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Release of Substance P by Intrathecal KK-3, a Newly Synthesized Leu-Enkephalin Derivative

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TAKAHASHI, M., S. TOKUYAMA AND H. KANETO. *Release of substance P by intrathecal KK-3, a newly synthesized Leu-enkephalin derivative.* PHARMACOL BIOCHEM BEHAV 33(4) 843–846, 1989.—Intrathecal (IT) injection of KK-3, a leucine-enkephalin analogue with weak, naloxone-reversible analgesic effect, produced behavior consisting of scratching, biting, licking and characteristic convulsion-like symptoms in mice. Naloxone or Mr2266 did not affect the behavior, suggesting a difference in the mechanisms for the production of the behavior and the analgesic effect. The behavior, except the convulsive symptom and the lag time of a couple of min for onset of the behavior, are quite similar to that elicited by IT substance P (SP). D-Pro^2-D-Trp^{7,9}-substance P, a SP antagonist, completely suppressed all the behavior induced by KK-3, indicating that the behavior is attributable to SP. By radioimmunoassay, it was found that SP was released by stimulation with KK-3 from the isolated spinal cord preparation of newborn rat. Intrathecal pretreatment with capsaicin, a depleter of SP, suppressed the KK-3-induced behavior, but did not affect the SP-evoked behavior. These results suggest that KK-3 acts as a releaser of SP in the spinal dorsal horn, and consequently produces SP-like behavior. Thus, a novel pharmacological action, the release of substance P from spinal cord by the IT injection of endogenous opioid peptide analogue, was demonstrated.

KK-3 Substance P D-Pro^2-D-Trp^{7,9}-substance P Intrathecal injection Substance P releaser Capsaicin Substance P-induced behavior

WE conducted a series of experiments to investigate the structure-activity relationship of enkephalin analogues with a Tyr moiety on the N-terminal, a Phe-group on the C-terminal, and connected with various lengths of methylene chain. We found that the analgesic effect of these compounds was dependent on the distance between both moieties and tended to decrease by increasing the number of methylene groups. At the same time we found that these compounds produced convulsion-like behavior and/or rotation ipsilateral to the injection side at a high intracerebroventricular dose, and the behavioral effect was maximal in KK-3 which possesses 3 methylene chains (16). During further studies on KK-3, we observed peculiar behavior characterized by scratching, biting, licking, grooming and specific convulsion-like behavior after intrathecal injection. These behavioral changes are similar to those elicited by intrathecally administered substance P (SP) (6).

Since SP is well recognized as a pain transmitter in the spinal cord (9–11), a comparative study was made on the characteristics of SP and KK-3, and the possibility that KK-3 acts as a releaser of SP from the spinal cord was demonstrated.

METHOD

Animals

Male ddY strain mice weighing 18–20 g (Otsubo Exp. Animals, Nagasaki) were housed as a group of 10 animals in plastic cages with free access to food and water. They were kept in a temperature-controlled room at 22 ± 1°C, and after reaching 23 to 26 g they were used for experiments.

Compounds

KK-3 (tyrosyl-N-methyl-γ-aminobutylyl-phenylalaninol, synthesized and supplied from Prof. K. Kawasaki of Kobe Gakuin University), U-50,488H (trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzene-acetamide methansulfonate hydrate, gift from Upjohn), Mr2266 [(−)-2-(3-furylmethyl)-norethazocine, gift from Boehringer Ingelheim], ^{3}H-substance P (40 Ci/mmol, New England Nuclear), substance P, D-Pro^2-D-Trp^{7,9}-substance P and dynorphin (Protein Res. Found.), capsaicin, picrotoxin (Nakarai Chemicals) and naloxone-HCl (Sigma) were used. Capsaicin was dissolved in 5% tween 80 and 5% ethanol and all other drugs were freshly prepared with saline.

Injection Procedure

Intrathecal (IT) injection was carried out according to the method described by Hylden and Wilcox (6). Briefly, a lumbar puncture was performed using a 30-gauge needle directly con-

nected to a microsyringe. The needle was inserted between L5 and L6, so that the dose was contained in a volume of 10 μl/mouse.

Release of Substance P From the Isolated Spinal Cord of Newborn Rat

An isolated spinal cord preparation of newborn rat was suspended in a plastic perfusion chamber according to the method of Akagi *et al.* (1). A modified Krebs-Ringer bicarbonate buffer [127 mM NaCl/3.73 mM KCl/1.8 mM $CaCl_2$/1.18 mM KH_2PO_4/1.18 mM $MgSO_4$/20 mM $NaHCO_3$/D-glucose (2 g/liter)] previously saturated with O_2/CO_2, 95:5 (v/v), was perfused at a flow rate of 0.67 ml/min. The solution temperature inside the chambers was kept at 37°C. The spinal cord was perfused with the buffer for 1 hr, then followed by the buffer containing 10^{-5} M and 10^{-4} M KK-3, and 10^{-6} M capsaicin, in turn.

After the application of the compound, the preparation was washed for 1 hr before the following application. The flow rate was 2 ml/3 min and 2 ml fractions were collected for 15 min, and then lyophilized. The SP in each sample was determined by radioimmunoassay.

Radioimmunoassay for SP

The reaction mixture in 0.5 ml of 50 mM phosphate buffered saline (pH 7.0) contained diluted antiserum, 20 nmol of bacitracin, normal bovine γ-globulin (0.5 mg as protein) and ^{3}H-SP (0.2 pmol, 17,000 dpm). After incubation at 4°C overnight the mixture was half saturated with polyethylene glycol No. 6000 and allowed to stand at 4°C for 1 hr. The precipitate was separated by centrifugation and dissolved in 1 ml of phosphate-buffered saline and 9 ml of scintillation cocktail (ACS II, Amersham) for counting.

Statistical Analysis

The statistical significance of the differences was determined by Student's or Welch's *t*-test and Fisher's exact probability test.

RESULTS

Behavioral Changes Induced by Various Compounds

KK-3. Intrathecal administration of KK-3 dose-dependently evoked unique behavioral changes characterized by scratching, biting and licking. The behavior was quite similar to the behavior induced by IT SP, but was accompanied by a characteristic convulsion-like behavior: a contracture of the caudal portion of the body. At a dose of 10 nmol/mouse, the behavior started with a lag of about 2 min and lasted intermittently for 10 min.

SP. SP, 15 pmol, injected IT elicited scratching, biting and licking, which appeared immediately after the injection and persisted for about 1 min.

Capsaicin. A few seconds after IT injection of capsaicin, 15 nmol/mouse, strong contracture of the caudal portions of the body, scratching, biting, squealing and licking were produced. This severe behavior was observed intermittently for about 10 min.

Dynorphin and U-50,488H. Intrathecal dynorphin and U-50,488H, selective opioid κ-receptor agonists (2, 4, 8, 15) at doses up to 10 nmol/mouse, did not elicit any behavioral changes.

Leucine-enkephalin. Even at a high dose, 50 nmol/animal, leucine-enkephalin did not induce scratching, biting, licking or any other specific behavior after IT administration.

Picrotoxin. Intrathecal administration of picrotoxin at a dose of 5 μg/mouse induced clonic convulsions lasting for 10 min.

Throughout the experiments, no characteristic behavior was produced by the IT injections of vehicle and saline.

TABLE 1

BEHAVIORAL CHARACTERISTICS ELICITED BY VARIOUS COMPOUNDS

Compound	Dose (IT)	Behavioral Characteristics	Incidence (%)
KK-3	5 nmol/mouse	SP-like behavior	6/7 (86)
	10 nmol/mouse		12/13 (92)
Substance P	15 pmol/mouse	Scratching Biting, Licking	12/12 (100)
Capsaicin	15 nmol/mouse	Scratching Biting, Licking	7/7 (100)
Dynorphin (1–13)	10 nmol/mouse	No effect	0/5 (0)
U-50488H	10 nmol/mouse	No effect	0/12 (0)
Leucine-enkephalin	50 nmol/mouse	No effect	0/8 (0)
Picrotoxin	5 μg/mouse	Clonic convulsion	5/5 (100)

The results of the behavioral tests are summarized in Table 1.

The onset and duration of bizarre behavior induced by KK-3 and SP were also compared and are shown in Table 2.

Effect of Capsaicin Pretreatment on the Behavioral Changes Induced by KK-3, SP and Picrotoxin

The behavior induced by the injection of KK-3 was significantly suppressed by capsaicin, 15 nmol/mouse, given 96 hr before KK-3 (5 nmol/mouse, $p<0.05$; 10 nmol/mouse, $p<0.01$; Fisher's exact probability test). However, the pretreatment could not prevent the production of behavior induced by SP. Capsaicin was also ineffective in protecting against the clonic convulsions caused by IT administered picrotoxin (Table 3).

Effect of Naloxone or Mr 2266 Pretreatment on the Behavior Induced by KK-3

A selective μ-antagonist naloxone or a selective κ-antagonist Mr2266, given 10 min prior to the administration of KK-3, did not

TABLE 2

COMPARISON OF ONSET AND DURATION OF THE BEHAVIOR INDUCED BY KK-3 AND SUBSTANCE P

Compound	Dose (nmol/mouse)	Onset (sec)	Duration (sec)	Incidence
KK-3	1	—	—	0/5
	2.5	188 ± 20.2	55.3 ± 16.7	3/5
	5	161 ± 14.2	75.8 ± 13.0	5/5
	10	124 ± 19.9	248 ± 14.4	10/10
Substance P	0.005	—	—	0/5
	0.015	11.9 ± 2.3*	48.4 ± 5.7*	7/7
	0.075	13.3 ± 5.6*	49.8 ± 3.9*	5/5

Figures indicate the mean ± S.E.M. *$p<0.001$, compared with 10 nmol KK-3-treated group (Student's or Welch's *t*-test).

TABLE 3

EFFECT OF CAPSAICIN PRETREATMENT ON THE BEHAVIOR ELICITED BY VARIOUS COMPOUNDS

Capsaicin	Drugs	Dose	Route	Incidence	(%)
	KK-3	5 nmol/mouse	IT	6/7	(86)
		10 nmol/mouse	IT	12/13	(92)
−	Substance P	15 pmol/mouse	IT	6/6	(100)
	Picrotoxin	5 μg/mouse	IT	5/5	(100)
	KK-3	5 nmol/mouse	IT	1/7†	(14)
		10 nmol/mouse	IT	2/8*	(25)
+	Substance P	15 pmol/mouse	IT	7/7	(100)
	Picrotoxin	5 μg/mouse	IT	5/5	(100)

Each compound was administered 96 hr after IT vehicle or capsaicin, 15 nmol/mouse. *$p<0.01$, †$p<0.05$, compared with corresponding vehicle-pretreated group (Fisher's exact probability test).

modify the behavior induced by KK-3 (Table 4).

Antagonism by SP Antagonist, D-Pro^2-D-Trp^{7,9}-Substance P, of the KK-3-Induced Behavior

Incidence of the KK-3-induced behavior was reduced and completely blocked by the concurrent treatment with IT D-Pro^2-D-Trp^{7,9}-substance P, 1 nmol and 3.3 nmol/mouse, respectively. The antagonist also completely suppressed the behavior induced by SP (Table 5).

Estimation of SP in Newborn Rat Spinal Cord

KK-3, 10^{-6} M and 10^{-5} M, as well as capsaicin 10^{-6} M,

TABLE 4

EFFECT OF PRETREATMENT WITH NALOXONE AND Mr2266 ON THE KK-3-EVOKED BEHAVIOR

Pretreatment	Drug	Dose, Route	Incidence
Saline 0.1 ml/10 g, IP	KK-3	10 nmol/mouse, IT	8/8
Naloxone 2 mg/kg, IP	KK-3	10 nmol/mouse, IT	13/13
Mr2266 2 mg/kg, IP	KK-3	10 nmol/mouse, IT	10/10

Naloxone and Mr2266 were given 10 min prior to KK-3.

TABLE 5

EFFECT OF PRETREATMENT WITH D-Pro^2-D-Trp^{7,9}-SUBSTANCE P ON THE KK-3- AND SUBSTANCE P-EVOKED BEHAVIORS

Pretreatment	Drug	Dose, Route	Incidence
1 nmol/mouse, IT	KK-3	10 nmol/mouse, IT	2/6
3.3 nmol/mouse, IT	KK-3	10 nmol/mouse, IT	0/6
3.3 nmol/mouse, IT	Substance P	15 pmol/mouse, IT	0/6

D-Pro^2-D-Trp^{7,9}-substance P was given 10 min prior to KK-3 and substance P.

TABLE 6

EFFECT OF KK-3 AND CAPSAICIN ON RELEASE OF ENDOGENOUS SUBSTANCE P FROM NEWBORN RAT SPINAL CORD IN VITRO

		Perfusion Period (min)				
Compound	Concentration	0–3	4–6	7–9	10–12	13–15
KK-3	10 μM	0.41*	0.72	1.34	2.43	1.09
	100 μM	0.56	0.68	73.0	122	105
Capsaicin	1 μM	0.57	0.56	20.1	57.2	27.4

*Immunoreactive substance P (pmol)/100 mg tissue/3 min.

released SP from isolated newborn rat spinal cord (Table 6). KK-3 and capsaicin, per se, did not cross-react with the antiserum.

DISCUSSION

Intrathecal KK-3 dose-dependently produced characteristic behavior which consisted of vigorous scratching, biting, licking and peculiar convulsions in mice. The KK-3-evoked behavior was similar to that induced by IT SP, except for the convulsive symptom. Another difference from that of SP was the lag of the onset of the KK-3-induced behavioral changes. The delay in the onset may suggest that KK-3 might exert its behavioral effect indirectly through the release of SP from its storage site in the spinal dorsal horn. Actually, we could identify the amount of radioimmunoassayable SP released in the perfusate after the stimulation of newborn rat spinal preparation by KK-3, as well as capsaicin, a depleter of SP (5,17). The hypothesis is also compatible with the behavioral similarities with capsaicin, when applied directly through the IT route. Other evidence to support the hypothesis includes the observation that pretreatment with capsaicin attenuated the behavioral changes of IT KK-3, but failed to modify the effect of SP applied directly to the spinal cord. In fact, pretreatment with D-Pro^2-D-Trp^{7,9}-substance P, a potent and selective SP antagonist (3), suppressed the KK-3-induced behavior. The difference between KK-3 and SP in the convulsion-like symptom suggests the existence of other mechanisms. This might include mediation through specific SP receptor subtypes or the release of different transmitters, such as somatostatin. Somatostatin also produces similar behavior after IT injection (12); however, the possible involvement of that peptide could be excluded, since D-Pro^2-D-Trp^{7,9}-substance P completely abolished the behavior. The possible involvement of other endogenous active peptides in the spinal cord, however, could not be excluded completely.

We have suggested in our previous report (16) that KK-3 may produce its analgesic effect through the opioid κ-receptor; however, intrathecal dynorphin or U-50,488H (both are specific opioid κ-receptor agonists), did not produce any behavioral changes at analgesic doses, 10 nmol/mouse.

Leucine-enkephalin, the precursor peptide of KK-3 and a typical opioid δ-receptor agonist, is known to be epileptogenic and produces typical EEG seizure in experimental animals (13,14). However, we could not find any abnormal behavior by IT injection up to 50 nmol. In addition, KK-3-induced behavior was different from the clonic convulsions induced by picrotoxin, suggesting no involvement of spinal GABAergic mechanisms mediated through a chloride channel in the expression of KK-3-induced behavior. The difference between KK-3 and picrotoxin convulsive behaviors is also evident from the fact that pretreatment with capsaicin reduced the appearance of KK-3-induced behavior, but could not modify the convulsions elicited by picrotoxin. Lack of naloxone

and Mr2266 antagonism to the behavior induced by KK-3 suggests that opioid μ- and κ-receptor mechanisms are not involved in the process.

SP is a pain transmitter in the spinal cord (9–11). The inhibition of its release is supposed to be a basis for the analgesic effect of morphine (7). Temporary excitation and the succeeding long-lasting analgesia induced by capsaicin are due to abrupt release and subsequent depletion of SP from its storage site (5,17). KK-3 produces SP-like scratching, biting and licking, followed by a short-lasting antinociceptive effect after IT and ICV (16) injection. The possibility that KK-3 produced a temporary depletion of SP from spinal dorsal horn, as is demonstrated in the present experiment, suggests the same mechanism as capsaicin in the production of the antinociceptive effect.

In conclusion, although the structures of KK-3 and capsaicin are completely different, the behavior of KK-3 is produced through fundamentally the same mechanisms as capsaicin: the release of SP. Thus, the novel pharmacological action, the release of substance P from spinal cord, by the IT injection of endogenous opioid peptide analogue was demonstrated.

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