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Inhibitors of Ca^{2+}-dependent endopeptidases modulate morphine-induced effects in rats

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Abstract

The effects of inhibitors of Ca^{2+}-dependent endopeptidases (antipain and leupeptin) on morphine analgesia, reinforcing properties of morphine and on the development of opiate physical dependence were studied. Male Wistar rats were used. The analgesic action of morphine in the tail-immersion test was increased significantly by combined injection of morphine with antipain or leupeptin. Antipain or leupeptin alone had no analgesic action. The combination of morphine with antipain or leupeptin led to the reduction of morphine-induced place preference and the development of physical dependence. A single injection of antipain diminished the opiate-withdrawal signs in morphine-dependent rats. These results suggest a possible inhibitory effect of antipain or leupeptin on the Ca^{2+}-dependent endopeptidases of neurons that mediate analgesia, reinforcing properties of morphine, development of opiate dependence and withdrawal.

Keywords: Morphine analgesia; Reinforcing property; Morphine dependence; Withdrawal; Antipain; Leupeptin

1. Introduction

Opiates, particularly morphine, are widely used in medical practice due to their analgesic characteristics. However, continuing use of morphine is accompanied with pathological psychological and physical dependence on the drug with the consequences of withdrawal after abrupt abolition of the drug.

One of current, widely circulated, theories describes opiate dependence and tolerance as a reflection of adaptations that come about on the cellular level. There are numerous studies demonstrating the involvement of opiate, noradrenergic, dopamine, glutamate, histamine and GABA receptors in the pharmacological action of opioids. Many investigations have been focused on the role of adaptation in the cAMP system in mechanisms of opiate tolerance, dependence and withdrawal (Koski and Klee, 1981; Duman et al., 1988; Nestler and Tallman, 1988; Guitart and Nestler, 1989; Rasmussen et al., 1990; Nestler et al., 1991; Guitart et al., 1992). In recent years, much has been learned about the molecular mechanisms by which an extracellular stimulus, such as administration of opiate, might be expected to regulate neural gene expression (Morgan and Curran, 1991; Sheng et al., 1991).

Studies of inhibition of Ca^{2+}-dependent endopeptidases by antipain and leupeptin (Aoyagi et al., 1969; Suda et al., 1972; Yoshihara et al., 1975; Umezawa, 1976) indicated that Ca^{2+}-dependent endopeptidases play an important role in the function of glutamate receptors (Lynch and Baudry, 1984; Yoneda and Ogita, 1987), histamine H_1 receptors (Siman et al., 1983) and GABA receptors (Majewska and Chuang, 1984) of neurons. Ca^{2+}-dependent endopeptidases regulate the concentration of cytosol Ca^{2+} (Malik et al., 1983; Yoshida et al., 1983; Smith et al., 1994). Various studies have provided evidence that Ca^{2+}-dependent endopeptidases are involved in processes of gene expression regulation (Meyn et al., 1977; Goodman, 1990; Sheng et al., 1991).

All these findings encouraged us to study the possible effects of inhibitors of Ca^{2+}-dependent endopeptidases (antipain and leupeptin) on morphine analgesia, reinforcing properties and dependence in vivo since the results could broaden our knowledge of the interactions between opioids and functions of Ca^{2+}-dependent endopeptidases.

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2. Materials and methods

2.1. Animals

Male Wistar rats (180–200 g) were obtained from the Animal Clinic 'Stolbovaya' of the Russian Academy of Science and were used in all experiments. The animals were housed in groups of no more than 10 rats, maintained at 21 ± 1°C with a 12:12-h light:dark cycle and free access to food and water for at least 7 days after arrival. All procedures were carried out between 10:00 and 17:00 h to minimize diurnal variation.

2.2. Drugs

Antipain and leupeptin were purchased from Sigma (USA), morphine hydrochloride was obtained from Chimkent Chemical factory (Kazakhstan). All the drugs were disolved in normal saline immediately before experiments.

2.3. Analgesia

The animals were restrained in plastic holders and their nociceptive thresholds were established with the 'tail-immersion' method using hot water (56°C) as previously described (Janssen et al., 1963). Nociceptive responses were assessed from latencies of the tail-withdrawal reflex. The animals were divided into treatment groups ($7 < n < 12$/group). The rats were injected i.p. with morphine (2, 10 mg/kg), antipain or leupeptin (0.5, 1, 5 mg/kg), the combination of morphine (2 mg/kg) with antipain or leupeptin (0.5, 1, 5 mg/kg), the combination of morphine (10 mg/kg) with antipain or leupeptin (0.5 mg/kg). The controls were injected with saline. Tail-withdrawal latencies were determined 30 min after drug administration, because the maximum severity of the morphine analgesia was observed at this time. The cut-off time (removal from the hot water) was 60 s.

2.4. Place preference

A conditioned place preference procedure using a visual cue (illuminated compartment vs. dark compartment) was employed. A 6-day schedule, in which the rats were tested on the 1st day to determine their initially preferred compartment. Animals which initially preferred the dark compartment were used in conditioned place preference procedures. The rats were divided into treatment groups ($n = 10$). Saline, morphine (5 mg/kg), antipain (0.5 mg/kg), leupeptin (0.5 mg/kg), combined injections of morphine (5 mg/kg) with antipain (0.5 mg/kg) or morphine (5 mg/kg) with leupeptin (0.5 mg/kg) were administered i.p. and 10 min after treatment rats were placed individually in the less-preferred (illuminated) compartment for a period of 20 min. This schedule was repeated 4 × at 24-h intervals. On the 6th day, the animals were tested: the rats were free to select a drug-paired (illuminated) compartment or a drug-unpaired compartment (initially preferred dark compartment). The time spent in the conditioned (illuminated) compartment was determined in a 20-min period of testing.

2.5. Physical dependence and withdrawal

Experiments were conducted on eight groups of animals ($n = 10$). Each animals received an i.p. injection of some solution twice a day (09:00 and 19:00 h) for 12 days. Group 1 received saline injections; groups 2 and 3 injections of antipain or leupeptin (0.5 mg/kg); rats of groups 4, 5, 6, 7 and 8 groups were injected with increasing doses of morphine (10–60 mg/kg). The solution of morphine, injected to rats of groups 5 and 6 contained antipain or leupeptin (0.5 mg/kg). Treatments were suspended on the 12th day in order to induce spontaneous withdrawal. Observation of the withdrawal was performed 36 h after the last injection of morphine or other solution. Rats of group 7 were additionally injected with 10 mg/kg of antipain 1 h before the last injection of morphine and rats of group 8 received the same injections but 1 h before testing. The animals were placed individually into a plastic chamber (41 × 41 × 51 cm) for 3 min to record any alterations in normal behavioural reactions and the presence of specific withdrawal reactions. Alterations in ambulation and rearing, presence and recurrence of wet dog shakes, diarrhea, dyspnea, piloerection, rhinorrhea, paw shakes, tooth chattering, posture disturbance were recorded. The global individual index of withdrawal was calculated as a sum of particular signs, multiplied by the appropriate coefficient (Sudakov et al., 1991). Body weight was determined after the last dose of morphine and 36 h after the disruption of morphine administration.

2.6. Statistics

Statistical comparison between different treatments and dosages was performed by one-way ANOVA. The results were considered significant when the P level was < 0.05.

3. Results

The tail-withdrawal latency in the morphine-treatment group (2 mg/kg) was not significantly different from that in the control group ($F = 0$, $P > 0.05$). Alone, antipain and leupeptin did not produce analgesia at the doses tested (0.5, 1, 5 mg/kg) ($F = 0.33$, $P > 0.05$; $F = 0.501$, $P > 0.05$); only the combination of morphine (2 mg/kg) with antipain or leupeptin (0.5, 1, 5 mg/kg) produced analgesia ($F = 5.12$, $P < 0.05$; $F = 6.11$, $P < 0.05$). Coadministration of antipain or leupeptin (0.5 mg/kg) with morphine (10 mg/kg) increased morphine antinociception ($F = 9.6$, $P < 0.01$; $F = 15.07$, $P < 0.01$) (Table 1).

Table 1
The effect of morphine, antipain or leupeptin and of combined injections of morphine with antipain or leupeptin on tail-withdrawal latencies of rats

Treatment groups	Dose (mg/kg)	Rats (n)	Latency (mean ± S.E.M.) (s)
Saline	11	11	4.25 ± 0.26
Antipain	0.5	9	5.03 ± 0.73
	1	8	4.62 ± 0.87
	5	8	5.06 ± 0.74
Leupeptin	0.5	9	4.40 ± 0.33
	1	8	4.75 ± 0.69
	5	8	5.20 ± 0.71
Morphine	2	8	4.25 ± 0.31
+ Antipain	0.5	9	6.67 ± 0.38 [a]
	1	9	6.96 ± 0.94 [a]
	5	9	7.75 ± 0.66 [a]
+ Leupeptin	0.5	9	6.38 ± 0.90 [a]
	1	9	6.78 ± 0.80 [a]
	5	9	7.00 ± 0.53 [a]
Morphine	10	11	21.27 ± 4.32 [a]
+ Antipain	0.5	10	34.67 ± 8.8 [b]
+ Leupeptin	0.5	10	54.3 ± 6.03 [b]

[a] $P < 0.05$ vs. morphine (2 mg/kg); [b] $P < 0.05$ vs. morphine (10 mg/kg).

Repeated treatment with saline, antipain or leupeptin (0.5 mg/kg) failed to affect the time spent in the conditioned (illuminated) compartment because the rats preferred to stay in the dark compartment. The morphine-treated group (5 mg/kg) preferred to stay in the drug-paired compartment of the experimental chamber. The time spent in the conditioned compartment was significantly reduced in groups of rats that had been injected with morphine (5 mg/kg) with antipain or leupeptin (0.5 mg/kg) ($F = 6.906$, $P < 0.01$; $F = 9.614$, $P < 0.001$) (Fig. 1).

Rats treated with morphine displayed marked withdrawal signs 36 h after the last dose of morphine. The global withdrawal index, characterizing all common behavioural and specific reactions of the animals, ranged from 8 to 12 (mean = 10.13). The global withdrawal index in rats that had received coadministration of morphine with antipain or leupeptin (0.5 mg/kg) was significantly reduced ($F = 18.521$, $P < 0.001$; $F = 43.071$, $P < 0.0001$) due to diminution of the signs of withdrawal (wet dog shakes, tooth chattering, paw shakes, alterations in ambulation and rearing) and suppression of dyspnea, piloerection, posture disturbance, rhinorrhea. The body weight losses in these groups of rats were less than in morphine control animals (Table 2). Repeated injections of antipain (0.5 mg/kg) or leupeptin (0.5 mg/kg) did not produce morphine-like physical dependence. A single injection of antipain (10 mg/kg) 1 h before the last dose of morphine significantly suppressed withdrawal signs (piloerection, posture disturbance, rhinorrhea, diarrhea, body weight loss). The administration of antipain (10 mg/kg) 1 h before testing of spontaneous morphine abstinence suppressed withdrawal signs (piloerection, posture disturbance, rhinorrhea) in morphine-dependent rats (Table 2). The global withdrawal indexes were significantly decreased in these groups of rats compared to the morphine control animals ($F = 7.258$, $P < 0.01$; $F = 23.195$, $P < 0.001$). No differences in the global withdrawal index were observed between the three groups of animals: the animals

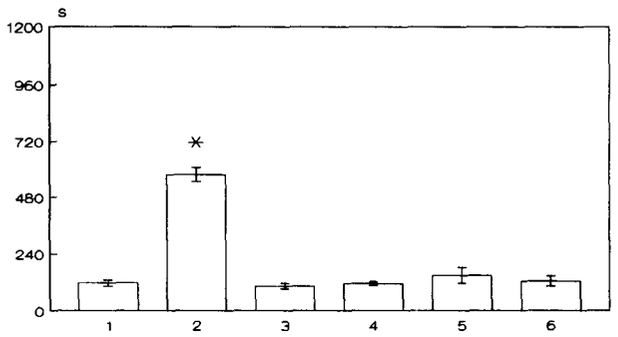

Fig. 1. Time spent in the conditioned (illuminated) compartment. Rats were conditioned with an i.p. injection of saline (1); morphine (5 mg/kg) (2); antipain (0.5 mg/kg) (3); leupeptin (0.5 mg/kg) (4); combined injections of morphine (5 mg/kg) with antipain (0.5 mg/kg) (5) or morphine (5 mg/kg) with leupeptin (0.5 mg/kg) (6). $n = 10$ on each group. * $P < 0.05$ vs. saline. Ordinate: time (s).

Table 2
Withdrawal signs determined in the testing of morphine-dependent rats

Withdrawal signs	Treatment groups (n = 10)				
	M	A_M	L_M	A_{LI}	A_W
Wet dog shakes	11.6 ± 1.1	4.2 ± 1.4 [a]	5.4 ± 0.9 [a]	6.6 ± 0.8 [a]	4.1 ± 0.7 [a]
Tooth chattering	3.6 ± 0.6	1.1 ± 0.4 [a]	0.8 ± 0.3 [a]	1.2 ± 0.6 [a]	0.7 ± 0.5 [a]
Dyspnea	0.4 ± 0.3	–	–	–	0.4 ± 0.2
Piloerection	2.3 ± 0.4	–	–	–	–
Posture disturbance	0.4 ± 0.2	–	–	–	–
Rhinorrhea	3.2 ± 0.1	–	–	–	–
Paw shakes	5.5 ± 0.8	3.3 ± 0.4 [a]	3.1 ± 0.5 [a]	2.8 ± 0.6 [a]	1.1 ± 0.5 [a]
Diarrhea	0.3 ± 0.1	–	–	–	0.2 ± 0.1
Inhibition of locomotor activity	132 ± 17	173 ± 4.3 [a]	177 ± 2.6 [a]	137 ± 11	156 ± 13
Rearing	9.9 ± 1.8	13 ± 3.4	14 ± 1.9 [a]	13 ± 1.2	10 ± 1.8
Body weight loss (g)	23 ± 5.3	15 ± 4.2 [a]	14 ± 3.7 [a]	17 ± 3.9	22 ± 4.8

Values are mean ± S.E.M. The letter of the group designations refers to the treatment that rats received (M, morphine; A_M, antipain (0.5 mg/kg) with morphine; L_M, leupeptin (0.5 mg/kg) with morphine; A_{LI}, antipain (10 mg/kg) 1 h before the last dose of morphine; A_W, antipain (10 mg/kg) 1 h before testing). [a] $P < 0.05$ vs. morphine.

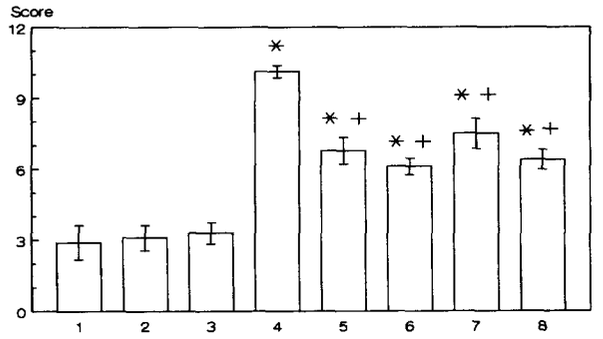

Fig. 2. Global withdrawal index defined in morphine-dependent rats. Rats received chronic i.p. treatment with saline (1), antipain (0.5 mg/kg) (2), leupeptin (0.5 mg/kg) (3), morphine (4), morphine + antipain (0.5 mg/kg) (5), morphine + leupeptin (0.5 mg/kg) (6). Antipain (10 mg/kg) was given to morphine-dependent rats 1 h before the last dose of morphine (7) or 1 h before testing (8). $n = 10$ in each group. * $P < 0.05$ vs. saline. $^{+}$ $P < 0.05$ vs. morphine. Ordinate: global withdrawal index (score).

that received antipain (10 mg/kg) 1 h before the last dose of morphine, the animals that received antipain (10 mg/kg) 1 h before testing of morphine withdrawal and the animals that had been injected with antipain (0.5 mg/kg) during the 12 days of morphine-dependence development ($F = 0.196$, $P > 0.05$) (Fig. 2).

4. Discussion

The main finding of the present study was that antipain and leupeptin increased morphine analgesia, reduced the development of physical dependence on this drug and diminished withdrawal. Thus, we provide evidence that Ca^{2+}-dependent endopeptidases are involved in these morphine-induced effects.

It is possible that the inhibition of Ca^{2+}-dependent endopeptidases with antipain and leupeptin in certain doses leads to an increase of the morphine analgesic action. Several studies have revealed the important role of neuronal Ca^{2+} in the processes of opioid analgesia, tolerance and dependence (Yamamoto et al., 1978; Ross and Cardenos, 1979; Chapman and Way, 1982; Baeyens et al., 1987; Antkiewich-Michaluk et al., 1990). Smith et al. (1994) hypothesized that compounds which release Ca^{2+} from intracellular pools would block the analgesic effects of morphine. Inhibition of Ca^{2+}-dependent endopeptidases by antipain or leupeptin apparently economizes cytosol Ca^{2+} and, thus, may promote morphine-induced analgesia. Nevertheless, it is possible that changes in the analgesic action of morphine that are induced by antipain and leupeptin may be determined through an effect on some other neurochemical systems which participate in opiate antinociception and use the endopeptidase mechanisms.

In our experiments, antipain and leupeptin decreased morphine-induced conditioned place preference. This action, with several reservations, may reflect the positive reinforcing properties of the conditioning drug. There are many reports suggesting that the opiate reinforcement results from the activation of different receptors in the CNS: μ- and δ-opioid receptors (Bilsky et al., 1990; Suzuki et al., 1991a, b; Heidbreder et al., 1992) or serotonin and dopamine receptors (George and Ritz, 1991; Koob, 1992). Recent finding support a physiological role of dihydropyridine binding sites of the brain in the reinforcement by opiates and psychostimulants (Dilullo and Martiniverson, 1992). We suppose that antipain and leupeptin may influence the activation mechanisms of some of these receptors and decrease the positive reinforcing property of morphine. However, the place preference procedure with animals is connected with their learning process. Moreover, in our design, when the animals were conditioned with morphine in the non-preferred compartment, the possible anxiogenic effect of antipain and leupeptin may decrease the time spent in the conditioned compartment. Although there is no evidence of an amnestic or anxiogenic action of inhibitors of Ca^{2+}-dependent endopeptidases, we cannot exclude these types of mechanisms of action of antipain and leupeptin on place preference behaviour. In order to investigate the action of antipain and leupeptin on the positive reinforcing property of morphine, we plan to study the effects of these compounds on morphine i.v. self-administration.

Our study showed that antipain and leupeptin may decrease the development of the withdrawal syndrome in morphine-dependent rats, when they are administered at different times. The results of experiments on the administration of morphine with antipain or leupeptin during the development of physical dependence led us to suggest that inhibition of some peptidases may prevent the development of physical dependence. This process involves not only activation of opioid receptors but changes in the cAMP, G-protein system and gene expression (Koski and Klee, 1981; Duman et al., 1988; Nestler and Tallman, 1988; Guitart and Nestler, 1989; Sheng et al., 1991; Guitart et al., 1992). In our earlier studies, we found significant increases of mRNA synthesis in different structures of rat brain slices after incubation with morphine (Lyupina et al., 1994; Yarygin et al., 1994). This increase was diminished when slices were incubated with morphine in the presence of antipain (unpubl. data). On the other hand, there is evidence that Ca^{2+}-dependent endopeptidases are involved in processes of gene expression (Meyn et al., 1977; Goodman, 1990; Sheng et al., 1991). We, thus, suggest that the combination of morphine with antipain or leupeptin may protect the action of morphine on gene expression and, thus, decrease the development of physical dependence. However, even a single injection of antipain 1 h before the last injection of morphine or 1 h before the testing of morphine withdrawal, when dependence has already developed, significantly decreases withdrawal signs. These data led us to the conclusion that some antipain-sensitive endopeptidases participate in the devel-

opment of opiate withdrawal. Apparently, some of these endopeptidases take part in the proteinase-receptor interaction described by Lynch and Baudry (1984).

Thus, our results suggest a possible inhibitory effect of antipain and leupeptin on Ca^{2+}-dependent endopeptidases of neurons that mediate analgesia, development of opiate dependence and withdrawal and, possibly, reinforcing properties of morphine. Antipain and leupeptin are prospective compounds for the design of drugs curing opiate withdrawal and to be used as supplement to morphine, increasing its analgesic effect and diminishing addiction.

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