Brain Research, 380 (1986) 317–324
Elsevier

BRE 11932

Electroacupuncture and Morphine Analgesia Potentiated by Bestatin and Thiorphan Administered to the Nucleus Accumbens of the Rabbit

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(Accepted December 31st, 1985)

Key words: electroacupuncture — morphine — enkephalin — analgesia — naloxone — thiorphan — bestatin — nucleus accumbens

The endogenous opioid peptide enkephalin (EK) is known to be degraded mainly by two enzymes, the dipeptidyl carboxypeptidase 'enkephalinase' and aminopeptidase. Microinjection of the enkephalinase inhibitor thiorphan or the aminopeptidase inhibitor bestatin into the nucleus accumbens of the rabbit produced a dose-dependent analgesic effect. This analgesic effect was totally reversed by the narcotic antagonist naloxone or by antibodies against [Met^5]enkephalin (MEK) administered to the same site. Antibodies against [Leu^5]enkephalin were not effective. Moreover, microinjection of thiorphan or bestatin into the nucleus accumbens resulted in a marked potentiation of the aftereffect of electroacupuncture (EA) produced analgesia, as well as the analgesia induced by a small dose of morphine. It is concluded that the analgesic effect elicited by EA and morphine is mediated, at least in part, by MEK-like immunoreactive substance(s) in the nucleus accumbens.

INTRODUCTION

A whole decade has passed since the discovery of enkephalins in 1975, and more than 20 endogenous opioids have been characterized that can be grouped into 3 gene product families. Compared to advances in molecular pharmacology, relatively little is known about the functional aspects of endogenous opioids. Most data has been obtained from two major experimental sources, namely, the assessment of functional changes in response to the administration of exogenous opioids, and the changes induced by the opioid antagonist naloxone. While these methods have been useful for preliminary studies, the information obtained therefrom provides only rough estimates of the real functions. Thus, the analgesic effect of centrally administered enkephalin (EK) has been thought to be very weak, only 2–3% of that of morphine on molar basis[7,25]. This might well be an underestimate, if one considers the rapid degradation of EK en route from the cerebroventricle or spinal subarachnoid space to its active sites of action in the central nervous system. The opioid antagonist naloxone is certainly a powerful tool for distinguishing opioid from non-opioid effects, but it is not specific enough to discriminate effects elicited by different kinds of opioids.

In studies of the analgesic effect of endogenous EK, bestatin[3] and thiorphan[13] have been shown to be very useful pharmacological tools for inhibiting aminopeptidase and enkephalinase, respectively, thus protecting EK from rapid degradation. A recent development in methodology is the central administration of specific antibodies to bind the released opioids, thus preventing them from activating the opioid receptors[9,10,16,23].

In an attempt to locate the sites of action for cerebral EK to produce an analgesic effect, we have found the nucleus accumbens to be an important candidate for the following reasons: (1) microinjection of morphine[4,29] or [D-Ala^2,D-Leu^5]enkephalin (DADLE)[24] into the nucleus accumbens produces marked analgesia; and (2) administration of naloxone into the nucleus accumbens antagonizes morphine analgesia and electroacupuncture (EA) analgesia[5,27].

The aims of the present study were: (1) to explore the importance of EK in mediating morphine analgesia and EA analgesia in the nucleus accumbens by lo-

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cal administration of EK degrading enzyme inhibitors; and (2) to differentiate the relative importance of [Met^5]enkephalin (MEK) and [Leu^5]enkephalin (LEK) by adopting the antibody microinjection techniques.

MATERIALS AND METHODS

Animal preparations

Male rabbits weighing 2.2–2.6 kg were implanted with intracerebral guide cannulae directed to the bilateral nuclei accumbens under nembutal anesthesia. Stainless steel cannulae of 0.65 mm o.d. were introduced into the brain at the stereotaxic coordinate[14] A 5.0, L 1.2, and H 8.7 mm from the surface of the skull, and fixed in situ with dental acrylic. One week was allowed for recovery.

Nociceptive test

The rabbit was fixed in a hammock with its eyes covered. Radiant heat emitted from a 12.5 W projection bulb was focused on the skin around the nostrils, and the latency of the head withdrawal reaction was recorded as the escape response latency (ERL). The values for the first 3 assessments (5 min apart) were averaged as the basal ERL, usually in the range of 5–7 s. Measurement of ERL was repeated at 10 min intervals for 1 h, and the subsequent values were expressed as the percentage changes from the basal ERL, with +200% as the cutoff limit to avoid damage of the skin. Details of the method have been described elsewhere[11].

Drugs and their injection

Intracerebral injection was performed through a stainless steel tube (0.3 mm diameter) extending 2.0 mm beyond the tip of the guide cannula to reach the nucleus accumbens. Drugs were dissolved in normal saline and injected via a constant infusion pump (Palmer). The injection volume was 1 μl to be finished within 8 min, and the tube was kept in place for another 2 min. Drugs contained in 1 μl of solution included: thiorphan (a gift from Drs. Berger and Chipkin), 1, 2 or 4 μg; bestatin (Sigma), 1, 2, or 4 μg; naloxone (NX, Endo Laboratories), 2 μg; immunoglobulin G against MEK (MEK-IgG), 10 μg; IgG against LEK (LEK-IgG), 10 μg; normal rabbit serum IgG (control IgG), 10 μg. Antisera directed to MEK or LEK were obtained from rabbits[20], and the titers for radioimmunoassay were 1:6000 and 1:8000, respectively. Crossreactivities of MEK antiserum with LEK, β-endorphin, and MEK-Arg^6-Phe^7 were 1.1, 0.8 and 0.1%; crossreactivities of LEK antiserum with MEK and β-endorphin were 3.7 and 0.09%, respectively. The affinity chromatography purification of IgG from antisera or normal rabbit serum was performed in a Protein-A Sepharose CL-4B column (Pharmacia), with a yield of 9–11 mg IgG per ml of serum. The IgG showed the same immunoreactivity as the authentic antisera in the radioimmunoassay system.

Morphine HCl is a product of Qinhai Drug Company (China). It was dissolved in normal saline and injected intravenously (i.v.) at a dose of 1.0 or 2.0 mg/kg, the injection volume being 0.5 ml/kg. Control animals were injected with the same volume of normal saline.

Characterization of the site of injection

At the conclusion of the experiment, a stainless steel tube of the same length as the injection tube was inserted into the guide cannula. The animal was then sacrificed and its head was removed to be fixed in 10% formalin for 10 days. The sites of injection were identified in serial frozen sections of 0.5 mm. The data from behavioral experiments were grouped according to the site of intracerebral injection. Results from animals with injection sites outside of the nucleus accumbens were designated the drug control group. The data were expressed as mean ± S.E.M. and the significance of difference between two groups was assessed by Student's t-test.

RESULTS

The analgesic effect of thiorphan and bestatin injected into the nucleus accumbens of the rabbit

Fig. 1 shows the sites of injection of bestatin (2 or 4 μg) or thiorphan (2 or 4 μg) in 4 coronary brain sections. Analgesia was produced in 27 out of 29 cases when bestatin or thiorphan was injected into the nucleus accumbens; whereas in 12 cases with injection of the drug outside of the nucleus accumbens, analgesia was apparent only in one case.

Table I shows the time course of the analgesic effect produced by unilateral injection of 2 or 4 μg of

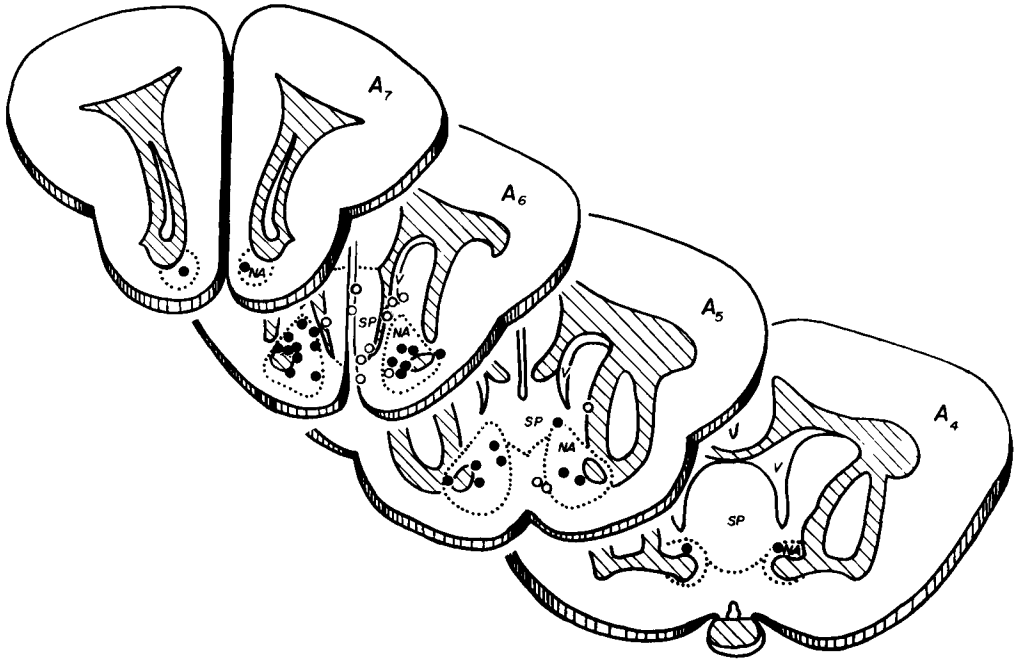

Fig. 1. Diagram showing the sites of injection of bestatin or thiorphan in 4 coronary brain sections, and their corresponding effects of analgesia. SP, septum; NA, nucleus accumbens; V, anterior horn of the lateral ventricle. The mean % change of escape response latency (ERL) plus two standard deviations in the saline control group where 1 μl of normal saline was injected into the nucleus accumbens, was taken as the criteria of analgesia. Black dots indicate an effect of analgesia, circles indicate that the changes of ERL are within the range of the saline control group.

TABLE I

Analgesic effect of thiorphan and bestatin administered to the nucleus accumbens

In, the site of injection is within the nucleus; Out, the injection site is outside the nucleus.

			n	*Basal ERL (s)*	*% change of ERL after intracerebral injection*				
					10 min	*20 min*	*30 min*	*40 min*	*50 min*
NS	1 μl	In	8	5.4 ± 0.4	1 ± 1	4 ± 3	1 ± 2	3 ± 1	1 ± 2
Thiorphan	1 μg	In	7	6.0 ± 0.2	44 ± 22	21 ± 16	−3 ± 5	−2 ± 5	2 ± 3
		Out	1	6.3	−16	−21	−8	−21	3
	2 μg	In	8	6.8 ± 0.4	32 ± 3**	46 ± 10**	103 ± 24***	51 ± 19*	8 ± 9
		Out	5	6.4 ± 0.6	−6 ± 3	5 ± 9	−5 ± 3	17 ± 17	12 ± 12
	4 μg	In	7	6.4 ± 0.7	94 ± 19***	97 ± 21***	117 ± 21***	77 ± 19**	49 ± 13**
		Out	5	6.6 ± 0.8	18 ± 4	26 ± 18	17 ± 19	13 ± 6	1 ± 2
Bestatin	1 μg	In	7	6.0 ± 0.9	38 ± 11**	28 ± 6**	15 ± 7	4 ± 2	2 ± 2
		Out	1	5.6	4	23	29	21	7
	2 μg	In	10	6.2 ± 0.6	74 ± 17***	85 ± 23**	33 ± 23	20 ± 18	6 ± 3
		Out	4	5.4 ± 0.8	−1 ± 8	14 ± 17	2 ± 11	9 ± 17	−2 ± 4
	4 μg	In	8	7.0 ± 0.5	88 ± 21***	140 ± 23***	81 ± 23**	78 ± 22**	45 ± 14
		Out	3	5.8 ± 0.8	15 ± 10	12 ± 3	5 ± 8	1 ± 2	4 ± 6

*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$ as compared to the NS control group.

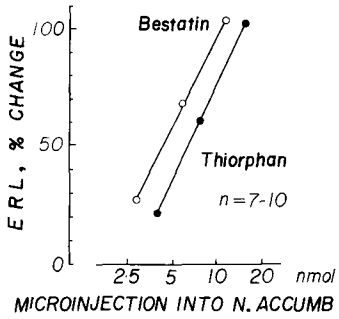

Fig. 2. Dose–response relationship of the analgesic effect of bestatin and thiorphan microinjected into the nucleus accumbens of the rabbit. ERL, escape response latency. The average of 3 postdrug measurements (10 min apart) is shown, compared to the baseline ERL assessed before drug administration.

thiorphan into the nucleus accumbens. The analgesic effect appeared in 10 min and lasted for 40–50 min. No significant analgesia was detected when the same amount of thiorphan was injected into the vicinity of the nucleus accumbens, showing the site specificity of the action. Administration of 1 μl of NS to the nucleus produced little effect on ERL. A similar effect was produced when bestatin, 2 or 4 μg, was injected.

In order to compare the analgesic potency of bestatin with that of thiorphan, the percentage changes in ERL for the first 3 postdrug measurements in each animal were averaged and used to construct dose–response curves on molar basis, as shown in Fig. 2. The two regression lines show a similar slope, favoring a similar mechanism of action. Bestatin seems to be more potent than thiorphan, although the difference is statistically insignificant.

Effect of naloxone on the analgesia elicited by thiorphan or bestatin

A group of 16 rabbits were given intracerebral injections of thiorphan (4 μg/1 μl), which produced a marked increase in pain threshold in 10 min. They were then given 1 μl of NS (n = 8) or 2 μg/1 μl of naloxone (n = 8) through the same guide cannula. As can be seen in Fig. 3A, the analgesic effect elicited by thiorphan was completely abolished by naloxone within 10 min, whereas in the NS control group the analgesic effect remained apparent for at least 1 h.

A similar experiment was performed using bestatin (4 μg/1 μl) instead of thiorphan. The results shown in Fig. 3B indicate clearly that the analgesia elicited by bestatin can also be completely reversed by the opioid antagonist naloxone, suggesting that the analgesia is operative via opioid receptors.

Effect of enkephalin antibodies on the analgesia elicited by thiorphan or bestatin

Since thiorphan and bestatin protect both MEK and LEK, it is not evident from the naloxone experiment whether MEK or LEK plays a major role in

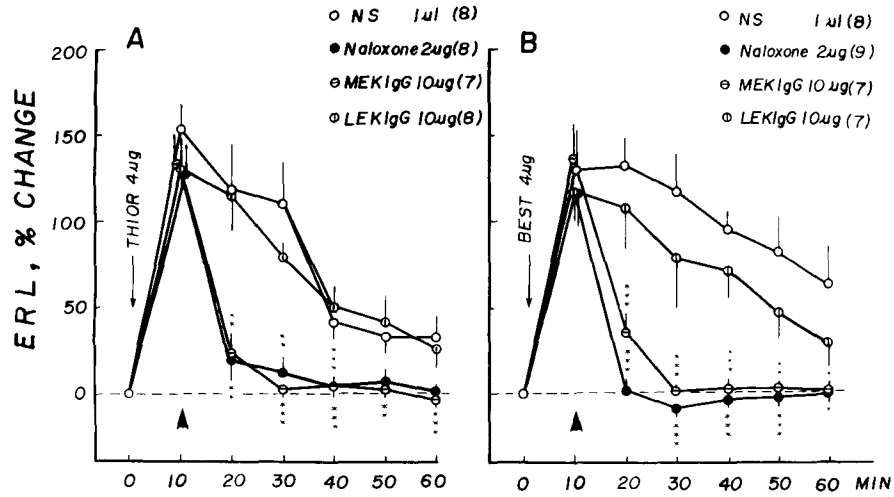

Fig. 3. The influence of naloxone, MEK-IgG and LEK-IgG on the analgesic effect elicited by thiorphan (A) and bestatin (B) in the nucleus accumbens of the rabbit. The arrows indicate microinjection of thiorphan or bestatin, 4 μg in 1 μl, into the nucleus accumbens. The arrowheads indicate a second injection (1 μl) into the same site; the drugs and their doses are indicated in upper right corner of each panel. Numerals in parentheses are the numbers of animals used. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, as compared to the NS control group.

producing analgesia in this nucleus. We therefore decided to use the antibody microinjection technique to study this problem.

Rabbits were given intracerebral injections of 4 μg/1 μl of thiorphan or bestatin, which was followed by intracerebral injections of 10 μg/1 μl of MEK-IgG, LEK-IgG or control IgG. The results of MEK-IgG and LEK-IgG are shown in Fig. 3A and B. The effect of MEK-IgG was very similar to that of NX in both the thiorphan and bestatin experiments. LEK-IgG was effective in neither case.

In order to make comparisons between the two enzyme inhibitors and the 5 different treatments (NS, NX, control IgG, MEK-IgG and LEK-IgG), changes in pain threshold during a 30 min period after the second intracerebral injection were normalized and are shown in Fig. 4. The results of the control IgG group are virtually the same as those of NS group. LEK-IgG produced a very weak attenuation of the analgesia, which is not significantly different from that of NS or control IgG group. In contrast, MEK-IgG produced an almost complete abolishment of the analgesia, as is seen in the NX group.

Potentiation of electroacupuncture (EA) analgesia by thiorphan or bestatin administered to the nucleus accumbens

From Table I it is evident that while 2 and 4 μg of thiorphan produced a marked analgesic effect, 1 μg of thiorphan produced only a minimal increase in ERL that lasted for 10–20 min. We therefore decided to use 1 μg doses of thiorphan to assess its effect on EA analgesia. A group of 12 rabbits were given EA stimulation for 10 min. The EA was then temporarily withdrawn for measurement of ERL to make sure that the stimulation was effective in producing an increase in ERL. Animals with an increase in ERL of less than 50% were discarded. The EA was then continued for another 10 min. In the meantime, half of the animals were given an intracerebral injection of thiorphan and the rest of the animals an injection of NS. The same experiment was repeated after 1 week but each group of animals received the opposite drug treatment. The results were pooled and shown in Fig. 5A. In the NS control group, EA stimulation produced a 150% increase in ERL, which faded away after the cessation of EA stimulation and approached baseline level in 50 min. In animals given thiorphan (1 μg), the aftereffect of EA analgesia was markedly potentiated. A significant analgesia can still be seen 50 min after termination of the EA stimulation.

Similar experiments were performed in rabbits using 1 μg of bestatin instead of thiorphan. The results shown in Fig. 5B indicate clearly a potentiation of the aftereffect of EA analgesia. The difference between the bestatin and NS control groups is very significant 50 min after the termination of EA stimulation (68 ± 15% vs 5 ± 4%, $P < 0.001$).

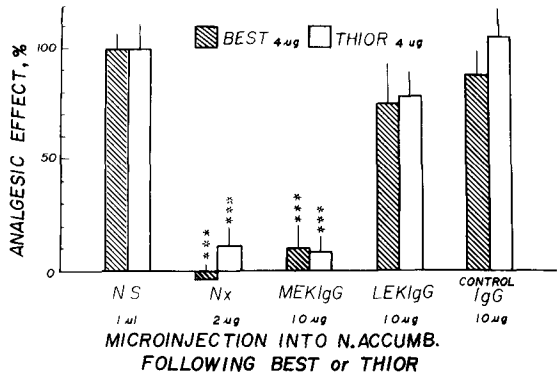

Fig. 4. Analgesia elicited by bestatin or thiorphan was antagonized by naloxone and MEK-IgG, but not LEK-IgG and control IgG. The analgesic effect represents the average of 3 post-drug measurements. The results are normalized for comparison between the two enzyme inhibitors thiorphan and bestatin, and between the 5 different treatments. ***$P < 0.001$, as compared to the corresponding NS control group.

Potentiation of morphine analgesia by thiorphan or bestatin administered to the nucleus accumbens

The effect of thiorphan on morphine analgesia is shown in Table II. In control animals receiving 1 μl of NS in the nucleus accumbens (n = 9), i.v. injection of 2 mg/kg of morphine produced an increase in pain threshold that peaked at 10 min and disappeared in 50 min. A significant potentiation of morphine analgesia was seen in animals receiving thiorphan (1 μg) injected in the nucleus accumbens (n = 12), but not in those animals with the same amount of thiorphan injected into the vicinity of the nucleus accumbens (n = 8).

The effect of bestatin on morphine analgesia is shown in Fig. 6. Sixteen rabbits were given intracerebral injections of either 1 μl of NS (n = 8) or 1 μg of bestatin (n = 8). Ten min later each rabbit was given an i.v. injection of 1 mg/kg of morphine. The same experiment was repeated 1 week later but each group

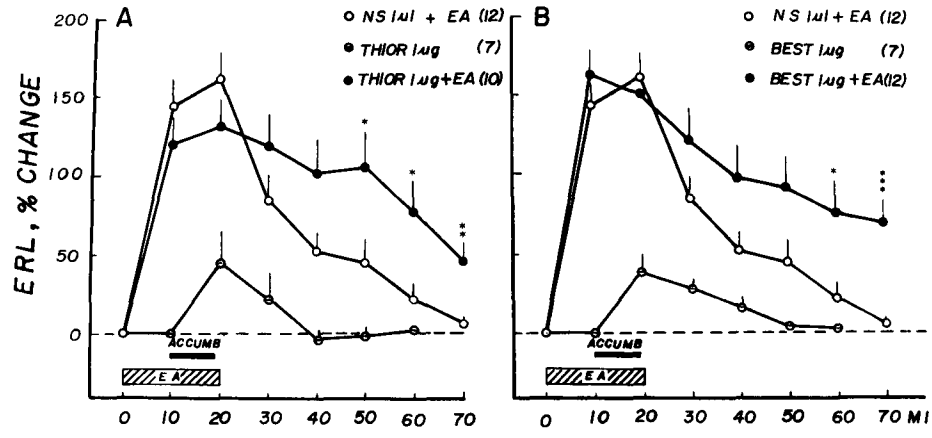

Fig. 5. Potentiation of the aftereffect of EA analgesia by thiorphan (A) and bestatin (B) administered into the nucleus accumbens. The dark bar indicates the period of microinjection (1 μl); the shaded box indicates the period of EA stimulation. The significance of difference between the thiorphan plus EA group and the NS plus EA group is shown as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

of animals received the opposite drug treatment. A similar experiment was also performed in which the dose of morphine was increased to 2 mg/kg.

As can be seen from Fig. 6, 1 mg/kg of morphine, which was non-effective by itself, became strongly analgesic when used in combination with bestatin. In animals injected with 2 mg/kg of morphine, bestatin doubled the analgesic effect. No significant potentiation of morphine analgesia was found when bestatin was injected outside of the nucleus (data not shown).

DISCUSSION

More than 5 enzymes have been characterized that are known to be involved in the degradation of enkephalins, among which the aminopeptidase and dipeptidyl carboxypeptidase (enkephalinase) seem to be the most important ones[12,15]. I.c.v. injection of thiorphan, the enkephalinase inhibitor, produced an increase in brain MEK level and a naloxone reversible analgesic effect in mice[13,26]. I.c.v. injection of bestatin, the aminopeptidase inhibitor, resulted in a marked potentiation of the analgesic effect elicited by exogenously administered MEK or LEK in mice[2,3], but not for enkephalins in which Gly^2 was replaced by D-Ala^2 (DADLE)[2]. We have reported that i.c.v. injection of bestatin (0.6 μmol) or thiorphan (0.4 μmol) in rabbits increased the pain threshold by more than 100%, this effect was completely reversed by a small dose of naloxone (0.125 mg/kg)[28]. The results suggest that inhibition of EK degrading enzymes in the brain may result in an accumulation of EK to manifest a profound analgesic effect.

Morphological studies have shown that the nucleus accumbens is a neuronal structure heavily innervated by enkephalinergic terminals[18]. Microinjection of bestatin or thiorphan into the nucleus accumbens produced dose-dependent analgesia, suggesting that endogenous EK in this nucleus may play a role in pain modulation if a sufficient amount is released and

TABLE II

Effect on morphine analgesia of thiorphan administered to the nucleus accumbens

Thiorphan was given 10 min prior to morphine. Legends same as in Table I.

			n	Basal ERL (s)	% change of ERL after morphine (2 mg/kg i.v.)				
					10 min	20 min	30 min	40 min	50 min
NS	1 µl	In	9	5.0 ± 0.3	93 ± 17	79 ± 18	57 ± 16	32 ± 8	16 ± 8
Thiorphan	1 µg	In	12	5.0 ± 0.3	115 ± 22	128 ± 20	120 ± 17*	89 ± 18**	53 ± 16
		Out	8	5.1 ± 0.4	61 ± 45	48 ± 39	46 ± 27	67 ± 34	62 ± 39

$^{*}P < 0.05$; $^{**}P < 0.01$ as compared to the NS group.

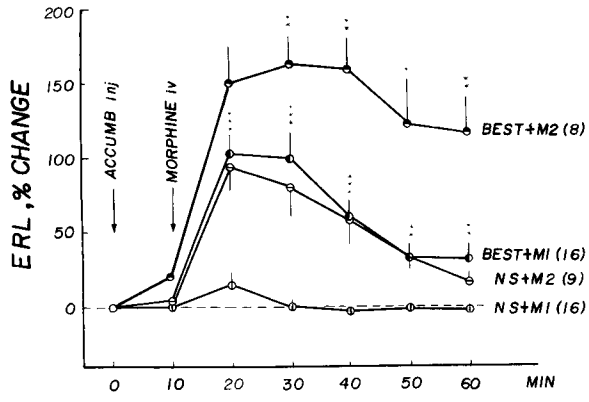

Fig. 6. Potentiation of morphine analgesia by bestatin (1 μg) administered to the nucleus accumbens. M1, morphine 1 mg/kg, s.c.; M2, morphine 2 mg/kg. The significance of difference between the bestatin and the corresponding NS group is shown as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

accumulated within the nucleus.

Concerning the relative importance of aminopeptidase and enkephalinase in degrading EK, it is interesting to note that while thiorphan is more potent than bestatin in producing analgesia after i.c.v. administration[28], bestatin is at least equipotent or even more potent than thiorphan when it is administered directly into the nucleus accumbens (cf. Figs. 2 and 3; also compare Table II and Fig. 6). To produce an equipotent analgesic effect, the dose ratio of i.c.v./accumbens administration is 30 for thiorphan and 60 for bestatin, indicating that bestatin is more effective than thiorphan in the nucleus accumbens. This may reflect an uneven distribution of the two enzymes in different brain areas. According to Sullivan et al.[17], the activity of aminopeptidase in the nucleus accumbens of the rat (11.9 ± 2.4 pmol/mg tissue/h) is significantly higher than the corresponding value for enkephalinase (6.6 ± 0.7). From nociceptive test data, one would anticipate that the same distribution pattern may also exist in the rabbit.

We have reported in a previous study that EA stimulation produced an increase in cerebral content of both MEK and LEK. However, only the MEK content displayed a positive correlation with the analgesic effect of EA[21]. The results indicate that, so far as analgesia is concerned, MEK seems to play a more important role than LEK. This idea is supported by the results obtained in the present study. The analgesia elicited by bestatin or thiorphan in the nucleus accumbens can be blocked only by antibodies directed to MEK and not by those recognizing LEK. This may prove to be the first evidence of functional differences between MEK and LEK in the nucleus accumbens.

In a mapping study, we found that microinjection of naloxone (2–4 μg) into bilateral nuclei accumbens resulted in a dramatic attenuation of EA analgesia both in rabbits[27] and in rats[5]. If endogenous opioids in the nucleus accumbens do contribute to mediate the effect of acupuncture, then blockade of EK degradation in the nucleus accumbens should result in a strengthening and prolongation of the acupuncture effect. Fig. 4 shows that both bestatin and thiorphan produced marked augmentation of the aftereffect of EA analgesia. In this experiment, a small dose (1 μg) of thiorphan or bestatin was used so that it would not significantly change the ERL under baseline conditions when the release of EK is low. However, when the release is accelerated by EA stimulation, an accumulation of EK may ensue, resulting in a stronger aftereffect.

There is a growing body of evidence showing that exogenously administered morphine is capable of releasing endogenous opioids to produce analgesia[1,6,8,19,22]. Hachisu et al. have studied 19 analogues of bestatin for in vitro inhibition of enkephalinase in order to compare it with their in vivo potentiation of morphine analgesia, and found a positive correlation between these two parameters[8], supporting the idea that the analgesic effect of morphine is mediated, at least in part, by EK.

The results obtained in the present study point to the nucleus accumbens as an important site at which morphine seems to release EK. We have shown in a recent study that the analgesia induced by microinjection of morphine into the periaqueductal gray (PAG) of the rabbit could be reversed by naloxone or MEK-IgG injected into the nucleus accumbens[24]. Further study revealed that this was attributable to the serotonergic neuronal pathway from the PAG of the nucleus accumbens, which in turn connected with an enkephalinergic interneuron within the nucleus accumbens (to be published).

In summary, microinjection of EK-degrading enzyme inhibitors into discrete brain areas is a powerful tool with which to examine the physiological functions of endogenous EK in a particular area. Further differentiation between MEK, LEK or other EK-re-

lated peptides, such as MEK-Arg^6-Phe^7 will be possible through the use of the antibody microinjection technique[9,10]. In the present study, a combination of the two approaches revealed that, in the nucleus accumbens, MEK is an important link for mediating EA analgesia as well as morphine analgesia.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Health, People's Republic of China. The authors thank Drs. Berger and Chipkin for a gift of thiorphan, and Endo Laboratories for naloxone.

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