Brain Research, 322 (1984) 289–296
Elsevier

BRE 10401

Met-Enkephalin-Arg^6-Phe^7-Like Immunoreactive Substances Mediate Electroacupuncture Analgesia in the Periaqueductal Gray of the Rabbit

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(Accepted March 20th, 1984)

Key words: Met-enkephalin — Met-enkephalin-Arg^6-Phe^7 — periaqueductal gray — electroacupuncture — analgesia — captopril —naloxone — antiserum

The present study was undertaken to investigate whether the C-terminal extended Met-enkephalin heptapeptide (Met-enkephalin-Arg^6-Phe^7, MEAP) played a role in mediating the analgesic effect of electroacupuncture in rabbits. MEAP and its degrading enzyme inhibitor captopril as well as antiserum against MEAP were injected into the periaqueductal gray (PAG) via a previously implanted cannula. Their effects on nociception were tested by the escape response latency (ERL) elicited by radiant heat applied on the skin of the snout. (1) Microinjection of MEAP (30–240 nmol) into PAG produced a dose-dependent analgesic effect which was 2.5 times more potent than Met-enkephalin (MEK) and 3 times less potent than morphine. The complete reversal of the analgesia elicited by 240 nmol of MEAP by a small dose of naloxone (0.1 mg/kg, i.v.) indicates that the effect of MEAP is mediated by naloxone sensitive opioid receptors. (2) In rabbits, a dose-dependent analgesia was elicited by an intra-PAG injection of captopril (60–240 nmol). A single dose of 240 nmol captopril increased ERL by more than 100%. This effect could be reversed by 30 nmol of naloxone injected into the same site, or by antiserum recognizing MEAP (1 μl, titer 1:1500) but not by antiserum recognizing MEK (1 μl, 1:8000) suggesting that captopril was able to protect MEAP from degradation. (3) Intra-PAG injection of 60 nmol of captopril significantly potentiated the after effect of electroacupuncture (EA) induced analgesia. This effect could be blocked either by 30 nmol (but not 7.5 nmol) of naloxone, or by 1 μl (but not 0.1 μl) of MEAP antiserum. These results suggest that MEAP may cause analgesia by acting in PAG, and could be operative in EA analgesia.

INTRODUCTION

Met-enkephalin-Arg^6-Phe^7 (MEAP) is an endogenous compound encoded in proenkephalin[11,15,19], as the carboxyterminus. It is structurally a carboxyextended precursor of Met-enkephalin (MEK) and was first isolated from bovine adrenal medullary granules[25]. It was found that the regional distribution of MEAP in CNS as revealed by radioimmunoassay approximates that of MEK[7–9,31], that MEAP-like immunoreactivity exists not only in neuronal perikarya but also in nerve termini[8,31], and that a K^+-stimulated Ca^{2+}-dependent release of MEAP has been shown in striatal slices[31], one might infer that MEAP is a putative neurotransmitter or neuromodulator that very often coexists with MEK in the same neurons.

Radioimmunoassay (RIA) and immunocytochemical studies have revealed the existences of MEAP-like immunoreactivity in periaqueductal gray (PAG)[27], which has been considered as one of the most important neuronal structures in relation with pain and analgesia[17,21,29,33]. The precise cellular and molecular mechanisms within PAG in pain modulation remain to be clarified.

Inturrisi et al.[16] reported an analgesic effect after intracerebroventricular (i.c.v.) injection of MEAP in mice. Zhang et al.[32] confirmed their result and showed that this action could be enhanced by captopril, a specific inhibitor of the angiotensin converting enzyme (ACE). In the present study, we have used antibody microinjection techniques to demonstrate that in the rabbit PAG endogenous MEAP is opera-

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tive in pain modulation, and that MEAP-like immunoreactive substances seem to play an important role in mediating EA analgesia.

MATERIALS AND METHODS

Chemicals

MEAP (Tyr-Gly-Gly-Phe-Met-Arg-Phe) was purchased from the Sigma company. The MEAP antiserum, a gift from Dr. H-Y. T. Yang (NIMH, Washington D.C.), was raised in rabbit against MEAP (Peninsula). The final dilution used for RIA was 1:1250–1:1500. It cross reacted with Phe-Met-Arg-Phe by about 10%, but not with the tripeptide Met-Arg-Phe or with Phe-Met-Arg-Phe-NH_2, Met-enkephalin, Met-enkephalin-Arg^6 in a concentration of 10 μM[31]. Met-enkephalin antiserum with a titer of 1:8000 in RIA was obtained by immunizing the rabbits with MEK (Sigma). Cross-reactivity with Leu-enkephalin, β-endorphin, dynorphin and MEAP was 1.1%, 0.8%, 0.02%, and 0.1%, respectively. Captopril (1-(D-3-mercapto-2-methylpropanoyl)-L-proline, SQ14225) was kindly offered by Squibb Chemical Company. Naloxone HCl was a gift from Endo Laboratories.

Animals

Healthy male rabbits weighing 2.0–2.6 kg were used. Stainless steel cannulae of 0.8 mm diameter were implanted under pentobarbital anesthesia (30 mg/kg, i.v.) directed to PAG bilaterally in the coordinates P 9.5, L 1.0, H 10.0 according to Sawyer et al.[24]. The cannulae were permanently fixed on the skull with dental resin. Kanamycin sulfate was given during the first 3 days after the surgical operation. One week was allowed for surgical recovery and the animals were then given a training session of repeated nociceptive tests so as to normalize animal responsiveness and adapt them to laboratory environment.

Electroacupuncture experiment

The details of the protocol for EA experiments were described elsewhere[14]. Stimuli of biphasic pulses of 0.3 ms duration at 1 V amplitude from a 57-6D electronic stimulator were applied to the following points: (1) the site corresponding to 'Zusanli' in humans, located in the hind limb, 8 mm lateral to the anterior tubercle of the tibia, with the needle passing the space between tibia and fibula; and (2) the site corresponding to the 'Quenlun' point in humans, at the proximal part of the Achilles tendon, with the needle passing in front of the tendon. The frequency shifted from 2 to 15 Hz every 5 s. EA stimulation continued for 20 min in each experiment.

Intracerebral injection

During the experiment, an injection tube of outer diameter 0.36 mm was inserted into the cannula protruding 2 mm beyond the tip of the cannula to reach PAG. Micro-injection was performed through a constant rate injection apparatus (Palmer) (1–2 μl in 8 min).

Verification of the injection points

Experiments were performed within 2–5 weeks after the operation. The animals were then sacrificed and the heads removed to be fixed in formalin for 1 week with the injection tubes in place. The site of injection was verified by the tract of the injection tube in cryostat brain sections.

Nociceptive test

The latency of the head jerk (escape response latency, ERL) elicited by radiant heat focused on the rabbit snout was measured and taken as the nociceptive index. The average value of 3 measurements made at 5 min intervals at the beginning of the experiment was taken as the basal ERL (100%). Subsequent measurements were performed every 10 min for a total of 1 h. The values were expressed as percentage changes of the basal ERL, with 200% increase as the cut-off limit to avoid skin burning.

Data are shown as mean ± S.E. Significance of differences between groups were tested with Student's *t*-test (two-tailed).

RESULTS

Analgesia elicited by MEAP injected into PAG

As shown in Fig. 1, unilateral MEAP injection of 30, 60, 120 and 240 nmol caused an elevation of the nociceptive threshold by 16 ± 6, 41 ± 6, 47 ± 16 and 52 ± 41%, respectively. The *P* values of the 4 groups were less than 0.05, 0.001, 0.05 and 0.01, respectively, when compared with the saline control animals.

Fig. 2. shows that MEAP analgesia could be re-

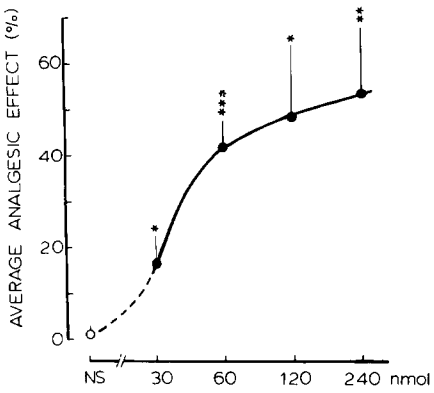

Fig. 1. The analgesic effect caused by unilateral intra-PAG injection of MEAP. Ordinate: the average percentage increase of escape response latency (ERL) in 6 measurements after the start of injection. Abscissa: the dose of MEAP (nmol). n = 6–8 in different groups injected with MEAP (●) or normal saline (NS) (○). Vertical bars indicate standard errors. Significance of difference between MEAP and NS group is expressed as * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

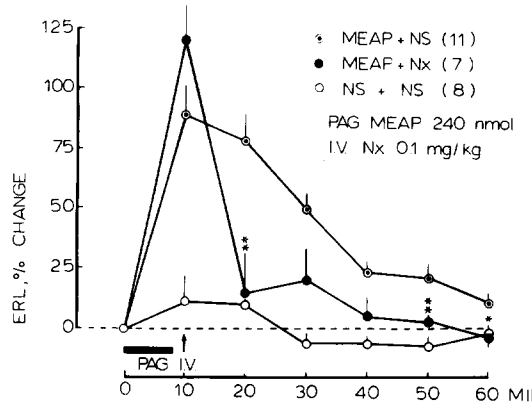

Fig. 2. The analgesic effect induced by intra-PAG injection of MEAP was reversed by naloxone. Abscissa: time after the starting of PAG injection (min). Each group received a serial intra-PAG and intravenous injection of MEAP 240 nmol and naloxone 0.1 mg/kg (●), MEAP and NS (⊙), NS and NS (○). The rectangle and the arrow represent the duration of intra-PAG injection and the time of intravenous injection, respectively. Significance of difference between the experimental (●) and control (⊙) groups: * $P < 0.05$ and ** $P < 0.01$. Numbers of animals in parentheses.

versed by i.v. injection of 0.1 mg/kg of naloxone. An abrupt drop of ERL was seen 10 min after naloxone administration. The difference between naloxone group (15 ± 16%) and saline treated group (78 ± 11%) was statistically very significant ($P < 0.01$).

The analgesic effect of captopril injected into PAG

Intra-PAG injection of 60, 120 and 240 nmol of captopril increased ERL by 38 ± 10, 81 ± 17 and 137 ± 13% (Fig. 3). The analgesic effect of 240 nmol of captopril lasted for 90 min (data not shown). A linear dose–response relationship was obtained when the average analgesic effect within 60 min was plotted against log dose of captopril as shown in the inset of Fig. 3.

Fig. 4 shows the location of the captopril microinjections (240 nmol) and their analgesic potency. No analgesia was obtained when captopril was injected outside the PAG area.

Reversal of captopril analgesia by naloxone or MEAP antiserum injected into PAG

Three groups of rabbits were given intra-PAG injection of 240 nmol of captopril to produce an increment of ERL by more than 100%. These rabbits were then given via the same cannula: (1) normal saline 1 μl; (2) naloxone 7.5 nmol; or (3) naloxone 30 nmol. The percentage increases of ERL measured 10 min after the second intracranial injection were 92 ± 27, 57 ± 17, and 11 ± 7, respectively (Fig. 5). The results indicate that the captopril analgesia could be reversed by 30 nmol ($P < 0.01$), but not by 7.5 nmol ($P > 0.05$) of naloxone. Administration of naloxone (30 nmol) alone failed to change ERL significantly.

In another experiment, 4 groups of rabbits receiving 240 nmol of captopril into PAG were given via the same cannula: (1) control serum from normal rabbit 1 μl; (2) MEAP antiserum (1:1500) 0.1 μl diluted with normal saline into 1 μl; (3) MEAP antiserum (1:1500) 1 μl; and (4) MEK antiserum (1:8000) 1 μl. The percentage increases of ERL over baseline level measured 10 min after the second intra-PAG injection were 127 ± 27, 70 ± 20, 41 ± 12 and 88 ± 17, respectively. A blockade of captopril analgesia was seen only in the rabbits receiving MEAP antiserum 1 μl ($P < 0.01$ as compared to control serum 1 μl group), but not in those receiving MEAP antiserum 0.1 μl or MEK antiserum 1 μl ($P > 0.05$). No significant changes of ERL were recorded when the same amount of normal saline, MEAP antiserum or MEK antiserum alone were injected into the same sites. These findings indicate that MEAP-like immunoreactive peptides in PAG are operative in the analgesic effect elicited by captopril.

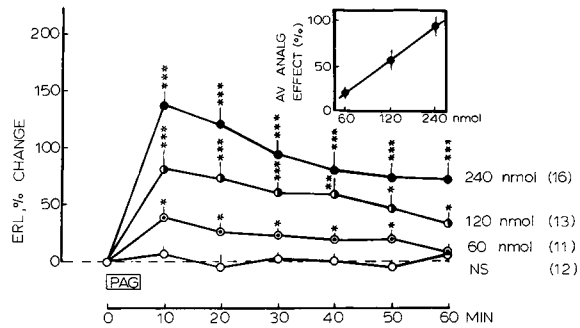

Fig. 3. The analgesic effect elicited by intra-PAG injection of captopril. The period of intra-PAG injection is shown in the left lower part of the figure. Doses of captopril injected into PAG are shown at the right side of the curves, with NS as the control. Inset is a plot of the average analgesic effect over a period of 60 min versus the dose of captopril in nmol.

Potentiation of electroacupuncture analgesia by captopril injected into PAG

Two groups of rabbits were given EA stimulation for 20 min. The increases of ERL in the two groups after 10 min of EA were 140 ± 14 and 166 ± 11%, respectively, showing no significant differences between groups. The EA stimulation was allowed to continue for another 10 min. In the mean time the animals received an intra-PAG injection of: (1) normal saline 1 μl; or (2) captopril 60 nmol. As can be seen from Fig. 6, the EA analgesia lasted much longer in the captopril group than in the saline control group. Injection of 60 nmol of captopril alone produced only a weak analgesic effect.

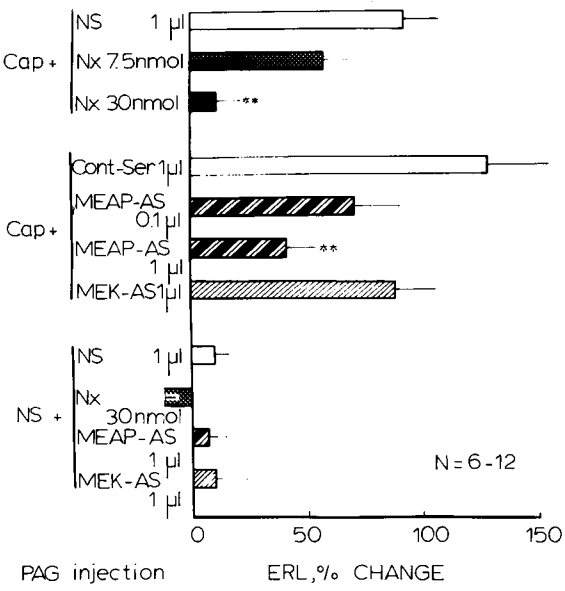

Fig. 5. The analgesia induced by intra-PAG injection of captopril was reversed by naloxone or by MEAP antiserum. Abscissa: percentage change of ERL 10 min after the intra-PAG injection of naloxone or MEAP antiserum. The dose of captopril and NS were 240 nmol and 1 μl respectively. Horizontal bars indicate standard errors. The significance of difference between experimental (dotted or shaded columns) and control (clear columns) groups is indicated by ** $P < 0.01$.

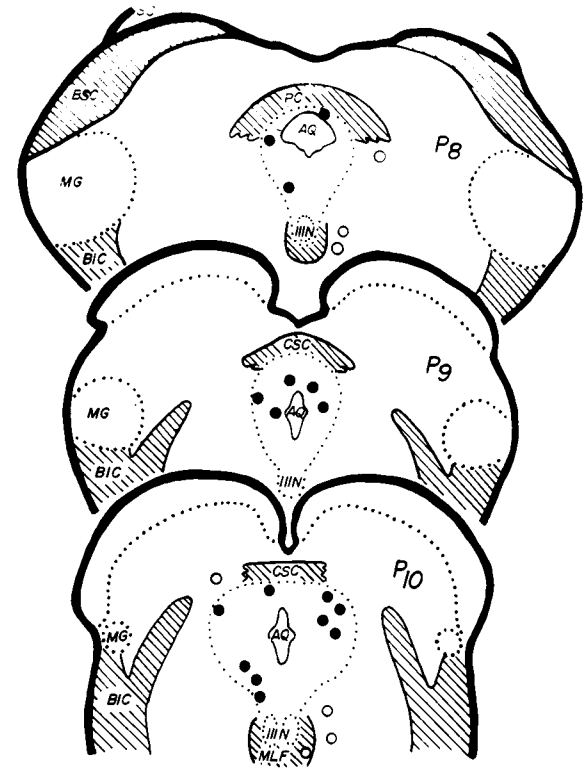

Fig. 4. Sites of microinjection of captopril into periaqueductal gray (PAG) of the rabbits and its relationship to the analgesic effect. Three coronary sections P8, P9 and P10 of rabbit brainstem show the sites of captopril injection (240 nmol). Black dots indicate a significant analgesic effect, i.e. the increase of ERL was greater than the mean ± 2 S.D. of the score of saline treated rabbits. Circles indicate that the change of ERL was within the range of mean ± 2 S.D. of the control group. Abbreviations: AQ, aqueductus mesencephali; SC, superior colliculus; BSC, brachium of superior colliculus; MG, medial geniculate body; BIC, brachium of inferior colliculus; CSC, commissure of superior colliculus; MLF, medial longitudinal fasciculus; IIIN, nuclei of oculomotor nerve.

The facilitatory effect of captopril on EA analgesia was blocked by naloxone injected into PAG

The results are shown in Fig. 7. The experimental procedures were similar to those shown in Fig. 6 ex-

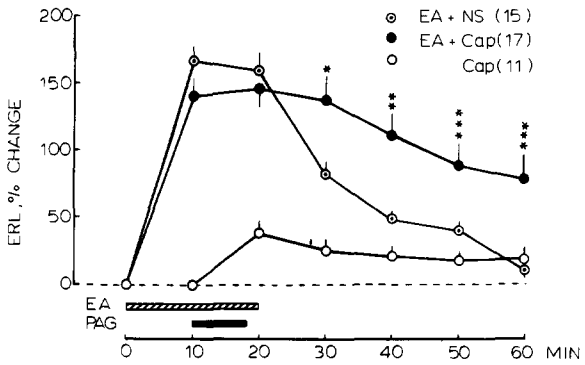

Fig. 6. Potentiation of EA analgesia by intra-PAG injection of captopril. The shaded area indicates the duration of EA stimulation. Black bar indicates intra-PAG injections of captopril (60 nmol) or NS (1 μl).

cept that the injection of captopril (60 nmol/1 μl) was completed within 4 min, which was followed by an injection of naloxone (30 nmol/1 μl) or normal saline (1 μl). Administration of naloxone into PAG not only abolished the facilitatory effect of captopril on EA analgesia, but also lowered the analgesic effect of EA to a level less than that seen in normal control animals. Microinjection of captopril 60 nmol and naloxone 30 nmol into PAG per se did not cause any significant change in ERL.

The facilitatory effect of captopril on EA analgesia was blocked by MEAP antiserum injected into PAG

Experiments were performed in a similar procedure as was shown in Fig. 7 except that MEAP antiserum was used instead of naloxone. The results were expressed in terms of average analgesic effect and shown in Fig. 8. EA analgesia was significantly po-

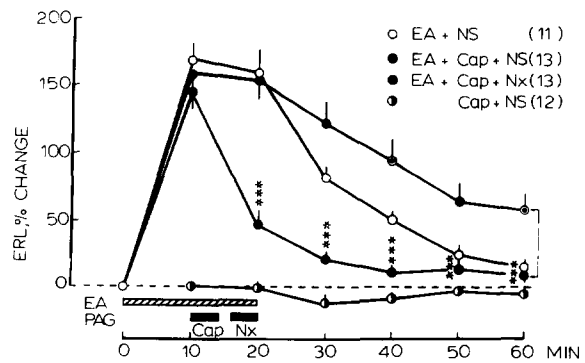

Fig. 7. Potentiation of EA analgesia by intra-PAG injection of captopril was blocked by naloxone. Intra-PAG injection of captopril 60 nmol/1 μl was followed by naloxone 30 nmol/1 μl injected in the same site.

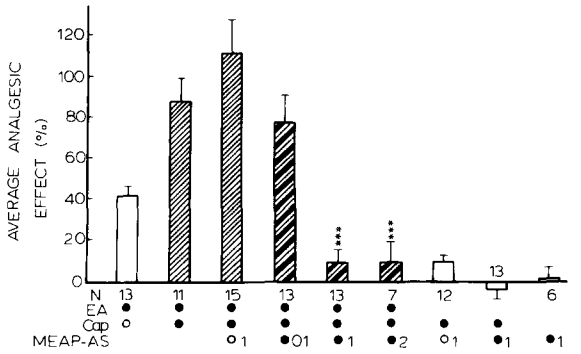

Fig. 8. Potentiation of EA analgesia by intra-PAG injection of captopril was blocked by MEAP antiserum. Ordinate indicates percentage change of ERL from the 4 nociceptive tests after the cessation of EA stimulation. Black dots indicate the application of EA or intra-PAG injection of captopril (60 nmol) or MEAP antiserum. Circle means injection of NS (1 μl) instead of captopril, or normal serum instead of MEAP antiserum. Numerals indicate the volume of normal serum injected in μl. *** $P < 0.001$ as compared with the control EA group (the first clear column).

tentiated by captopril injected into PAG and was markedly attenuated by intra-PAG injection of 1 μl of MEAP antiserum, but not by 1 μl of MEK antiserum (not shown in the figure). The antagonistic effect of MEAP antiserum was no longer prominent when the dose was reduced to 0.1 μl. However, increase of the dose of MEAP antiserum to 2 μl did not produce a stronger effect, indicating that the optimal dose is between 0.1 and 1 μl. The administration of captopril 60 nmol and/or MEAP antiserum 1 μl did not influence the ERL in a significant manner.

DISCUSSION

The central analgesic effect of MEAP

From the results of in vitro studies obtained from bioassay on guinea pig ileum and mouse vas deferens preparations[22,23], and from the binding assay to replace [^{3}H]D-Ala^2-Leu-enkephalin or[^{3}H]etorphine from rat brain membrane[23], the potency of the opioid activity of MEAP is of the same order as enkephalins. The analgesic effect of MEAP assessed from in vivo systems was, however, much stronger than enkephalins. Injected i.c.v. to mice, MEAP was reported to be 8 times more potent than MEK in producing analgesia as assessed by hot plate[32] and tail flick test[16]. In our hand, intra-PAG injection of 120 and 240 nmol of MEAP produced an increase of radiant

heat induced ERL by 75 and 140%, which was 2.5 times more potent than MEK[28] on a molar basis.

The question of the types of opioid receptors relevant to MEAP activities is still in debate. The δ- and κ-receptors have been suggested by some authors[1,5,23], and rejected by others[23]. So far as its analgesic effect in PAG of the rabbit is concerned, κ-receptor does not seem to be the most appropriate candidate for the following reasons: (1) intra-PAG injection of dynorphin A, the endogenous κ agonist, did not produce any analgesic effect[12]; (2) the analgesic effect of MEAP could be easily reversed by 0.1 mg/kg of naloxone, which was only 1/100 of the dose needed to reverse κ-receptor mediated analgesia found in spinal cord of the rat[20]. However, it has been pointed out recently that κ_2-receptor, a newly found κ-receptor subtype, is capable of mediating a naloxone sensitive physiological effect[3] and MEAP can be a candidate ligand for κ_2-receptor[4]. This would certainly be a matter of interest for future study.

Mechanisms of antinociceptive effect of captopril injected into PAG

In vitro studies have shown that MEAP is readily hydrolized by angiotensin converting enzyme (ACE) to generate MEK[6,18,30]. As a powerful and specific inhibitor of ACE, captopril has been demonstrated to increase the level of cerebral MEAP after its i.c.v. injection in mice[32], and to exert an analgesic effect after its i.c.v. injection[2], systemic injection[26] or oral administration[10]. However, no data are available concerning its exact site of action in the CNS, nor is there any unequivocal evidence to show that MEAP is the peptide relevant to captopril analgesia. Results obtained in this study provide strong support to the contention that PAG is one of the strategic sites for captopril to exert its analgesic effect. That captopril analgesia could be blocked by MEAP antiserum injected into PAG but not by MEK antiserum injected into the same site indicates that the analgesic effect was a result of accumulation of MEAP-like immunoreactive substances rather than MEK-like substances.

Evidence for the participation of MEAP in EA analgesia

EA stimulation has been shown to release enkephalins and β-endorphin in the PAG area[13]. Since MEAP and the enkephalins are derived from the same precursor — proenkephalin A, it is reasonable to expect that MEAP will be released concomitantly with enkephalins. The problem is how important is the MEAP in mediating EA analgesia. To explore this, we have chosen intra-PAG injection of: (1) captopril (60 nmol) to protect endogenously released MEAP from enzymatic degradation; (2) a highly specific antibody to bind with MEAP thus preventing the peptide from reaching the receptor sites; and (3) naloxone to block opioid receptors. As expected, captopril significantly prolonged the duration of EA analgesia, probably by increasing the lifetime of MEAP. This increment was blocked by intra-PAG injection of naloxone, a result quite similar to that obtained from the rat experiment using the i.c.v. route for administration of captopril and naloxone[32]. An issue of interest is that the injection of MEAP antibody not only abolished the increment elicited by captopril, but also reduced EA analgesia to a level much less than that seen in the normal control animals, implying that this heptapeptide is effective in physiological conditions to induce analgesia when it is released from the storage pool.

Advantages and limitations of antibody microinjection technique

The conventional method to study the possible involvement of opioid peptides in a given reaction is to look at their sensitivity to naloxone blockade. Being a good pharmacological tool for a preliminary survey, naloxone is not able to differentiate which member of the opioid family is actually involved. Taking advantage of the high specificity of antibody–antigen reaction, we have used the antibody micro-injection technique[13] to study the endogenous opioid peptides implicated in EA analgesia, and found that all 3 opioid systems[12,13] are involved although they play different roles in different regions of the CNS. While enkephalins play an important part in brain (PAG) and spinal cord[13], β-endorphin and dynorphin are only of regional importance; the former functions in PAG but not in spinal cord[13], the latter in spinal cord but not in PAG[12].

The limitations of this method are at least twofold. Owing to the molecular size of the IgG, it can be administered only by an intracranial or intrathecal

route. Whether the Fab fraction of the IgG is capable of penetrating the blood–brain barrier to reach the CNS sites of interest remains to be elucidated. The cardinal factor determining the successful application of the method is the specificity of the antiserum. The MEAP antiserum used in the present study exhibited no cross-reactivity with the pentapeptide Met-enkephalin, the hexapeptide ME-Arg^6 and ME-Lys^6, and the heptapeptide ME-Arg^6-Lys^7. However, it did cross-react with the C-terminal tetrapeptide of MEAP, Phe-Met-Arg-Phe, by 10%. This tetrapeptide and other potentially existing peptides with MEAP-like immunoreactivity must be taken into account before a final conclusion can be drawn.

ACKNOWLEDGEMENTS

We thank Dr. E. Costa and Dr. H-Y. T. Yang (NIMH, Washington D.C. U.S.A.) for the generous gift of MEAP antiserum used in this study. The donation of naloxone from Endo Laboratories (Garden city, NJ, U.S.A.) and captopril from the Squibb Institute (Princeton, NJ, U.S.A.) are also cordially acknowledged.

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