ALGESIogenic AND ANALGESic ACTIVITIES OF SYNTHETIC SUBSTANCE P

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Abstract—The objective of our study was to determine whether the pure synthetic substance P (SP) is algesiogenic or analgesic when administered centrally or peripherally. The relationships between SP-induced analgesia and the content of morphine-like factor (MLF) in the brain were also studied. Intracarotid arterial administration of SP (20–200 μg) produced no pseudo-affective responses to pain in six out of nine rats, but in the remaining three, there was an exhibition of these responses. Chlorpheniramine pretreatment antagonized these responses. On cantharidin blister base experiments in humans, SP (10⁻⁴ g/ml) produced slight pain and an itchy sensation. SP given intracerebroventricularly produced an analgesia in mice in a dose of 5 ng/mouse, as determined by the acetic acid-induced writhing and hot plate methods. These SP-induced analgesia were antagonized by naloxone pretreatment. SP did not alter the content of MLF in the mouse whole brain. However, SP₅₋¹¹ not only produced an analgesia but also increased the content of MLF. These results suggest that SP has a slight algesiogenic activity which might be mediated by histamine and a slight analgesic activity which might be mediated by MLF.

Substance P (SP) is unevenly distributed in the central nervous system (1) and it may be a transmitter of primary afferent nerves (2). Although SP is reportedly a potent algesiogenic substance (3–5), there are discrepancies in the literature (6). Synthetic SP was first reported to be hyperalgesic (7) yet later found to be analgesic, having a morphine-like property, when administered not only intraventricularly but also intraperitoneally to mice (8). These different results were probably attributed to contamination of kinins into the crude preparation. The purpose of the present study was to determine whether the pure synthetic SP is algesiogenic or analgesic when administered centrally or peripherally. Relationships between SP-induced analgesia and the content of morphine-like factor (MLF) in the brain were also studied.

MATERIALS AND METHODS

1. Algesiogenic activity of SP

1) Intracarotid arterial administration to rats

Technique of the cannulation was that described by Deffenu et al. (9) and Blane (10). Male Wistar strain rats, weighing 250–300 g were anesthetized with ether and a polyethylene cannula (internal diameter 0.8 mm) was inserted into the right carotid artery with the tip pointing towards the heart. As a definite sign of the pseudo-affective responses to pain, appearance of either dextro-rotation of the head, flexion of the right forelimb or squeaking occurred after the administration of the inciting agent.
through the cannula.

2) Intrafemoral arterial administration in rabbits

Rabbits of both sexes weighing 2.5–3.0 kg were anesthetized with ether. A polyethylene tubing with a tightly fitting cap was inserted into the right femoral artery. As a sign of the pseudoaffective responses to pain, holding or dragging of the cannulated extremity and vocalization occurred after the administration of the inciting agent through the cannula.

3) Cantharidin blister base experiment in humans

According to Armstrong et al. (11) and Inoki et al. (12), a cantharidin plaster containing 0.3% cantharidin was applied circularly on the skin of the forearm about 1 cm in a diameter for 5–6 hr before experiment. The test solution was usually applied to the blister so as to cover the whole area as quickly as possible. Pain sensation was previously graded into 5, that is, 0: no pain, 1: slight pain, 2: moderate pain, 3: severe pain and 4: very severe pain. The grades were recorded oscillographically by pulling a lever in accordance with the strength of pain.

2. Analgesic activity of SP

1) Writhing behavior of mice

Male dd-strain mice, weighing 10–15 g, were used. The intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) administration of SP were performed 15 min and 5 min prior to an acetic acid injection, respectively, and after 5 min had elapsed from the acetic acid injection, the number of writhings was counted for the following 10 min. The i.c.v. administration of SP in a volume of 0.05 ml saline containing an appropriate dose of the agent was given in accordance with the method of Haley and McCormick (13).

2) Hot plate method in mice

Male dd-strain mice, weighing 10–15 g, were used. The hot plate was constantly warmed at 55±0.5°C. The time until the appearance of the licking behavior of forelimb after the placement onto the hot plate was measured as licking latency. The i.p. and i.c.v. administration of SP were performed 30 min and 10 min prior to the experiment, respectively.

3) Measurement of MLF

a) Extraction of MLF

The procedure of Pasternak et al. (14) was used. Immediately after decapitation microwaves were applied to the brain. The area of the brain required for investigation was homogenized with Polytron in 30 volumes of the iced 10 mM Tris-HCl buffer (pH 7.7), heated in boiling water for 15 min and then centrifuged at 100,000×g for 1 hr after which supernatant was lyophilized.

b) Receptor binding assay

1) Preparation of the membraneous fraction:

Male Spraque-Dawley rats were decapitated and then the whole brain except for the cerebellum was homogenized in 30 volumes of 50 mM Tris-HCl buffer (pH 7.7). The suspension was centrifuged at 49,000×g for 15 min and the pellet obtained was re-suspended in an adequate volume of the same buffer. The resuspension was then incubated at 37°C for 30 min and was re-centrifuged. The pellet was finally re-suspended in 30 volumes of 50 mM Tris-HCl buffer.

2) Assay:

The receptor binding assay was used for MLF measurement. 3H-methionine enkephalin (2 p mole, sp. act. 50 Ci/m mole) was added to 400 μl of brain membraneous pellet suspension in the presence of bacitracin (50 mg/ml) and aliquots of the lyophilized brain extract (100 or 200 μl) were added and the final volume in each test tubing was adjusted to 1,000 μl by 50 mM Tris-HCl buffer. The mixture were incubated for 40 min at 25°C and then filtered through Whatmann GF/B Glass fibre disci. Radioactivities of discus were counted using a liquid scintillation counter. Quantity of MLF
was calculated as the percent of the brain extract which prevented binding of \(^{3}H\)-methionine enkephalin to the brain membraneous fraction. Protein volume was estimated by the method of Lowry et al. (15).

4) Drugs used in this experiment were synthetic substance P (Protein Research Foundation), synthetic bradykinin (Protein Research Foundation), substance P\(_{5-11}\) (H-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH\(_{3}\)) (Peninsula Laboratories), chlorpheniramine maleate (Kowa), naloxone chloride (Sankyo), acetylcholine chloride (Daichi), morphine chloride (Shionogi) and \(^{3}H\)-methionine enkephalin (New England Nuclear).

RESULTS

1) Algesiogenic activity of SP

1) Intracarotid arterial administration in rats: Bradykinin in doses of 0.5–2 µg always produced typical pseudoaffective responses such as dextro-rotation of the head and flexion of the right forelimb. However, SP even in a high dose of 200 µg produced neither ipsilateral rotation of the head nor flexion of the ipsilateral forelimb, in six out of nine rats. The pseudoaffective responses exhibited in three rats were antagonized by the pretreatment with the i.p. administration of 5 mg/kg of chlorpheniramine. Subcutaneous administration of 8 mg/kg of morphine completely inhibited the pseudoaffective responses induced by bradykinin (Table 1).

2) Intrafemoral arterial administration in rabbits: Intrafemoral arterial administration of bradykinin in doses of 0.4–1 µg always produced pseudoaffective responses such as holding or dragging of the ipsilateral hindlimb (n=3). However, SP even in high doses of 20–200 µg produced no response (n=6).

3) Cantharidin blister base in humans: Algesiogenicity of SP was compared with several putative pain producing substances such as bradykinin and acetylcholine. Bradykinin (10\(^{-5}\) g/ml) produced severe pain and SP (10\(^{-3}\) g/ml) produced slight pain and an itchy sensation. On the other hand, SP\(_{5-11}\) (10\(^{-4}\) g/ml) which was dissolved in Hartmann's solution produced no pain sensation. The characteristics of pain elicited by both bradykinin and SP were pain sensation with properties of increasing intensity which persisted during the application and even after washing with saline. Acetylcholine (10\(^{-4}\) g/ml) temporarily elicited moderate to severe pain with a short latency (Table 2).

2. Analgesic activity of SP

1) Writhing behavior in mice: SP produced an analgesia in mice after both the i.c.v. administration of 5 ng/mouse (Fig. 1)

### Table 1. Effect of substance P on the appearance of the pseudoaffective responses following the intracarotid arterial administration in rats

| Agents         | Dose/Animal µg, i.a. | Pseudoaffective responses |
|----------------|----------------------|--------------------------|
|                |                      | Dextrorotation of the head | Flexion of the right forelimb | Squeaking |
| Substance P    | 20                   | (0/3)                      | (0/3)                         | (0/3)     |
|                | 200                  | (3/9)*                     | (3/9)*                        | (1/9)     |
| Bradykinin     | 0.5–2                | (14/14)                    | (14/14)                       | (1/14)    |
| Morphine (8 mg/kg, s.c.) + Bradykinin | 1                   | (0/3)                      | (0/3)                         | (0/3)     |

*Reversal by the pretreatment with chlorpheniramine (5 mg/kg, i.p.) + and − represent appearance and no appearance of each response, respectively. Numbers in parenthesis represent number of the rats exhibiting each response against the number of experimental rats. Morphine was given s.c. 30 min prior to bradykinin.
and i.p. administration of 10 mg/kg (percent of inhibition: 79.5±6.8, N=8, P<0.01), when acetic acid was used as an inciting agent. SP-induced analgesia was partially antagonized by the pretreatment with naloxone (1 mg/kg, i.p.).

2) Hot plate method in mice: SP slightly prolonged the licking latency of mice after the i.c.v. administration of 5 ng/mouse (Fig. 2), but not after the i.p. administration of 10 mg/kg (percent of control: 107.7±14.4, N=17). SP also slightly prolonged the

Table 2. Algesiogenic activity of several agents on cantharidin blister base in humans

| Agents       | g/ml     | Score | Subject |
|--------------|----------|-------|---------|
| Bradykinin   | 10⁻⁵     | A     | B       | C       | D       | E       |
| Substance P  | 10⁻³     | 1*    | 1-2    | 1-2*    | 1*      | 0*      |
| Substance P₅₁ | 10⁻³     | 0     | 0      | 0       | 0       | 0       |
| ACh          | 10⁻⁴     | 0     | 2-3    | 0-1     | 2-3     | 3       |
| NaCl         | 0.9%     | 1     | 0-1    | 0       | 2       | 0       |

*Itchy sensation rather than pain. Score: 0: no pain, 1: slight pain, 2: moderate pain, 3: severe pain and 4: very severe pain.

Fig. 1. Effect of substance P (SP) on acetic acid-induced writhing following i.c.v. administration. Inhibitory percent of acetic acid-induced writhing represents 0 following physiological saline i.c.v. administration. Closed circles represent the value of the inhibitory percent of acetic acid-induced writhing at 10 min after SP administration for 10 min. Open circles represent the effect of naloxone pretreatment at 15 min before the experiment on SP-induced inhibition of acetic acid-induced writhing. Vertical bars represent S.E.M. (N=6-28, **P<0.01 Student’s t-test or Fisher-Behrens test).

Fig. 2. Effect of substance P(SP) on licking latency following i.c.v. administration. Effect of physiological saline on the licking latency represents 100 percent. Closed circles represent the value of the percent of latency at 10 min after SP administration. Open circles represent the effect of naloxone pretreatment at 20 min before the experiment on SP-induced prolongation of the licking latency. Vertical bars represent S.E.M. (N=9-15, *P<0.05 Student’s t-test or Fisher-Behrens test).
licking latency after the i.c.v. administration of 5 ng/mouse (Fig. 3). SP and SP₅₋₁₁-induced analgesia was completely antagonized by the pretreatment with naloxone (1 mg/kg, i.p.).

3) Change of the content of MLF in the mouse whole brain: SP did not alter the content of MLF in the mouse brain after both the i.c.v. administration of 5 ng/mouse and i.p. administration of 10 mg/kg. However, SP₅₋₁₁ after the i.c.v. administration of 5 ng/mouse increased the content of MLF (Fig. 4).

DISCUSSION

Armstrong et al. (3) demonstrated that SP produced a painful sensation similar to that produced by bradykinin or several other peptides. Zetler (5) also demonstrated that SP reduced the threshold to pain. Furthermore, Potter et al. (4) reported that purified SP in the dog produced more potent pseudoaffective responses than did bradykinin when injected intraarterially. Juan and Lembeck (16) used the circulatory isolated but neurologically innervated rabbit ear and found that intra-arterial administration of some extracts of SP but not synthetic SP mimicked such responses as a reflex fall in arterial pressure and an increase in respiration, elicited by a painful pinch to the same ear. Lembeck and Gamse (6) also reported that SP was devoid of an algesic effect on paravascular pain receptors and concluded that the algesic action shown by SP was attributed to a contamination with other peptides, especially bradykinin-like peptides. In our experiments, intracarotid arterial administration of bradykinin in doses of 0.5–2 μg in rats and 0.4–1 μg in rabbits always produced pseudoaffective responses. SP in a large dose of 200 μg produced no responses in six out of nine rats. However, pseudoaffective responses did occur in three rats and these responses could be antagonized by the pretreatment with chlorpheniramine (5 mg/kg, i.p.). As Abe et al. (17) and Tallarida et al. (18) have discussed, bradykinin may produce pseudo-
affective responses as a result of direct stimulation on the paravascular pain receptors. Our results suggested that histamine may participate in the pseudoaffective responses elicited by SP in some animals. Moreover, when SP was given in a high concentration, slight pain and an itchy sensation occurred. It was also reported that intradermal administration of SP released histamine (19), which also produced both pain and itchy sensations in cantharidin blister base experiments, as already demonstrated by Armstrong et al. (11) and Rosenthal and Minard (20). SP$_{5-11}$, a peptide which has a shorter sequence than SP, even in the high concentration of 10$^{-4}$ g/ml produced no pain or itchy sensations in our cantharidin blister base experiment. Johnson and Erdős (21) reported that release of histamine by SP strongly depended upon the existence of arginine and lysine at the first and third positions in the amino-acid sequence of SP. As there is a lack of these two amino acids at these positions in SP$_{5-11}$, such may explain why SP$_{5-11}$ does not induce the release of histamine. This result also suggests that pain and itchy sensations of SP are caused by the action of histamine. We found herein that the algesiogenicity of SP was a considerably weak and the pseudoaffective responses elicited by a high dose of SP were exhibited by the released histamine. Stewart et al. (8) found that SP was a potent analgesic with morphine-like properties in mice, when administered both i.c.v. and i.p. In the present study, the analgesic property of SP was demonstrated with the i.c.v. administration of 5 ng/mouse and i.p. administration of 10 mg/kg, as assessed by the acetic acid-induced writhing syndrome method. The analgesic effect of SP as determined by the hot plate method was slight. These results also suggest that analgesic action of SP is weak. With regard to the mechanism of SP-induced analgesia, it should be considered that the analgesic action of SP was not exhibited as the result of binding to opiate receptors but rather by mediation through the action of MLF, since SP$_{5-11}$ induced analgesia could be antagonized by naloxone. The opiate receptor cannot be bound by SP directly (22). Although the content of MLF did not correspond in time to the analgesic effect, the possibility that SP$_{5-11}$ induced analgesia was to some extent involved in action of MLF cannot be ruled out, since SP did not alter the content of MLF in the mouse brain after the i.c.v. and i.p. administration. SP$_{5-11}$ reportedly has much higher depolarizing actions on rat motoneurons than does SP and competes to a greater extent than SP with $^3$H-SP binding sites in synaptic membranes (23). SP$_{5-11}$ not only produces analgesia but also increases the content of MLF after the i.c.v. administration. Moreover, the analgesic effect of SP was only observed with a low dose of 5 ng/mouse and not at all with the high doses. These results also suggest that SP may induce a release of MLF to produce the analgesic effect in low dose, but nevertheless antagonizes the effect of MLF by initiating some nociceptive neural activity in the case of a high dose, as Frederickson et al. (24) already demonstrated that SP could antagonize the effect of MLF. Since the inhibitory effect of SP in a dose of 10 mg/kg, i.p. on acetic acid-induced writhing behavior was not completely antagonized by the pretreatment of naloxone, other factors may be involved in the appearance of SP$_{5-11}$-induced analgesia.

In conclusion, SP, only in high doses, had a slight algesiogenic activity and this activity may be mediated by histamine. On the other hand, SP had a weak analgesic activity in a low dose when given i.c.v. and in a high dose when given i.p. It was also suggested that SP$_{5-11}$-induced analgesia was in part mediated by MLF in the brain.
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