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Possible Involvement of the Spinal Substance P System in Pilocarpine-Induced Scratching in Mice

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SAKURADA, T., Y. MANOME, K. TAN-NO, Y. MATSUNAGA, S. SAKURADA AND K. KISARA. *Possible involvement of the spinal substance P system in pilocarpine-induced scratching in mice.* PHARMACOL BIOCHEM BEHAV **44**(2) 439–445, 1993.—IT administration of pilocarpine in the spinal subarachnoid space of mice produced a dose-related hindlimb scratching. When coadministered with substance P IT, the pilocarpine-induced scratches were enhanced by high doses of substance P but not by subthreshold doses. This characteristic behavioral response was inhibited dose dependently by IT coadministration of spantide [D-Arg[1], D-Trp[7,9], Leu[11]] substance P. Significant antagonistic effects of [D-Phe[7], D-His[9]] substance P (6-11), a selective antagonist for substance P receptors, and substance P (1-7), a substance P *N*-terminal fragment, were observed against the pilocarpine-induced scratching. Pretreatment with substance P antiserum resulted in the reduction of the response to pilocarpine. When coadministered IT with pilocarpine, atropine potently inhibited pilocarpine-induced scratching. These results demonstrate that not only muscarinic receptors but also substance P-containing neurons in the mouse spinal cord may be involved in elicitation of the scratching behavior following IT injection of pilocarpine.

Pilocarpine Reciprocal hindlimb scratching Intrathecal injection Substance P antagonist Substance P antiserum Substance P (1-7) Mouse

THERE is strong evidence implicating substance P as a potential neurotransmitter of nociceptive primary neurons. The undecapeptide is mostly contained in nerve terminals of small-diameter primary afferents (4) and excites preferentially nociceptive neurons of the dorsal horn of the spinal cord (14). When injected IT into the lumbar region of the mouse or rat, substance P elicits a characteristic behavioral response consisting of reciprocal hindlimb scratching, licking, and biting reactions (7,10).

It has been reported that peripherally administered pilocarpine led to a variety of pharmacological actions in animals. For example, pilocarpine at relatively small doses induced yawning (3,28) and at large doses catalepsy and seizures (2,27,35). A number of articles indicate that systemically administered cholinergic agonists could change the response to noxious stimuli. Muscarinic agonists given IT to animals resulted in a dose-dependent increase in the thermal nociceptive response latency (36). Recently, IT-administered pilocarpine has also been shown to produce a severe reciprocal hindlimb scratching response (23). The scratching response induced by pilocarpine is reversed by coadministration of atropine (23), suggesting that pilocarpine-induced scratching is mediated spinally through muscarinic receptors. The duration of pilocarpine-induced scratching was longer than that of the substance P-induced behavioral response, which disappeared 5 min following IT injection of substance P. Hence, to see the involvement of neurons containing substance P in the production of pilocarpine-induced scratching the effects of substance P analogs, substance P (1-7), and antiserum against substance P were examined on the hindlimb scratching elicited by IT administration of pilocarpine to mice.

Interactions of substance P and cholinergic neurons have been reported previously. For example, substance P can be hydrolyzed by acetylcholinesterase (1). The coexistence of substance P with acetylcholine was also found in the ascending cholinergic reticular system (31). Substance P evokes the release of acetylcholine from the isolated spinal cord of the newborn rat and somatic cholinergic nerve endings (9,32).

The purpose of this study was to determine whether substance P-containing afferent systems in the spinal cord are involved in pilocarpine-induced scratching in mice.

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METHOD

Animals

Male ddY mice (Shizuoka Laboratory Center, Japan) weighing 22–27 g were used in these experiments. Animals were housed in groups of thirty with food and water available ad lib. Behavioral experiments were carried out in a quiet temperature- and humidity-controlled room (23°C, 50–60% relative humidity) in which a 12 L : 12 D cycle was maintained (light 9:00 a.m.–9:00 p.m.).

Injection Procedure

The IT injection procedure was adapted from the method described by Hylden and Wilcox (6). The needle was inserted between lumbar 5 and lumbar 6 in unanesthetized mice, and drugs were given in a volume of 5 μl. When spantide, [D-Phe^7, D-His^9] substance P (6-11), substance P (1-7), and substance P were tested, they were coinjected with pilocarpine in a total volume of 5 μl. In the experiment with substance P antiserum, mice received two separate IT injections, each a volume of 5 μl. A slight flick of the tail was used as an indication that the needle had penetrated the dura.

Behavioral Observation

One hour prior to IT injection, animals were adapted to an individual plastic cage (22.0 × 15.0 × 12.5 cm) that served as the observation chamber. Immediately following IT injection of pilocarpine, each mouse was replaced into this cage and behavioral testing was begun. Scratching behavior was defined as a back and forth movement of the hindlimb, making contact with the animal's flank. Animals were determined to be either responders or nonresponders. When the mouse displayed hindlimb scratching behavior, the observer started to count the number of reciprocal hindlimb scratches.

Drugs

The commercial drugs used were: pilocarpine (Hoei Chemical Co., Osaka, Japan), atropine sulphate (Nichidoku Chemical Co., Japan), substance P, spantide (Peptide Institute, Osaka, Japan), and substance P (1-7) (Peninsula Laboratories, Inc., Belmont, CA). [D-Phe^7, D-His^9] substance P (6-11) was synthesized by solid-phase peptide methodology (20). All substances were dissolved in sterile artificial cerebrospinal fluid (CSF) reported elsewhere (16). The doses of spantide, [D-Phe^7, D-His^9] substance P (6-11), and substance P (1-7) were chosen on the basis of their potency as antagonists of substance P-induced licking, biting, and scratching (19,20). Substance P antiserum for IT injection was obtained from rabbits by repeated intradermal injection of substance P coupled to bovine serum albumin by glutaraldehyde. The substance P antiserum was diluted in artificial CSF and injected IT 5 min prior to IT injection of pilocarpine. The K_d value of substance P antiserum (titer 1: 100,000) was 1×10^{-10} M. The cross-reaction was 10% for eledoisin, 9.0% for physalaemin, 8.0% for neurokinin (NK) B, 6.0% for substance P (6-11), and 4.0% for NK A. Substance P (1-7), Met-enkephalin, Leu-enkephalin, and β-endorphin showed less than 0.1% cross-reaction.

Data Analysis

The results are expressed as the percentage of mice that demonstrated hindlimb scratching and the number of hindlimb scratches within the 5-min observation period, except in the time course experiment. The ID_{50} (dose of antagonist that inhibited the number of pilocarpine-induced scratches by 50%) was determined from linear regression analysis of probit plots. Two different statistical tests were applied to the data: Fisher's exact test was used for the number of mice in each treatment group that displayed reciprocal hindlimb scratching and the Mann–Whitney *U*-test was applied to the number of scratches. For all statistical tests, effects were considered significant at $p < 0.05$. All results are given as means ± SEM.

RESULTS

Pilocarpine-Induced Scratching

When injected IT into mice, pilocarpine elicited a behavioral response predominantly consisting of reciprocal hindlimb scratching toward the flanks. The incidence of scratching was much higher than that of biting or licking. Mice displayed dose-dependent scratching over the range of pilocarpine doses between 1.25–10.0 nmol (0.31–2.45 μg). These effects were dose dependent when calculated both from the percentage of mice displaying hindlimb scratching and from the number of scratches during the 5-min observation period (Fig. 1). The induced behavior was rapid in onset (approximately within 30 s after injection) and lasted for at least 15 min with a dose of 10.0 nmol. The frequency of the induced behavior declined gradually until 15 min after IT injection of pilocarpine. IT coadministration of atropine (5.0–10.0 pmol, equivalent to 3.5–6.9 ng) significantly reduced pilocarpine-induced scratching in a dose-dependent manner (data not shown).

Substance P, when injected IT at doses of 6.25–50.0 pmol (10.1–80.9 ng), elicited behaviors with hindlimb scratching directed at the flank predominating (Table 1). Coadministered with pilocarpine (2.5 nmol), high doses (25.0 and 50.0 pmol) of substance P elevated the response to pilocarpine but subthreshold doses (6.25 and 12.5 pmol) of substance P were ineffective.

Inhibition of Pilocarpine-Induced Scratching by Substance P Analogs, Substance P (1-7), and Antiserum Against Substance P

As shown in Fig. 2A, spantide (1.0–2.0 nmol), coadministered with pilocarpine (10.0 nmol), produced a dose-related inhibition of pilocarpine-induced scratching. Coadministered with pilocarpine (10.0 nmol), [D-Phe^7, D-His^9] substance P (6-11) (0.5–2.0 nmol) also produced a dose-related inhibition of the induced scratching (Fig. 2B). The pilocarpine-induced scratching was dose dependently decreased by small doses of substance P (1-7) (0.5–2.0 pmol) (Fig. 2C). The ID_{50} values for spantide, [D-Phe^7, D-His^9] substance P (6-11), and substance P (1-7) were 1.6 (1.3–1.9) nmol, 1.6 (0.9–2.7) nmol, and 1.0 (0.5–2.2) pmol, respectively. As shown in Table 2, mice receiving only [D-Phe^7, D-His^9] substance P (6-11) or substance P (1-7) in the absence of pilocarpine scarcely displayed hindlimb scratching. Spantide induced a few one-sided scratches and IT injection of artificial CSF elicited no scratching at all.

Pretreatment with antiserum against substance P reliably reduced pilocarpine-induced scratching in a dilution-related manner (Table 3). IT injection of pilocarpine into mice pretreated with IT CSF produced hindlimb scratching to almost the same degree as a single injection of pilocarpine. IT injection of antiserum against substance P elicited no scratching.

DISCUSSION

The primary finding of this study was that substance P-containing afferent systems in the spinal cord were partially

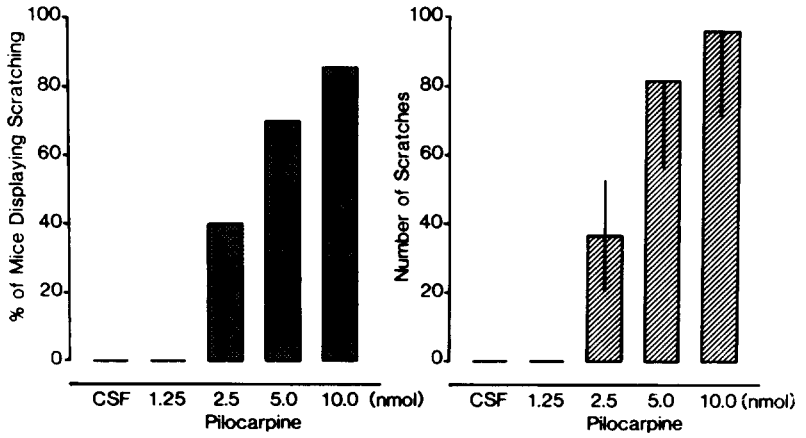

FIG. 1. Dose-related scratching behavior induced by intrathecal administration of pilocarpine in mice. Pilocarpine was administered IT and mice were observed over a 5-min period starting immediately after injection. The percentage of animals that displayed reciprocal hindlimb scratching and the number of scratches are indicated. The data are mean ± SEM from 10–20 mice in each group. CSF, cerebrospinal fluid.

involved in induction of the scratching response following IT injection of pilocarpine.

Previous studies revealed that substance P, when injected IT into mice in picomole amounts, can elicit a behavioral syndrome indicative of a nociceptive behavioral response such as scratching, biting, and licking (7,11,16,17,20,26). In the present study, subthreshold doses of substance P (6.25 and 12.5 pmol) failed to elevate pilocarpine-induced scratching. Inferring from fmol amounts of spontaneously released substance P in the spinal cord (37), the data suggest that the released substance P may not interact synergically with pilocarpine at a postsynaptic site. Substance P in higher doses (25.0 and 50.0 pmol) than subthreshold doses resulted in an elevation of pilocarpine-induced scratching. This behavioral observation suggests that there may exist an interaction between substance P and muscarinic receptors in the spinal cord, resulting in an elevation of the behavioral response. The substance P-induced behavioral response was found to be antago-

TABLE 1

EFFECT OF SUBSTANCE P ON PILOCARPINE-INDUCED SCRATCHING IN MICE

Treatment	Dose	Incidence (%)	Number of Scratches
Pilocarpine	1.25 nmol	0	0
	2.5 nmol	40	37.3 ± 17.0
Substance P	6.25 pmol	30	0.5 ± 0.7
	12.5 pmol	20	0.8 ± 0.7
	25.0 pmol	30	3.5 ± 1.9
	50.0 pmol	80	15.9 ± 3.3
Pilocarpine + Substance P	2.5 nmol + 6.25 pmol	50	44.4 ± 17.1
Pilocarpine + Substance P	2.5 nmol + 12.5 pmol	40	27.4 ± 13.2
Pilocarpine + Substance P	2.5 nmol + 25.0 pmol	80	62.2 ± 13.7*
Pilocarpine + Substance P	2.5 nmol + 50.0 pmol	70	72.6 ± 18.9*

Pilocarpine in combination with substance P was coadministered IT in a total volume of 5 μl. Scratching behavior was observed over a 5-min period starting immediately after IT injection. The data are mean ± SEM from 10 mice in each group.

*$p < 0.01$ compared to pilocarpine (2.5 nmol) alone.

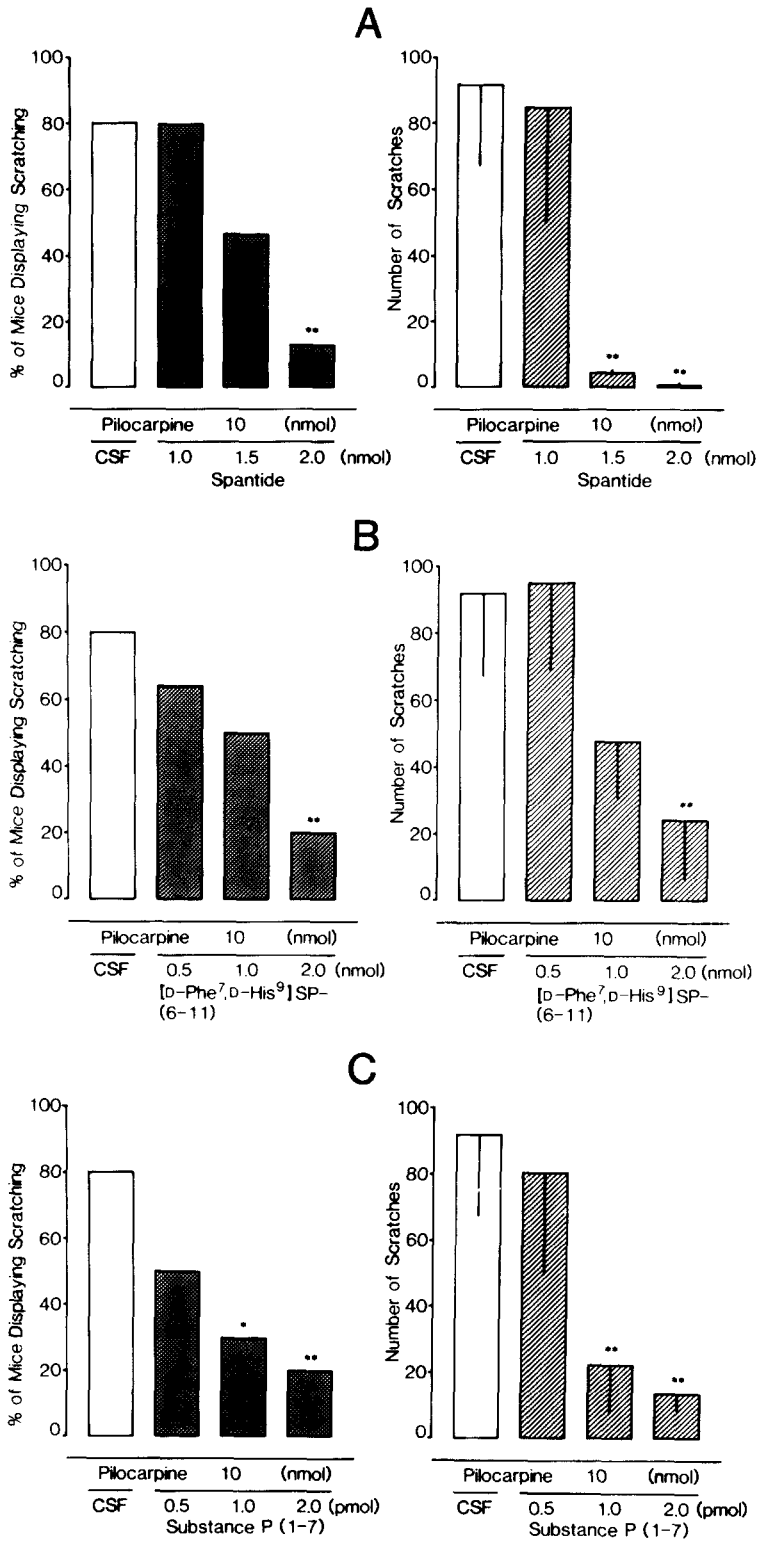

FIG. 2. Antagonistic effects in mice of spantide (A), [D-Phe^7, D-His^9] substance P (6–11) (B), and substance P (1–7) (C) on the scratching behavior induced by IT administration of pilocarpine. Each peptide was coadministered IT with pilocarpine in a total volume of 5 μl. Scratching behavior was observed over a 5-min period starting immediately after IT injection. The data are mean ± SEM from 8–12 mice in each group. $*p < 0.05$, $**p < 0.01$, significant difference from corresponding cerebrospinal fluid (CSF) control. All statistics are given in the text.

TABLE 2

BEHAVIORAL EFFECT OF SPANTIDE, [D-Phe^7, D-His^9] SUBSTANCE P (6-11), AND SUBSTANCE P (1-7)

Peptides	Dose	Number of Scratches
CSF control		0
Spantide	1.0 nmol	2.4 ± 1.7
	2.0 nmol	1.6 ± 0.5
[D-Phe^7, D-His^9] substance P (6-11)	0.5 nmol	0.1 ± 0.1
	2.0 nmol	0.6 ± 0.3
Substance P (1-7)	0.5 pmol	0.6 ± 0.6
	2.0 pmol	0.1 ± 0.1

The peptides were administered IT without coadministration of pilocarpine and mice were observed over a 5-min period starting immediately after injection. The doses of spantide, [D-Phe^7, D-His^9] substance P (6-11), and substance P (1-7) were chosen from the experiment of coadministration with pilocarpine. The data are mean ± SEM from 10 mice in each group.

nized by a number of substance P (1-11) or (6-11) analogs and a substance P *N*-terminal fragment, substance P (1-7) or (1-8) (16,19,20,29,30). [D-Pro^2, D-Trp^{7,9}] substance P has been found to block the pilocarpine-induced behavioral response (23). It should be mentioned, however, that [D-Pro^2, D-Trp^{7,9}] substance P may have nonspecific actions on the spinally mediated response (5,8,15). Spantide has often been used as a tachykinin antagonist, although the specificity of this peptide has been questionable (22,25,34). In the behavioral assay, IT administration of spantide resulted in a significant reduction of the behavioral response produced by not only NK-1 receptor agonists (substance P, physalaemin, and septide) but also by the other tachykinins, including eledoisin and NK A, or somatostatin and NMDA (18,20). However, it was found that higher doses of spantide were needed to exert an antagonistic action against NK A than against substance P (20,21). In the present investigation, pilocarpine-induced scratching was significantly reduced by coadministration of spantide with an ID_{50} of 1.6 (1.3-1.9) nmol. Previously, we reported that spantide was equally effective in the substance P behavioral assay; the ID_{50} for inhibition of 0.1 nmol substance P was 1.0 (0.51-2.00) nmol (20).

We recently demonstrated that a novel antagonist of substance P, [D-Phe^7, D-His^9] substance P (6-11), selectively inhibited the substance P-induced behavioral response without affecting the other NK-1 receptor agonists (physalaemin and septide), an NK-2 agonist (NK A), an NK-3 agonist (eledoisin), and non-NK peptides such as somatostatin, bombesin, and NMDA (18,20,21). The use of this hexapeptide antagonist led us to speculate that two different subtypes of the NK-1 receptor may exist in the mouse spinal cord (21) because this antagonist has an ability to discriminate substance P- and the other NK-1 receptor-mediated actions. Of particular interest in the present study is that [D-Phe^7, D-His^9] substance P (6-11), coadministered IT with pilocarpine, was effective in inhibiting the pilocarpine-induced scratching. Based upon this finding,

TABLE 3

EFFECT OF PRETREATMENT WITH SUBSTANCE P ANTISERUM ON PILOCARPINE-INDUCED SCRATCHING IN MICE

Intrathecal Injection				
First	Second	Number of Mice	Incidence (%)	Number of Scratches
None	Pilocarpine	20	80	94.6 ± 20.6
CSF	Pilocarpine	20	80	98.9 ± 18.0
Substance P antiserum 1:8,192	Pilocarpine	10	80	96.5 ± 23.1
Substance P antiserum 1:512	Pilocarpine	10	50	59.1 ± 32.2
Substance P antiserum 1:32	Pilocarpine	10	20*	38.5 ± 25.7†

The antiserum against substance P was preinjected IT 5 min prior to IT injection of pilocarpine (10.0 nmol). First and second injections were done in a 5-μl volume, separately. Hindlimb scratching behavior was observed over a 5-min period starting immediately after the second IT injection. The data are mean ± SEM from 10 mice in each group.

*$p < 0.05$ compared to pilocarpine alone and CSF plus pilocarpine-treated group (Fisher's exact test).

†$p < 0.05$ compared to pilocarpine alone and CSF plus pilocarpine-treated group (Mann-Whitney *U*-test).

it seems reasonable to presume that the hindlimb scratching elicited by pilocarpine may be partially due to activation of neurons containing substance P in the spinal cord.

Recently, we found that a substance P *N*-terminal fragment, substance P (1-7), in picomole amounts significantly antagonized the scratching, biting, and licking responses induced by NK-1 receptor agonists, substance P (100.0 pmol), physalaemin (2.0 pmol), and septide (5.0 pmol) (19). The behavioral antagonism produced by the substance P *N*-terminal fragment was also found to be limited to the NK-1 receptor at the spinal cord level. It seems that enzymatically generated *N*-terminal fragments, in particular substance P (1-7), may act as inhibitors of substance P. Substance P (1-7) also antagonized pilocarpine-induced response to almost the same degree as the response to substance P (19). Together, these data support the concept that pilocarpine may indirectly interact with the NK-1 receptor, with which substance P acts endogenously as an agonist. The results presented with the substance P analogs and the *N*-terminal fragment suggest that IT-administered pilocarpine may stimulate substance P receptors indirectly, possibly through activation of the function of neurons containing substance P in the spinal cord. This suggestion is supported by the present data that pretreatment with the antiserum against substance P resulted in a significant reduction of the pilocarpine-induced hindlimb scratching. In addition, an interaction of substance P neurons and the cholinergic system has recently been suggested by the fact that substance P levels in the dorsal horn are reduced significantly by IT administration of carbachol, a direct-acting cholinergic agonist (24).

In agreement with previous works, the present study demonstrates that IT injection of pilocarpine elicited a dose-dependent reciprocal hindlimb scratching of the flanks that lasted for 15 min at a relatively large dose (10.0 nmol). We also confirmed that IT coadministration of atropine produced a potent inhibition of pilocarpine-induced scratching, suggesting that the induced response was mediated by activation of muscarinic receptors in the spinal cord. Of muscarinic receptor subtypes, the M_1 type receptor sensitive to pirenzepine, a selective M_1 antagonist, is involved in mediation of pilocarpine-induced scratching (12,13). On the other hand, peripherally administered pilocarpine and other muscarinic agonists can also produce antinociception. This antinociception seems to be mediated by muscarinic M_2 type receptors, which are pirenzepine insensitive (3). The autoradiographic and radioligand studies indicated that a high concentration of muscarinic receptors is located in the substantia gelatinosa of the dorsal horn (38), as well as the hippocampus and corpus striatum, in the rat (33). Based upon these previously reported results, it seems evident that the processing of sensory information is mediated by spinal muscarinic receptors. In addition, Scott et al. (23) also reported that the induced behavior elicited by pilocarpine was not changed by phentolamine, yohimbine, or naloxone. These data indicate that the spinal adrenergic and opioid mechanism may not be involved in mediating the pilocarpine-induced scratching.

In conclusion, the mechanism underlying the behavioral event of pilocarpine-induced hindlimb scratching appears to involve substance P-containing neurons in addition to muscarinic receptors in the spinal cord.

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