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Cardiovascular and behavioural effects of centrally administered tachykinins in the rat: characterization of receptors with selective antagonists

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1 The effects of intracerebroventricular (i.c.v.) injection of selective and potent NK_1 (RP 67580), NK_2 (SR 48968) and NK_3 (R 486, [Trp^7, β-Ala^8]NKA(4-10)) receptor antagonists were assessed on the cardiovascular and behavioural responses elicited by the i.c.v. injection of substance P (SP), neurokinin A (NKA) or [MePhe^7]neurokinin B ([MePhe^7]NKB) in the conscious freely moving rat.

2 SP, NKA and [MePhe^7]NKB (5–650 pmol) evoked dose-dependent increases in mean arterial blood pressure (MAP) and heart rate (HR) with the rank order of potency SP > NKA > [MePhe^7]NKB. The cardiovascular responses were accompanied by excessive face washing, grooming and wet dog shakes.

3 The cardiovascular effects and face washing behaviour induced by SP (25 pmol) were significantly reduced by the pre-injection (i.c.v., 5 min earlier) of RP 67580 (6.5 nmol). However, this antagonist failed to affect the central effects of 25 pmol NKA or [MePhe^7]NKB.

4 The cardiovascular and behavioural responses (except for wet dog shakes) elicited by NKA (25 pmol) were significantly reduced by 6.5 nmol SR 48968. However, the latter antagonist had no effect on the SP or [MePhe^7]NKB-mediated responses.

5 Both cardiovascular and behavioural effects produced by either SP or NKA (25 pmol) were completely abolished when rats were pretreated with a combination of RP 67580 (6.5 nmol) and SR 48968 (6.5 nmol), yet this combination of antagonists failed to modify the central effects of [MePhe^7]NKB.

6 R 486 (6.5 nmol) inhibited the cardiovascular effects as well as wet dog shakes produced by [MePhe^7]NKB, but it was inactive against the responses induced by either SP or NKA.

7 None of the tachykinin receptor antagonists or agonists caused motor impairment or respiratory distress. All antagonists blocked in a reversible manner and were devoid of intrinsic activity except R 486 (6.5 nmol) which produced a transient increase of MAP and HR.

8 These results suggest that the central effects of SP, NKA and [MePhe^7]NKB are primarily mediated by central NK_1, NK_2 and NK_3 receptors, respectively. However, a minor activation of NK_2 receptors by SP and NK_1 receptors by NKA was seen during blockade of both receptors. This study therefore supports the existence of functional NK_1, NK_2 and NK_3 receptors in the adult rat brain.

Keywords: Substance P; neurokinin A; neurokinin B; tachykinin receptors; tachykinin antagonists; central effects; cardiovascular system; behaviour

Introduction

The mammalian tachykinins, substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) are widely distributed in the central nervous system (CNS) and peripheral tissues. They are believed to play several neurotransmitter functions in central cardiovascular regulation, motor activity, and on sensory, autonomic and endocrine systems (for review see Otsuka & Yoshioka, 1993). So far, three tachykinin receptors termed NK_1, NK_2 and NK_3, have been cloned and pharmacologically characterized. The rank order of potency of tachykinins is SP > NKA > NKB for the NK_1 receptor, NKA > NKB > SP for the NK_2 receptor and NKB > NKA > SP for the NK_3 receptor (Guard & Watson, 1991; Maggi *et al.*, 1993b; Mussap *et al.*, 1993).

Intracerebroventricular (i.c.v.) injection of SP or NKA in the conscious freely moving rat leads to increase in mean arterial blood pressure (MAP), heart rate (HR), cardiac output, and to enhanced locomotor activity, awareness, scratching and face washing behaviour (Unger *et al.*, 1988; Itoi *et al.*, 1992; Tschöpe *et al.*, 1992). On the other hand, administration of NKB or the NK_3-selective agonist, senktide, into the lateral ventricle of the conscious rat also induces increases in blood pressure and HR, but evokes a unique behavioural pattern, the wet dog shake (Itoi *et al.*, 1992). Although all three tachykinins have been reported to act mainly on hypothalamic neurones (Itoi *et al.*, 1991; Massi *et al.*, 1991), the cardiovascular responses induced by either SP or NKA have been associated with an increased sympathoadrenal activity (Unger *et al.*, 1981; Takano *et al.*, 1990), while those induced by i.c.v. injection of NK_3 agonists would result mainly from the release of vasopressin from the hypothalamus and to a minor extent by activation of the sympathetic nervous system (Polidori *et al.*, 1989; Takano *et al.*, 1990; 1993).

Tachykinin antagonists may represent useful pharmacological tools to characterize central tachykinin receptors and to define better the role of these neuropeptides in central cardiovascular regulation. In a recent study, we provided evidence for the existence of distinct populations of functionally active NK_1 and NK_2 receptors in the adult rat brain with the use of selective tachykinin antagonists, namely (±)-CP 96345 to block the NK_1, MEN 10207, MEN 10376 and R 396 to inhibit the NK_2 receptor. It was concluded that the cardiovascular and behavioural effects of i.c.v. SP and NKA are mediated by NK_1 and NK_2 receptors, respectively (Tschöpe *et al.*, 1992). The role of NK_1 receptors in the central action of SP remains, however, to be confirmed as the quinuclidine antagonist (±)-CP 96345 may act as an

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antagonist of L-type calcium channels in rat cardiac and brain tissue (Schmidt *et al.*, 1992; Guard *et al.*, 1993) and as a blocker of voltage-dependent sodium currents in rat neocortical neurones (Caeser *et al.*, 1993). Hence, non-specific effects produced by (±)-CP 96345, probably not related to an interaction with tachykinin receptors, could not be excluded in our earlier study (Tschöpe *et al.*, 1992). In this respect, the perhydroisoindolone compound, RP 67580, is a new, potent and promising non-peptide antagonist selective for the NK_1 receptor showing higher potency in rat than in guinea-pig and man (Garret *et al.*, 1992; Carruette *et al.*, 1992; Rouissi *et al.*, 1993). Moreover, the new selective NK_2 receptor non-peptide antagonist, SR 48968, has higher affinity and stability than the former peptide antagonists (MEN 10207, MEN 10376, R 396, MDL 28564) (Advenier *et al.*, 1992; Emonds-Alt *et al.*, 1992). SR 48968 blocks in a dose-dependent and reversible manner the hyperalgesic response to NKA(4-10) (selective NK_2 agonist) but not that induced by [Sar^9, $Met(O_2)^{11}$]SP (selective NK_1 agonist) in the rat spinal cord (Picard *et al.*, 1993). RP 67580 and SR 48968 are potent antagonists which exert specific, competitive, reversible, yet non toxic antagonism and should therefore be suitable for studying the function of central NK_1 and NK_2 receptors (Advenier *et al.*, 1992; Rouissi *et al.*, 1993; Maggi *et al.*, 1993a; Picard *et al.*, 1993). Up to now, the lack of a potent and selective NK_3 receptor antagonist had limited our understanding of the role played by this receptor in central cardiovascular regulation. However, R 486, R 487 and GR 138676 belong to the first generation of peptide antagonists selective for the NK_3 receptor (Regoli *et al.*, 1991; Stables *et al.*, 1993). R 487 blocks in a specific manner the antinociceptive effect of intrathecally injected [$MePhe^7$]NKB (selective NK_3 agonist) in the rat tail-flick test (Couture *et al.*, 1993).

The purpose of the present study was twofold: firstly, to confirm the participation of NK_1 and NK_2 receptors in central cardiovascular and behavioural effects of SP and NKA, by using newly developed, non-peptide, receptor selective antagonists (RP 67580 and SR 48968). Secondly, to determine the participation of central NK_3 receptors in the cardiovascular and behavioural effects of tachykinins by using a selective agonist ([$MePhe^7$]NKB) and antagonist (R 486) of the NK_3 receptor. A preliminary account of this work has been presented elsewhere (Picard *et al.*, 1992).

Methods

Animal preparation

Male Wistar rats (Charles River, St. Constant, Québec, Canada) weighing 300–350 g were used. The animals were allowed free access to food and water and maintained on a 12 h light/dark cycle (lights on 06 h 00 min–18 h 00 min).

Rats were anaesthetized with an intraperitoneal (i.p.) injection of 65 mg kg^{-1} sodium pentobarbitone (Somnotol; M.T.C. Pharmaceuticals, Cambridge, Ont. Canada) and an i.c.v. polyethylene cannula (PE-20; Intramedic, Clay Adams, NJ, U.S.A.) was implanted into the right brain ventricle by use of a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, U.S.A.) and fixed to the skull with dental cement (Reliance Dental MFG. Co., Worth, IL, U.S.A.). The co-ordinates were 1.3 mm lateral to the midline, 0.6 mm caudal to the bregma and 5.0 mm vertical from the skull surface. The angle of the head was adjusted according to the horizontal plan with respect to bregma and lambda reference points. The animals were then placed in individual plastic cages (40 cm × 23 cm × 20 cm). After a recovery period of 24 h, rats were anaesthetized again and a second polyethylene cannula (PE-50) was inserted through a femoral artery into the abdominal aorta for measurement of blood pressure and heart rate. The pre-siliconized intraarterial catheter was filled with physiological saline containing heparin sodium salt (100 i.u. ml^{-1}), passed through a subcutaneous tunnel and emerged at the back of the neck. Experiments were conducted 24 h after the intravascular surgery on conscious unrestrained rats kept in their resident cage.

Rats were injected i.c.v. with 25 pmol angiotensin II (AII) to verify the potency of the i.c.v. cannula. Only those animals which responded by an immediate sharp rise of blood pressure associated with an intense dipsogenic activity as reported earlier (Thunhorst & Johnson, 1993) were included in the study. The correct position of the i.c.v. cannula was verified histologically by post-mortem dissection.

Measurement of cardiovascular and behavioural responses

The arterial pressure was monitored through the intra-arterial catheter with a Statham pressure transducer (P231D) while the heart rate was measured with a cardiac tachometer (model 7P4) and both variables were displayed on a Grass polygraph model 79D (Grass Instruments Co., Quincy, MA., U.S.A.). Experiments started when the animal was in a resting state and basal MAP and HR were stable.

The behavioural activity was recorded in their resident plastic cage over a 30 min period starting immediately with the i.c.v. injections. During the course of these experiments, the grid cage top was removed. The frequency of the individual behavioural responses: face washing and grooming, was determined according to the 15 s sampling procedure of Gispen *et al.* (1975). During every consecutive period of 15 s, a score 1 or 0 was given systematically depending on whether the animal showed the specific type of behaviour or not, whatever its frequency, intensity or duration during that period. Summation of scores for 30 min following the i.c.v. injection gave the behavioural scores for face washing and grooming in each experiment. The maximal theoretical score was 120 (15 s intervals × 30 min). The wet dog shake was measured according to the number of episodes (less than 1 s each) during the 30 min period, whatever the intensity.

Experimental protocols

In the first series of experiments, the effects of three to four doses (5 pmol, 25 pmol, 65 pmol, 325 pmol or 650 pmol) of SP, NKA or [$MePhe^7$]NKB on MAP and HR were measured following i.c.v. administration. Only one peptide was administered to a rat at increasing doses; each dose was administered at intervals of 24 h to avoid tachyphylaxis (Itoi *et al.*, 1992), in a volume of 1 µl of artificial cerebrospinal fluid (CSF; composition in mM: NaCl 128.6, KCl 2.6, $MgCl_2$ 2.0 and $CaCl_2$ 1.4; pH adjusted to 7.2). The catheter was then flushed with 4 µl of CSF over a period of 20–30 s and the cardiovascular responses were measured for 30 min. Control animals were injected with 5 µl CSF only.

In the second series of experiments, the animals received randomly a single i.c.v. injection of either 25 pmol SP, NKA or [$MePhe^7$]NKB (1 µl of peptide solution flushed with 4 µl of CSF) and the cardiovascular and behavioural responses were measured over a period of 30 min. The vehicle CSF containing dimethylsulphoxide (DMSO), used to dissolve the tested antagonist, was injected i.c.v. 5 min prior to the agonist. On the second day, CSF or one of the three antagonists (RP 67580, SR 48968 and R 486) were randomly administered i.c.v., at 6.5 or 65 nmol, 5 min prior to SP, NKA or [$MePhe^7$]NKB. Only one antagonist was administered to each animal. On the third day, the tested agonist was injected alone to evaluate the reversibility of any inhibition produced by the antagonist. No tachyphylaxis to SP, NKA or [$MePhe^7$]NKB was seen on MAP, HR, face washing, grooming and wet dog shakes when each agonist (25 pmol) was injected i.c.v. on three consecutive days (Figure 1). The intrinsic activity of the antagonists was tested in separate experiments. Baseline MAP and HR values were calculated 1 min before the injection of 25 pmol SP, NKA or [$MePhe^7$]NKB.

Peptides and non-peptides

The non-peptide NK_1 antagonist, RP 67580 (racemic form of 7,7-diphenyl-2[1-imino-2(2-methoxy-phenyl)-ethyl] perhydro-isoindol-4-one (3aR, 7aR); mol. wt: 475,0 for the hydrochloride salt) was a gift from Dr C. Garret, Rhône-Poulenc Rorer, Paris, France. The NK_2 antagonist SR 48968 ((S)-N-methyl-N[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)-butyl]benzamide; mol. wt: 570,0) was a gift from Dr J.-C. Brelière, Sanofi, Montpellier, France. R 486 (H-Asp-Ser-Phe-Trp-β-Ala-Leu-Met-NH_2; mol. wt.: 868.1) and [MePhe^7]-NKB (H-Asp-Met-His-Asp-Phe-Phe-MePhe-Gly-Leu-Met-NH_2) were synthesized in the laboratory of Dr D. Regoli at Sherbrooke University, Sherbrooke, Canada by conventional solid-phase methods. SP, NKA and AII were purchased from Hükabel Scientific Ltd, Montréal, Canada. Heparin sodium salt Grade II from porcine intestinal mucosa was purchased from Sigma chemicals (St-Louis, MO, U.S.A.). The antagonists and [MePhe^7]NKB were dissolved in DMSO (Fisher) and CSF was added to obtain the desired solution (the final solution contained a maximum of 30% of DMSO). SP, NKA and AII were dissolved directly in CSF. The stock solutions (1–10 mg ml^{-1}) of peptides and non-peptides were divided into 100 µl aliquots and stored at – 20°C until used. Daily dilutions were made in CSF before each experiment.

Statistical analysis of data

The results are expressed as mean ± s.e.mean. Statistical differences were evaluated with Student's *t* test for paired samples or Wilcoxon–Mann–Whitney (U) test for unpaired samples on non parametric values (behaviour frequency). When more than one comparison was made, the significance of differences among groups was evaluated with a two-way analysis of variance (ANOVA) in conjunction with Bonferroni confidence intervals. Only probability values (*P*) smaller than 0.05 were considered to be statistically significant.

Results

Central cardiovascular and behavioural effects induced by SP, NKA and [MePhe^7]NKB

The i.c.v. injection of 5 pmol SP, NKA or [MePhe^7]NKB failed to cause significant cardiovascular changes when compared with CSF values (Figure 2). However, at 25 pmol all three agonists induced significant increases of MAP and HR ($P<0.001$) which reached a maximum at 3–5 min and returned gradually to pre-injection levels within 30 min (Figures 2 and 3). The doses of 65 pmol and 325 pmol SP produced further increases of MAP and HR. However, cardiovascular responses were maximal at 25 pmol NKA or [MePhe^7]NKB as higher doses (325 and 650 pmol) failed to cause further increases of MAP and HR (Figure 2). Thus, the agonists evoked maximal cardiovascular changes (3–5 min post-injection) with the rank order of potency SP>

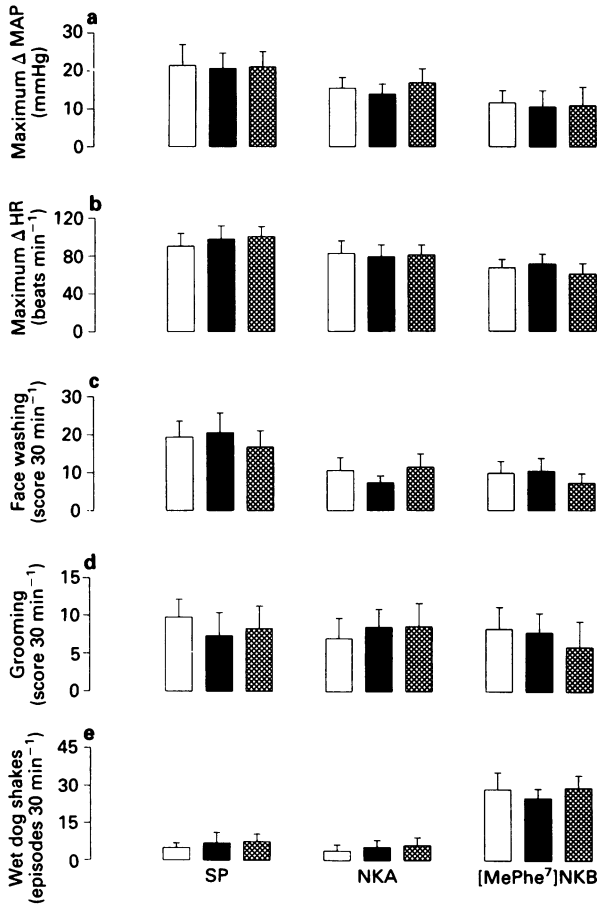

Figure 1 Cardiovascular and behavioural effects of 25 pmol substance P (SP), neurokinin A (NKA) and [MePhe^7]NKB on three consecutive days in conscious rats. The agonist was injected i.c.v. on day 1 (open columns), day 2 (solid columns) and day 3 (cross-hatched columns). The increases in (a) mean arterial blood pressure (MAP) and (b) heart rate (HR) represent maximal values at 3–5 min post-injection. Individual behaviours were measured for a period of 30 min (c, d and e). Each value represents the mean ± s.e.mean of 8 rats for each agonist. There is no statistical difference between days 1, 2 and 3 for each agonist.

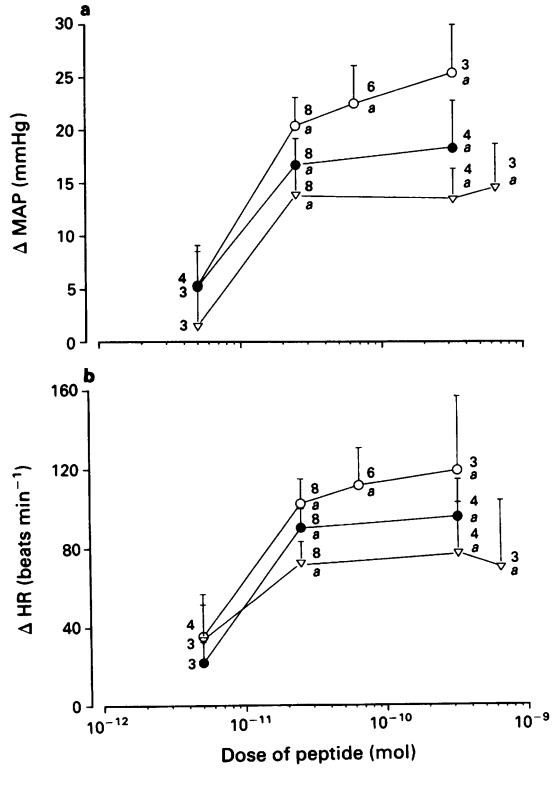

Figure 2 Effects of several doses of substance P (○), neurokinin A (●) and [MePhe^7]NKB (▽) injected intracerebroventricularly in conscious rats. Maximal increases in (a) mean arterial blood pressure (MAP) and in (b) heart rate (HR) measured at 3–5 min post-injection are shown. Each point represents the mean ± s.e.mean of several rats indicated by numbers. Statistically significant difference compared with CSF values (2.1 ± 1.8 mmHg and 15.1 ± 7.4 beats min^{-1}) is indicated by $^aP<0.001$.

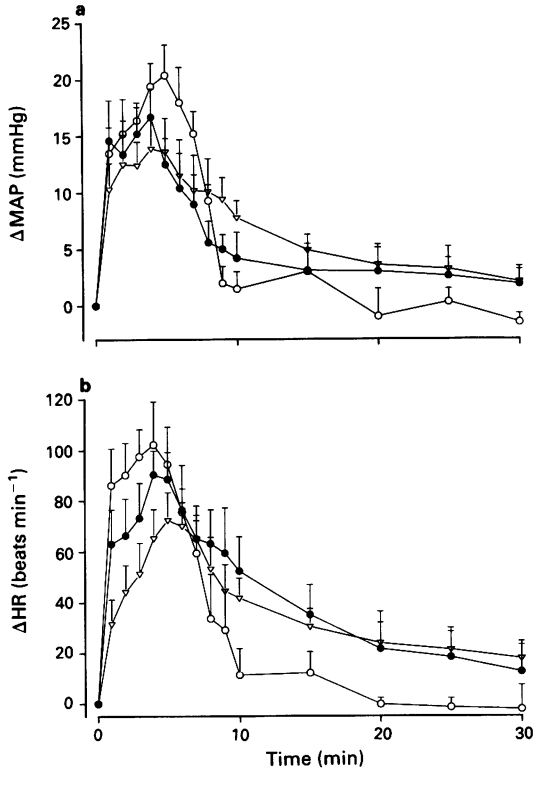

Figure 3 Time course of (a) mean arterial blood pressure (MAP) and (b) heart rate (HR) changes evoked by the i.c.v. injection of 25 pmol substance P (○), neurokinin A (●) and [MePhe^7]NKB (▽) in conscious rats. Each point represents the mean ± s.e.mean of 8 rats.

NKA > [MePhe^7]NKB (Figure 2). The dose selected for each agonist in the further experiments was 25 pmol.

The cardiovascular responses elicited by SP and NKA were accompanied by excessive face washing and grooming/biting of hindlimbs (Table 3). These behavioural effects occurred simultaneously with the cardiovascular responses and presented a similar time course. On the other hand, i.c.v. injected [MePhe^7]NKB induced not exclusively but mainly uninterrupted wet dog shakes for the 30 min observation period; this behaviour was not parallel to the cardiovascular effects (Tables 1–3). When injected i.c.v., CSF (5 μl) produced no appreciable cardiovascular (Tables 1 and 2) or behavioural (Table 3) effects.

Effects of tachykinin receptor antagonists on the responses to SP, NKA and [MePhe^7]NKB

Cardiovascular responses as well as face washing but not grooming and wet dog shake induced by 25 pmol SP were significantly reduced (by about 50–70%) when animals were pretreated with 6.5 nmol RP 67580 (Tables 1–3). The inhibitory effect of the antagonist was no longer observed when the agonist was reinjected 24 h later. The cardiovascular response to SP was not further inhibited by higher doses of RP 67580. Maximal ΔMAP and ΔHR induced by 25 pmol SP in the absence of RP 67580 were 17.3 ± 4.2 mmHg ($n = 8$) and 93.7 ± 13.8 beats min^{-1} ($n = 8$) while in the presence of 65 nmol RP 67580, they were reduced to 4.0 ± 2.5 mmHg ($n = 5$) and 40.9 ± 10.3 beats min^{-1} ($n = 5$), respectively. The residual responses to SP in the presence of 6.5 nmol (Tables 1 and 2) and 65 nmol RP 67580 were not statistically different from each other. In contrast, the NK_1 antagonist (6.5 nmol) was inactive against the central cardiovascular and behavioural effects induced by 25 pmol NKA or [MePhe^7]NKB (Tables 1–3).

At 6.5 nmol, SR 48968 significantly reduced by approximately 60% the central cardiovascular response and abolished the behavioural effects (but not wet dog shakes) induced by 25 pmol NKA. The inhibition was reversible 24 h later, yet SR 48968 had no effect on the responses to 25 pmol SP or [MePhe^7]NKB (Tables 1–3). The i.c.v. injection of 65 nmol SR 48968 did not reduce further the cardiovascular effects evoked by NKA. Maximal ΔMAP and ΔHR induced by 25 pmol NKA in the absence of SR 48968 were 13.4 ± 3.6 mmHg ($n = 8$) and 88.7 ± 10.5 beats min^{-1} ($n = 8$) while in the presence of 65 nmol SR 48968, they were reduced to 3.1 ± 2.0 mmHg ($n = 5$) and 25.3 ± 10.1 beats min^{-1} ($n = 5$), respectively. The residual responses to NKA in the presence of 6.5 nmol (Tables 1 and 2) and 65 nmol SR 48968 were not statistically different from each other.

RP 67580 (6.5 nmol) and SR 48968 (6.5 nmol) were co-injected i.c.v. and tested against the agonist-mediated effects. This combination of antagonists completely abolished the increases in MAP and HR as well as the face washing, grooming and wet dog shakes induced by SP or NKA (25 pmol), yet this antagonist mixture did not affect the cardiovascular and behavioural responses to [MePhe^7]NKB (Tables 1–3). The blockade of the SP or NKA mediated cardiovascular responses by RP 67580 plus SR 48968 was no longer observed when SP or NKA was re-administered alone 24 h later (Figure 4).

I.c.v. pretreatment with the selective NK_3 receptor antagonist, R 486 (6.5 nmol), inhibited the pressor and tachycardiac responses as well as wet dog shake induced by 25 pmol [MePhe^7]NKB (Tables 1–3). The same pretreatment with R 486 failed, however, to alter both cardiovascular and behavioural responses induced either by SP or NKA (Tables 1–3). The cardiovascular effects of [MePhe^7]NKB were entirely recovered 24 h after treatment with R 486 (Figure 4). There were no significant differences in MAP and HR basal values among the experimental groups (Tables 1–2). None of the tested antagonists or agonists showed any apparent toxic effects. Moreover, at 6.5 nmol, RP 67580 or SR 48968 had no significant effect on MAP or HR (Figure 5). On the other hand, the NK_3 antagonist, R 486 (6.5 nmol), caused a small transient increase of MAP and HR that lasted less than 5 min (Figure 5). Furthermore, the three antagonists had no direct effects on the individual behaviours namely face washing, grooming and wet dog shake (Table 4).

Discussion

The intracerebroventricular injection of SP, NKA or [MePhe^7]NKB elicited dose-dependent increases in mean arterial blood pressure and heart rate accompanied by specific behavioural manifestations in conscious rats. SP and NKA prevailing responses were grooming and face washing, while activation of the NK_3 receptor induced mainly wet dog shake behaviour. Even though it is difficult to associate these behaviours with physiological correlates, the SP/NKA behaviours are typical behaviours observed during the defence reaction (Unger *et al.*, 1988; Itoi *et al.*, 1991).

The hypothalamus may be the site of action of tachykinins, since microinjections of SP into the anterior and ventromedial parts of hypothalamus evoked cardiovascular changes similar to those produced by i.c.v. injection of SP or NKA (Itoi *et al.*, 1991). Moreover, the magnocellular part of the rat hypothalamic paraventricular nucleus was identified as a site of action for the central effect of tachykinins (NK_3 agonists) on the release of vasopressin (Massi *et al.*, 1991; Takano *et al.*, 1993). However, it would be premature to reach any conclusions regarding the exact localization of the tachykinin receptors activated by i.c.v. SP, NKA or [MePhe^7]NKB. Nonetheless, the fast onset of the response to i.c.v. tachykinin agonists leads one to suggest that receptor sites must be localized in the circumventricular organs or in adjacent periventricular structures. A peripheral site of action is unlikely since i.v. injections of SP, NKA or [MePhe^7]NKB

Table 1 Effects of selective tachykinin receptor antagonists on changes in mean arterial blood pressure (MAP) elicited by the i.c.v. injection of 25 pmol substance P (SP), neurokinin A (NKA) or [MePhe^7]NKB in the conscious rat

				ΔMAP (mmHg) Time (min) after agonist injection				
Antagonist	*Agonist*	n	*Baseline MAP* (mmHg)	3	5	10	20	30
–	CSF	10	97.6 ± 3.6	1.5 ± 0.6	1.0 ± 0.6	1.2 ± 0.7	0.5 ± 0.3	0.4 ± 0.5
–	SP	10	109.3 ± 7.7	18.5 ± 4.0***	19.2 ± 2.9***	3.3 ± 2.0	1.5 ± 1.6	1.1 ± 1.3
RP 67580	SP	10	103.2 ± 6.0	5.8 ± 2.6*†	8.3 ± 4.0*†	2.1 ± 3.5	1.7 ± 1.3	−0.5 ± 0.6
–	NKA	6	98.5 ± 5.6	13.9 ± 3.1**	12.5 ± 2.4**	8.4 ± 1.3**	4.2 ± 1.3*	2.6 ± 0.9*
RP 67580	NKA	6	105.6 ± 4.3	10.3 ± 2.5*	9.3 ± 2.9**	6.1 ± 2.1*	3.6 ± 1.9*	1.5 ± 1.8
–	[MePhe^7]NKB	7	93.6 ± 6.7	10.2 ± 1.9**	7.3 ± 2.5*	4.3 ± 2.0*	4.6 ± 1.6*	3.2 ± 1.2*
RP 67580	[MePhe^7]NKB	7	103.1 ± 7.2	12.1 ± 5.3**	11.9 ± 3.4**	8.2 ± 2.4**	3.6 ± 2.7*	1.9 ± 2.1
–	SP	8	106.5 ± 6.1	18.0 ± 4.3***	20.5 ± 3.0***	3.1 ± 1.9	0.5 ± 0.4	−0.3 ± 1.1
SR 48968	SP	8	105.2 ± 5.1	12.9 ± 3.2**	17.1 ± 1.9***	2.1 ± 2.2	−1.3 ± 1.6	−2.1 ± 1.9
–	NKA	8	101.4 ± 4.1	12.5 ± 3.3**	12.6 ± 3.9**	9.0 ± 3.8*	3.9 ± 2.3*	2.2 ± 1.3
SR 48968	NKA	8	97.1 ± 9.1	4.3 ± 1.8†	5.8 ± 2.5*†	4.4 ± 3.1	2.5 ± 1.3*	2.4 ± 2.0
–	[MePhe^7]NKB	8	99.1 ± 5.6	9.8 ± 3.0**	12.5 ± 1.7**	5.4 ± 2.8*	3.6 ± 2.0*	2.8 ± 0.6*
SR 48968	[MePhe^7]NKB	8	102.5 ± 6.3	12.1 ± 4.1**	13.5 ± 2.9**	4.8 ± 1.9*	2.2 ± 1.4	2.5 ± 1.7
–	SP	7	94.8 ± 5.6	15.9 ± 2.4***	19.7 ± 3.3***	3.6 ± 1.8*	4.0 ± 1.9*	1.6 ± 0.7
RP 67580 + SR 48968	SP	7	97.5 ± 4.3	1.4 ± 2.5††	2.1 ± 0.9†††	0.5 ± 1.9†	1.3 ± 0.4†	0.1 ± 0.9
–	NKA	8	95.1 ± 3.8	13.5 ± 2.0**	10.9 ± 1.6**	8.5 ± 1.0**	3.2 ± 1.4*	2.8 ± 0.6*
RP 67580 + SR 48968	NKA	8	100.8 ± 4.4	−0.3 ± 1.8†††	1.2 ± 1.3††	0.6 ± 0.8††	2.1 ± 0.5*	2.2 ± 0.8*
–	[MePhe^7]NKB	5	97.6 ± 6.8	9.9 ± 3.6**	8.1 ± 2.4**	5.3 ± 2.1*	4.7 ± 2.2*	4.8 ± 1.8*
RP 67580 + SR 48968	[MePhe^7]NKB	5	95.2 ± 4.5	8.5 ± 2.3**	5.3 ± 2.9*	4.1 ± 3.1	2.3 ± 1.9	1.6 ± 1.6
–	SP	7	102.6 ± 5.1	16.5 ± 2.3***	20.3 ± 3.1***	2.9 ± 0.5*	0.2 ± 0.9	−1.1 ± 0.3
R 486	SP	7	107.1 ± 6.3	17.5 ± 3.6***	18.9 ± 5.1***	6.0 ± 3.2*	1.5 ± 1.6	1.9 ± 1.0
–	NKA	6	106.3 ± 5.3	13.2 ± 3.5**	11.0 ± 2.0**	6.1 ± 1.2*	2.1 ± 3.4	2.5 ± 1.6
R 486	NKA	6	108.0 ± 9.1	13.5 ± 3.2**	12.0 ± 2.2**	3.9 ± 1.6*	1.6 ± 1.8	−0.2 ± 1.3
–	[MePhe^7]NKB	8	100.7 ± 7.0	11.0 ± 1.6**	8.8 ± 1.5**	6.0 ± 1.5*	4.3 ± 3.9	3.0 ± 2.3
R 486	[MePhe^7]NKB	8	98.3 ± 5.1	3.7 ± 3.1†	1.2 ± 2.6†	−0.3 ± 2.9†	−0.9 ± 2.6	0.7 ± 2.8

Values represent the means ± s.e.mean of (n) rats. The antagonists were injected at the dose of 6.5 nmol, 5 min prior to the agonist. Statistical comparison to CSF (*) or to the agonist in the absence of antagonist (†) was calculated with a two-way ANOVA: *,†$P<0.05$; **,††$P<0.01$; ***,†††$P<0.001$.

Table 2 Effects of selective tachykinin receptor antagonists on changes in heart rate (HR) elicited by the i.c.v. injection of 25 pmol substance P (SP), neurokinin A (NKA) or [MePhe^7]NKB in the conscious rat

				ΔHR (beats min^{-1}) Time (min) after agonist injection				
Antagonist	*Agonist*	n	*Baseline HR*	3	5	10	20	30
–	CSF	10	311.4 ± 19.5	7.3 ± 5.2	5.1 ± 9.3	8.2 ± 8.9	3.5 ± 6.1	4.8 ± 7.0
–	SP	10	305.6 ± 15.6	98.1 ± 12.1***	95.1 ± 9.4***	17.3 ± 6.9	−2.0 ± 8.5	−5.4 ± 7.1
RP 67580	SP	10	318.0 ± 21.3	43.1 ± 9.5**†	41.2 ± 12.5**†	10.3 ± 8.2	12.5 ± 9.0	0.8 ± 7.2
–	NKA	6	293.1 ± 22.3	78.5 ± 9.3***	89.1 ± 11.6***	43.9 ± 8.3**	28.1 ± 10.0*	20.1 ± 5.2*
RP 67580	NKA	6	301.4 ± 17.6	82.5 ± 12.0***	87.3 ± 9.4***	50.1 ± 9.1**	20.5 ± 5.2*	12.3 ± 4.8
–	[MePhe^7]NKB	7	306.8 ± 16.3	60.1 ± 7.3**	76.2 ± 10.1**	38.4 ± 7.8**	29.3 ± 6.1*	25.4 ± 3.8*
RP 67580	[MePhe^7]NKB	7	319.5 ± 18.2	55.4 ± 8.0**	81.2 ± 6.3***	40.3 ± 8.8**	17.9 ± 7.3*	25.1 ± 4.1*
–	SP	8	311.9 ± 17.1	103.2 ± 11.4***	96.4 ± 10.3***	25.1 ± 12.0	7.8 ± 9.5	−2.6 ± 5.7
SR 48968	SP	8	298.1 ± 14.3	93.6 ± 8.6***	79.5 ± 9.4***	15.4 ± 7.1	−1.9 ± 7.2	−4.4 ± 6.0
–	NKA	8	305.0 ± 16.8	65.3 ± 8.1**	93.1 ± 10.5***	51.1 ± 6.8**	34.1 ± 7.4**	12.5 ± 8.0
SR 48968	NKA	8	303.8 ± 17.4	28.0 ± 9.3*†	36.3 ± 7.0*†	30.1 ± 4.2*†	28.1 ± 4.1**	17.3 ± 6.3*
–	[MePhe^7]NKB	8	301.6 ± 18.3	63.1 ± 9.1**	69.7 ± 7.8**	51.3 ± 6.7**	33.4 ± 9.0**	10.1 ± 7.7
SR 48968	[MePhe^7]NKB	8	289.1 ± 16.3	58.4 ± 7.5**	59.2 ± 8.3**	43.2 ± 5.4**	27.1 ± 4.3**	7.0 ± 8.1
–	SP	7	303.4 ± 22.1	87.5 ± 8.4***	80.6 ± 7.7***	12.3 ± 9.1	5.3 ± 3.8	−5.6 ± 6.1
RP 67580 + SR 48968	SP	7	305.1 ± 23.7	3.5 ± 6.8††	5.3 ± 8.1††	−3.5 ± 6.7	−0.8 ± 4.7	−7.9 ± 5.6
–	NKA	8	287.1 ± 16.2	80.3 ± 13.5***	92.1 ± 9.1***	60.3 ± 7.2**	31.4 ± 7.5**	12.3 ± 8.1
RP 67580 + SR 48968	NKA	8	296.1 ± 18.5	10.3 ± 5.3††	14.1 ± 6.0††	3.6 ± 8.0††	7.3 ± 5.1†	4.3 ± 3.6
–	[MePhe^7]NKB	5	294.1 ± 16.0	55.1 ± 12.3**	73.1 ± 9.9**	40.6 ± 8.3**	21.6 ± 8.0*	18.5 ± 6.3*
RP 67580 + SR 48968	[MePhe^7]NKB	5	304.1 ± 14.2	48.3 ± 9.3**	64.1 ± 8.3**	34.2 ± 7.5**	18.3 ± 5.1*	7.3 ± 7.8
–	SP	7	309.1 ± 17.8	95.3 ± 12.7***	98.3 ± 10.9***	23.4 ± 7.1*	8.6 ± 5.9	−5.8 ± 4.9
R 486	SP	7	301.2 ± 17.5	93.4 ± 13.0***	98.1 ± 8.3***	17.3 ± 10.8	9.1 ± 6.1	7.6 ± 4.8
–	NKA	6	297.0 ± 14.9	78.1 ± 9.6***	87.3 ± 10.7***	61.2 ± 8.5**	34.1 ± 7.9**	26.4 ± 5.8*
R 486	NKA	6	299.3 ± 18.0	62.9 ± 10.8**	90.1 ± 9.3***	53.1 ± 8.0**	27.1 ± 5.6**	16.9 ± 8.1*
–	[MePhe^7]NKB	8	316.2 ± 18.9	61.3 ± 9.7**	89.1 ± 5.3***	43.8 ± 5.7**	39.1 ± 7.3**	21.5 ± 6.0*
R 486	[MePhe^7]NKB	8	320.1 ± 20.5	23.1 ± 8.2*†	19.0 ± 7.0*††	14.7 ± 7.1†	12.1 ± 9.0†	8.3 ± 4.1†

See footnote to Table 1.

Table 3 Effects of selective tachykinin receptor antagonists on behavioural responses elicited by the i.c.v. injection of 25 pmol substance P (SP), neurokinin A (NKA) or [MePhe^7]NKB in the conscious rat

Antagonist	*Agonist*	n	*Face washing score* (30 min^{-1})	*Grooming score* (30 min^{-1})	*Wet dog shake episodes* (30 min^{-1})
–	CSF	10	1.3 ± 0.4	1.2 ± 0.8	2.0 ± 1.3
–	SP	10	17.2 ± 3.1**	9.3 ± 2.6*	5.6 ± 2.8
RP 67580	SP	10	5.3 ± 2.4*†	8.3 ± 3.0*	6.8 ± 3.7
–	NKA	6	9.2 ± 2.6*	6.6 ± 3.0*	4.0 ± 1.6
RP 67580	NKA	6	7.5 ± 2.7*	5.1 ± 1.2*	7.4 ± 2.4*
–	[MePhe^7]NKB	7	12.5 ± 3.6*	9.3 ± 3.6*	22.6 ± 7.1**
RP 67580	[MePhe^7]NKB	7	9.6 ± 3.7*	8.2 ± 3.4*	26.4 ± 8.3**
–	SP	8	10.7 ± 3.6*	8.5 ± 2.4*	5.1 ± 3.2
SR 48968	SP	8	12.5 ± 4.1*	8.1 ± 2.0*	5.9 ± 2.1
–	NKA	8	8.9 ± 2.9*	7.0 ± 1.5*	9.1 ± 4.3*
SR 48968	NKA	8	0.9 ± 0.2†	1.2 ± 1.0†	10.3 ± 2.9*
–	[MePhe^7]NKB	8	9.7 ± 2.1*	6.3 ± 1.2*	25.6 ± 5.4**
SR 48968	[MePhe^7]NKB	8	10.1 ± 2.5*	7.0 ± 2.5*	21.6 ± 7.2**
–	SP	7	15.7 ± 2.9**	10.3 ± 2.6*	8.0 ± 2.8*
RP 67580 + SR 48968	SP	7	2.0 ± 0.9††	3.2 ± 0.7†	4.1 ± 2.0†
–	NKA	8	13.2 ± 2.6*	7.0 ± 3.6*	5.1 ± 2.6
RP 67580 + SR 48968	NKA	8	1.3 ± 0.8††	2.9 ± 1.1†	2.6 ± 1.9
–	[MePhe^7]NKB	5	10.5 ± 1.9*	6.5 ± 1.6*	30.6 ± 7.1**
RP 67580 + SR 48968	[MePhe^7]NKB	5	7.8 ± 1.4*	5.8 ± 2.0*	28.5 ± 6.7**
–	SP	7	15.4 ± 3.2*	8.1 ± 1.2*	12.5 ± 5.1*
R 486	SP	7	17.3 ± 2.7*	7.2 ± 2.1*	10.3 ± 3.1*
–	NKA	6	9.3 ± 1.2*	7.3 ± 2.4*	8.5 ± 2.5*
R 486	NKA	6	7.3 ± 2.0*	6.0 ± 2.0*	9.1 ± 2.3*
–	[MePhe^7]NKB	8	5.2 ± 1.7*	5.3 ± 2.0*	22.8 ± 8.5**
R 486	[MePhe^7]NKB	8	4.4 ± 1.4*	3.4 ± 1.5	4.5 ± 1.5†

Values represent the frequency of individual behaviour for 30 min and are indicated by the mean ± s.e.mean of (n) rats. The antagonists were injected at a dose of 6.5 nmol, 5 min prior to the agonist. Statistical comparison to CSF (*) was evaluated with a Wilcoxon–Mann–Whitney (U) test, while comparison to the agonist in the absence of antagonist (†) was calculated with Student's t test for paired samples; *, †$P<0.05$; **, ††$P<0.01$.

cause decreases in blood pressure (Couture *et al.*, 1989). Furthermore, the possibility of a spinal activation can also be excluded since intrathecal injection of 65 nmol [MePhe^7]NKB produced no cardiovascular effect while 6.5 nmol of SP injected intrathecally was necessary to induce an increase of 10–15 mmHg (Hassésian *et al.*, 1988).

Immunocytochemistry, *in situ* hybridization and radioimmunoassay studies have shown discrete and abundant distribution of SP, NKA and NKB (and their preprotachykinin mRNAs) in all major subdivisions of the rat brain (Warden & Young, 1988; Harlan *et al.*, 1989; Marksteiner *et al.*, 1992; Merchenthaler *et al.*, 1992; Lucas *et al.*, 1992). Whereas SP is found throughout the rat brain, NKB is distributed more to forebrain than to brainstem structures (Cuello & Kanazawa, 1978; Lucas *et al.*, 1992; Merchenthaler *et al.*, 1992). A high density and widespread distribution of SP and NKA was reported in the rat hypothalamus (Larsen *et al.*, 1992). Also, the paraventricular and supraoptic nuclei of the hypothalamus and the substantia nigra have higher contents of NKB-like immunoreactivity than any other CNS areas (Tateishi *et al.*, 1989; Merchenthaler *et al.*, 1992). Both the NK_1 and NK_3 receptors have been found in moderate to high density in the paraventricular and supraoptic nuclei of the rat hypothalamus (Dam *et al.*, 1990a,b; Larsen *et al.*, 1992; Maeno *et al.*, 1993). However, the presence of NK_2 receptor binding sites in the rat brain remains controversial (Mantyh *et al.*, 1989; Quirion *et al.*, 1991; Takeda & Krause, 1991; Mussap *et al.*, 1993).

Our results support our earlier finding that i.c.v. SP acts primarily through the activation of NK_1 receptors to induce cardiovascular and behavioural changes; this conclusion was reached on the basis of results obtained with CP 96345 (Tschöpe *et al.*, 1992). These results needed to be confirmed with RP 67580, a novel NK_1 antagonist (Carruette *et al.*, 1992; Rouissi *et al.*, 1993) which does not have the non-specific action of CP 96345 on calcium and sodium channels (Schmidt *et al.*, 1992; Guard *et al.*, 1993; Caeser *et al.*, 1993). In addition, the present study provides an explanation for the failure of CP 96345 (Tschöpe *et al.*, 1992) or RP 67580 (even at high doses) to abolish the central effects of SP. The persisting residual cardiovascular and behavioural responses to SP measured after pretreatment with the NK_1 receptor antagonist (RP 67580) were blocked when RP 67580 was co-administered with the NK_2 antagonist, SR 48968. These results suggest that SP can activate NK_2 receptors during NK_1 receptor blockade, unmasking the non-selectivity of the natural peptide SP. Since individual pretreatments with SR 48968 and R 486 failed to modify the central effects of SP, it appears that SP acts preferentially on the NK_1 receptor when the latter is functional.

The central NKA effects were very similar to those mediated by SP. However, NKA seems to activate mainly NK_2 receptors since only the NK_2 receptor antagonist SR 48968 reduced the biological effects of NKA, while the NK_1 (RP 67580) or NK_3 (R 486) receptor antagonists were inactive against NKA. These data confirm a previous study in which we concluded that the cardiovascular and behavioural effects of i.c.v. NKA are mediated by a NK_2 receptor that was sensitive to R 396 (NK_2 selective antagonist) but not to CP 96345 (NK_1 selective antagonist) (Tschöpe *et al.*, 1992). However, a higher dose of SR 48968 (65 nmol) was unable to block entirely the NKA-mediated responses, and as was the case with SP administration, the residual effects of NKA were completely abolished when SR 48968 was co-injected with the NK_1 selective antagonist, RP 67580. These results

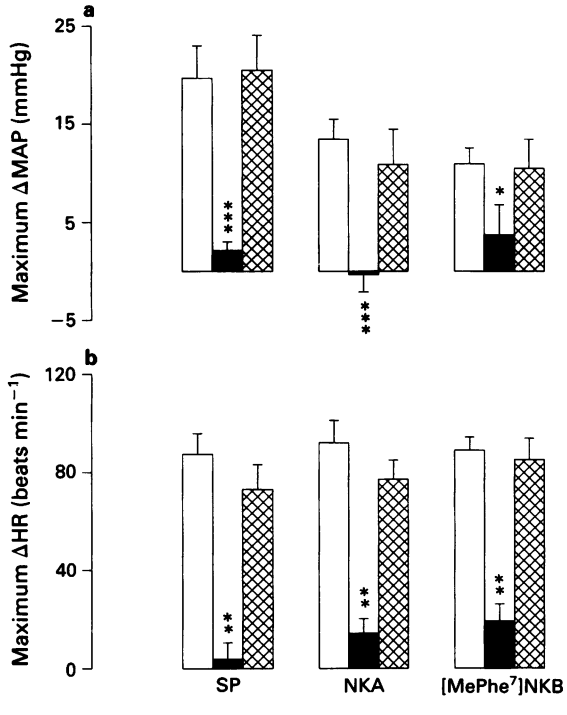

Figure 4 Effects of selective tachykinin receptor antagonists on maximal changes in (a) mean arterial blood pressure (MAP) and (b) heart rate (HR) induced by the i.c.v. injection of 25 pmol substance P (SP), neurokinin A (NKA) or [MePhe^7]neurokinin B ([MePhe^7]NKB) in conscious rats. The agonist was injected alone on day 1 (open columns), 5 min after the antagonist on day 2 (solid columns) or alone on day 3 (cross-hatched columns). SP and NKA were tested in the presence of RP 67580 plus SR 48968 (6.5 nmol each) while [MePhe^7]NKB was tested in the presence of 6.5 nmol R 486. Values represent the means ± s.e.mean of 7–8 rats. Statistically significant difference compared to the agonist alone (open columns) is indicated by $*P<0.05$; $**P<0.01$; $***P<0.001$.

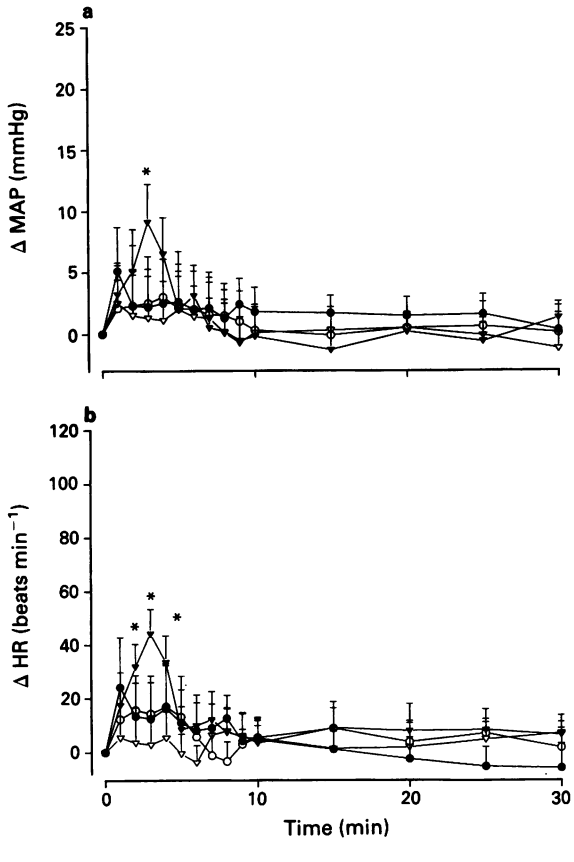

Figure 5 Direct effects of 6.5 nmol RP 67580 (○), 6.5 nmol SR 48968 (●), 6.5 nmol R 486 (▼) or CSF (▽) on (a) mean arterial blood pressure (MAP) and (b) heart rate (HR) after i.c.v. injection in conscious rats. Values represent the means ± s.e.mean of 7–8 rats. Statistically significant difference compared to CSF values is indicated by $*P<0.05$.

Table 4 Effects of selective tachykinin receptor antagonists on behavioural responses in the conscious rat

Treatment	*Dose* (nmol)	*Face washing score* (30 min^{-1})	*Grooming score* (30 min^{-1})	*West dog shake episodes* (30 min^{-1})
CSF	–	1.5 ± 0.6	1.3 ± 1.0	1.9 ± 1.4
RP 67580	6.5	2.1 ± 1.1	2.5 ± 1.3	1.4 ± 0.8
SR 48968	6.5	2.3 ± 1.6	2.4 ± 1.5	2.9 ± 1.0
RP 67580 + SR 48968	6.5 each	2.6 ± 1.6	1.9 ± 1.2	2.0 ± 1.3
R 486	6.5	1.5 ± 0.7	1.7 ± 0.9	3.1 ± 1.8

Values represent the frequency of individual behaviour for 30 min and are indicated by the mean ± s.e.mean of 8 rats in each group. No statistical difference was found when compared to CSF values.

can be interpreted in the same way as the SP-mediated responses, but in this case, NKA activates primarily NK_2 receptors. During NK_2 receptor blockade, a non selective activation of NK_1 receptors has been revealed with NKA.

The most prominent behavioural response (wet dog shakes) evoked by i.c.v. [MePhe^7]NKB injection is indicative of a different central neuronal pathway and confirms results obtained with senktide (NK_3 agonist), injected either subcutaneously or intracisternaly (Stoessl *et al.*, 1988) or i.c.v. (Itoi *et al.*, 1992) in the rat. Senktide also induced pressor responses through vasopressin release when injected either i.c.v. or directly into the hypothalamic paraventricular nucleus of the anaesthetized rat (Takano *et al.*, 1990; 1993). As expected, [MePhe^7]NKB induced cardiovascular and wet dog shake responses were selectively blocked by the NK_3 receptor antagonist, R 486 (Drapeau *et al.*, 1990; Regoli *et al.*, 1991) and were unaffected by RP 67580 or SR 48968 administered individually or in combination, thus assigning the NK_3 receptor as the sole functional tachykinin receptor site mediating [MePhe^7]NKB responses. Hence, our study confirms the presence of functionally active supraspinal NK_3 sites involved in the central cardiovascular and behavioural effects of tachykinins.

The inhibitory effect of the three antagonists was reversible and not related to motor deficits or to changes of baseline parameters. No residual agonists activity was shown with i.c.v. injection of SR 48968 or RP 67580 which is consistent with *in vitro* studies (Advenier *et al.*, 1992; Carruette *et al.*, 1992). On the other hand, the NK_3 antagonist, R 486, exhibited a direct central stimulatory effect which might be due to a residual agonistic activity on both NK_1 and NK_2 receptors (Regoli *et al.*, 1991). Since R 486 blocked selec-

tively the [MePhe^7]NKB-induced effects, this antagonist appears suitable for investigating the functional role of central NK_3 receptors. A similar NK_3 receptor antagonist, R 487 ([Phe^7, β-Ala^8]NKA(4-10), 6.5 nmol) was also found to block the central cardiovascular and behavioural effects of [MePhe^7]NKB in a selective and reversible manner (Picard *et al.*, 1992).

In summary, selective and potent antagonists of NK_1, NK_2 and NK_3 receptors have been used to characterize the receptor subtypes which are responsible for the central cardiovascular and behavioural effects of SP, NKA and [MePhe^7] NKB. SP activates mainly NK_1 but also NK_2 receptors whereas NKA-mediated effects are secondary to NK_2 and to a lesser extent NK_1 receptor activation. The interactions of SP and NKA with the NK_1 and NK_2 receptors were seen at doses as low as 25 pmol. The central effects of [MePhe^7]NKB are mediated by specific tachykinin receptors which are not identical with those activated by SP or by NKA and which belong to NK_3 receptor subclass. Hence, our data provide pharmacological evidence for the existence of distinct populations of functionally active NK_1, NK_2 and NK_3 receptors in the adult rat brain.

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