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ABSTRACT

((2S,3S)-(cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine) (CP-96,345) noncompetitively inhibits substance P (SP) binding at the neurokinin-1 (NK-1) site and has been widely used to determine the extent of NK-1 activity in nociception. To test the selectivity of this compound in vivo regarding other putative nociceptive transmitters, such as excitatory amino acids, we compared the actions of CP-96,345 to those of ((2R,3R)-(cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine), a less active isomer, on behavioral responses induced by SP, N-methyl-D-aspartate (NMDA) and kainic acid (KA) injected intrathecally in mice. When injected intrathecally, SP, NMDA or KA produce a caudally directed biting and scratching behavior that lasted for approximately 60 to 90 sec. At a dose as high as 2 nmol, CP-96,345 had no effect on responses induced by a single injection of 22.5 pmol of SP. In contrast, NMDA-induced behaviors were inhibited by CP-96,345 in a dose-related fashion beginning at a dose as low as 0.02 nmol. There was also an inhibitory effect of CP-96,345 on KA-induced activity that was not dose related. The more potent inhibitor of $[^3]$H SP binding, (+)-(2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine (CP-99,994), was approximately 10 times more potent in inhibiting NMDA-induced activity than CP-96,345. CP-99,994 also inhibited NMDA-induced activity at doses that failed to inhibit SP-induced behavior. Also attenuated by CP-96,345 was the development of sensitization to the behavioral effects produced by repeated injections of KA and desensitization to repeated injections of SP, phenomena linked to an action of the N-terminus of SP. NMDA-induced behaviors and sensitization to KA were found to be sensitive to verapamil, consistent with their mediation by calcium. These results indicate that either CP-96,345 and CP-99,994 do not inhibit NK-1-induced activity in the mouse spinal cord, or that exogenously administered SP does not induce behavioral responses by an interaction with NK-1 receptors. Whether CP-96,345 acts by a mechanism that involves inhibition of calcium channels and/or SP N-terminal activity requires further testing.

The peripheral and central roles of SP in nociceptive transmission have been extensively studied. SP and EAAs are coexpressed in small diameter primary afferent fibers (Battaglia and Rustioni, 1988; DeBiasi and Rustioni, 1988) and are thought to be involved in nociception (Cruwys et al., 1995; Levine et al., 1993; Meller et al., 1992; Mjellem-Jolly et al., 1992; Aanonsen and Wilcox, 1987; Hylden and Wilcox, 1981). SP has a high affinity for NK-1 receptors that are present in abundance in the superficial layers of the dorsal horn of the spinal cord (Liu et al., 1995; Mantyh et al., 1995). SP is released into the cerebrospinal fluid after stimulation known to depolarize primary afferent nociceptors (Levine et al., 1993). Activation of NK-1 sites by i.t. injection of SP results in a transient hyperalgesia (Radhakrishnan et al., 1995) although inhibition, using various SP antagonists, results in antinociception (Ohkubo et al., 1990).

When injected i.t., SP also produces a series of behaviors characterized by biting and scratching of the hindlimbs (Hylden and Wilcox, 1981). This behavior can also be elicited by administration of KA, NMDA and capsaicin (Sun and Larson, 1991), compounds thought to activate receptors involved

__ABBREVIATIONS:__ CP-96,344, ((2R,3R)-(cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine); CP-96,345, ((2S,3S)-(cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine); CP-99,994, (+)-(2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine; DPDT-SP, [o-Pro$^2$, o-Phe$^3$] substance P; EAA, excitatory amino acid; i.t., intrathecal; KA, kainic acid; MK-801, dizocilpine or (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; NK-1, neurokinin-1; NMDA, N-methyl-D-aspartate; POP, phenacyclidine; SP, substance P.
in nociception. Caudally directed biting and scratching behavior has been suggested to reflect activation of a variety of pathways including neurons involved in the transmission of pain resulting from a local, spinally mediated mechanism (Piercey et al., 1981). Although it is still unclear whether this behavior reflects pain perception, the number of these behaviors permits a reproducible and quantifiable measure of the degree of activation of receptor populations believed to be involved in nociception (Hornfeldt et al., 1994).

CP-96,345 has been found to be a potent inhibitor of SP binding (Snider et al., 1991). Although originally proposed to act as a competitive antagonist at NK-1 sites, CP-96,345 interacts with a site on the NK-1 receptor that has been found to be distinct from the binding site for the C-terminus of SP (Fong et al., 1992b). Because of the difference among species in the amino acid sequence at residues 116 and 290 of the NK-1 receptor, some species are significantly more or less susceptible to the antagonistic action of CP-96,345 at NK-1 sites relative to its nonselective ability to inhibit calcium channels (Fong et al., 1992a). The guinea pig is the most sensitive to CP-96,345, although the rat and mouse each have only a 10-fold difference in the concentration of CP-96,345 that inhibits SP binding and the concentration that produces a nonstereoselective inhibition of calcium channels (Schmidt et al., 1992). Whether CP-96,345 produces its effects in vivo via inhibition of SP or inhibition of calcium flux is dependent on the model tested.

Compared to CP-96,344, its 2R,3R enantiomer, CP-96,345 has been found to inhibit a variety of phenomena associated with nociceptive transmission, including late responses to noxious thermal stimulation and iontophoretically applied SP in the cat spinal cord (Radhakrishnan and Henry, 1991), SP-mediated slow excitatory postsynaptic potentials in cat dorsal horn neurons (De Koninck and Henry, 1991), late discharges of superficial dorsal horn neurons carrying nociceptive input in the rat spinal cord (Toda and Hayashi, 1993), SP-induced plasma extravasation in the guinea pig skin (Nagahisa et al., 1992), plasma extravasation induced by antidromic C-fiber stimulation in the rat hindpaw (Xu et al., 1992) and SP-induced excitation of locus ceruleus neurons in the guinea pig (McLean et al., 1991). CP-96,345 and CP-96,344 are equally effective in their inhibition of carrageenin-induced foot edema, carrageenin-induced hyperalgesia and the second phase response of the formalin-induced paw licking in the rat (Nagahisa et al., 1992), suggesting a possible involvement of calcium channel activity rather than inhibition of the NK-1 receptor.

Although the selectivity of CP-96,345 on NK-1 vs. NK-2 and NK-3 receptor binding has been well studied (McLean et al., 1991; Snider et al., 1991), its selectivity with respect to other receptors, including EAAs receptors, has not. The goal of our study was to examine the selectivity of the inhibitory effect of CP-96,345 on the behavioral response produced by i.t. injections of SP compared to that produced by NMDA and KA. The effect of CP-96,345 was then compared to that of CP-96,344 and verapamil, compounds that inhibit calcium channel activity with less or no affinity to inhibit NK-1 receptors, respectively. We also used CP-99,994, a newer and more potent NK-1 antagonist than CP-96,345, for comparison to the effects of CP-96,345.

Materials and Methods

Animals. Male Swiss-Webster mice (15–20 g, Sasco Inc., Omaha, NE) were housed four per cage in animal facilities with controlled temperature and 12-hr light and dark cycles. This facility is cleaned daily. Animals were allowed free access to food and water, allowed to acclimate for at least 24 hr before use and always used during the light period of the cycle. Animals were used strictly in accordance with the Guidelines of the University of Minnesota Animal Care and Use Committee and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication (NIH) 78-23, revised 1978].

Drug administration. Except where indicated, all injections were administered i.t. in mice at approximately the L5-L6 intervertebral space using a 30-gauge, 0.5-inch disposable needle on a 50-μl Luer tip Hamilton syringe (Fisher Scientific, Pittsburgh, PA). A volume of 5 μl was used for all i.t. injections as it is readily measured and appears to elicit sensitization to repeated injection of KA that is identical to that using 2 μl per injection (Sun and Larson, 1991). SP was administered in 0.85% NaCl containing 0.01 N acetic acid (pH 3.4) to minimize absorption to synthetic surfaces (Hall and Stewart, 1983). KA and NMDA were routinely administered in saline (pH 7.4). Doses of 25 pmol SP, 300 pmol NMDA and 22.5 pmol of KA were used as these doses were found to produce an intensity of behavioral responses that allows us to monitor either increases or decreases in the response. CP-96,345, CP-96,344, CP-99,994 and verapamil were diluted in normal saline (pH 7.4) and administered 30 min before injection of SP or EAAs, except where indicated.

Experimental protocol for behavioral responses. Immediately after i.t. injection of KA, NMDA or SP, animals were placed in a large glass cylinder containing approximately 2 cm of bedding. The total number of caudally directed bites and scratches over a 2-min interval were recorded. Injection of the same volume of vehicle over this time interval has been shown to elicit no increase in behaviors above noninjected control mice and to have no effect on the normal exploratory behavior of the mice (Sun and Larson, 1991). Four consecutive injections of KA and SP (or NMDA) were used in all experiments involving testing of sensitization and desensitization, respectively. After the first injection, behaviors were counted during a 2-min interval. At the end of the 2-min interval, the animal was repositioned and behaviors counted for 2 min. The same procedure was repeated for the third and fourth injections. Sensitization was defined as an increase in the number of behaviors measured during the 2-min interval after the fourth injection of a compound. Desensitization was defined as a decrease in the number of the biting and scratching behavior during the 2-min interval after the fourth injection.

Drugs. CP-96,345, CP-96,344 and CP-99,994 were generously provided by Pfizer Central Research (Groton, CT). SP was purchased from Peninsula Laboratories (Belmont, CA). KA, NMDA and verapamil were purchased from Sigma Chemical Company (St. Louis, MO).

Data analysis. Mean (± S.E.M.) of the data are presented in all figures. Throughout the experiments, each group represents at least six mice. Statistical analysis of the number of behaviors 2 min after the first and fourth injection was performed using analysis of variance followed by the Scheffé F test of significance using the Macintosh StatView version 1.02 software. P < .05 were used as the cut off to indicate a significant difference between the test group and control values.

Results

Injection of 0.002 to 2 nmol of CP-96,345 30 min before testing, failed to inhibit the number of caudally directed biting and scratching behaviors induced by a single injection of 22.5 pmol of SP (fig. 1A), despite the sensitivity of this
response to the inhibitory effect of 1 nmol of DPDT-SP when injected 5 min before SP (Mousseau et al., 1994). In contrast, pretreatment with CP-96,345 produced a dose-related inhibition of NMDA-induced behaviors (fig. 1C). KA-induced behaviors were uniformly but weakly inhibited by all doses of CP-96,345 tested (fig. 1B). The lack of a dose-relatedness suggests a nonspecific action on this non-NMDA-induced activity.

Whereas CP-96,345 failed to alter the number of behavioral responses to a single injection of SP, it prevented the development of desensitization to the behavioral effect of SP that results from repeated injections of SP. The effect of CP-96,345 was to attenuate, in a dose-related fashion, the normal decrease in the intensity of behaviors (i.e., behavioral desensitization) induced by each injection of SP as indicated by a dose-related decrease in the response to the fourth injection of SP (fig. 2). In addition, the increased number of responses to each of four injections of KA (i.e., behavioral sensitization) was inhibited by pretreatment with CP-96,345 over a dose-range similar to that preventing behavioral desensitization to SP (fig. 2).

To determine whether the effect of CP-96,345 was via its ability to inhibit calcium channels, we first examined the effect of CP-96,344, an isomer of CP-96,345, that is equal to CP-96,345 in its ability to inhibit calcium channels activity, but less potent than CP-96,345 in its ability to inhibit NK-1 activity. Our results indicate that CP-96,344 differed only slightly from CP-96,345, in its ability to inhibit the development of desensitization to SP (fig. 3A), sensitization to repeated injections of KA (fig. 3B) or to inhibit NMDA-induced behaviors (fig. 3C). When administered i.p., the response to each of these isomers did not differ from each other in their influence on responses to four injections of KA (fig. 4). The magnitude of the effects produced by 40 µg/kg of CP-96,345, which is approximately 1.6 to 2 nmol per mouse, was also very similar to the effect elicited by an i.t. injection of 2 nmol of CP-96,345 (fig. 3B).

To test the possibility that inhibition of calcium flux produces effects similar to the inhibitory effect of CP-96,345, we compared its action to that of verapamil, which has no ability to inhibit SP binding. Injected at a dose of 5 pmol 30 min before testing, verapamil did not inhibit the response to a single injection of SP or to repeated injections of SP (fig. 5A). However, verapamil inhibited the development of sensitization to repeated injections of KA, suggesting a calcium-sensitive component in this phenomenon (fig. 5B). Verapamil also inhibited the response to a single injection of NMDA,
which is known to be brought about by activation of a calcium channel (fig. 5C).

We also found that pretreatment with CP-99,994, a more potent nonpeptide NK-1 antagonist, failed to inhibit biting and scratching produced 30 min later by a single injection of 22.5 pmol SP (fig. 6A) but was effective in inhibiting NMDA-induced behaviors at that time (fig. 6B).

To determine if the previously reported inhibitory effect of CP-96,345 on SP-induced biting and scratching (Sakurada et al., 1994) may be due to a difference in the dose of SP or the experimental design, we also examined the effects of CP-96,345 using the same time of pretreatment, measurement interval and dose of SP as that used previously by Sakurada’s group. We coadministered 2 nmol CP-96,345 and 100 pmol SP i.t. and measured the number of behaviors over a period of 2 and 5 min. Although 2 nmol of CP-96,345 failed to affect the behavioral response to 22.5 pmol of SP (fig. 1A), this same dose of CP-96,345 was sufficient to inhibit the number of behaviors elicited over a 5-min interval by the higher dose of SP (fig. 7), consistent with previously reported studies of CP-96,345 (Sakurada et al., 1994). When the same data from the first 2-min interval are analyzed, inhibition of SP by CP-96,345 was not significant using our statistical criteria.

**Discussion**

The inability of CP-96,345 to inhibit caudally directed biting and scratching produced in mice by a single i.t. injection of SP presents an interesting paradox concerning the identity of receptors with which this compound interacts to elicit its effects in vivo. This is especially true as the doses of CP-96,345 tested readily inhibited other effects, including NMDA activity, desensitization to the behavioral effects of SP and sensitization to KA. If SP-induced behaviors are exclusively the result of NK-1 activation, CP-96,345 and CP-99,994 would be expected to inhibit the response to SP as they are potent inhibitors of [3H]SP binding (Snider et al., 1991). Our data suggest that either the response to a single injection of SP is not mediated by activation of NK-1 sites, or that CP-96,345 and CP-99,994 also bind, with an even greater affinity, to a site distinct from the NK-1 receptor to affect EAA activity.

Although it is possible that SP induces a behavioral response in mice that is not mediated by NK-1 activity (Matsumura et al., 1985), DPDT-SP, a D-analog of SP and a NK-1 antagonist, is able to inhibit the behavioral response produced by i.t. injections of SP (Mousseau et al., 1994). In addition, SP(5-11), a SP fragment that contains the tachykinin sequence homology necessary to interact with NK receptors, mimics the behavioral effect of SP. In fact, the ability of SP(5-11) to elicit biting and scratching behaviors is roughly...
equipotent to that of SP(1-11) (Igwe et al., 1990c). This is in contrast to their relative affinity at NK-1 binding sites in vitro where increasing the length of the amino terminal sequence from SP(5-11) to SP(1-11) enhances the affinity of the SP fragment for the NK-1 peptide binding site about 1000-fold (Cascieri et al., 1992). This indicates that the amino terminal portion of SP is essential for affinity as well as selectivity of binding. However, SP N-terminal fragments, such as SP(1-7) by themselves do not compete for binding at NK-1 sites and produce no behavioral response when injected i.t. (Sun and Larson, 1991). The discrepancy between the relative potency of SP(1-11) and SP(5-11) in vivo vs. their affinity for NK-1 binding may be due to inhibitory effects of SP N-terminal metabolites that are believed to accumulate in vivo (Nyberg et al., 1984; Sakurada et al., 1985) and interact at distinct binding sites (Igwe et al., 1990b). SP(1-11) may have a higher affinity for NK-1 binding than SP(5-11) in vitro, however, SP(1-11) may contribute to the generation of
a pool of amino terminal metabolites that are inhibitory to NK-1-induced activity in vivo.

Consistent with the absence of any possible inhibitory SP N-terminal activity associated with SP(5-11), there is no desensitization to the behavioral effects produced by repeated injections of SP(5-11). In contrast, desensitization develops readily to SP(1-11) (Igwe et al., 1990a; Moorchala and Sawynok, 1984). Thus, the inability of CP-96,345 to inhibit the response to a single injection of SP yet attenuate the development of desensitization to SP argues for a possible interaction of CP-96,345 with a site that is normally modulated by activity of the N-terminus of SP. Based on the poor ability of compounds such as SP(1-7) to compete for [3H]SP binding (Yukhananov and Larson, 1994), modulation of NK-1 activity by SP N-terminal fragments likely occurs by an indirect route involving a SP amino terminal binding site (Igwe et al., 1990b) that is distinct from the NK-1 receptor.

In support of the possibility that CP-96,345 inhibits SP N-terminal activity, CP-96,345 inhibits both SP desensitization and KA sensitization, responses that have been proposed to be mediated by SP N-terminal activity (Sun and Larson, 1991; Larson and Sun, 1992). Substance P N-terminal fragments have also been found to inhibit and potentiate NMDA-induced behaviors, depending on the time of administration relative to injection of NMDA (Hornfeldt et al., 1994). As with CP-96,345, D-SP(1-7), the D-analog of a commonly occurring SP metabolite, prevents behavioral desensitization to repeated injections of SP (Igwe et al., 1990c) and inhibits sensitization to KA (Larson and Sun, 1992) although SP N-terminal fragments, such as SP(1-7), inhibit SP-induced behaviors (Igwe et al., 1990c) and potentiate the response to KA (Larson and Sun, 1992). Thus, CP-96,345 and CP-99,994 may affect SP, KA and NMDA activity by interfering with the mechanism by which the N-terminus of SP normally elicits its modulatory effects.

Based on the ability of verapamil to inhibit NMDA-induced activity as well as behavioral sensitization to KA, these phenomena appear to be dependent on calcium channel activity. Binding studies suggest that there is only a 10-fold difference in the inhibition of NK-1 sites by CP-96,345 compared to nonselective calcium channel inhibition, as demonstrated by the use of CP-96,344. The difference between the effects of CP-96,345 and CP-96,344 on NMDA activity and on KA sensitization in our study and the sensitivity of these phenomena to verapamil suggest that the majority of the effect of CP-96,345 appears to result from inhibition of calcium channels. One possibility is that SP N-terminal binding sites are directly or indirectly linked to calcium channel activity. Although we found no inhibition of SP-induced behaviors, a racemic mixture of (+)-CP-96,345 has been previously found to inhibit responses elicited by an i.t. injection of 200 nmol of SP in mice (Lecce et al., 1991). Results using a racemic mixture and such a large dose of SP must be considered carefully. Because a behavioral response can be readily evoked by 25 pmol of SP, it is unclear why such a large and potentially nonspecific dose of SP (8000 times the dose used in our study) was used. Sakurada et al. (1994) have also achieved a partial inhibition of SP-induced behaviors in mice by coadministration of 2 nmol of CP-96,345 with 100 pmol of SP, a dose that is approximately four times greater than those used in our investigation. Although one would expect that lower doses of an agonist would be more readily antagonized than higher doses, when we repeated the experiments as done by Sakurada’s group, testing the effect of 2 nmol CP-96,345 coadministered with 100 pmol SP, CP-96,345 inhibited SP-induced behaviors evoked over a 5-min interval (fig. 7). Time of administration of CP-96,345 relative to challenge with SP was not the critical factor in the ability of this drug to inhibit the effect of 100 pmol of SP as coadministration of 2 nmol of CP-96,345 with 22.5 pmol of SP, rather than as a pretreatment, had no effect on SP-induced behaviors (fig. 7) and still inhibited NMDA-induced responses (data not shown). The ability of CP-96,345 to inhibit the effects of a high but not a low dose of SP suggests that CP-96,345 may have inhibited a nonselective action of SP or an event downstream from SP receptor activity. Because SP can induce release of EAA’s in the dorsal spinal cord (Skilling et al., 1990), the inhibitory effect of CP-96,345 on a high, but not a low dose of SP may have been brought about by inhibition of NMDA-induced activity after EAA release.

Pain transmission is believed to involve activity among primary afferent C-fibers resulting in the release of SP and EAA’s (Urban et al., 1994). Although NMDA activity is proposed to play a role in nociception (Mjellem-Joly et al., 1992) and hyperalgesia (Aanonsen and Wilcox, 1987), KA sensitization may also reflect a component of nociceptive processing. If true, KA sensitization would be expected to be sensitive to those compounds that inhibit pain or prevent the development of hyperalgesia. In support of such a relationship, MK-801 and phencyclidine, which inhibit KA sensitization, also inhibit hyperalgesia that develops in response to a variety of stimuli (Nishihara et al., 1995). In a similar fashion, capsaicin pretreatment in the adult animal not only prevents KA sensitization, but attenuates nociception (Cruys et al., 1995) and prevents the development of hyperalgesia (Meller et al., 1992). The antinociceptive effects of CP-96,345 observed by numerous investigators (Lecce et al., 1991; Nagahisa et al., 1992; Yu et al., 1992) may thus reflect the ability of CP-96,345 to inhibit NMDA and KA activity. One might postulate that CP-96,345 attenuates NMDA activity indirectly by inhibiting SP released in response to activation of NMDA receptors located on primary afferent C-fibers (Liu et al., 1994). However, it remains unclear why NMDA-induced release of SP would be sensitive to the inhibitory effect of CP-96,345 whereas exogenously administered SP would not.

In summary, CP-96,345 produced a dose-dependent inhibition of the behavioral response to an i.t. injection of NMDA at doses that failed to inhibit the behavioral response to SP. CP-96,345 also inhibited the development of sensitization to KA-induced behaviors. Thus, the inhibitory effect of CP-96,345 on the activity of NMDA and sensitization to KA may be responsible for the antinociceptive effect of CP-96,345. These effects may be brought about, at least in part, by inhibition of calcium channels involved in NMDA activity and KA sensitization and perhaps by inhibition of SP N-terminal activity. These possibilities require further investigation.

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References
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