The Synergistic Antinociceptive Interactions of Endomorphin-1 with Dexmedetomidine and/or S(+)-Ketamine in Rats

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Spinal administration of the endogenous μ-opioid agonist peptide, endomorphin-1, results in antinociception in rodents, but there are few data about its interaction with other antinociceptive drugs. We investigated the antinociceptive interactions at the spinal level of endomorphin-1 with the *N*-methyl-D-aspartate antagonist S(+)-ketamine, the α_2-adrenoceptor agonist dexmedetomidine, or both in awake rats. Nociception was assessed by the tail-flick test. Dose-response curves were determined for endomorphin-1 (0.6–50 μg), for dexmedetomidine (0.1–10 μg), for mixtures of S(+)-ketamine (30 or 100 μg) with endomorphin-1 (2–18 μg) or of endomorphin-1 with dexmedetomidine in a fixed ratio (4:1), and for the triple combination of the three drugs after intrathecal administration. Endomorphin-1 and dexmedetomidine both produced dose-dependent antinociception. The coadministration of 100 μg S(+)-ketamine significantly enhanced the antinociceptive effect of 6 μg endomorphin-1. Isobolographic analysis of the combinations of endomorphin-1 and dexmedetomidine revealed a synergistic interaction between these drugs. The 80% effective dose for the triple combination was significantly less than that for either binary combination. These data indicate that S(+)-ketamine and dexmedetomidine, acting via different receptors, produce synergistic antinociceptive interaction with endomorphin-1 at the spinal level. Furthermore, the triple combination of an opioid agonist, an α_2-adrenoceptor agonist, and an *N*-methyl-D-aspartate receptor antagonist shows potent antinociceptive activity.

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The spinal cord is an important neuronal structure for pain transmission, and it is one of the pharmacologic sites of action for the antinociceptive effects of different drugs. The intrathecal administration of both opioids and α_2-adrenoceptor agonists produces spinal analgesia in animals and humans, and these drugs show synergistic antinociceptive interaction (1–6). Numerous studies performed at the level of the spinal cord have shown that *N*-methyl-D-aspartate (NMDA) receptor activation plays a major role in the transmission of nociceptive information (7–10). We have previously shown that the S(+)- isomer of the NMDA antagonist ketamine potentiated morphine- and dexmedetomidine-induced antinociception on the tail-flick test (11).

Endomorphin-1 and endomorphin-2 are tetrapeptides with high affinity and selectivity for the μ-opioid receptor and have been proposed as the endogenous ligands for the μ-opioid receptor (12). Endomorphins are potent antinociceptive agents spinally, supraspinally, and peripherally; therefore, they might have potential clinical significance. In contrast to morphine, however, their effects are short lasting, and the data indicate the development of acute tolerance (or tachyphylaxis) against both endomorphins (13–15). There is also some evidence suggesting a plateau effect at 40%–50% of maximum possible effect (%MPE) in the acute heat-pain test (16). One way to overcome these problems might be a combination with other drugs. The aim of this study was to investigate the interaction of endomorphin-1 with dexmedetomidine or S(+)-ketamine on acute heat-pain sensation after intrathecal administration in awake rats. No data are available about the antinociceptive potency of the triple combination of an opioid agonist, an α_2-adrenoceptor agonist, and an NMDA receptor antagonist. Therefore, our second goal was to determine the antinociceptive effect of the triple combination of these drugs.

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Methods

After institutional approval had been obtained from the Animal Care Committee of the University of Szeged, Faculty of Medicine, male Wistar rats weighing 250–350 g were studied. For spinal drug administration, the rats were surgically prepared under ketamine plus xylazine anesthesia (72 and 8 mg/kg intraperitoneally, respectively). An intrathecal catheter (PE-10 tubing) was inserted through a small opening in the cisterna magna and passed 8.5 cm caudally into the intrathecal space, as described previously (17). After surgery the rats were housed individually, had free access to food and water, and were allowed to recover for at least 4 days before use. Rats that exhibited postoperative neurologic deficits were not used. All experiments were performed during the same period of the day (8:00 to 11:00 AM) to exclude diurnal variations in pharmacologic effects. The animals were randomly assigned to treatment groups (n = 5–11 per group), and the observer was blinded to the treatment administered. Each animal was studied twice in an experimental series, with 6- to 8-day intervals between studies. After experimental use, rats were killed with an overdose of pentobarbital, and 1% methylene blue was injected to confirm the position of the catheter and the probable spread of the injectate.

The drugs used were ketamine hydrochloride (Ketalar; Parke-Davis, Vienna, Austria), xylazine hydrochloride (Rompun; Bayer, Leverkusen, Germany), S(+)-ketamine hydrochloride (a generous gift from Gödecke/Parke-Davis Ltd., Vienna, Austria), and dexmedetomidine (a generous gift from Orion-Farmos, Turku, Finland). Endomorphin-1 was synthesized by a solid-state method and purified by means of high-performance liquid chromatography in the Isotope Laboratory of the Biological Research Center of the Hungarian Academy of Sciences. Drugs were dissolved in sterile physiological saline. The intrathecally administered drugs were injected over 30 s in a volume of 5 μL, followed by a 10-μL flush of physiological saline. Physiological saline served as a control.

Acute nociceptive threshold was assessed by the tail-flick test. The reaction time in the tail-flick test was determined by immersing the lower 5-cm portion of the tail in hot water (51.5°C) until a tail-withdrawal response was observed (cutoff time, 20 s). The tail-flick latencies were obtained immediately before and then 10, 30, and 60 min after the drug injections.

The first series of experiments was performed to determine the dose-response and time course for intrathecally administered endomorphin-1 and dexmedetomidine (Table 1). According to our earlier results (11), which indicated no antinociceptive activity of S(+)-ketamine on the tail-flick test, we did not determine the dose-response curve for S(+)-ketamine again, but used 30 and 100 μg in the interaction studies. The second series of experiments was performed with fixed doses of S(+)-ketamine (30 or 100 μg) with different doses of endomorphin-1 (2–18 μg) to determine the effect of S(+)-ketamine on endomorphin-1-induced antinociception (Table 1). The third series of experiments determined the type of interaction between dexmedetomidine and endomorphin-1 after their coadministration in a fixed-dose ratio (4:1) (Table 1). The final series of experiments investigated the interaction of a triple combination of endomorphin-1 and dexmedetomidine at a ratio of 4:1 plus 100 μg S(+)-ketamine (Table 1).

Analgesic latencies were converted to %MPE by using the following formula:

$$\%\text{MPE} = [(\text{observed latency} - \text{baseline latency})]/[(\text{cutoff} - \text{baseline latency})] \times 100.$$

Data are presented as mean ± SEM. Because all drugs or their combinations generally resulted in an increase in withdrawal latency, with the peak effect occurring at 10 min, the values obtained at 10 min were used for dose-effect curves and the linear regression analysis. Dose-effect curves were constructed for each drug or drug combination. The 50% effective dose (ED_{50}) was defined as the dose that yielded 50% MPE. Because a higher level of the effect might also be important for therapeutic practice, we also determined ED_{80}. The ED_{50} and ED_{80} values with 95% confidence intervals were calculated by linear regression. Data sets were examined by one- and two-way analyses of variance. *Post hoc* comparison was performed with the Newman-Keuls test. A P value <0.05 was considered significant.

Isobolographic analysis of the interactions between dexmedetomidine and endomorphin-1 was performed by using the procedure of Tallarida and Raffa (18). Theoretical simple additive ED_{50} and ED_{80} for each ratio of two drugs was then generated from the equation

$$Z_{add} = Z_1^{\circ}/(p_1 + R \times p_2),$$

where Z_{add} is the total additive dose, Z_{1° is the ED_{50} or ED_{80} of endomorphin-1, R is the potency ratio of two drugs, p_1 is the proportion of endomorphin-1 in the total dose, and p_2 is that of dexmedetomidine. The confidence intervals for the drug components of the theoretical additive ED_{50} or ED_{80} were obtained from the variances about ED_{50} and ED_{80} for each drug administered alone. This theoretical additive point was compared with the experimentally derived values for the mixture by a t-test. A significant potency ratio with the experimental ED_{50} and ED_{80} significantly less than the theoretical additive ED_{50} and ED_{80} indicated a synergistic interaction.

Table 1. Doses of Endomorphin-1, Dexmedetomidine, and S(+)-Ketamine (μg) Used to Determine the Antinociceptive Interactions

Group	Endomorphin-1 (μg)	Dexmedetomidine (μg)	S(+)-Ketamine (μg)
Endomorphin-1	0.6		
	2		
	6		
	18		
	50		
Dexmedetomidine		0.1	
		0.3	
		1	
		3	
		6	
		10	
Endomorphin-1/	1	0.25	
dexmedetomidine = 4:1	2	0.5	
	4	1	
	8	2	
Endomorphin-1 +	0.1		100
S(+)-ketamine	0.6		100
	2		30
	2		100
	6		30
	6		100
	20		30
	20		100
Endomorphin-1 +	0.04	0.01	100
Dexmedetomidine +	0.12	0.03	100
S(+)-ketamine	0.4	0.1	100
	1	0.25	100

Results

There was no significant difference in tail-flick latency between the groups (by using Student's *t*-test) before any drug administration (6.6 ± 0.10 s for all animals). The tail-flick latency in the Control group did not change significantly during the investigation.

Endomorphin-1 resulted in a dose-dependent increase in thermal withdrawal latency, with the peak effect occurring at 10 min (Fig. 1 upper panel). The largest dose (50 μg) caused close to 100% MPE and also caused temporary motor dysfunction (rigidity). The antinociceptive effect of smaller doses of endomorphin-1 was short lasting: it caused antinociception only at 10 min. The largest dose of endomorphin-1 produced a longer-lasting effect.

Dexmedetomidine at smaller doses (0.1–3 μg) produced a slight and short-lasting increase in %MPE (23%–26%); the two larger doses (6 and 10 μg) caused very effective (50%–100% MPE), long-lasting antinociception (Fig. 1, middle panel). Dexmedetomidine in larger doses (3–10 μg) was associated with substantial diuresis and sedation (decreased spontaneous exploring activity, but the animals were still responsive to acoustic or tactile stimuli). ED_{50} and ED_{80} and the confidence intervals for endomorphin-1 and dexmedetomidine are listed in Table 2.

Time-course and dose-response curves for endomorphin-1 and S(+)-ketamine coadministration (Fig. 2) revealed that smaller doses of S(+)-ketamine did not influence the antinociceptive effect of endomorphin-1 at any time. Coadministration of 100 μg S(+)-ketamine slightly potentiated (at 10 min) the antinociceptive effect of endomorphin-1, which was significant with the 6-μg endomorphin-1 combination (Fig. 2). The dose-effect curve of endomorphin-1 shifted to the left when it was combined with 100 μg S(+)-ketamine (Fig. 3, lower panel).

In the special case of a drug's lacking pharmacologic effect [in this case, S(+)-ketamine], any statistically significant decrease in the ED_{50} of the other, active component (i.e., endomorphin-1) denotes synergism. The larger dose of intrathecal S(+)-ketamine significantly reduced the ED_{50} of endomorphin-1 (Table 2). The decrease observed in ED_{50} after coadministering the smaller dose of S(+)-ketamine (30 μg) with endomorphin-1 was not significant. Animals receiving the combinations exhibited no unusual behavior (sedation or motor dysfunction).

Intrathecal coadministration of dexmedetomidine and endomorphin-1 in a fixed ratio (4:1) resulted in a significant increase in the tail-flick latency following a dose-dependent fashion (Fig. 4). Isobolographic

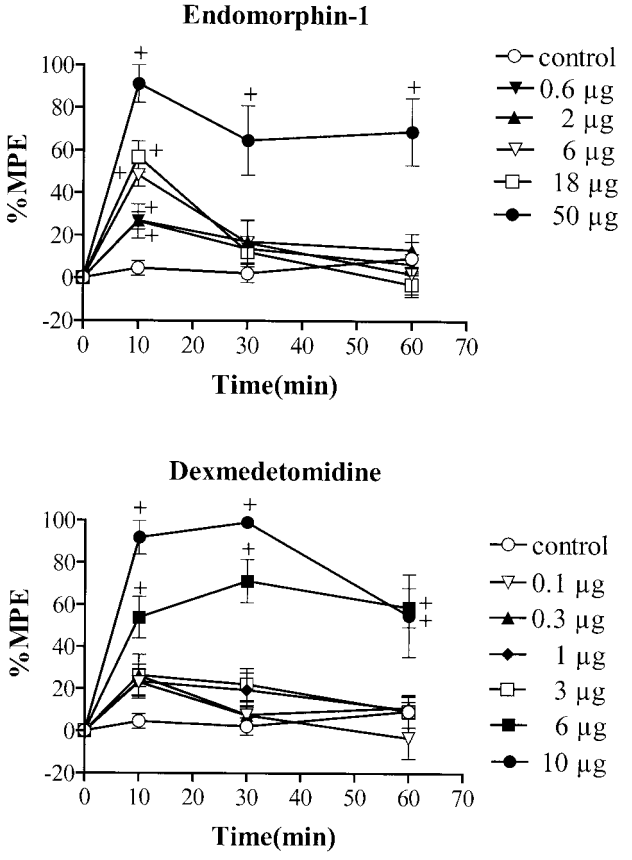

Figure 1. Antinociceptive effect (%MPE) of various doses of intrathecally administered endomorphin-1 (upper panel) and dexmedetomidine (lower panel). Each point represents the mean ± SEM for 5–11 animals. $+P<0.05$ versus control with the Newman-Keuls test.

analysis demonstrated that this interaction was synergistic, because the doses of dexmedetomidine and endomorphin-1 necessary to produce 50% or 80% MPE were significantly less than those calculated to be necessary for an additive interaction (Fig. 4 and Table 2). Therefore, the dose-effect curve of endomorphin-1 shifted to the left when it was combined with dexmedetomidine (Fig. 3, lower panel). Animals receiving the combinations exhibited no unusual behavior, except for one group (8 µg endomorphin-1 and 2 µg dexmedetomidine) that showed sedation.

Coadministration of endomorphin-1, dexmedetomidine, and S(+)-ketamine caused a dose-dependent antinociception, and a slightly prolonged effect was observed at larger doses (Fig. 3, upper panel). The dose-effect curve of endomorphin-1 was shifted to the left when it was given in the triple combination (Fig. 3, lower panel).

Previously we determined the interaction of dexmedetomidine with S(+)-ketamine (11). The ED_{50} and ED_{80} values for this drug combination are shown in Table 2. We could, therefore, compare ED_{50} and ED_{80} for the triple combination with the values for either of the double combinations. The ED_{50} for the triple combination (endomorphin-1, dexmedetomidine, and S(+)-ketamine) did not differ significantly from the two binary combinations [S(+)-ketamine and dexmedetomidine or S(+)-ketamine and endomorphin-1], although the confidence interval decreased. The statistical significance for the differences in the degree of synergism reached the level of $P < 0.05$ between the third binary combination (endomorphin-1 and dexmedetomidine) on one side and the triple combination on the other. In contrast, ED_{80} for the triple combination was significantly less than that for either binary combination (Table 2).

Discussion

This study has revealed three main findings: first, that combined intrathecal administration of S(+)-ketamine and endomorphin-1 caused a significant decrease in ED_{50} for endomorphin-1; second, that combined intrathecal administration of small to moderate doses of dexmedetomidine and endomorphin-1 produced stronger antinociception than would be expected if these effects were simply additive; and third, that the synergism exhibited by binary combinations was further improved by the addition of the third component. Therefore, the dose-effect curve of endomorphin-1 shifted to the left when it was given in double or triple combinations.

The importance of opioids in pain control is undisputed. The antinociceptive effects of opioids are caused by the activation of opioid receptors at supraspinal, spinal, and peripheral levels. Opioids exert both pre- and postsynaptic control of the nociceptive primary afferent input into the cord (19). Endomorphin-1 and endomorphin-2 are recently discovered µ-opioid receptor ligands whose antinociceptive effects have been observed by several authors (12,14,15,20). In all cases, endomorphins displayed µ-opioid antagonist reversible antinociceptive effects, although the potencies of the drugs and the duration of the effects seemed to depend on the species, on the applied pain tests, and on the route of administration. Some differences from morphine were also observed; i.e., they are more potent than morphine in neuropathic pain (20). One study also found that endomorphin-induced antinociception exhibited a steady plateau at approximately 40% MPE in acute pain tests (16). It has been suggested that the different patterns of G-protein activation observed for the agonists at µ-opioid receptors might account for this low efficacy exhibited by endomorphins in the production of µ-opioid receptor-mediated antinociception, although further studies are needed to clarify these controversies (16). Few interaction studies have been performed on endomorphin in respect to antinociception. Beneficial interactions have been described between endomorphin-1 and spinal nociceptin (21) (the endogenous ligand

Table 2. ED_{50} and ED_{80} for Dose-Response Curves of Intrathecal Drugs

Drugs	ED_{50} (μg) (95% confidence interval)		ED_{80} (μg) (95% confidence interval)	
	Predicted	Observed	Predicted	Observed
Endomorphin-1		14.2 (9.1–19.3)		39.4 (31.3–47.8)
Endomorphin-1 + 30 μg S(+)-ketamine		6.2 (1.8–14.2)		
Endomorphin-1 + 100 μg S(+)-ketamine	14.2 (9.1–19.3)	0.8 (0.1–5.1)[a]	39.4 (31.3–47.8)	13.6 (5.6–21.6)[a]
Dexmedetomidine		4.9 (3.81–6.14)		9.3 (7.33–11.33)
Dexmedetomidine + 100 μg S(+)-ketamine	4.9 (3.81–6.14)	0.15 (0.01–1.54)[a]	9.3 (7.33–11.33)	4.9 (2.5–7.4)
Endomorphin-1 + dexmedetomidine = 4:1	9.63 (6.2–13.1)	2.4 (1.0–3.8)[a]	21.8 (17.3–26.4)	7.1 (5.8–8.4)[a]
Endomorphin-1 + dexmedetomidine = 4:1 + 100 μg S(+)-ketamine		0.41 (0.19–0.64)[b]		0.94 (0.6–1.2)[c]

ED = effective dose.
[a] Significantly different from the predicted ED_{50} or ED_{80} values.
[b] Significantly different from the endomorphin-1 + dexmedetomidine combination.
[c] Significantly different from all the binary combinations. $P < 0.05$ is considered significant.

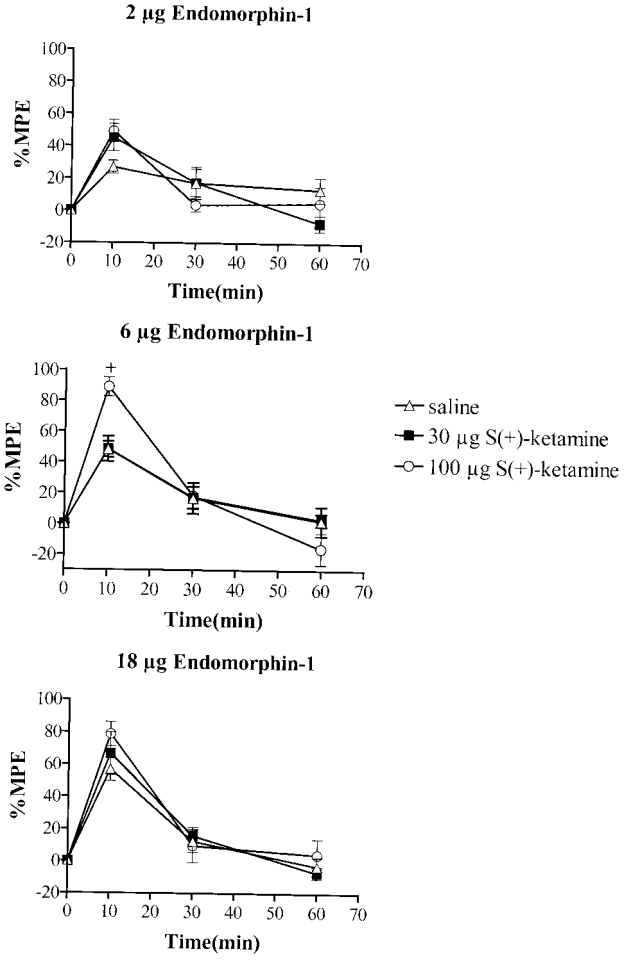

Figure 2. Antinociceptive effect (%MPE) of various mixtures of intrathecally administered endomorphin-1 (EM) with different doses of S(+)-ketamine. Each point represents the mean ± SEM for 5–11 animals. $+P<0.05$ versus saline with the Newman-Keuls test.

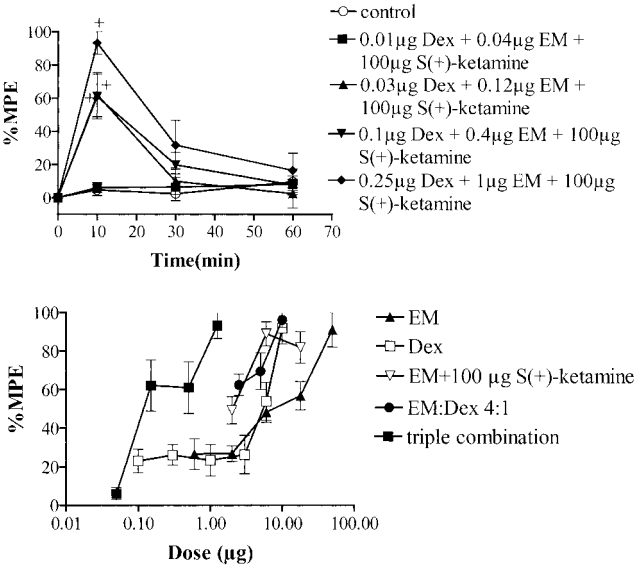

Figure 3. Time course of the antinociceptive effect (%MPE) of intrathecally coadministered endomorphin-1, dexmedetomidine, and S(+)-ketamine, where the dose ratio of endomorphin-1 (EM) and dexmedetomidine (Dex) is 4:1 and this is combined with 100 μg S(+)-ketamine (upper panel). Dose-response curves for the antinociceptive effect of the single-drug treatment, double or triple combination at 10 min (lower panel). In the case of coadministration of endomorphin-1 plus dexmedetomidine, Dose indicates the sum of the doses of the two drugs. Each point represents the mean ± SEM for 5–11 animals. $+P < 0.05$ versus control with the Newman-Keuls test.

of the opioid receptor-like orphan receptor), as well as with lidocaine (22) and clonidine (23).

NMDA receptors are likewise concentrated in the superficial dorsal horn, with the largest concentration in lamina II. Their activation plays a major role in the transmission of nociceptive information (8,19,24). Blockade of the NMDA receptor produces only weak or no antinociception against acute thermal or mechanical stimuli in uninjured rats (10,25), but it causes antinociception in various models of persistent pain (8,26). In contrast, various studies have already indicated the beneficial interaction between opioids and NMDA receptor antagonists both in acute and chronic pain (11,27–29), in agreement with our results. Dickenson (30) suggested that only seconds after C-fiber stimulation, spinal NMDA receptor activation

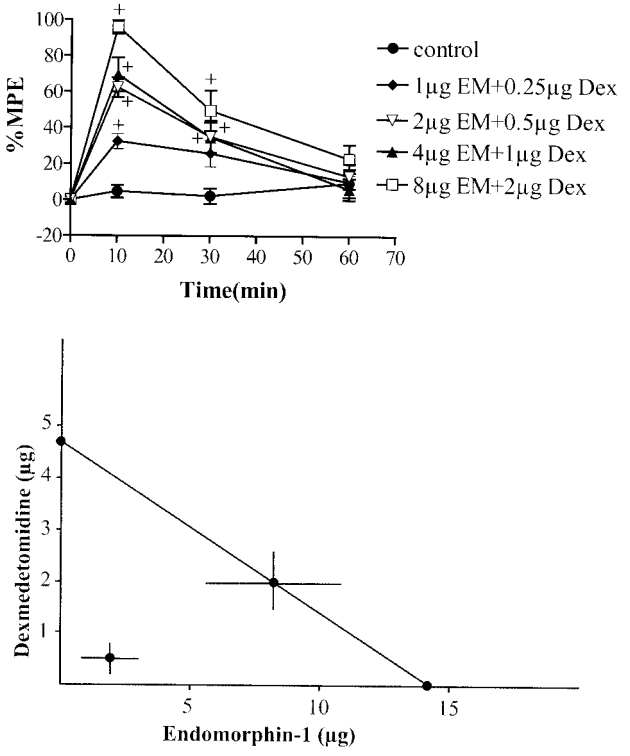

Figure 4. Time course of the antinociceptive effect (%MPE) of intrathecally-administered endomorphin-1 (EM) with dexmedetomidine (Dex) in a fixed (4:1) dose ratio (upper panel). Each point represents the mean ± SEM for 5–11 animals. $+P < 0.05$ versus saline with the Newman-Keuls test. Isobologram indicating the interaction between intrathecally administered endomorphin-1 and dexmedetomidine (lower panel). Values on the axes represent 50% effective dose (ED_{50}) values obtained from the drugs administered separately. The diagonal line connecting the values shows the additive interaction line. The points on this line represent the theoretical additive ED_{50} values. The points below the line show the experimentally derived ED_{50} values. The vertical and horizontal bars through the points represent the 95% confidence intervals for the values. The points below the line indicate synergistic interaction between endomorphin-1 and dexmedetomidine.

occurs, and the inhibition of this activation by ketamine might be responsible for the potentiation.

The α_2-adrenergic ligands are also preferentially bound to laminae I and II in the spinal cord (19). Intrathecal α_2-adrenoceptor agonists produce antinociception by decreasing the release of glutamate from primary afferent nerve terminals (31) and by suppressing the noxiously evoked activity of wide dynamic range neurons (32). There is a body of evidence revealing that opioid agonist-induced antinociception is modulated by spinal α_2-adrenergic agonists (3,5). Electrophysiological evidence has shown that clonidine potentiates the inhibitory action of intrathecal morphine on electrically evoked C-fiber activity (33). Sullivan et al. (33) investigated the location of α_2-adrenoceptor and opioid binding sites by using *in vitro* autoradiography with selective ligands, and they demonstrated that both opioid and α_2-adrenoceptors are present within the same superficial layers of the dorsal horn (laminae I and II), the site of entry of afferent A-δ and C pain-transmitting fibers into the central nervous system, which provides anatomic evidence for a possible interaction between the two systems.

When the mechanism of interaction of these three drugs is considered, there are several possibilities for a synergistic interaction among endomorphin-1, S(+)-ketamine, and dexmedetomidine. Because all of the receptor types on which these drugs act are abundant in the superficial laminae of the dorsal horn (19), their coeffect on these receptors produces a decrease in the sensation of pain in small doses. An important mechanism of spinal opioid agonists, α_2-adrenoceptor agonists, and NMDA receptor antagonists in the antinociception is the inhibition of transmitter release from C-fiber primary afferent terminals, though they have inhibitory effects on interneurons and projecting neurons as well (31). These dual actions at both pre- and postsynaptic sites may synergize via the inhibition of primary afferent transmitter release and reduced postsynaptic depolarization.

Moreover, it could not be excluded that the augmented activity resulted in part from a decreased clearance, because the duration of action of the dexmedetomidine and endomorphin-1 combinations were longer than those of endomorphin-1 or dexmedetomidine alone. S(+)-ketamine however, did not prolong the antinociceptive effect of endomorphin-1, and this suggests a mainly pharmacodynamic interaction between the two drugs.

In summary, this study shows that endomorphin-1, similarly to morphine, shows synergistic interaction with both the NMDA antagonist S(+)-ketamine and the α_2-adrenoceptor agonist dexmedetomidine. The synergistic interaction between these drugs may be of therapeutic significance in the future by allowing a decrease of the dose of either drug required to achieve an acceptable level of analgesia.

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