive responses. Intrathecal pretreatment with selective δ-opioid antagonist 7-benzylidene naltrexamine significantly enhanced both the first and second phases of nocifension. Intrathecal pretreatment with CTOP also increased the second but not the first phase of the nocifension. However, i.t. pretreatment with the selective δ-opioid antagonist naltrexone or nor-binaltrophimine did not affect the second phase of nocifension. Intrathecal pretreatment with antisera against Leu-enkephalin, Met-enkephalin, or dynorphin A(1–17), but not β-endorphin, enhanced only the second phase of nocifensive response to formalin. It is concluded that the blockade of ε- and μ-receptors, but not δ- or κ-receptors, at the supraspinal sites enhanced the second phase of formalin-induced nocifension. In the spinal cord, Leu-enkephalin, and to a lesser extent, Met-enkephalin and dynorphin A(1–17) and μ- and δ-opioid receptors, but not δ2- or κ-opioid receptors, are involved in modulating the feedback inhibition of the second phase of formalin-induced nocifension.

The formalin test is one of the most frequently used models to evaluate the nocifensive response to noxious stimulation because it closely resembles human responses to painful stimuli (Dubuisson and Dennis, 1977; Murry et al., 1988; Abbott et al., 1999). Two phases of nocifensive responses induced by intraplantar injection of formalin are observed. The first phase of acute nocifensive behavior lasts for 10 min and the subsequent second phase of tonic nocifensive responses starts from 15 min and ends at 50 to 60 min after intraplantar injection of a formalin solution (Dubuisson and Dennis, 1977; Tjølsen et al., 1992). The first phase may result from a direct activation of myelinated and unmyelinated fibers, both low-threshold mechanoreceptive and nociceptive types, and the second phase may be caused by the activation of central sensitized neurons due to peripheral inflammation stimuli as well as ongoing activity of primary afferents (Hunskaar and Hole, 1987; Puig and Sorkin, 1996; Dubner and Ren, 1999).

Peripheral noxious stimuli may lead to central endogenous opioid changes. An increase of β-endorphin immunoactivity in supraspinal site resulting from peripheral formalin-induced inflammation has been observed and i.c.v. pretreatment with antisera against β-endorphin markedly increases the nocifensive response to formalin in the rat and mouse (Porro et al., 1991; Wu et al., 2001). Zangen et al. (1998) demonstrated that the extracellular levels of β-endorphin in the arcuate nucleus are increased markedly, corresponding to the nocifensive response to intraplantar injection of formalin. These results indicate that central β-endorphin is activated in modulating the formalin-induced nocifensive response.

Other endogenous opioid peptides dynorphins and enkephalins are also involved in modulating the hyperalgesic response. Ossipov et al. (1996) demonstrated that intrathecal (i.t.) pretreatment with antisera against dynorphin or Leu-enkephalin, but not Met-enkephalin enhances the nocifensive responses to formalin injection, indicating the increased

ABBREVIATIONS: i.c.v., intracerebroventricular; i.t., intrathecal; CTOP, d-Phe-Cys-Tyr-Orn-Thr-Pen-Thr-NH₂; NTI, naltrindole; nor-BNI, nor-binaltrophimine; NTB, naltrexone; BNTX, 7-benzylidene naltrexamine; DAMGO, [d-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin; ANOVA, analysis of variance; PAG, periaqueductal gray.
releases of dynorphin and Leu-enkephalin to inhibit the nociceptive response to formalin. Le Bars et al. (1987) suggested that spinal enkephalins are involved not only in inhibitory control but also in the detection of pain. The inflammatory hyperalgesia induced by intraplantar injection of carrageenan or complete Freund’s adjuvant activates the dynorphin biosynthesis and increases the spinal immunoreactive dynorphin A(1–8) (Iadarola et al., 1988) and intraplantar formalin injection also increases immunoactive Met-enkephalin in the spinal cord (Bourgoin et al., 1990). The inflammatory pain induced by intraplantar inoculation of Mycobacterium butyricum is modulated by κ-, but not µ- or δ-opioid receptors in the spinal cord (Millan and Colpaert, 1991). However, Murry and Cowan (1991) suggested that κ- and µ-, but not δ-opioid receptors modulate the tonic pain perception at both spinal and supraspinal sites in mice.

Abbott et al. (1986) suggested that the antinociception induced by opioids is mediated primarily by the stimulation of µ-opioid receptors with the thermal pain models, whereas in nonthermal tests, κ-effects predominate. We have previously shown that not only β-endorphinergic but also Leu-enkephalinergic systems are activated at the supraspinal sites to attenuate the nociceptive responses to formalin stimulation (Wu et al., 2001). The present experiments were therefore designed to systemically investigate the roles of various endogenous opioid peptides at both supraspinal and spinal sites that might be involved in the modulation of nociceptive response to formalin stimulation.

Materials and Methods

Animals. Male ICR mice weighing 25 to 30 g (Charles River Laboratories, Inc., Wilmington, MA) were used. The animals were housed five per cage in a room maintained at 22 ± 0.5°C with an alternating 12-h light/dark cycle. Food and water were available ad libitum. The animals were used only once. All experiments were approved by and conformed to the guidelines of the Medical College of Wisconsin Animal Care Committee.

Drug Injection and Assessment of Nocifensive Responses. Intracerebroventricular injection was performed according to the method described by Haley and McCormick (1957), by using a 25-µl Hamilton syringe with a 26-gauge needle. Intrathecal injection was performed according to the procedure of Hylden and Wilcox (1980), by using a 25-µl Hamilton syringe with a 30-gauge needle. Injection volumes for i.c.v. and i.t. were 4 and 5 µl, respectively.

To measure the nociceptive response induced by formalin, mice were gently held and injected subcutaneously with 25 µl of 0.5% formalin solution into the plantar surface of the right hind paw (Shibata et al., 1989) with a microsyringe and 26-gauge needle. After formalin injection, mice were then observed for licking behavior of their injected hind paw in translucent plastic observation chambers (12 × 12 × 25 cm). The time spent in licking the injected paw was counted continuously every 5 min, starting immediately after the formalin injection for 60 min (Hunskaar et al., 1985). The nociceptive responses had two phases, the first and second phase (Dubuisson and Dennis, 1977), which were defined as between 0 to 10 min and 10 to 60 min after formalin injection, respectively.

Experimental Protocols. Mice were placed into translucent plastic observation chambers for adaptation 1 h before the experiments. The mice were then pretreated i.c.v. with various doses of β-endorphin (1–27), D-Phe-Cys-Tyr-Orn-Thr-Pen-Thr-NH₂ (CTOP), or naltrindole (NTI) 10 min or nor-binaltorphimine (nor-BNI) 24 h (Spanagel et al., 1994) before s.c. injection of formalin solution into the plantar surface of the right hind paw. In other experiments, mice were pretreated i.t. with the antiseraum against β-endorphin, Leu-enkephalin, Met-enkephalin, dynorphin A(1–17), or normal rabbit serum 1 h (Wu et al., 2001) before intraplantar injection of formalin. In another experiment, mice were pretreated i.t. with selective opioid antagonist, CTOP, NTI, naltriben (NTB), or 7-benzylindene naltrexamine (BNTX) 10 min, or nor-BNI 24 h before intraplantar injection of formalin. Groups pretreated with vehicle before s.c. formalin injections served as controls. All mice were then observed for nocifensive response for 60 min immediately after formalin injection.

Drugs and Antisera. BNTX, NTB, NTI, and nor-BNI were synthesized in H. Nagase’s laboratory (Pharmaceutical Research Laboratories, Kamakura, Japan). CTOP was purchased from Bachem Biosciences (King of Prussia, PA). BNTX, nor-BNI, and NTI were dissolved in 0.9% NaCl for i.c.v. and i.t. injections. CTOP, NTI, and β-endorphin (1–27) were dissolved in 0.9% NaCl containing 0.01% Triton X-100 for i.c.v. and i.t. injections. The doses of β-endorphin (1–27), CTOP, BNTX, NTB, NTI, and nor-BNI used in this study were determined based on the results of the previous studies that these doses of antagonists when given i.c.v. effectively block the antinociception with the tail-flick test induced by i.c.v. administration of respective selective ε-, µ-, δ₁-, δ₂-, and κ-opioid receptor agonists β-endorphin, DAMGO, deltorphin II, and U50,488H (Suh et al., 1988; Calcagnotti and Holtzman, 1991; Mizoguchi et al., 1995, 2000).

The antisera against β-endorphin, Leu-enkephalin, Met-enkephalin, and dynorphin (A(1–17)) were produced by repeated intradermal injection of male New Zealand White rabbits with opioid peptide coupled to bovine thyroglobulin according to the method described previously (Hollt et al., 1978). Radioimmunoassay or enzyme-linked immunoabsorbent assay characterized the specificities of these antisera. The antisera against β-endorphin did not cross-react with Leu-enkephalin, Met-enkephalin, or dynorphin (A(1–17)). The antisera against Leu-enkephalin did not immunoreact with β-endorphin or dynorphin A(1–17). It did show 14% cross-immunoreactivity with Met-enkephalin. The antisera against Met-enkephalin did not cross-react with dynorphin A(1–17) or β-endorphin. However, it showed 29.4% cross-immunoreactivity with Leu-enkephalin. The antisera to dynorphin (A(1–17)) did not show cross-immunoreactivity with β-endorphin, Leu-enkephalin, or Met-enkephalin.

Statistical Analysis. The behavioral test data are presented as the means ± S.E.M. for different 5-min time bins during the first and the second phases. Analysis of variance (ANOVA) followed by Dunnnett’s test was performed to test the difference among groups. A value of P < 0.05 was considered statistically significant.

Results

Effects of i.c.v. Pretreatment with Selective Opioid Antagonists on Nocifensive Responses to Intraplantar Injection of Formalin. Intraplantar subcutaneous injection of 25 µl of 0.5% formalin produced a typical biphasic nocifensive paw licking with an initial acute phase, which started immediately after injection for 5 min and the second phase, which started 10 to 15 min, peaked at 20 to 25 min and dissipated in 30 min after formalin injection in mice pretreated with saline. The i.c.v. pretreatment with 2 nmol of β-endorphin (1–27) caused a marked increase between 25 and 30 min of the second phase of the nocifensive responses to formalin compared with mice pretreated with vehicle (Fig. 1A). The total time spent in licking during the second phase of nocifension was increased by 76.8% compared with mice pretreated with vehicle (Fig. 1A). As shown in Fig. 2, A and B, i.c.v. pretreatment with various doses of β-endorphin (1–27) (0.3–2 nmol) dose dependently enhanced the second phase of nocifensive response to formalin. Low doses (0.3, 0.6, and 1 nmol) of β-endorphin (1–27) pretreatment did not affect the first phase of the nocifensive response to formalin. However, β-endorphin (1–27) at a high dose (2...
nmol) significantly attenuated the first phase of the nocifensive response to formalin (Figs. 1B and 2B).

Pretreatment with CTOP (150 pmol) given i.c.v. enhanced significantly between 15 and 20 min the second phase of nocifensive response to formalin, and the duration of the nocifensive response of the second phase to formalin was also significantly prolonged (Fig. 1, A and B). The total time spent in licking during the second phase of nocifensive response was increased by 43.1% compared with mice pretreated with saline. However, the first phase of the nocifensive response to formalin was not affected by CTOP pretreatment (Fig. 1B).

As shown in Fig. 3, A and B, pretreatment with various doses of CTOP (50–200 pmol) enhanced dose dependently the second phase, but not the first phase, of the nocifensive response and the increase of the nocifensive response reached its peak effect at 150 pmol of CTOP. Thus, the magnitude of the increase of the formalin nocifensive response caused by 150 pmol of CTOP treatment was significantly lower than that caused by 2 nmol of β-endorphin(1–27) pretreatment. Pretreatment with 11.1 nmol of NTI or 13.3 nmol of nor-BNI given i.c.v. did not affect either the first or the second phase of the nocifensive response to formalin (Fig. 4, A and B).

**Effects of i.t. Pretreatment with Selective Opioid Antagonists on Nocifensive Response to Intraplantar Injection of Formalin.** Intrathecal pretreatment with 2.0 nmol of BNTX significantly increased both the first and second phase of nocifensive responses to formalin by 34.1 and 111.8%, respectively, whereas i.t. pretreatment with NTI (11.1 nmol) did not increase both the first and second phase of the nocifensive response to formalin (Fig. 5A). Pretreatment with CTOP (150 pmol) given i.t. increased the second but not the first phase of nocifensive response to formalin. Intrathecal pretreatment with NTB (18.8 nmol) did not affect the second phase, but attenuated the first phase of the nocifensive response to formalin (Fig. 5B). Nor-BNI (13.3 nmol) given i.t. did not affect the first and the second phase of the nocifensive response to formalin (Fig. 5C).
Effects of i.t. Pretreatment with Antisera against β-Endorphin, Leu-Enkephalin, Met-Enkephalin, or Dynorphin A(1–17) on the nocifensive response to intraplantar injection of formalin. Intrathecal pretreatment with antisera against Met-enkephalin, Leu-enkephalin, or dynorphin A(1–17) (200 μg each) significantly enhanced with different magnitude the second phase, but not the first phase of the nocifensive response to formalin. The increases of the time spent in licking during the second phase of the nocifensive response were found to be 54.5, 88.3, and 36.1% for mice pretreated with antisera against Met-enkephalin, Leu-enkephalin, and dynorphin A(1–17), respectively, compared with mice pretreated with normal rabbit serum. However, i.t. pretreatment with antisera against 200 μg of β-endorphin did not affect either the first or the second phase of the nocifensive response to formalin. (Fig. 6).

Discussion

Enhancement of Formalin-Nocifensive Response by Supraspinal Pretreatment with β-Endorphin(1–27). β-Endorphin(1–27) has been demonstrated to be an antagonist and blocks the antinociception induced by β-endorphin (Tseng, 2001). This is evidenced by the findings that intracerebroventricular coadministration of β-endorphin(1–27) with β-endorphin effectively attenuates the β-endorphin-induced tail-flick inhibition in mice (Hammonds et al., 1984; Suh et al., 1988; Tseng, 2001). In rats, cointracerebral administration of β-endorphin(1–27) with β-endorphin microinjected into periaqueductal gray (PAG), raphe obscurus nucleus, posterior nucleus accumbens, medial preoptic area, or acuate hypothalamic nucleus effectively attenuates the tail-flick inhibition induced by β-endorphin (Tseng and Tang, 1990; Tseng, 2001). In the present study, we found that i.c.v. pretreatment with β-endorphin(1–27) markedly enhanced the second phase, but not the first phase of nocifensive response to formalin stimulation. This finding is in line with the previous reports that pretreatment with antisera against β-endorphin enhances the nocifensive response to formalin in rats and mice, indicating an increased release of β-endorphin during the second phase of formalin-induced nocifension (Porro et al., 1991; Wu et al., 2001). It is proposed that the nociceptive stimulation by intraplantar injection of formalin evokes the release of β-endorphin, which subsequently stimulates the putative e-opioid receptors to inhibit the nocifensive response and the blockade of the e-opioid receptors by β-endorphin(1–27) results in an enhancement of formalin-induced nocifension. Intraplantar injection of formalin increases the release of β-endorphin from the hypothalamic arcuate nucleus at times corresponding to the second phase of nocifension (Zangen et al., 1998) and increases the β-endorphin immunoreactivities of the ventral PAG and ventromedial hypothalamus, the brain regions important for pain and pain control (Porro et al., 1991; Facchinetti et al., 1992). The nocifensive response to formalin is enhanced in rats with lesions in the arcuate nucleus of the hypothalamus, the region that is rich in β-endorphin neurons (Hamba, 1988). These findings strongly indicate that formalin stimulation activates the β-endorphinergic system at the supraspinal sites and induces the release of β-endorphin, which, in turn, exerts an inhibitory effect on the nocifensive response.

We have previously demonstrated that coadministration of β-endorphin(1–27) given intracerebroventricularly in mice selectively blocks the antinociception induced by β-endorphin, but not μ-opioid agonists DAMGO or morphine, δ-opioid agonists [d-Pen²,d-Pen⁵]-enkephalin or [d-Ala²,d-Leu⁵]-enkephalin, or κ-opioid receptor agonist U-50,488H. In addition, supraspinal β-endorphin and morphine in mice fail to develop antinociceptive cross-tolerance (Suh and Tseng, 1990). This unique receptor for β-endorphin and β-endorphin(1–27) has been putatively classified as the e-opioid receptor (Tseng, 2001). The selective blocking effect of β-endorphin(1–27) on e-opioid receptors found in mice was also confirmed in rats. Coadministration of β-endorphin(1–27) microinjected into PAG, nucleus raphe obscurus, or nucleus accumbens selectively blocks the antinociception induced by β-endorphin, but not by μ-opioid agonists morphine or DAMGO (Tseng, 2001). We suggest that these β-endorphin-
increase of the \([^{35}S]guanosine-5'-O-(3-thio)triphosphate\) binding stimulated by \(\beta\)-endorphin is attenuated by both \(\mu\)-opioid receptor antagonist \(\beta\)-funaltrexamine and \(\beta\)-endorphin (1–27), indicating that \(\beta\)-endorphin stimulates both \(\mu\)- and putative \(\epsilon\)-opioid receptors. \(\beta\)-Endorphin (1–27) at high doses produces antinociception with the tail-flick test, which is much less potent and has a much shorter duration of action than that produced by \(\beta\)-endorphin (Hammonds et al., 1984). We found in the present study that \(\beta\)-endorphin (1–27) at 2 nmol given i.c.v. attenuated the first phase of nocifensive response to formalin. The inhibition of the first phase of the formalin response probably is due to the agonistic property of \(\beta\)-endorphin (1–27).

**Effects of Opioid \(\mu\), but not \(\delta\)- or \(\kappa\)-Receptor Antagonists Given Supraspinally on Formalin-Induced Nocifensive Response.** Pretreatment with selective \(\mu\)-opioid antagonist CTOP given i.c.v. enhanced the paw licking induced by formalin, indicating that the \(\mu\)-opioid receptors are activated to inhibit the formalin-induced nocifension. However, the magnitude of the enhancement by CTOP pretreatment is lower than that by \(\beta\)-endorphin (1–27) pretreatment. Stimulation of \(\mu\)-opioid receptors by supraspinal administration of \(\mu\)-opioid agonists or endogenous \(\mu\)-ligands, endomorphins, attenuates the nocifensive response to formalin (Murry and Cowan, 1991; Soignier et al., 2000). Thus, intraplantar injection of formalin may lead to the release of endomorphins acting on \(\mu\)-opioid receptors to modulate the nocifension. In addition, \(\beta\)-endorphin also possesses a high affinity to \(\mu\)-opioid receptors and some other effects of \(\beta\)-endorphin have been reported to be mediated in part by the stimulation of \(\mu\)-opioid receptors (Monroe et al., 1996). The antinociception induced by \(\beta\)-endorphin microinjected into PAG of rats or injected intracerebroventricularly in mice is also blocked by \(\mu\)-opioid receptor antagonist CTOP (\(\beta\)-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH\(_2\)), indicating that \(\mu\)-opioid receptors are also involved in \(\beta\)-endorphin-induced antinociception (Shook et al., 1988; Monroe et al., 1996). It is possible that the released \(\beta\)-endorphin may also stimulate \(\mu\)-opioid receptors to attenuate the formalin-induced nocifension and the blockade of \(\mu\)-opioid receptors by CTOP therefore enhances the response.

Blockade of opioid \(\delta\)- or \(\kappa\)-receptors at the supraspinal sites by i.c.v. pretreatment with \(\delta\)-receptor antagonist NTI or \(\kappa\)-receptor antagonist nor-BNI did not affect the nocifensive response, indicating that \(\delta\)- and \(\kappa\)-opioid receptors may not be involved in formalin-induced nocifension at the supraspinal sites. This conclusion is consistent with the finding by Murry and Cowan (1991) that \(\delta\)-opioid receptors do not modulate the formalin pain at the supraspinal sites. We have previously reported that i.c.v. pretreatment with antisera against Met-enkephalin or dynorphin A(1–17) does not affect the nocifensive response to formalin (Wu et al., 2001).

**Effects of \(\delta_1\), \(\delta_2\), \(\mu\), or \(\kappa\)-Opioid Receptor Antagonists Given Spinally on Formalin-Induced Nocifensive Response.** Hammond et al. (1998) previously have demonstrated that i.t. administration of \(\delta\)-opioid agonists \(\beta\)-(Pen\(_2\)-\(^{5}\))enkephalin or deltorphin II attenuates the first and the second phase of formalin-induced nocifension in rats. The formalin-induced nocifension is enhanced by intraperitoneal administration of nonselective opioid receptor antagonist naloxone or \(\delta\)-receptor antagonist NTI in rats (Ossipov et al., 1996), indicating that \(\delta\)-opioid receptors are activated by formalin injection in this animal. We found in the present study that the blockade of \(\delta_1\)-opioid receptors in the spinal
cord by i.t. administration of BNTX markedly potentiated both the first and the second phase of the nocifensive response induced by formalin injection. On the other hand, pretreatment with δ-opioid receptor antagonists NTB or NTI did not affect either the first phase or the second phase of the formalin response. Our results indicate that δ₁-, but not δ₂-receptors are activated during the first and second phase of the formalin stimulation. The reason for the attenuation of the first phase of formalin nocifension by δ₂-opioid receptor antagonist NTB is not clear at present time.

We found in the present study that the blockade of μ-opioid receptors in the spinal cord by i.t. pretreatment with CTOP potentiated the second phase of nocifensive response induced by formalin. Murry and Cowan (1991) demonstrated that μ-opioid receptors are activated to modulate the formalin-induced nocifension at both supraspinal and spinal sites. Endogenous μ-opioid peptides endomorphins are distributed in the dorsal horn of the spinal cord (Martin-Schild et al., 1999). Thus, intraplantar formalin injection may cause the release of endomorphins to modulate the nocifensive response to formalin at spinal cord.

The blockade of κ-opioid receptors in the spinal cord by i.t. pretreatment with nor-BNI did not affect the nocifensive behavior induced by formalin. The finding indicates that κ-opioid receptors in the spinal cord are not activated by formalin stimulation in mice. The result of our study in mice is different from that of the study in rats by Ossipov et al. (1996). They reported that the nocifension induced by formalin in rats is enhanced by intraperitoneal pretreatment with κ-opioid receptor antagonist nor-BNI, but not μ-opioid receptor antagonist β-funaltrexamine. The reason for the discrepancy is not clear at this time. It may be due to the different species used.

Formalin-Induced Nocifensive Response Is Enhanced by i.t. Pretreatment with Antiserum against Leu-Enkephalin, Met-Enkephalin, or Dynorphin A(1–17), but not by Antiserum against β-Endorphin. Enkephalins have been proposed to be the neurotransmitters for δ-opioid receptors. The finding that δ₁-opioid receptors are activated after formalin injection leads us to speculate that the enkephalins are released during the formalin-induced

Fig. 5. Effects of i.t. pretreatment with NTI or BNTX (A), CTOP or NTB (B), or nor-BNI (C) on the paw-licking nocifensive response to intraplantar injection of formalin. Groups of mice were pretreated i.t. with 11.1 nmol of NTI, 2.0 nmol of BNTX, 150 pmol of CTOP, or 19.0 nmol of NTB for 10 min, or 13.3 nmol of nor-BNI 24 h before formalin injection and the paw-licking responses were then observed. The data are expressed as means ± S.E.M.; error bars indicated S.E.M. One-way ANOVA followed by Dunnett’s test was used to test the difference among groups; *P < 0.001 compared with vehicle group; n = 8 to 15/group.

Fig. 6. Effects of i.t. pretreatment with normal rabbit serum (NRS) or antiserum (A/S) against β-endorphin, Leu-enkephalin, Met-enkephalin, or dynorphin A(1–17) on the paw-licking nocifensive response to intraplantar injection of formalin. Groups of mice were pretreated with antiserum against β-endorphin, Leu-enkephalin, Met-enkephalin, or dynorphin A(1–17) (200 μg), 1 h before subcutaneous intraplantar injection of 25 μl of 0.5% formalin. Paw-licking responses were then observed. The data are expressed as means ± S.E.M.; error bars indicated S.E.M. One-way ANOVA followed by Dunnett’s test was used to test the difference among groups; *P < 0.001; **P < 0.05 compared with normal rabbit serum; n = 8 to 9/group.
nocifensive. We found that formalin-induced nocifensive was enhanced by i.t. pretreatment with Leu-enkephalin antiserum, and to a lesser extent, with Met-enkephalin or dynorphin A(1–17) antiserum. Our results indicate that Leu-enkephalin, and to a lesser extent, Met-enkephalin and dynorphin A(1–17) are released after formalin stimulation. The results are consistent with the observation by Ossipov et al. (1996) that i.t. pretreatment with Leu-enkephalin or dynorphin A(1–17) antiserum potentiates formalin-induced nocifensive in rats. However, they fail to observe any increase of nocifensive after pretreatment with Met-enkephalin antiserum. Our finding is consistent with the observation by Bourgon et al. (1990) that formalin stimulation induces the release of Met-enkephalin from the rat spinal cord and supports the view that β-endorphin is released from the supraspinal sites after formalin stimulation and subsequently induces the release of Met-enkephalin in the spinal cord (Tseng, 2001).

Pretreatment with antiserum against β-endorphin did not affect the nocifensive response to formalin injection. Although there is a direct β-endorphinergic projection from the arcuate nucleus of the hypothalamus to the spinal cord (Tsou et al., 2001), this spinal bet-endorphinergic pathway appears not to play a major role in formalin-induced nocifensive.

The question arises as to the source of Leu-enkephalin, which stimulates δ-opioid receptors. Activation of supraspi- nal β-endorphin causes the release of Met-enkephalin rather than Leu-enkephalin from the spinal cord (Tseng, 2001). Both Leu-enkephalin and Met-enkephalin share the same precursor, preproenkephalin, with a proportion of one to five copies, respectively. However, pretreatment with antiserum against Leu-enkephalin caused more enhancements than with antiserum against Met-enkephalin of the formalin-induced nocifensive. Thus, it is unlikely that the source of Leu-enkephalin comes from preproenkephalin. Silberring et al. (1992) described a dynorphin convertase, which degraded dynorphins into Leu-enkephalin-Arg⁶. A carboxypeptidase then acted on Leu-enkephalin-Arg⁶ to form Leu-enkephalin. Thus, Leu-enkephalin could have come from preprodynorphin. This view is also supported in the present study that the formalin-induced nocifensive behavior is enhanced by pretreatment with antiserum against dynorphin A(1–17). This effect is not mediated by the stimulation of κ-opioid receptors because the blockade of κ-opioid receptors in the spinal cord by nor-BNI given i.t. did not affect formalin-induced nocifensive response.

Conclusion

It is concluded that the blockade of κ- and μ- receptors, but not δ- or κ-opioid receptors at the supraspinal sites enhances the second phase of formalin-induced nocifensive. At the spinal site, the blockade of spinal μ- and δ₁-receptors, but not δ₂- or κ-opioid receptors as well as the binding of the extracellular enkephalins or dynorphin A(1–17) by antiserum enhances the second phase of the formalin-induced nocifensive. It is proposed that intraplantar injection of formalin induces the release of β-endorphin at supraspinal site, which subsequently acts on μ- and/or putative ε-receptors to exert a feedback inhibition of the formalin-induced nocifensive. Formalin stimulation also induces the release of Leu-enkephalin, Met-enkephalin, and dynorphin A(1–17) from the spinal cord and subsequently act on μ- and δ₁-receptors, but not δ₂- or κ-receptors for the feedback inhibition of the formalin response in the spinal cord.

References

Spanagel R, Almeida OF, and Shippenberg TS (1994) Evidence that nor-
binaltorphimine can function as an antagonist at multiple opioid receptor sub-
Suh HH and Tseng LF (1990) Lack of antinociceptive cross-tolerance between in-
tracerebroventricularly administered β-endorphin and morphine or DPDPE in
Suh HH, Tseng LF, and Li CH (1988) β-Endorphin-(1–27) antagonizes β-endorphin-
but not morphine-, D-Pen²-D-Pen⁵-enkephalin- and U50,488H-induced analgesia in
Tseng LF and Tang R (1990) Different mechanisms mediate β-endorphin- and
morphine-induced inhibition of the tail-flick response in rats. J Pharmacol Exp
Ther 252:546–551.

Tseng LF (2001) Evidence for e-opioid receptor-mediated β-endorphin-induced anal-
zation of pro-opiomelanocortin-derived peptides in adults rat spinal cord. Brain
endogenous opioids increase the nocifensive response to formalin: demonstration

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