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Effect of Tachykinin Receptor Inhibition in the Brain on Cardiovascular and Behavioral Responses to Stress

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ABSTRACT

The neurokinins, substance P (SP) and neurokinin A (NKA) represent natural, nonspecific ligands of NK_1 and NK_2 receptors. In our study in conscious rats, we tested the hypothesis that neurokinins, especially SP, are used by neuronal circuits to generate cardiovascular and behavioral responses to stress by using the selective, high-affinity, nonpeptide antagonists of NK_1 and NK_2 receptors, CP-96, 345, RP 67580 and SR 48968, respectively, Formalin injected s.c. through a chronically implanted catheter in the region of the lower leg was used as a stress stimulus. The antagonists and their inactive enantiomers, RP 68651 and SR 48965, as a control for nonspecific activity, were injected intracerebroventricularly (i.c.v.) 10 min before the s.c. injection of formalin. Formalin (2.5%, 50 μl, s.c.) induced a marked increase in mean arterial pressure (MAP) and heart rate (HR) as well as hind limb grooming/biting (HG) as the dominant behavioral manifestation. Pretreatment with the NK_1 receptor antagonist, CP-96,345 (5 nmol, i.c.v.), significantly attenuated only the HR (-54%; $P < .01$) but not the MAP response to formalin. The NK_1 receptor antagonist, RP 67580, injected i.c.v. at doses of 100, 500 and 2500 pmol significantly reduced both, the MAP and HR responses to formalin by maximally 63% ($P < .01$) and 52% ($P < .01$), respectively. In a separate set of experiments, we compared the effect of the individual and simultaneous blockade of central NK_1 and NK_2 receptors on the cardiovascular and behavioral responses to formalin stress. Pretreatment with RP 67580 (100 pmol, i.c.v.) attenuated the MAP (-30%; $P < .05$), HR (-40%; $P < .01$) and HG ($P < .05$) responses to formalin. The NK_2 receptor antagonist, SR 48968 (650 pmol, i.c.v.), affected neither the cardiovascular nor the behavioral responses. I.c.v. pretreatment with both tachykinin receptor antagonists (RP 67580: 100 pmol; SR 48968: 650 pmol) reduced the MAP, HR and HG responses to formalin to the same extent as RP 67580 alone. Pretreatment with the inactive enantiomers, RP 68651 (100 pmol, i.c.v.) and SR 48965 (650 pmol, i.c.v.) did not alter the cardiovascular and behavioral responses to formalin. Our results demonstrate that centrally administered NK_1 receptor antagonists inhibit the cardiovascular and behavioral reactions in response to a noxious stimulus. They provide first pharmacological evidence that endogenous SP acts as mediator of stress responses in the brain.

SP belongs to the neurokinins, the mammalian members of the tachykinin family of peptides (Guard and Watson, 1991). The principal neurokinins, SP, NKA and NKB, are unevenly distributed in the central nervous system. SP represents the most abundant neuropeptide in the rat brain (Minamino *et al.*, 1984; Arai and Emson, 1986; Jessop *et al.*, 1990). Along with other neurokinins, this peptide substantially contributes to the central cardiovascular and endocrine regulations and control of behavior (Itoi *et al.*, 1988; Otsuka and Yoshioka, 1993).

SP has been postulated to act as a natural, nonspecific ligand on NK_1 receptors, while NKA and NKB are the preferential agonists for NK_2 and NK_3 receptors, respectively (Guard and Watson, 1991; Regoli *et al.*, 1994). However, recent findings have demonstrated that SP and NKA in the brain are capable of interacting with both, NK_1 and NK_2 receptors (Culman *et al.*, 1993; Picard *et al.*, 1994).

Substantial evidence from *in vitro* and *in vivo* studies indicates that SP serves as a pain neurotransmitter in the primary afferent neurons (Otsuka and Yanagisawa, 1987; Otsuka and Yoshioka, 1993). Noxious stimuli represent classical threatening events that activate neuronal circuits in the brain to generate a complex pattern of cardiovascular, endocrine and behavioral responses. Although the question concerning the neurotransmitter specificity of these neuronal circuits has yet not been answered, several attempts have been made to link brain neurokinins, especially SP, with central stress reactions. In conscious rats, SP administered centrally induces an integrated pattern of cardiovascular, behavioral and endocrine responses. The cardiovascular part

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ABBREVIATIONS: AUC, area under the curve; BP, blood pressure; DMN, dorsomedial nucleus; FW, face washing/head scratching; HG, hind limb grooming/biting; HR, heart rate; i.c.v., intracerebroventricular(ly); MAP, mean arterial pressure; NKA, neurokinin A; NKB, neurokinin B; PAG, periaqueductal gray; PVN, paraventricular nucleus; SP, substance P; VMN, ventromedial nucleus; WDS, wet dog shakes; ANOVA, analysis of variance.

of this response is brought about by increased sympathoadrenal activity and comprises an increase in BP, HR as well as mesenteric and renal vasoconstriction and hindlimb vasodilatation. The behavioral response is characterized by increased locomotion and grooming behavior. The endocrine component to centrally injected SP consists of a marked, dose-dependent release of oxytocin but not vasopressin or corticotrophin into the circulation (Unger *et al.*, 1985; Unger *et al.*, 1988). Because this response pattern to SP closely resembles the integrated stress response to nociceptive stimuli in rodents, we speculated that SP may be important for the generation of an integrated cardiovascular and behavioral response pattern within the efferent pathways of the reaction to nociceptive stimuli (Unger *et al.*, 1988). However, the direct evidence in favor of this hypothesis has not yet been provided. Several authors have demonstrated rapid changes in SP content and its receptors in distinct brain areas on various stress stimuli (Bannon *et al.*, 1986; Takayama *et al.*, 1986; Siegel *et al.*, 1987; Rosén *et al.*, 1992) but the results of these studies are rather equivocal, most likely due to the fact that the effects of different stress situations on diverse brain tachykinin systems were analyzed that cannot be directly compared. Moreover, because the physiological significance of changes in neurotransmitter contents is difficult to interpret in the context of neurotransmitter release, the nature of the SP actions in the brain with respect to central responses to stress has remained obscure.

One of the major reasons for the slow progress in this field was the lack of selective, high-affinity antagonists of tachykinin receptors. This situation has greatly improved by the recent development of several selective, nonpeptide NK_1 and NK_2 receptor antagonists (Snider *et al.*, 1991; Garret *et al.*, 1991, Emonds-Alt *et al.*, 1992). In our study, the selective, high-affinity, nonpeptide antagonists for NK_1 receptors, CP-96,345 and RP 67580, and NK_2 receptors, SR 48968, were used to test the hypothesis that brain neurokinins play a role in the generation of the cardiovascular and behavioral responses to stress. The tachykinin receptor antagonists were administered i.c.v. before rats were exposed to a modified formalin stress. Formalin was injected s.c. through a chronically implanted catheter in the area of the lower leg of the rat resting in the test cage. The effects of the active enantiomers of the NK_1 and NK_2 receptor antagonists, RP 67580 and SR 48968, respectively, on the cardiovascular and behavioral responses to formalin were compared with those of their inactive enantiomers, RP 68651 and SR 48965 as a control for nonspecific activity. With the help of these newly developed tachykinin receptor antagonists, we can now show for the first time that brain neurokinins, in particular SP, are involved in central pathways generating an integrated cardiovascular and behavioral stress responses to a noxious stimulus.

Methods

Male Wistar rats weighing 300 to 350 g obtained from Dr. Karl Thomae GmbH (Biberach/Riss, Germany) were used.

Surgical Methods

For i.c.v injections, chronic polyethylene cannulae (PP 20; LHD, Heidelberg, Germany) were implanted under chloralhydrate anesthesia (400 mg/kg, i.p.) into the left lateral brain ventricle 7 to 10 days before the experiment (Unger *et al.*, 1981). The stereotaxic coordinates were: 1.3 mm lateral to the midline, 0.6 mm posterior to the bregma and 5 mm vertical from the skull surface. Five days after surgery, rats were injected i.c.v. with 25 pmol angiotensin II. Only those animals that responded by an immediate drinking were included in further experiments. The animals were anesthetized, and a polyethylene catheter (PP 50, LHD, Heidelberg, Germany) filled with heparinized saline was inserted through one femoral artery into the abdominal aorta, passed through a s.c. tunnel, sealed and secured at the back of the neck. At the same time the s.c catheter was implanted. For this purpose, polyethylene catheters (PP 50; LHD) of an entire length 20.5 were used. The inner volume of the catheters was 60 μl. After a small incision through the skin in the upper part of the lower leg, a narrow s.c. tunnel about 2.5 cm of length was made in the direction of the hind paw. The end of the catheter was inserted into the s.c. tunnel and sutured to the skin. The catheter was then passed through a s.c. tunnel and exteriorized at the back of the neck. After surgery, rats were housed individually in plastic cages under controlled temperature and humidity on a 12-hr light/dark cycle and were allowed free access to food and water. Experiments were performed 48 hr after the implantation of the femoral and s.c. catheters. The correct position of the i.c.v. cannulae was verified histologically by postmortem dissection at the end of each experiment.

General procedures. All experiments were carried out in conscious, freely moving rats. On the test day, rats were placed in the test cages, which were of the same size as the home cages, and were habituated to the new environment at least for 1 hr. Then the femoral artery catheter was connected to the blood pressure transducer. A PP 50 catheter connected to a syringe and filled with 2.5% formaldehyde solution (weight/weight in physiological saline) was connected to the s.c. catheter.

The experiments were started when the animals were resting and when basal MAP and HR were stable. The tachykinin receptor antagonists or vehicle were injected i.c.v. in a volume of 1 μl and flushed with 4 μl of physiological saline (CP-96,345 and SR 48968) or with 4 μl of phosphate-buffered saline, pH 7.4 (RP 67580) (see "Materials"). Ten min later, formalin (2.5%) was injected s.c. through the implanted catheter, and the cardiovascular and behavioral responses were recorded over a period of 15 min. A total volume of 110 μl of formalin was injected. Because the inner volume of the s.c. catheter was exactly 60 μl, the animals received 50 μl of formalin s.c.

Measurements of MAP and HR were performed via the femoral arterial catheters using a Statham p23Dc pressure transducer and a Gould Brush pressure computer coupled to a Gould Brush 2400 recorder. Analogue output signals of MAP and HR from the blood pressure computer were digitalized and then processed using a computerized program developed in our laboratory. This program permits sampling of hemodynamic data from experimental animals directly onto the hard disk of the computer and subsequent analysis with an interactive and graphic program. The hemodynamic data are sampled and stored continuously in real time during the entire experiment (Stauss *et al.*, 1990). MAP and HR are expressed as AUC (AP: mmHg $\times$ min; HR: b.p.m $\times$ min). The same computerized program was used to determine maximal increases in MAP and HR in the last set of experiments (see below).

Behavioral responses were recorded in test cages with grid tops removed over a 15-min period at 15-sec intervals starting immediately after the s.c. formalin injection. The frequency of the following behavioral manifestations, 1) FW, 2) HG and 3) wet dog shakes, was determined according to the 15-sec sampling procedure of Gispen *et al.* (1975). During each consecutive period of 15 sec, a score 1 or 0 was given depending on whether the animal showed the specific type of behavioral manifestation or not, regardless of the frequency, intensity or duration of the response during that period. Summation of scores for the 15-min period after the formalin injection gave the total behavioral score.

Experimental Protocols

Effect of i.c.v. treatment with the NK_1 receptor antagonist, CP-96,345, on the cardiovascular responses to s.c-injected formalin. A group of rats ($n = 7$) received an i.c.v injection of physiological saline (vehicle treated controls). Another group of rats ($n = 7$) was i.c.v. injected with the NK_1 antagonist, CP-96,345 (5 nmol), dissolved in physiological saline. In previous studies, this dose of CP-96,345 had been shown to inhibit the cardiovascular response to 25 pmol SP injected i.c.v. The antagonist injected i.c.v. alone was devoid of intrinsic cardiovascular and behavioral activity (Tschöpe *et al.*, 1992). Ten min after the i.c.v. injection of the NK_1 antagonist or physiological saline, formalin (2.5%, 50 μl) was injected s.c., and the cardiovascular response was recorded. A second control group ($n = 8$) received an i.c.v. injection of physiological saline but no formalin injection, and 10 min after the i.c.v. injection, the cardiovascular response was recorded.

Effect of i.c.v treatment with the NK_1 receptor antagonist, RP 67580, or with its inactive enantiomer, RP 68651 on the cardiovascular response to s.c. formalin. Six groups of rats were used in this set of experiments. Control rats ($n = 7$) received injections i.c.v. with vehicle (1 μl of acidic saline, pH 4, injected together with 4 μl of phosphate-buffered saline, pH 7.4) (see "Materials"). Ten min later, the recording of the cardiovascular parameter was commenced. Formalin was not injected s.c. in this group. The remaining groups were injected i.c.v. either with vehicle (one group, $n = 11$), the NK_1 antagonist, RP 67580, (three groups) or its inactive enantiomer, RP 68651 (one group). Three doses of RP 67580 (100 pmol, $n = 8$; 500 pmol, $n = 12$ and 2500 pmol, $n = 8$) and one dose of RP 68651 (2500 pmol, $n = 7$) were tested. The dose of 100 pmol RP 67580 had been shown previously to almost completely abolish the cardiovascular and behavioral responses to 25 pmol SP injected i.c.v. (Culman *et al.*, 1995). Each rat received only one dose of RP 67580 or RP 68651. Ten min after the i.c.v. treatment, formalin (2.5%, 50 μl) was injected s.c. through the implanted catheter and the cardiovascular response was recorded. RP 67580 and RP 68651 injected i.c.v. alone are without appreciable effects on cardiovascular or behavioral parameters (Culman *et al.*, 1995).

Comparison of individual i.c.v. treatments with the NK_1 and NK_2 receptor antagonist, RP 67580 and SR 48968, respectively, and of simultaneous i.c.v. treatment with both antagonists on the cardiovascular and behavioral responses to s.c. formalin. To each group of rats treated with the active enantiomer(s) of the tachykinin receptor antagonists, a group of rats treated with an equimolar dose of the inactive enantiomer(s) was assigned. Eight groups of rats were used. Control rats ($n = 9$) received vehicle i.c.v. (see "Materials") and, 10 min later, without s.c. formalin injection, the cardiovascular and behavioral responses were recorded over a period of 15 min. The remaining seven groups of rats received the following i.c.v. treatment: 1) vehicle ($n = 10$); 2) RP 67580 (100 pmol, $n = 9$); 3) SR 48968 (650 pmol, $n = 8$); 4) RP 67580 (100 pmol) + SR 48968 (650 pmol), ($n = 9$); 5) RP 68651 (the inactive enantiomer of RP 67580, 100 pmol, $n = 9$); 6) SR 48965 (the inactive enantiomer of SR 48968, 650 pmol, $n = 6$) and 7) RP 68651 (100 pmol) + SR 48965 (650 pmol) ($n = 8$). Ten min after the i.c.v. injections, formalin (2.5%, 50 μl) was administered s.c. to all seven groups of rats, and the cardiovascular and behavioral responses were recorded over a 15-min period. The dose of 100 pmol RP 67580 was used because it had significantly reduced the cardiovascular response to s.c. formalin in the preceding set of experiments. The dose of the NK_2 antagonist was chosen on the basis of its capability to inhibit the cardiovascular and behavioral effects to i.c.v.-injected NKA (25 pmol) (Picard and Couture, 1994, our unpublished observations).

In addition to increases in MAP and HR responses expressed as AUC, maximal increases in MAP and HR, measured 1 to 5 min after the s.c. formalin injection, were also analyzed in this set of experiments. The experimental protocols had been approved by the Governmental Committee for Ethical Use of Animals (Regierungspräsidium Karlsruhe).

Materials

CP-96,345 (2S,3S)-cis-2-(diphenylmethyl)-N-(2-methoxyphenyl)-methyl-1-azabicyclo [2.2.2.] octan-3-amine (R)(-) mandelate was obtained as a gift from Dr. Jaw-Kang Chang, Peninsula Laboratories, Heidelberg, Germany. The substance was dissolved directly in physiological saline in the desired concentration. RP 67580 (3a**R**, 7a**R**)-7,7-diphenyl-2 1-imino-2(2-methoxyphenyl)-ethyl perhydroisoindol-4-one) and its (3a**S, 7aS**) enantiomer, RP 68651, kind gifts from Dr. C. Garret, Rhône-Poulenc Rorer, Vitry sur Seine, France, were dissolved in a small volume of 0.1 M HCl. Physiological saline was added to obtain the final volume in the stock solution (5000 pmol/μl). The NK_2 antagonist, SR 48968 (S)-N-methyl-N [4-(4-acetylamino-4-phenyl piperidino)-2-(3,4-dichlorphenyl)butyl] benzamide and its (R)-enantiomer, SR 48965, were kind gifts from Dr. X. Emonds-Alts, Sanofi Recherche, Montpellier, France. The antagonist and its inactive enantiomer were dissolved in a small volume of DMSO (Merck, Germany), and physiological saline was added to obtain the final volume in the stock solution (6500 pmol/μl). On the day of experiment, the stock solutions of the antagonists and their inactive enantiomers were further diluted with physiological saline to obtain the desired concentration of the compounds. One μl of the solution containing either RP 67580 or RP 68651 (approximate pH 4) was injected i.c.v. together with 4 μl of phosphate-buffered physiological saline, pH 7.4. The final pH of the injected solution was 7.3 to 7.4. The final solutions of SR 48968 and SR 48965 contained maximally 5% of DMSO and were injected i.c.v. in a volume of 1 μl together with 4 μl of physiological saline. Physiological saline was used as the vehicle in the experiment using CP-96,345. In all other experiments, control groups of rats and vehicle-treated, stressed rats were injected i.c.v. with the vehicle used for i.c.v. injections of RP 67580 and RP 68651, respectively. One μl of this vehicle (approximate pH 4) was injected i.c.v together with 4 μl of phosphate-buffered saline, pH 7.4. The final pH of the injected solution was 7.3 to 7.4. The cardiovascular and behavioral responses to the vehicle used for i.c.v. injections of SR 48968 and 48965, respectively, have been shown to be identical with those of physiological saline (Tschöpe *et al.*, 1995).

Statistics

All values are expressed as mean ± S.E. Data were subjected to an ANOVA followed by a *post hoc* Bonferroni test. A significance level of $P < .05$ was accepted.

Results

In general, the early phase of the response to formalin injected s.c. through a chronically implanted catheter was characterized by increases in MAP and HR that reached a maximum at 1 to 5 min and then returned gradually to basal values. The majority of animals reached control preinjection MAP and HR values within 10 min after the formalin injection. The cardiovascular responses were associated with a behavioral action comprising increased locomotion and grooming behavior. HG was far the most dominant behavioral manifestation. In most of the animals, an additional late phase of the response was observed that started 20 to 30 min after the formalin injection and was invariably associated with long-lasting increases in MAP and BP of diverse amplitudes. Only the early phase of the response to formalin was analyzed in our study with respect to the cardiovascular and behavioral responses.

Effect of i.c.v. treatment with the NK_1 receptor antagonist, CP-96,345, on the cardiovascular response to s.c.-injected formalin. Compared to rats receiving injec-

tions i.c.v. with physiological saline, CP-96,345 (5 nmol) significantly reduced only the HR response to s.c. formalin, whereas the MAP response was not affected (fig. 1). The maximal increases in blood pressure and HR induced by the s.c. injection of formalin were similar in both, the NK_1 antagonist- and the physiological saline-pretreated groups. The NK_1 receptor antagonist tended to attenuate HG induced by s.c.-injected formalin (data not shown).

Effect of i.c.v. treatment with the NK_1 receptor antagonist, RP 67580, or its inactive enantiomer, RP 68651, on the cardiovascular response to s.c. formalin. Compared to rats receiving injections i.c.v. with vehicle, an i.c.v. dose of 100 pmol RP 67580 was already capable of significantly attenuating both, the MAP and HR responses to the noxious stimulus (fig. 2). Intracerebroventricular treatment with 500 pmol of RP 67580 did not reduce the MAP and HR responses more effectively than did 100 pmol of the NK_1 antagonist. However, after i.c.v treatment with 2500 pmol of RP 67580, an additional reduction of the MAP response but not of the HR response to s.c. formalin was observed compared to 100 or 500 pmol of the antagonist. The inactive enantiomer, RP 68651, injected i.c.v. at a dose of 2500 pmol did not alter the cardiovascular response to s.c. formalin (fig. 2).

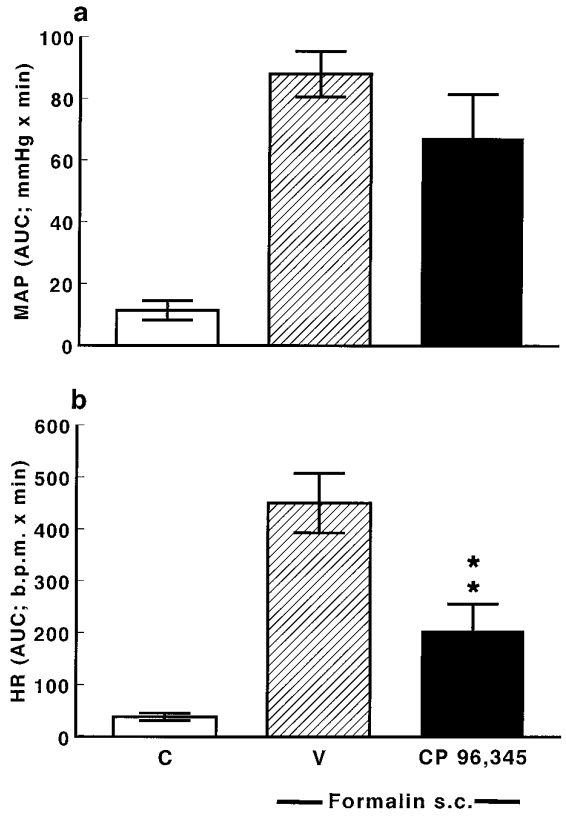

Fig. 1. Effects of i.c.v. pretreatment with vehicle (V) (hatched columns) and CP-96, 345 (solid columns) on MAP (a) and HR (b) responses to s.c.-injected formalin (2.5%, 50 μl). Controls (C) (open columns): MAP and HR response to i.c.v.-injected vehicle (physiological saline) when no s.c. formalin injection followed. MAP and HR are expressed as area under the curve (AUC). Values are expressed as means S.E. The MAP and HR values in formalin-injected groups differ significantly from controls (C) (significance not shown). **P < .01 statistical comparison to the vehicle pretreated, formalin injected group, calculated with a one-way ANOVA followed by a *post hoc* Bonferroni test.

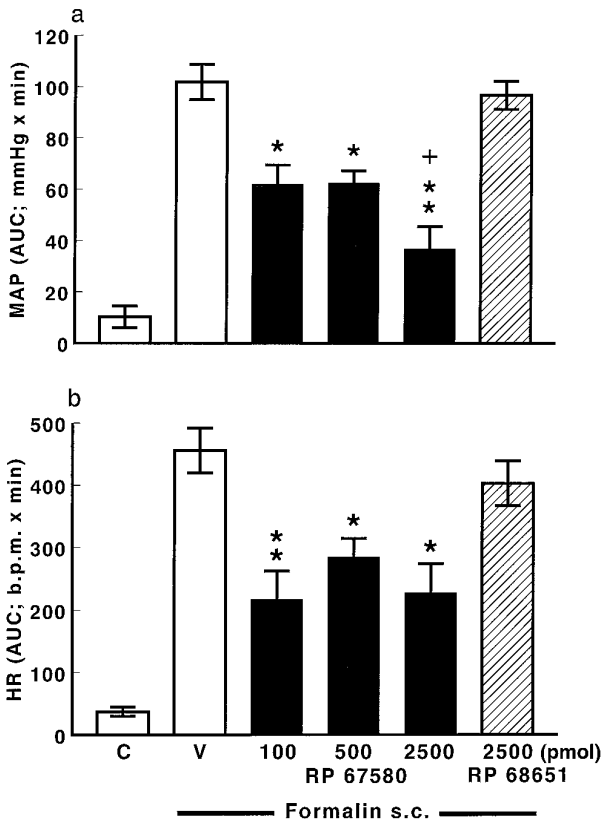

Fig. 2. Effects of i.c.v pretreatment with vehicle (V) (open columns), the NK_1 receptor antagonist, RP 67580 (100, 500 and 2500 pmol) (solid columns) and its inactive enantiomer, RP 68651 (2500 pmol)(hatched columns), on MAP (a) and HR (b) responses to s.c.-injected formalin (2.5%, 50 μl). Controls (C) (open columns): MAP and HR responses to i.c.v.-injected vehicle when no s.c. injection of formalin followed. The MAP and HR values in formalin injected groups differ significantly from controls (C) (significance not shown). *P < .05, **P < .01 statistical comparison to vehicle pretreated, formalin-injected group; +P < .05 statistical comparison to groups pretreated with 100 and 500 pmol RP 67580, respectively, calculated with a one-way ANOVA followed by a *post hoc* Bonferroni test. For further details see legend to figure 1.

Comparison of individual i.c.v. treatments with the NK_1 and NK_2 receptor antagonists, RP 67580 and SR 48968, respectively, and of simultaneous i.c.v. treatment with both antagonists on the cardiovascular and behavioral responses to s.c. formalin. Similar to the findings obtained in the previous set of experiments, the dose of 100 pmol RP 67580 effectively attenuated the cardiovascular response to s.c. formalin (fig. 3). The NK_1 antagonist also attenuated HG, the dominant behavioral manifestation induced by s.c. injection of formalin (table 1). Intracerebroventricular treatment with the NK_2 receptor antagonist, SR 48968, at a dose of 650 pmol altered neither the cardiovascular nor the behavioral response to s.c. formalin. Simultaneous i.c.v. treatment with both tachykinin receptor antagonists reduced the MAP, HR and HG responses to s.c. formalin to the same extent as did i.c.v. pretreatment with the NK_1 receptor antagonist alone (fig. 3; table 1). Because the cardiovascular responses to various treatments of experimental animals are often expressed as peak values of MAP and HR, the maximal increases in MAP and HR were also analyzed in this sets of experiments. In contrast to the respective MAP values expressed as AUC, the maximal increases in

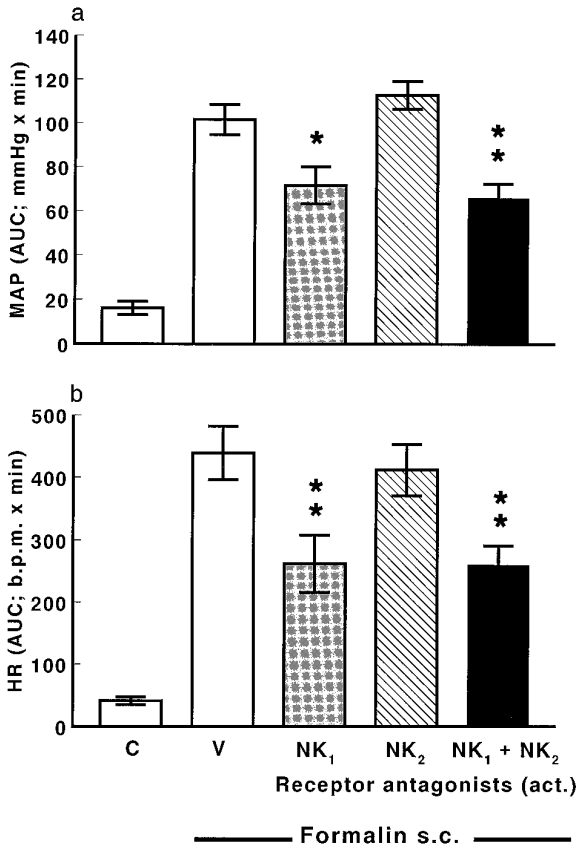

Fig. 3. Effects of i.c.v pretreatment with vehicle (V) (open columns), and active enantiomers (act.) of the NK_1 and NK_2 receptor antagonists, RP 67580 and SR 48968, respectively, on MAP (a) and HR (b) responses to s.c.-injected formalin (2.5%, 50 μl). Stippled columns: i.c.v. pretreatment with RP 67580 (100 pmol); hatched columns: i.c.v. pretreatment with SR 48968 (650 pmol); solid columns: simultaneous i.c.v. pretreatment with RP 67580 and SR 48968 (100 and 650 pmol, respectively). Controls (C) (open columns): MAP and HR responses to i.c.v.-injected vehicle when no s.c. injection of formalin followed. The MAP and HR values in formalin injected groups differ significantly from controls (C) (significance not shown). *$P < .05$, **$P < .01$ statistical comparison to vehicle pretreated, formalin-injected group calculated with a one-way ANOVA followed by a *post hoc* Bonferroni test. For further details see legend to figure 1.

MAP (ΔMAP) induced by s.c. formalin were identical in all groups, regardless of the substance used for i.c.v. treatment (table 2). Intracerebroventricular treatment with the NK_1 antagonist, RP 67580, alone tended to reduce the maximal increases in HR (ΔHR) to s.c formalin. However, a significant reduction of the HR increases after s.c. injection of formalin was only achieved in rats simultaneously treated i.c.v. with both tachykinin receptor antagonists (table 2).

Intracerebroventricular pretreatment with the inactive enantiomers of the NK_1 and NK_2 tachykinin receptor antagonists, RP 68651 and SR 48965, respectively, did not affect either the MAP and HR responses, expressed as AUC (fig. 4) or as maximal increases in MAP or HR (table 2), or the behavioral response to s.c. formalin (table 1).

Discussion

Threatening external or internal events such as noxious stimuli represent typical stress situations. Numerous studies have demonstrated neurokinins, especially SP, in the primary afferent neurons, to be involved in the transmission of information related to noxious stimulation (Otsuka and Yanagisawa, 1987; Otsuka and Yoshioka, 1993). Using selective and high-affinity, nonpeptide antagonists for NK_1 and NK_2 receptors developed recently, we now provide evidence demonstrating that SP, in addition to its function as a pain neurotransmitter in the spinal cord, acts as a mediator of stress responses in the brain and participates in the central generation of cardiovascular and behavioral responses to noxious stimuli.

The selective, high-affinity, nonpeptide NK_1 receptor antagonists, CP-96,345 and RP 67580 used in our study have been shown to possess a high affinity for NK_1 binding sites and to act as competitive inhibitors toward NK_1 receptor-mediated responses in various *in vitro* or *in vivo* tests (Snider *et al.*, 1991; Garret *et al.*, 1991, Maggi *et al.*, 1993). CP-96,345 was shown to be very potent in displacing of SP from guinea pig or bovine brain membrane preparations (K_i values of 3.4 and 0.5 nM, respectively); however, it was less potent in displacing of SP from binding sites in the rat forebrain (K_i value of 240 nM) (Snider *et al.*, 1991). The converse is true for RP 67580. This NK_1 antagonist is a potent inhibitor of SP-binding in rat brain (K_i value of 3.3 nM), but has reduced potency to displace SP from the guinea pig or human brain (K_i values of 41 and 21 nM, respectively) (Garret *et al.*, 1991; Fardin *et al.*, 1993). RP 67580 does not possess any appreciable affinity to NK_2 or NK_3 tachykinin receptors in concentrations up to 10 μM (Garret *et al.*, 1991). Both NK_1 antagonists selectively abolished the cardiovascular and behavioral responses induced by centrally administered SP, and left those of NKA or NKB unaffected (Tschöpe *et al.*, 1992; Culman *et al.*, 1995).

In our study, i.c.v. treatment with 5 nmol CP-96,345 attenuated only the HR response to s.c. formalin. However, both BP and HR responses were significantly attenuated when rats were i.c.v treated with a 50-fold lower dose of RP 67580. Species differences in binding affinities for these highly selective NK_1 receptor antagonits may explain the difference in their efficiencies to inhibit the cardiovascular response to stress. Although CP-96,345 showed a high selectivity for NK_1 compared with NK_2 and NK_3 binding sites (Snider *et al.*, 1991; McLean *et al.*, 1991), larger doses than that of 5 nmol were not used in our study. It has been demonstrated that CP-96,345 also displays high affinity for the L-type calcium channel and, moreover, the affinities of CP-96,345 for NK_1 receptors and calcium channels in the rat are quite similar (Guard *et al.*, 1993). A local anesthetic activity has also been reported although only at μM concentrations (Wang *et al.*, 1994). The effect of a large dose of CP-96,345 in *in vivo* experiments may, therefore, be a composite effect of NK_1 receptor antagonism and nonspecific actions that are not related to NK_1 receptors.

RP 67580 administered s.c. was reported to inhibit the nociceptive response to formalin injected into the hind paw of rats in a dose-dependent manner (Garret *et al.*, 1993). In our experiments, all three doses of RP 67580 (100, 500 and 2500 pmol) applied i.c.v. attenuated the HR response to s.c. injected formalin to the same extent. With respect to the BP response, the highest dose of the antagonist was more effective than two lower doses. We have shown previously that i.c.v. pretreatment with 100 pmol of RP 67580 most effectively inhibited the cardiovascular and behavioral responses

TABLE 1

Effects of i.c.v. pretreatment with the selective NK_1 and NK_2 receptor antagonists, RP 67580 and SR 48968, respectively, or their inactive enantiomers, RP 68651 and SR 48965, on behavioral responses induced by s.c. injection of formalin (2.5%, 50 μl)[a]

Pretreatment i.c.v.	Dose (pmol)	Formalin s.c.	*n*	Face Washing/Head Scratching	Hind Limb Grooming	Wet Dog Shakes
Vehicle		−	9	1.9 ± 0.8	0.9 ± 0.4	2.0 ± 0.9
Vehicle		+	10	2.0 ± 0.6	11.7 ± 1.1	2.1 ± 0.7
RP 67580	100	+	9	0.7 ± 0.3	7.3 ± 1.6[b]	4.5 ± 0.7
SR 48968	650	+	8	1.9 ± 0.7	10.9 ± 1.5	0.4 ± 0.3
RP 67580	100					
+		+	9	1.2 ± 0.3	7.5 ± 0.7[b]	3.3 ± 0.4
SR 48968	650					
RP 68651	100	+	9	2.1 ± 0.9	11.0 ± 1.5	4.3 ± 0.7
SR 48965	650	+	6	2.4 ± 0.2	14.4 ± 1.6	1.4 ± 0.6
RP 68651	100					
+		+	8	1.5 ± 0.4	15.0 ± 1.7	0.8 ± 0.3
SR 48965	650					

[a] Values represent the frequency of individual behavioral manifestations for 15 min and are indicated by the means ± S.E. of (*n*) rats. Vehicle and the antagonists, RP 67580, SR 48968 or their inactive enantiomers, RP 68651, SR 48965 were injected i.c.v. 10 min before s.c. injection of formalin (+).

[b] $P < .05$ statistical comparison to vehicle pretreated, formalin injected (+) group. Statistical differences were analysed with a one-way ANOVA followed by a *post hoc* Bonferroni test.

TABLE 2

Effects of i.c.v. pretreatment with the selective NK_1 and NK_2 receptor antagonists, RP 67580 and SR 48968, respectively, or their inactive enantiomers, RP 68651 and SR 48965, on maximal increases in arterial blood pressure (ΔMAP) and heart rate (ΔHR) in response to formalin injected s.c. (2.5%, 50 μl)[a]

Pretreatment i.c.v.	Dose (pmol)	*n*	Base-Line MAP (mmHg)	ΔMAP (mmHg)	Base-Line HR (b.p.m.)	ΔHR (b.p.m.)
Vehicle		10	92.6 ± 4.0	29.4 ± 2.6	325 ± 6	153 ± 21
RP 67580	100	9	101.3 ± 3.5	24.6 ± 2.5	337 ± 15	115 ± 12
SR 48968	650	8	92.5 ± 1.9	32.5 ± 2.7	302 ± 7	139 ± 12
RP 67580	100					
+		9	100.9 ± 2.0	24.0 ± 1.0	312 ± 7	109 ± 9[b]
SR 48968	650					
RP 68651	100	9	95.1 ± 2.0	32.0 ± 2.2	341 ± 10	151 ± 14
SR 48965	650	6	96.8 ± 1.5	29.6 ± 3.2	299 ± 10	138 ± 18
RP 68651	100					
+		8	97.0 ± 2.4	28.5 ± 2.7	317 ± 8	135 ± 13
SR 48965	650					

[a] Values represent the means ± S.E. of (*n*) rats. Vehicle and the antagonists, RP 67580, SR 48968 or their inactive enantiomers, RP 68651, SR 48965 were injected i.c.v. 10 before s.c. injection of formalin.

[b] $P < .05$, statistical comparison to vehicle, calculated with a one-way ANOVA followed by a *post hoc* Bonferroni test.

to i.c.v. SP. Lower doses of the antagonist were ineffective, and doses of RP 67580 of more than 100 pmol were less potent to antagonize the SP responses. This shape of the dose-response curve concerning the effects of RP 67580 on the cardiovascular and behavioral responses to SP might result from a specific, concentration-dependent interaction of the antagonist with NK_1 receptors in the circumventricular organs or periventricular, most probably hypothalamic, regions (Culman *et al.*, 1995). Because these regions need not necessarily belong to the neuronal circuits that are activated upon stress, higher doses of RP 67580 than that of 100 pmol were also used in our study with the purpose to achieve a sufficient blockade of NK_1 receptors localized in deeper brain structures. No data are available regarding the penetration of RP 67580 from the brain ventricular system into the surrounding neuronal tissue, and we have no evidence that RP 67580 injected i.c.v. at a dose of 100 pmol is capable to inhibit other NK_1 receptors than those localized in the close vicinity of the ventricular system. Because this dose of the antagonist did attenuate the cardiovascular response to s.c. formalin in our study, SP acting on NK_1 receptors in periventricular regions, most probably at the hypothalamic level (see below), may indeed be involved in the integration of the efferent output in response to noxious stimuli. The more effective reduction of the blood pressure response to formalin stress observed in our study after i.c.v. treatment with the highest dose of the NK_1 receptor antagonist may be due to an additional effect of the antagonist in lower brain stem areas involved in controlling the autonomic preganglionic neurons (Dampney, 1994). However, RP 67580, in addition to blocking NK_1 receptors, has been reported to exert at concentrations in the μM range actions unrelated to the inhibition of these receptors, including an interaction with calcium channels and nonspecific inhibitory effects on neurotransmission (Wang *et al.*, 1994; Lombet and Spedding, 1994). The nonspecific actions of RP 67580 might, at least partially, be responsible for the more effective inhibition of the blood pressure response observed after i.c.v. pretreatment with the highest dose of the antagonist.

As already mentioned above, SP serves as a mediator of nociception in the spinal cord (Otsuka and Yoshioka, 1993). Correspondingly, NK_1 receptor antagonists, such as RP 67580, were reported to exhibit antinociceptive effects. However, much higher doses of RP 67580 than those used in our study, administered peripherally or intrathecally, were required to induce antinociception (Garret *et al.*, 1993; Holzer-Petsche and Rordorf-Nikolic, 1995). Therefore, it is unlikely that the alteration of the cardiovascular response to s.c.

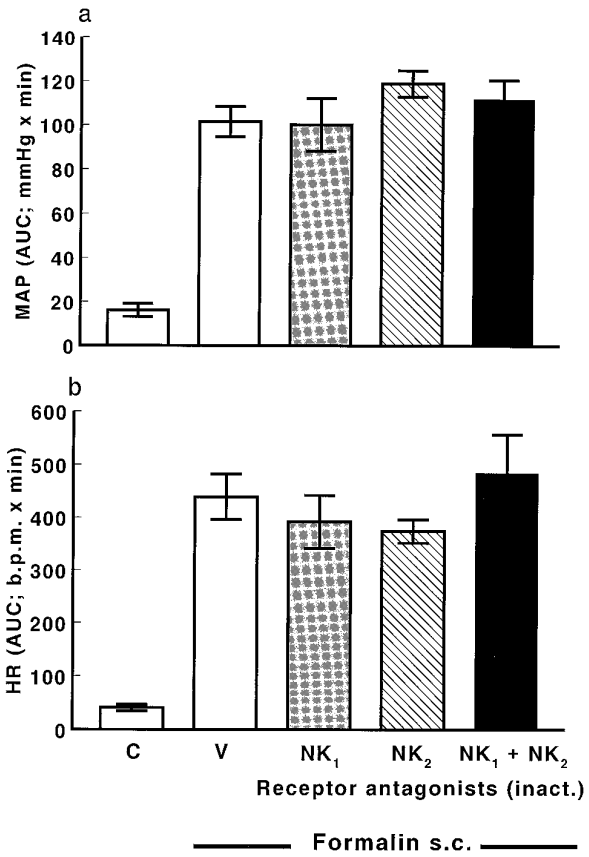

Fig. 4. Effects of i.c.v pretreatment with vehicle (V) (open columns) and inactive enantiomers (inact.) of the NK_1 and NK_2 receptor antagonists, RP 68651 and SR 48965, respectively, on MAP (a) and HR (b) responses to s.c.-injected formalin (2.5%, 50 μl). Stippled columns: i.c.v. pretreatment with RP 68651 (100 pmol); hatched columns: i.c.v. pretretament with SR 48965 (650 pmol); solid columns: simultaneous i.c.v. pretreatment with RP 68651 and SR 48965 (100 and 650 pmol, respectively). Controls (C) (open columns): MAP and HR responses to i.c.v.-injected vehicle when no s.c. injection of formalin followed. The MAP and HR values in formalin-injected groups differ significantly from controls (C) (significance not shown). The values in formalin-injected groups did not differ significantly. For further details see legend to figure 1.

formalin observed after i.c.v. treatment even with the highest dose of RP 67580 used in our study was due to a diffusion or transport of the antagonist from the forebrain ventricular system down to the spinal cord.

Recent pharmacological and autoradiographic studies using various selective peptide and nonpeptide NK_2 receptor antagonists have demonstrated the presence of functionally active NK_2 receptors in the adult rat brain (Tschöpe *et al.*, 1992; Hagan *et al.*, 1993; Picard *et al.*, 1994). NKA, a natural nonspecific ligand of NK_2 receptors, induces on central administration, a cardiovascular response comprising increases in BP and HR, and as with SP, this effect is brought about by peripheral sympathoadrenal activation (Takano *et al.*, 1990). The cardiovascular responses to equimolar doses of both peptides injected i.c.v. are virtually identical (Culman *et al.*, 1993). Therefore, it is conceivable that NKA acting on NK_2 receptors in certain brain areas may, in addition to SP, contribute to the initiation or modulation of central reaction activated upon stress. Moreover, both neurokinin peptides, SP and NKA, are capable of interacting with NK_1 and NK_2 receptors in the rat brain (Culman *et al.*, 1993; Picard *et al.*, 1994). Hence, in the last experimental setting, we compared the effects of the individual and simultaneous inhibition of central NK_1 and NK_2 receptors on the cardiovascular and behavioral responses to the noxious stimulus.

Intracerebroventricular pretreatment of rats with the NK_2 antagonist, SR 48968, did not affect any response elicited by s.c. formalin. SR 48968 has been reported to possess a high affinity for the rat NK_2 receptor in binding assays (K_i value of 0.51 nM). The affinity for the rat NK_1 and NK_3 receptors is much lower ($K_i > 5$ μM) indicating that SR 48968 is a specific, high affinity antagonist for NK_2 receptors (Emonds-Alt *et al.*, 1992). Because the dose of 650 pmol SR 48968 did not even tend to attenuate any responses to s.c. formalin, larger doses were not used. Moreover, as it occurs with other nonpeptide tachykinin receptor antagonists, SR 48968 at higher concentrations in the μM range is not devoid of nonspecific effects, that are not related to its interaction with the NK_2 receptor. Thus, SR 48968 can act as opiod agonist and interact with calcium channels (Martin *et al.*, 1993; Lombet and Spedding, 1994). When larger doses of the antagonist are used, these nonspecific actions may account for at least part of the observed effects.

In contrast to the inhibition of NK_1 receptors in the brain, a selective blockade of NK_2 receptors did not affect the cardiovascular and behavioral responses elicited by s.c. formalin. This finding suggests that brain neuronal circuits integrating stress reactions either do not possess functionally active NK_2 receptors or that these receptors are not activated during stress. Another possibility is that the receptors are not located in the vicinity of the ventricles and, therefore, could not be targeted by the antagonist. However, because NKA can also interact with the NK_1 receptor and may thus be responsible for a fine modulation of SP actions at this receptor (Culman *et al.*, 1993), our data do not allow any definite conclusions about the physiological relevance of NKA with respect to central processes activated upon stress.

Compared to the NK_1 antagonist alone, simultaneous i.c.v. pretreatment with both tachykinin receptor antagonists did not exert any additional inhibitory action on the cardiovascular and behavioral responses to s.c. formalin. This result further underlines the relevance of NK_1 receptors localized in the neuronal networks adjacent to the ventricle for the generating of central reactions activated upon stress.

Peak values of MAP and HR are commonly used to express the effects of various treatments on blood pressure and HR. Therefore, maximal increases in MAP and HR were also determined in the last set of experiments. Intracerebroventricular pretreatment with the NK_1 receptor antagonist, RP 67580, did not affect the amplitude of the maximal MAP increase, and only tended to reduce the amplitude of the maximal HR increase. This finding corresponds to our observation that the initial increases in MAP and HR in response to formalin stress in vehicle-treated and in RP 67580-treated rats were quite similar. However, the antagonist-treated rats reached the preinjection, control values of MAP and HR considerably faster than vehicle-treated rats after s.c. injection of formalin. Therefore, the marked reduction of both responses to formalin stress after i.c.v. pretreatment with RP 67580 is only evident when changes of MAP and HR amplitudes are integrated over the time of the response, *i.e.*, when AUC is used as a measure of MAP and HR. Combined i.c.v.

pretreatment with both tachykinin receptor antagonists reduced not only the duration of the cardiovascular response, but also the maximal HR increases in response to s.c.-injected formalin, suggesting that the simultaneous blockade of brain NK_1 and NK_2 receptors may indeed be more effective in inhibiting the cardiovascular responses to noxious stimuli than the blockade of NK_1 receptors alone. This assumption is consistent with the findings demonstrating that i.c.v.-injected SP even at relatively low doses (25 pmol) can interact with NK_2 receptors when NK_1 receptors are inhibited (Picard *et al.*, 1994).

Forebrain site of action of the tachykinin receptor antagonists. The inhibition of the formalin stress response by a comparatively low i.c.v. dose of the NK_1 receptor antagonist suggests that the targeted neuronal circuits lie in the vicinity of the brain ventricular system. Several pieces of evidence suggest that certain hypothalamic areas may represent the site of the antagonist action. The hypothalamus, especially its periventricular zone comprising, among others, the PVN along with the medial zone, containing the DMN and VMN, is known to play a crucial role in the integration of the autonomic, endocrine and behavioral responses to stress (Swanson, 1987). All these hypothalamic areas contain high densities of SP-immunoreactive networks and belong to the richest regions in the brain with respect to the SP content and the number of NK_1-binding sites (Brownstein *et al.*, 1976; Ljungdahl *et al.*, 1978; Cuello and Kanazawa, 1978; Buck *et al.*, 1986; Mantyh *et al.*, 1989; Jessop *et al.*, 1990; Bittencourt *et al.*, 1991). The dense SP innervation of these nuclei provides a neuroanatomical basis for the peptide to participate in central stress reactions. A number of studies indicate that SP in the hypothalamus might indeed be involved in the generation of central responses to stress. The cardiovascular response induced by SP microinjected into certain hypothalamic nuclei resembles the cardiovascular response to stress (Itoi *et al.*, 1991, 1994). Shaikh *et al.* (1993) have demonstrated that NK_1 receptors in the medial hypothalamus play a role in the facilitation of the feline defense rage behavior. Siegel *et al.* (1987) reported a depletion of SP content in the VMN and DMN and in the lateral hypothalamus after exposure of rats to foot shock stress. Electrolyte lesions of the PVN were shown to selectively abolish the tachycardia but not the BP response to stress (Callahan *et al.*, 1989). Assuming that SP is involved in the regulation of HR upon stress by acting on NK_1 receptors in the PVN, already low doses of NK_1 receptor antagonists injected i.c.v. should be able to sufficiently block these receptors to obtain a reduction of the stress-induced tachycardia. Higher doses of the antagonist would not induce any additional reduction of the HR response to stress. This would explain the shape of the dose-response curve obtained in our experiments.

The thalamus, especially its periventricular zone, and the PAG may represent another target regions for the NK_1 receptor antagonist to inhibit the cardiovascular and behavioral responses to stress. Both regions contain moderate to high densities of SP-positive nerve terminals and NK_1-binding sites (Ljungdahl *et al.*, 1978, Buck *et al.*, 1986; Mantyh *et al.*, 1989).

It has been demonstrated that SP is used as a neurotransmitter in the spinothalamic tract that conveys somatosensory signals including those of pain from the spinal cord to the ventral thalamus (Nishiyama *et al.*, 1995). Antagonists of NK_1 receptors can, therefore, block the transmission of nociceptive signals at the thalamic level thus preventing the activation of brain regions responding to stress. However, because the ventral thalamus does not lie in the vicinity of the ventricular system it is questionable whether and to which extent an i.c.v injected NK_1 receptor antagonist can interact with NK_1 receptors in this thalamic area.

Along with the important role of the PAG in antinociception and defense behavior, the PAG has also received attention as a region involved in central cardiovascular control (Dampney, 1994). This region was shown to play a key role in the generation of the defense-like reactions in the rat and cat (Hilton and Redfern, 1986; Bandler and Carrive, 1988). Different acute noxious stimuli were reported to alter the SP content in the PAG indicating that SP-system in this area responds to stress (Rosén *et al.*, 1992). Intracerebroventricular injections of dye revealed, that 10 min after the injection of the tachykinin receptor antagonist into the lateral ventricle, the antagonists reach the cerebral aqueduct and may, therefore, interact with the tachykinin receptors in the PAG. Although the PAG contains one of the highest levels of SP among the brain regions along with high quantitites of NK_1-binding sites (Douglas *et al.*, 1982; Dam *et al.*, 1990) the relevance of SP in this region for the generation of the cardiovascular responses to noxious stimuli has not yet been established.

In conclusion, selective, high affinity, nonpeptide antagonists of NK_1 and NK_2 receptors have been used to study the effect of brain tachykinin receptor inhibition on cardiovascular and behavioral responses to a noxious stimulus. Inhibition of central NK_1 receptors—possibly in periventricular structures at the hypothalamic level—attenuated both responses to formalin stress. Our data thus provide for the first time pharmacological evidence that endogenous neurokinins, especially SP, in the brain act as neurotransmitters or neuromodulators within neuronal circuits integrating the efferent output in response to noxious stimuli.

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