Laboratory Investigations

Intrathecal neurokinin-1 receptor antagonist reduces isoflurane MAC in rats

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Purpose: To study the effects of intrathecal administration of a neurokinin-1 (NK-1) receptor antagonist (CP96,345) on the minimum alveolar anaesthetic concentration (MAC) of isoflurane in anaesthetized rats, and on the locomotive activity of conscious rats.

Methods: Wistar rats ($n=36$) were fitted with indwelling intrathecal catheters, and the MAC of isoflurane was determined following the intrathecal administration of saline (control group) or the NK-1 receptor antagonist CP96,345 (CP) at 1, 10 and 100 μg . Subsequently a reversal dose of intrathecal Substance P (SP) at 1, 10 and 100 μg was administered and MAC isoflurane was redetermined. Conscious rats ($n=35$) were also examined for the presence of locomotor dysfunction following intrathecal administration of CP and SP. Animals were randomly assigned to each treatment group and the investigators were blinded.

Results: CP at 10 and 100 μg reduced MAC isoflurane by 9.9% and 15.3%, respectively ($P < 0.05$). Intrathecal administration of SP reversed the decreases in MAC by CP; however, locomotive activity was not changed.

Conclusion: These results suggest that the NK-1 receptor plays an important role in determining the MAC of isoflurane by inhibition of pain transmission in the spinal cord.

Objectif : Étudier les effets de l'administration sous-arachnoïdienne d'un antagoniste du récepteur neurokinine-1 (NK-1 le CP96 345) sur la concentration alvéolaire minimum (MAC) de l'isoflurane chez le rat anesthésié et sur l'activité locomotrice chez le rat conscient.

Méthodes : Des cathéters sous-arachnoïdiens ont été implantés chez des rats Wistar ($n=36$) et le MAC de l'isoflurane déterminé après l'administration sous-arachnoïdienne de sol. phys. (Groupe contrôle) ou 1, 10 et 100 μg de l'antagoniste du récepteur NK-1, le CP96 346 (CP). Par la suite, une dose sous-arachnoïdienne neutralisante de la substance P (SP) de 1, 10 et 100 μg était administrée et le MAC de l'isoflurane déterminé de nouveau. Chez les rats conscients, on a aussi recherché la présence d'un dysfonctionnement locomoteur après l'administration de CP et de SP. Les animaux étaient assignés aléatoirement à chaque groupe de traitement. L'observation des effets était réalisée à l'aveugle.

Résultats : Dix et 100 μg de CP diminuaient respectivement le MAC de l'isoflurane de 9,9% et 15,3% ($P < 0,05$). L'administration sous-arachnoïdienne de SP abolissait les diminutions du MAC provoquées par CP ; l'activité locomotrice demeurait toutefois inchangée.

Conclusion : Ces résultats suggèrent que le récepteur NK-1 influence nettement le MAC de l'isoflurane par inhibition médullaire de la transmission de la douleur.

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IN general anaesthesia, anaesthetists attempt to abolish consciousness, pain perception and memory reversibly. These are functions associated with the cerebral cortex. However, inhalational anaesthetics have an important effect on the spinal cord.¹ Recent studies report that the potency of anaesthetics is determined by their actions in the mid-brain, brain stem, and spinal cord.^{2,3} We have reported that intrathecal administration of N-methyl-D-aspartate (NMDA) receptor antagonists reduce MAC isoflurane.^{4,5} It is possible, then, that inhalational anaesthetics suppress noxious stimulus-evoked movement through actions upon the spinal cord.

Several lines of evidence have supported the hypothesis that Substance P (SP) serves as a neurotransmitter in the spinal cord mediating afferent neurotransmission of noxious stimuli.⁶⁻⁸ Based on bioassays and on a variety of binding studies, three classes of neurokinin recognition sites have been identified, NK-1, NK-2 and NK-3.^{9,10} Nociceptive neurons within the dorsal horn express NK-1 receptors which are excitatory. Moreover, the NK-1 receptor site shows preferential affinity for SP¹¹ suggesting that NK-1 receptor may modulate afferent pain pathways.

Intrathecal injection of agonists of the NK-1 receptor causes behavioural reactions including scratching, biting, and licking in mice, syndromes that indicate nociceptive sensation in conscious mice.¹² Recent behavioral studies have demonstrated that intrathecal injection of CP 96,345 (CP), a non-peptide antagonist of the NK-1 receptors¹³ was able to diminish the nociceptive response.^{14,15} These findings prompted us to investigate the effect of intrathecal administration of the SP receptor antagonist CP on MAC isoflurane. In addition, we examined the effect on the locomotive activity of conscious rats in order to exclude motor nerve dysfunction and sedation.

Methods

This investigation was conducted in accordance with a protocol approved by the Animal Care Committee of Gunma University. All of the experiments were performed randomized and blind.

Animal preparation

Male Wistar rats (280 to 320 g) were fitted with indwelling intrathecal catheters using methods described previously.¹⁶ In brief, the rats were mounted in a conventional stereotactic instrument under halothane anaesthesia. A midline scalp incision was made, extending from a line between the ears to a point 2 cm caudally, and the fascia and muscle were retracted from the skull and first vertebra. The atlanto-occipital membrane was then visualized. Indwelling intrathecal catheters were placed by passing a PE-10 catheter through an incision in the atlanto-occipital membrane to a position 8 cm caudal to the cisterna at the level of the lumbar enlargement. The catheter was externalized on the top of the skull, and the wound was closed with 3-0 silk sutures. We used only catheterized rats in which flaccid paralysis of the hindlimbs was observed after intrathecal administration of lidocaine 500 µg·10 µl⁻¹ on the day of surgery.¹⁷ Rats showing postoperative neurological deficits, inflammation, or weight loss were killed with a barbiturate overdose. Determination of isoflurane MAC was carried out four to nine days after the intrathecal implantation of the catheter.

The agents studied were: CP-96,345[(2*s*,3*s*)-cis-2(diphenylmethyl)-N-[2-methoxyphenyl]-methyl]-1-azabicyclo[2.2.2]octan-3-amine] courtesy of Pfizer, and SP (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂, Sigma). All the drugs were dissolved in normal saline, so that the final dose was administered in a volume of 10 µl. The animals were randomly assigned to one of six groups with six animals per group (Table I), including one control group and three SP

TABLE I Experimental design

	<i>M</i>		<i>M</i>		<i>M</i>		<i>M</i>	
saline group	saline	saline	saline	saline	saline	saline	saline	saline
saline-CP-SP group	saline	CP	1 µg	SP	1 µg*			
	saline	CP	10 µg	SP	10 µg*			
	saline	CP	100 µg	SP	100 µg*			
saline-SP group	saline	SP	1 µg*	SP	10 µg*	SP	100 µg*	
CP-SP group	CP 10 µg	SP	1 µg*	SP	10 µg*	SP	100 µg*	

M: Measurement of MAC. 20 min after administration of drug.

* Substance P was administered repeatedly just before measurement of MAC.

receptor antagonist groups; receiving either 1, 10, or 100 µg CP. After measurement of MAC following administration of the antagonist, a reversal dose of SP was administered, and the MAC measurement was repeated. In two other groups, rats were given either intrathecal saline or 10 µg of CP followed by increasing doses of SP (1, 10, and 100 µg). A reversal dose and then increasing doses of SP as required were administered just before each measurement of MAC.

Measurement of MAC

Anaesthesia was induced with halothane 4% in oxygen by placing the rats in an induction chamber for three to five minutes. After tracheostomy, the trachea was intubated with a 14-G polyethylene catheter. The rats breathed spontaneously through a non-rebreathing circuit. Then we used isoflurane instead of halothane, and the oxygen supply to the isoflurane vaporizer was kept at 2 L·min⁻¹ throughout the experiments. A polyethylene catheter (PE-50) was inserted through the wall of the ventilatory tubing, and the tip of the sampling tube was positioned distal to the tracheostomy. This arrangement permitted sampling of end-tidal gases and regulation of end-tidal CO₂ by management of the oxygen escape valve. For blood pressure monitoring and blood sampling, one femoral artery was catheterized with a polyethylene catheter (PE-50). Rectal temperature was maintained between 36.5 and 37.5°C by the use of a heating pad and thermometer system (CMA150, Microdialysis AB, Stockholm, Sweden). The end-tidal concentration of isoflurane was measured with a calibrated infrared absorption instrument (ULTIMA, Datex Co., Helsinki, Finland).¹⁸ The analyzer was warmed up for at least 90 min before use and was calibrated with a standard calibration gas mixture.

The MAC of isoflurane was determined according to the up-and-down method using the tail clamp technique.¹⁹ The lungs of each animal were ventilated initially for 20 min with isoflurane 1.5%. First, we injected saline to determine the standard MAC. A 20-cm rubber-shod haemostat was then applied with full ratcheted lock to the mid-portion of the rat's tail. The haemostat was applied continuously for 60 sec and then was removed. Any purposeful movement of the animal constituted a positive response, such as the movement of extremities, tail flick, or twitching. Non-purposeful movements, such as stiffening, grimacing, and changing of respiratory patterns were disregarded. The isoflurane concentration was then adjusted by 10%, and isoflurane was administered for 20 min at the new concentration before application of the

haemostat was repeated. The subsequent position of the tail clamp was proximal to any previous site of application. The MAC was defined as the mean of the lowest concentration at which a negative response was observed plus the highest concentration at which a positive response was observed. After measurement of control values, CP was infused intrathecally by a blinded investigator. Following measurement of MAC, SP was administered, and MAC was redetermined. Reversal doses of SP were administered at 20 min intervals just before measurement of MAC. We performed a blood gas sampling prior to each tail clamp.

Test of locomotor function

The rats which received this test were different from those for the determination of isoflurane MAC. The rats were examined for the presence of locomotor dysfunction using the following methods.²⁰ A blind investigator injected either saline, the NK-1 receptor antagonist, and/or SP in the manner described above in seven groups of five rats each. We then tested the 1) Placing reflex: The rat was held with the hindlimbs slightly lower than the forelimbs, and the dorsal surface of the hind-paws was brought into contact with the edge of a table. The experimenter recorded whether the hind-paws were placed on the table surface reflexively; 2) Grasping reflex: The rat was placed on a wire grid and the experimenter recorded whether the hind-paws grasped the wire upon contact; 3) Righting reflex: The rat was placed on its back on a flat surface and the experimenter noted whether it immediately assumed the normal upright position. Scores for placing, grasping, and righting reflexes were based on counts of each normal reflex exhibited in five trials.

Statistical analysis

Statistical comparisons were made by ANOVA, and the Bonferroni method was applied for multiple comparisons. Wilcoxon's signed rank sum test was performed on the MAC values within each group. Level of statistical significance was defined as $P < 0.05$. The values were expressed as mean $\pm$ SEM.

Results

The physiological variables were well controlled within the normal range (Table II). There were no differences in blood pressure, heart rate, blood gases, haemoglobin concentration, or body temperature among the groups. Table III and the Figure show the changes in the MAC of isoflurane. There were no differences among the four groups with respect to baseline MAC values. At a CP dose of 1 µg, two of six rats

TABLE II Mean (SEM) changes in physiological variable due to intrathecal administration of CP 96,345 and substance P following administration of CP 96,345.

	saline n=6	CP96,345 1 µg n=6	substance P 1 µg n=6	CP96,345 10 µg n=6	substance P 10 µg n=6	CP96,345 100 µg n=6	substance P 100 µg n=6
MAP (mmHg)	108 (5)	111 (8)	114 (4)	105 (9)	114 (8)	104 (4)	117 (7)
Heart rate (bpm)	353 (22)	373 (9)	387 (30)	380 (9)	417 (14)	364 (12)	415 (14)
PaO ₂ (mmHg)	380 (20)	322 (28)	372 (22)	360 (17)	392 (33)	382 (28)	395 (25)
PaCO ₂ (mmHg)	37.5 (0.9)	38.9 (1.4)	38.4 (0.5)	38.0 (0.9)	38.7 (1.2)	38.3 (0.9)	37.2 (0.9)
pH	7.40 (0.02)	7.40 (0.02)	7.41 (0.02)	7.39 (0.01)	7.39 (0.010)	7.43 (0.01)	7.40 (0.01)
HCO ₃ ⁻ (meq · L ⁻¹)	23.5 (0.5)	23.1 (0.7)	23.1 (0.7)	23.2 (0.8)	23.3 (0.7)	23.1 (0.7)	23.3 (0.8)
Body temperature (°C)	37.1 (0.1)	37.0 (0.1)	37.0 (0.1)	37.0 (0.2)	36.9 (0.2)	36.9 (0.1)	36.9 (0.1)
Hb (g · dL ⁻¹)	13.6 (0.4)	14.0 (0.5)	13.8 (0.7)	13.9 (0.5)	13.9 (0.5)	14.4 (0.3)	13.9 (0.4)

TABLE III Minimum Alveolar Anaesthetic Concentration of isoflurane caused by intrathecal administration of saline, CP96,345 and substance P. n=6

saline	+saline	+saline
1.45% (0.03)	1.45% (0.02)	1.43% (0.02)
saline	+CP96,345 1 µg	+substance P 1 µg
1.43% (0.04)	1.40% (0.05)	1.42% (0.03)
saline	+CP96,345 10 µg	+substance P 10 µg
1.43% (0.04)*	1.3% (0.02)*	1.37% (0.02)
saline	+CP96,345 100 µg	+substance P 100 µg
1.45% (0.04)*	1.23% (0.03)*†	1.38% (0.02)†

Results are given as Mean ± SEM; n=6

* $P < 0.05$ saline vs CP96,345.

† $P < 0.05$ CP96,345 vs CP96,345 + substance P.

showed a decrease in the MAC; at CP-96,345 doses of 10 µg and 100 µg, all the rats had a decrease in the MAC of isoflurane. CP at 10 µg and 100 µg, produced decreases in the MAC of isoflurane ranging from 9.9% to 15.3% of control values, respectively ($P < 0.05$). Administration of SP reversed the decrease in the MAC of isoflurane at 100 µg CP. The dose of SP (100 µg) reversed the MAC of isoflurane at the 10 µg, CP dose (Table IV). The effects of the agents on locomotive function tests are shown in Table V. There were no differences between the groups. Convulsions and hypermobility were not observed in association with the combination of CP plus SP.

Discussion

The non-peptide compound, CP, has been introduced as a potent and selective antagonist of the NK-1 receptor for use in experimental studies of the action of SP.¹³ Nociceptive responses to intraplantar formalin are suppressed in rats pretreated with CP, and the effect of CP is of long duration.¹⁴ The administered dose was determined according to antinociceptive effects observed in

FIGURE Minimum alveolar anaesthetic concentration of isoflurane due to intrathecal administration of saline, CP96,345, and substance P.

animal experiments.¹⁴ The reversal dose of SP was based on previous reports in which SP inhibited contraction of isolated ureter in a CP-containing solution¹⁵ and on CP antagonism SP-induced relaxation of isolated dog carotid artery previously contracted by exposure to nor-epinephrine.²¹ Intrathecal administration of SP increases activity of neurons in the spinal cord, but this activation of neurons decreases or disappears some 20 min later.²² As a peptide, SP is rapidly metabolized, and inactivated by angiotensin-converting enzyme, neural endopeptidase-24,11, and aminopeptidase M.²³ It was necessary to inject the reversal dose of SP repeatedly just before measurement of MAC in this study.

The present study demonstrated that administration of a NK-1 receptor antagonist, CP at 10 µg and 100 µg, decreased MAC isoflurane, and that the changes were reversed by the administration of SP. In this study, physiological variables were well controlled,

TABLE IV Minimum Alveolar Anaesthetic Concentration with isoflurane for intrathecal doses of substance P with saline or CP96,345, 10 µg

saline	saline + substance P 1 µg	saline + substance P 10 µg	saline + substance P 100 µg
1.45% (0.03)	1.45% (0.03)	1.47% (0.03)	1.50% (0.02)
CP96,345 10 µg	CP96,345 10 µg + substance P 1 µg	CP96,345 µg + substance P 10 µg	CP96,345 10 µg + substance P 100 µg
1.30% (0.02)*	1.32% (0.02)	1.37% (0.02)	1.42% (0.03)*

Results are given as Mean ± SEM; n=6

* $P < 0.05$ CP96,345 10 µg *vs* CP96,345 10 µg + substance P 100 µg

TABLE V Locomotion test score

Drug	Placing	grasping	righting
Saline	5 (0)	5 (0)	5 (0)
CP96,345 1 µg	5 (0)	5 (0)	5 (0)
CP96,345 10 µg	5 (0)	5 (0)	5 (0)
CP96,345 100 µg	4.8 (0.02)	4.6 (0.02)	5 (0)
CP96,345 1 µg + substance P 1 µg	5 (0)	5 (0)	5 (0)
CP96,345 10 µg + substance P 10 µg	5 (0)	5 (0)	5 (0)
CP96,345 100 µg + substance P 100 µg	5 (0)	5 (0)	5 (0)

Results are given as Mean ± SEM for 5 trials n=5

and the body temperature remained stable.²⁴ We also regulated the PaCO₂ by managing the escape flow; so that the PaCO₂ remained stable even when isoflurane was at high concentration. Therefore, it is presumed that the NK-1 receptor is responsible, at least in part, in determining the potency of inhalational anaesthetics.

It has been shown that the release of SP from primary afferents is not suppressed by inhalational anaesthetics. Go *et al.*⁶ have reported that SP was released in spinal cord by noxious stimulation during halothane anaesthesia. Moreover, a specific excitatory effect of SP on spinal nociceptive transmission has been demonstrated.¹² In the present study, a single administration of SP did not increase the MAC of isoflurane. We administered the doses which induce nociception in conscious rats.¹² Had we used much higher doses, MAC might have been increased.

Long-duration spinal cord responses have been measured intracellularly,²⁵ and these studies have a fast component with a 100 msec peak time, which is highly sensitive to NMDA receptor antagonist and to a lesser degree to an NK-1 receptor antagonist.²⁶ This component can be evoked by a strong stimulus enough to recruit an Aδ or C fibres. A slow component lasting 15 to 20 sec is sensitive predominately to an NK-1 receptor antagonist and is produced by stimulation of C fibres.²⁷ In the present study, measurement of MAC was performed by tail pinch which stimulates the Aδ and C fibres.²⁸ Activation of the Aδ

and C fibres results in the release of an excitatory amino acid and SP. In addition, an interaction between SP and excitatory amino acids in central nociceptive processing has been suggested.^{29–31} Substance P produces prolonged enhancement of the responses of dorsal horn neurons to iontophoretically applied glutamate or NMDA.²⁹ Combined treatment with SP and NMDA produces a profound enhancement of the responses of dorsal horn neurons to noxious stimulation, as well as the behavioral responses to noxious stimulation. Moreover, SP receptors have been found in dorsal horn, and SP increases the strength of excitatory amino acid-mediated primary afferents in the rat spinal dorsal horn.³⁰ Furthermore, the excitory effects of SP on the responses to NMDA were uniformly reversed by the NK-1 receptor antagonist, CP.³¹ Therefore, we suspected that the NK-1 receptor antagonist inhibits the enhancing effect of SP on the response to the NMDA receptor activation. We believe that intrathecal administration of the NK-1 receptor antagonist CP may act by postsynaptic inhibition of the binding site of the SP that is released by primary afferents in the spinal dorsal horn, thereby inhibiting the enhancement of SP on the NMDA receptor, and reducing the MAC of isoflurane.

We have previously reported that intrathecal administration of the competitive NMDA receptor antagonists APV(D-2-amino-5-phosphopentaoate), CPP(3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid), the non-

competitive antagonist MK801(dizocilpine) and antagonist at the glycine site on the NMDA receptor 7-chlorokynurenic acid, reduced MAC isoflurane.^{4,5} The degree of reduction in MAC with the NK-1 receptor antagonist was smaller than that obtained with the intrathecal administration of NMDA receptor antagonists. Stimulation of A δ and C fibres activates the NMDA receptor, thus it is not surprising that NMDA receptor antagonists reduce MAC isoflurane to a greater degree than NK-1 receptor antagonists. Moreover, NMDA receptor antagonists inhibited the movement immediately following tail pinch in the previous study, whereas in the present study, the NK-1 receptor antagonist inhibited the movement during the tail pinch. It is considered that the fast component consisting of NMDA receptor has more important role than the slow component consisting of NK-1 receptor in determining the MAC of isoflurane.

Substance P could not completely reverse MAC isoflurane following CP administration. The dissociation constant (K_d) of CP is 0.22 nM, and that of SP is 1.89 nM.²¹ Based on relative affinity for the NK-1 receptor, CP is more potent than SP, so SP could not completely reverse its effect. In addition, CP has an antinociceptive action, which is partially due to calcium channel blockade. Schmidt *et al.*³² have reported that CP acts as an antagonist at Ca²⁺ channels in addition to being a potent NK-1 receptor antagonist. Verapamil inhibits carrageenin-induced hyperalgesia as well as formalin-induced hyperalgesia.³³ We suspect that the incomplete reversal by SP reflect both the difference in affinity for the NK-1 receptor and Ca²⁺ channel block. One additional difficulty in this study is that CP is non-peptide and long-acting, while SP is a peptide which is short-acting. In our preliminary study, CP at 10 and 100 μ g could decrease MAC isoflurane, but the decrease in MAC caused by CP did not reverse reliably with a single administration of SP. We were able to reverse change in MAC by repeated administration of SP. Details of the incomplete reversal remain unknown.

In a previous study, intrathecal administration of CP at 400 μ g caused motor dysfunction in conscious rats.¹⁴ The motor dysfunction manifested as hindlimb ataxia, diminished placing, and stepping responses and weakness of the flexion response to traction placed on the paw. We examined the locomotive activity following intrathecal administration of CP at 1, 10, and 100 μ g, and it was normal. Hypermobility was not induced by treatment with SP following the administration of CP.

A new drug of NK-1 receptor antagonist is under development.^{34,35} This experiment suggests that NK-1 receptor antagonists decrease the need for inhalation-

al anaesthetics. Further investigation is necessary to define the role of the NK-1 receptor in determining the MAC of inhalational anaesthetics.

In summary, the MAC of isoflurane was depressed by intrathecal administration of the NK-1 receptor antagonist CP. Intrathecal administration of SP reversed the decreases in MAC. Locomotive activity was not affected.

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