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Characterization of tachykinin receptors in the uterus of the oestrogen-primed rat

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1 The aim of our study was to characterize the tachykinin receptor population in the oestrogen-primed rat uterus. For this purpose, we investigated the receptor type(s) responsible for tachykinin-induced contraction of longitudinally-arranged smooth muscle layer. The effects of substance P (SP), neurokinin A (NKA), neurokinin B (NKB) and several of their analogues with well-defined selectivities for tachykinin NK_1, NK_2 and NK_3 receptors were studied and their inhibition by the selective nonpeptide tachykinin receptor antagonists (**S**)1-(2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl)-4-phenyl-1-azoniabicyclo[2.2.2]octane chloride (SR 140333, NK_1-selective), (**S**)-N-methyl-N[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide (SR 48968, NK_2-selective) and (**R**)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl)- 4-phenylpiperidin- 4-yl)-N- methylacetamide (SR 142801, NK_3-selective) was evaluated. Additionally, expression of tachykinin receptor mRNA was examined by using the reverse transcription-polymerase chain reaction (RT-PCR).

2 SP, NKA, [Nle^{10}]-NKA(4-10), the analogue with selectivity at the tachykinin NK_2 receptor type, and NKB elicited concentration-dependent contractions of the rat uterus. The pD_2 values were 5.95 ± 0.19; 6.73 ± 0.21; 7.53 ± 0.12 and 5.76 ± 0.21, respectively. The selective agonist for the tachykinin NK_1 receptor [$Sar^9Met(O_2)^{11}$]-SP produced a small phasic response in the nanomolar concentration range. The selective tachykinin NK_3 receptor agonist [$MePhe^7$]-NKB failed to induce any significant contraction.

3 In the presence of the neutral endopeptidase inhibitor phosphoramidon (1 μM), the log concentration-response curves to exogenous tachykinins and their analogues were shifted significantly leftwards. The pD_2 values were 6.12 ± 0.10, 8.04 ± 0.07, 7.89 ± 0.03 and 6.59 ± 0.07 for SP, NKA, [Nle^{10}]-NKA(4-10) and NKB, respectively. In the presence of phosphoramidon (1 μM), [$Sar^9Met(O_2)^{11}$]-SP (1 nM – 0.3 μM) induced concentration-dependent contractions of increasing amplitude when only one concentration of drug was applied to each uterine strip and the pD_2 value was 7.61 ± 0.89. [$MePhe^7$]-NKB induced small, inconsistent contractions and, therefore, a pD_2 value could not be calculated.

4 In experiments performed in the presence of phosphoramidon (1 μM), SR 48968 (3 nM – 0.1 μM) caused parallel and rightward shifts in the log concentration-response curves of NKA. The calculated pK_B value was 9.16 ± 0.08 and the slope of the Schild regression was 1.28 ± 0.24. SR 48968 (0.1 μM) also antagonized responses to SP with an apparent pK_B value of 7.63 ± 0.13. SR 48968 (0.1 μM) inhibited contractions elicited by NKB (1 nM – 3 μM) and [Nle^{10}]-NKA(4 – 10) (0.1 nM – 3 μM) but had no effect on the response evoked by [$Sar^9Met(O_2)^{11}$]-SP (0.1 μM).

5 SR 140333 (0.1 μM) inhibited responses to SP with an apparent pK_B value of 7.19 ± 0.22. This compound did not significantly affect responses to NKA, [Nle^{10}]-NKA(4-10) and NKB, but suppressed [$Sar^9Met(O_2)^{11}$]-SP (0.1 μM)-induced contraction. SR 142801 (0.1 μM) had no effect on responses to natural tachykinins or their analogues.

6 Total RNA was extracted from some of the uteri used in functional studies. RT-PCR assays revealed single bands corresponding to the expected product sizes encoding cDNA for tachykinin NK_1 (587 base pairs) and NK_2 receptors (491 base pairs) ($n = 6$ different animals). A very low abundance transcript corresponding to the 325 base pairs product expected for the tachykinin NK_3 receptor was detected.

7 The present data show that functionally active tachykinin NK_1 and NK_2 receptors are expressed in the oestrogen-primed rat uterus. The NK_2 receptor type seems to be the most important one involved in the contractile responses elicited by tachykinins. NK_3 receptors are present in trace amounts and seem not to be involved in tachykinin-induced contractions.

Keywords: Tachykinins; [$Sar^9Met(O_2)^{11}$]-SP; [Nle^{10}]-NKA(4-10); [$MePhe^7$]-NKB; SR 140333; SR 48968; SR 142801; tachykinin receptor expression; rat uterus

Introduction

Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) are members of a family of structurally related peptides called the tachykinins which are widely distributed within the mammalian peripheral and central nervous system (Otsuka & Yoshioka, 1993; Regoli *et al*., 1994). These neuropeptides are localized in sensory nerves supplying a number of mammalian peripheral tissues where they may be released and exert local effect functions (Maggi *et al*., 1995; Lundberg, 1996). In the female reproductive tract, SP and NKA coexist with calcitonin

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gene-related peptide (CGRP) in capsaicin-sensitive primary afferent fibres, the presence of which has been demonstrated in virtually all mammalian species examined (Ottesen & Fahrenkrug, 1990; Papka & Shew, 1994). In the rat, nerve terminals are associated with the myometrial and vascular smooth muscle and are distributed throughout the endocervix (Traurig *et al*., 1984; 1988; Shew *et al*., 1991). However, the precise physiological role of tachykinins in the genital tract remains unclear.

Tachykinins interact with three distinct types of receptor, as suggested by pharmacological and radioligand binding experiments (Maggi *et al*., 1993; Regoli *et al*., 1994). These receptors have been designated NK_1, NK_2 and NK_3 and are activated preferentially by SP, NKA and NKB, respectively. However, the naturally occurring tachykinins are not highly selective and can activate each of the tachykinin receptor (Hastrup & Schwartz, 1996). In the oestrogen-primed rat uterus, SP, NKA and NKB induce contractile responses which seem to be mainly mediated by activation of a NK_2 receptor (Fisher *et al*., 1993; Pennefather *et al*., 1993) but the presence of a non-neuronal NK_3 receptor has also been suggested (Barr *et al*., 1991). The availability of novel, highly selective agonists and antagonists acting at the tachykinin receptor types provides an additional opportunity to characterize the relative contribution of NK_1, NK_2 and NK_3 receptors to tachykinin-induced responses. In addition, the tachykinin receptors have recently been cloned (Sasai & Nakanishi, 1989; Shigemoto *et al*., 1990; Hershey *et al*., 1991; Gerard *et al*., 1993), allowing an alternative approach to study the localization and quantification of these receptors in a tissue. Therefore, the aim of the present investigation was to determine the expression and function of the different tachykinin receptors in the oestrogen-primed rat uterus. For this purpose, we studied the mechanical responses produced by the endogenous ligands SP, NKA and NKB and the selective analogues [$Sar^9Met(O_2)^{11}$]-SP, [Nle^{10}]-NKA(4-10) and [$MePhe^7$]-NKB (Regoli *et al*., 1988; 1994), as well as the ability of the highly selective non-peptide antagonists SR 140333 (Nolpitantium, NK_1 receptor antagonist, Emonds-Alt *et al*., 1993), SR 48968 (Saredutant, NK_2 receptor antagonist, Emonds-Alt *et al*., 1992; Advenier *et al*., 1992) and SR 142801 (Osanetant, NK_3 receptor antagonist, Emonds-Alt *et al*., 1995) to antagonize the tachykinin-induced contractile responses. In addition, we analysed the expression of tachykinin NK_1, NK_2 and NK_3 receptors in the rat uterus, by using reverse transcription-polymerase chain reaction to determine whether mRNAs were present for these receptors in this preparation.

Methods

Animals

Virgin female Wistar rats (200–250 g) were treated with 17β-oestradiol benzoate dissolved in olive oil (50 μg kg^{-1}, 0.25 ml, i.p.) 24 and 48 h before being killed. The stage of the oestrous cycle was determined by microscopic examination of a vaginal smear. Animals which presented the vaginal exfoliate cytology of oestrous, with the presence of cornified cells lacking a nucleus and absence of leukocytes, were killed by decapitation and the uterine horns removed, trimmed of surrounding connective tissue and opened longitudinally.

Functional studies

Longitudinal strips of uterine smooth muscle (8–10 mm in length and 1–2 mm in diameter) were prepared and mounted in siliconized (with 5% dimethyl-dichlorosilane in chloroform; Sigma, St. Louis, U.S.A.) tissue baths containing 4 ml of physiological salt solution of the following composition (mM): NaCl 154, KCl 5.6, $CaCl_2$ 1.9, $MgCl_2$ 0.95, $NaHCO_3$ 5.95 and glucose 2.78, gassed with 95% O_2, 5% CO_2. Some experiments were carried out in a low Ca^{2+} (0.54 mM Ca^{2+}) medium. The bathing solution was maintained at 32°C. Mechanical responses were recorded isometrically by means of force-displacement transducers (Grass FT-03) connected to a LETICA amplifier and an ABB GOERZ SE 130 multichannel recorder. A resting tension of 0.5 g was applied to the tissue, and an equilibration period of 45 min was allowed, during which the preparation was washed with fresh solution every 15 min. After equilibration, the preparation was induced to contract two or more times by administration of a maximally effective concentration of acetylcholine (ACh, 1 mM) at 30 min intervals, until constant responses were obtained. Uterine strips were allowed to equilibrate for a further 60 min period and then non-cumulative log concentration-response curves to SP, NKA, NKB and to the selective analogues [$Sar^9Met(O_2)^{11}$]-SP, [Nle^{10}]-NKA(4-10) and [$MePhe^7$]-NKB were established. Each concentration of a tachykinin or analogue remained in contact with the tissue for 5 min and the tissue was then washed thoroughly and allowed to rest for 20 min before the addition of the next concentration. The log concentration-response curves to tachykinins and their analogues were constructed in the absence or in the presence of the neutral endopeptidase inhibitor phosphoramidon (1 μM),which was added to the bath 20 min before the application of an agonist and maintained in contact with the preparation throughout the experiment. A higher concentration of phosphoramidon (5 μM) did not further potentiate the contractile response evoked by the different agonists tested. Some experiments were carried out in uterine strips pretreated with phosphoramidon (1 μM) to assess the effect of the tachykinin receptor antagonists SR 140333 (NK_1-receptor selective), SR 48968 (NK_2-receptor selective) or SR 142801 (NK_3-receptor selective). In these experiments, tissues were incubated for 90 min with a single concentration of an antagonist (test strips) or its vehicle (time-matched paired control strips) before the concentration-response curve was constructed. Phosphoramidon and the antagonist or its vehicle were reapplied after each concentration of the agonist was washed out. Only one log concentration-response curve was constructed on each strip, the same compound being tested on parallel strips. At the end of the experiment, the preparation was washed repeatedly for 60 min before application of a maximally effective concentration of ACh (1 mM), to check the stability of tissue contractility. This last response served as an internal standard for all experiments.

In some experiments, two non-cumulative log concentration-response curves to SP, NKA or NKB were constructed on the same uterine strip with an interval of 60 min between curves, to check for desensitization. Another set of experiments was carried out to evaluate the possible non-specific effects of phosphoramidon and the tachykinin receptor antagonists. After reproducible responses to ACh (1 mM) had been obtained, non-cumulative log concentration-response curves to oxytocin (0.1 nM–1 μM) were constructed in parallel strips after equilibration for 20 min with phosphoramidon (1 μM), for 90 min with SR 140333 (0.1 μM), SR 48968 (0.1 mM) or SR 142801 (0.1 μM), or for the same time period with the corresponding vehicle (time-matched paired control strips). The effect of the compounds on the response to ACh (1 mM) was also assessed.

Statistical analysis of results

Contractile responses were measured as peak increases in force development or as areas under the force-time curve during the 5 min period that each concentration of an agent was in contact with the preparation. The responses are expressed as a percentage of the peak increase in force or of the area under the force-time curve measured during a 5 min period for each agonist or for ACh (1 mM). To measure the areas, polygraph tracings were scanned and then processed by using the Sigma-Scan software package (Jandel Scientific Corp., Erkrath, Germany). Maximal effects of agonist (E_{max}) were considered as 100% or expressed as a percentage of the response to ACh 1 mM. The pD_2 values were obtained from individual log concentration-response curves (areas), and are defined as the negative log of the concentration producing 50% of the maximal response to the corresponding agonist.

Competitive antagonism was assessed according to the method of Arunlakshana & Schild (1959). The affinity of competitive antagonists was expressed in terms of pK_B from the equation: $pK_B = \log (CR-1)$-log [antagonist concentration] (Arunlakshana & Schild, 1959), where CR is the dose-ratio. When a single concentration of antagonist was used, apparent pK_B values were determined from individual CR values by use of the same equation.

Values are expressed as mean ± s.e.mean for n number of experiments. Unless otherwise indicated, n represents the number of different experiments in n different animals. Statistical analysis was performed by Student's paired or unpaired t test, with a probability level of $P<0.05$ being considered significant.

Expression studies

RNA isolation Total RNA of approximately 20 mg of uterine tissue and cerebral cortex was isolated according to the method of Chomczynski & Sacchi (1987). The RNA pellet was resuspended in 50 μl of RNase-free water and quantified by spectrophotometric measurement at 260 μM. The integrity of the purified RNA collected with this method was confirmed by visualization of the 28S and 18S ribosomal RNA bands after the electrophoresis of RNA through a 1% agarose-formaldehyde gel.

Treatment of total RNA with DNase I To eliminate contaminating genomic DNA, total RNA samples were treated with FPLC pure DNase I (Pharmacia Biotech, Uppsala, Sweden). RNA samples (10 μg each) were incubated at 37°C for 10 min with 10 u of DNase I in DNase buffer (40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 10 u ribonuclease inhibitor RNasin (Promega Corp., Madison, U.S.A.). The reaction was stopped by extraction with phenol/chloroform (1:1 v/v), and RNA samples were ethanol-precipitated, then dried and resuspended in diethylpyrocarbonate (DEPC)-treated water and stored at −70°C until use.

Reverse transcription of RNA First strand cDNA was synthesized by use of random hexamers according to Pharmacia instructions (First-strand cDNA synthesis kit, Pharmacia Biotech, Uppsala, Sweden) in a 15 μl volume reaction containing 5 μg of DNase-treated total RNA.

Oligonucleotide primers Appropriate sense and antisense primer oligonucleotides were designed from the rat sequences of tachykinin receptors by using the OLIGO primer analysis software, version 4.0. Primers designed, their sizes and appropriate references are shown in Table 1. β-Actin served as an internal control. Sequences of sense and antisense primers for β-actin were 5′ CCTAGCACCATGAAGATCAA 3′ and 5′ TTTCTGCGCAAGTTAGGTTTT 3′, respectively, according to Makino *et al*. (1990). The expected size of the PCR product was 227 base pairs (bp).

Polymerase chain reaction PCR was used to detect the mRNAs for either tachykinin NK_1, NK_2 or NK_3 receptors by use of specific oligonucleotide primers for each receptor type. Amplification of the β-actin gene transcript was used to control the efficiency of RT-PCR among the samples. Equal aliquots of the RT solution (corresponding to 100 ng of total RNA) were serially half diluted and amplified for a fixed number of cycles with the β-actin primers. In the exponential range of amplification, the amount of PCR product in the sample is directly proportional to the concentration of target cDNA (Cullinan-Bove & Koos, 1993; Chelly & Kahn, 1994). Equal amounts of templates were then amplified for the three tachykinin receptors and β-actin. PCR mixes contained 0.2 μM primers, 1.5 u of Taq polymerase (Pharmacia Biotech, Uppsala, Sweden), the buffer supplied, 2.5 mM $MgCl_2$, 200 μM dNTP's and cDNA in 25 μl. Each experiment also contained two negative controls (one with the RT reaction containing no added RNA and the other one containing non retro-transcripted RNA) and a positive control (cDNA from rat cerebral cortex). Following heating at 94°C for 2 min, the parameters used for PCR amplification were as follows: denaturation, 30 s at 94°C; annealing, 30 s at 58°C; extension, 30 s at 72°C. Cycle numbers were 35 for tachykinin receptors and 24 for β-actin. PCR products were separated by gel electrophoresis on 1.7% agarose, stained with ethidium bromide and visualized and photographed under u.v. transiluminator (Fotodyne, Hartland, U.S.A.). DNA fluorescence in ethidium bromide-stained gels was densitometrically scanned by using a video documentation system and the image analysis software Intelligent quantifier (Bio Image Systems Corporation, Michigan, U.S.A.). Tachykinin receptor mRNA expression levels were normalized with respect to β-actin mRNA levels.

Table 1 Structures of the primers used

Receptor	*Primers*	*Sense*	*Size*	*References*
NK_1	5′-GGTCATCTTTGTCATCTGGG-3′	F	587 bp	Hershey *et al*. (1991)
	5′-CTGTGTCTGGAGGTATCGGG-3′	R		
NK_2	5′-CATCACTGTGGACGAGGGGG-3′	F	491 bp	Sasai & Nakanishi (1989)
	5′-TGTCTTCCTCAGTTGGTGTC-3′	R		
NK_3	5′-CATTCTCACTGCGATCTACC-3′	F	325 bp	Shigemoto *et al*. (1990)
	5′-CTTCTTGCGGCTGGATTTGG-3′	R		

Shown are the structures of forward (F) and reverse (R) primers of indicated receptor types. The size of the PCR-amplified product of each receptor mRNA and the references from which these primers were designed are shown.

Restriction enzyme analysis To confirm the identity of amplified cDNAs, restriction enzyme analysis of the PCR products was carried out. A PCR sample (10 μl) was ethanol-precipitated, redissolved in 5 μl water, and incubated with the enzymes *Hinf*I and *Rsa*I (Boehringer, Manheim, Germany) according to the manufacturer's instructions.

Drugs

The drugs used were SP, NKA, NKB, [Sar^{9}Met(O$_2$)^{11}]-SP, [Nle^{10}]-NKA(4-10) and [MePhe^7]-NKB (Bachem, Switzerland). Phosphoramidon (N-(α-L-rhamnopyranosyl-oxyhydroxyphosphinyl) - L - leucyl - L - tryptophan sodium salt), acetylcholine hydrochloride, oxytocin, (Sigma, St. Louis, U.S.A.). SR 140333 (Nolpitantium, (**S**)1-(2-[3-(3,4-dichlorophenyl) - 1 - (3 - isopropoxyphenylacetyl)piperidin-3-yl]ethyl)-4-phenyl-1-azoniabicyclo [2.2.2]octane chloride), SR 48968 (Saredutant, (**S**)-N-methyl-N[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide) and SR 142801 (Osanetant, (**R**)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl)-4-phenylpiperidin- 4 - yl) - N - methylacetamide) were synthesized by Sanofi Research Center (Montpellier, France). SP, NKA, [Sar^{9}Met(O$_2$)^{11}]-SP, [Nle^{10}]-NKA(4-10) and [MePhe^7]-NKB were dissolved in distilled water containing 10% acetic acid at a concentration of 0.1 to 1 mM and stored at -20°C until used. NKB was dissolved in dimethylsulphoxide at a concentration of 0.1 mM. Peptides were then diluted in physiological solution. Tachykinin receptor antagonists were dissolved in absolute ethanol and then diluted with deionized water to appropriate concentrations. The other agents were dissolved daily in deionized water. The vehicle for tachykinin receptor agonists or antagonists had no effect on baseline tension or mechanical responses when added at the same dilution in parallel strips.

Results

Effect of tachykinins on the mechanical activity of rat uterus

The log concentration-response curves to SP (1 nM – 0.1 mM), NKA (0.1 nM – 30 μM) and NKB (1 nM – 30 μM), expressed as areas under the force-time curve, are shown in Figure 1a, c and e. At the lower concentrations assayed, NKA ($\leqslant$30 nM) and NKB ($\leqslant$1 μM) induced rhythmic contractions developed on the basal tone of the preparation. At higher concentrations, both agonists elicited an initial phasic contraction followed by rhythmic contractions superimposed on an increase in baseline tension. After washing and a 60 min equilibration period, a second log non-cumulative concentration-response curve to NKA or NKB could be obtained in the same uterine strip, even when maintaining each tachykinin concentration in contact with the preparation for a longer time period (10 min). This suggests no significant desensitization of responses to these agonists. The E_{max} values (areas, expressed as a percentage of the maximal response to the agonist in the first curve) in the second log non-cumulative concentration-response curves were 106.29 $\pm$ 4.78 (n = 3, P > 0.05) and 105.29 $\pm$ 2.71 (n = 3, P > 0.05) for NKA and NKB, respectively. The pD_2 values determined for both tachykinin receptor agonists are shown in Table 2.

The response to SP appeared to be biphasic (Figure 1a). SP (1 nM – 0.1 μM) induced a transient rapid response sometimes followed by a few, small rhythmic contractions. The threshold concentration for contraction was 1 nM in three of seven myometrial strips and 3 nM in the other four tissues. The peak increase in force reached about 15% of the control contraction induced by ACh 1 mM, although its contribution to the log concentration-response curve for SP, in terms of area, was minimal (Figure 1a). Within this concentration range, responses did not increase in a concentration-dependent manner, with a higher, smaller or no contraction observed after addition of higher concentrations. In contrast, SP caused concentration-dependent contractions at concentrations above 0.1 μM (Figure 1a). Responses to SP were not reproducible in the same preparation and this was particularly evident in the low concentration range (1 nM – 0.1 μM).

The endopeptidase 24.11 inhibitor phosphoramidon (1 μM) markedly increased the myometrial contraction induced by SP (>0.1 μM), NKA and NKB (Figure 1a, c, e). The pD_2 values in the absence or presence of the peptidase inhibitor were significantly increased for NKA and NKB but not for SP (Table 2). Comparison of pD_2 values obtained in the absence of phosphoramidon indicated a rank order of potency NKA > SP = NKB whereas in the presence of phosphoramidon, the order of potency was NKA > NKB > SP.

In the presence of phosphoramidon (1 μM), the responses evoked by low concentrations of SP (1 nM – 0.1 μM) were similar in pattern to those observed in the absence of the inhibitor. The threshold concentration for contraction was 1 nM in eight strips and 3 nM in nine strips (n = 17) and the peak increase in force reached about 20% of the maximal contraction (peak tension) induced by ACh 1 mM.

Effect of selective tachykinin analogues

[Nle^{10}]-NKA(4-10) (0.1 nM – 3 μM) produced concentration-dependent contractile responses in the oestrogen-primed rat uterus (Figure 1d). The pD_2 values determined for this NK_2-selective agonist in the absence or in the presence of phosphoramidon are shown in Table 2. In the presence of the peptidase inhibitor, [Nle^{10}]-NKA(4-10) showed a similar apparent affinity (pD_2) and E_{max} to NKA (Figure 1 and Table 2). The tachykinin NK_1 receptor selective analogue [Sar^{9}Met(O$_2$)^{11}]-SP (1 nM – 3 μM) produced contractions characterized by bell-shaped log concentration-response curves, similar in pattern to those observed with low concentrations of SP (Figure 1b). Responses were more consistently produced in the presence of phosphoramidon. Although [Sar^{9}Met(O$_2$)^{11}]-SP induced contraction in all preparations tested, there was a high variability between preparations in (a) the threshold concentration for contraction (1 nM in two strips, 3 nM in five strips and 10 nM in four strips, n = 11) and (b) the maximum amplitude and the area under the force-time curve for each particular concentration. Concentration-dependent contractions of increasing amplitude could be obtained by applying only one concentration of [Sar^{9}Met(O$_2$)^{11}]-SP on each preparation, suggesting that [Sar^{9}Met(O$_2$)^{11}]-SP-induced response was subject to rapid desensitization. In these conditions, the E_{max} value (peak increase in tension) in the presence of phosphoramidon was 33.75 $\pm$ 6.83% of the maximal response (peak tension) to ACh 1 mM and the pD_2 value was 7.61 $\pm$ 0.89 (n = 6).

The tachykinin NK_3 receptor selective analogue [MePhe^7]-NKB (1 nM – 10 μM) failed to induce any significant contractile response in uterine strips without pretreatment with phosphoramidon (n = 5, Figure 1f). In the presence of phosphoramidon, [MePhe^7]-NKB was a very weak constrictor in the rat uterus and induced rhythmic contractions which were identical at all concentrations assayed (n = 5, Figure 1f). The response to this NK_3-selective agonist appeared in about 60% of the preparations tested and could not be characterized.

Influence of external Ca^{2+} on the effects of the natural and selective tachykinin agonists

Some experiments were carried out in a low Ca^{2+} (0.54 mM Ca^{2+}) solution, to avoid development of myometrial spontaneous activity and to study the influence of external Ca^{2+} on tachykinin-induced contractions. In this medium, the log concentration-response curves to NKA, [Nle^{10}]-NKA (4-10) and NKB were shifted to the right by approximately 0.5 log units without significant modification of the E_{max} values. The pD_2 values were 6.21 ± 0.12 ($n=6$) and 7.60 ± 0.09 ($n=15$) for NKA and 7.12 ± 0.25 ($n=5$) and 7.29 ± 0.38 ($n=5$) for [Nle^{10}]-NKA(4-10) in the absence or presence of phosphoramidon, respectively. The pD_2 value for NKB in the presence of phosphoramidon was 5.88 ± 0.16 ($n=12$). Due to the limited availability of the peptides, only a partial log concentration-response curve was obtained for NKB (1 nM – 3 μM) in the absence of phosphoramidon and the pD_2 value could not be calculated.

In a low Ca^{2+} medium, the responses to SP were significantly reduced ($P<0.05$) at all concentrations assayed (1 nM – 3 μM). The E_{max} values (areas) at 3 μM were

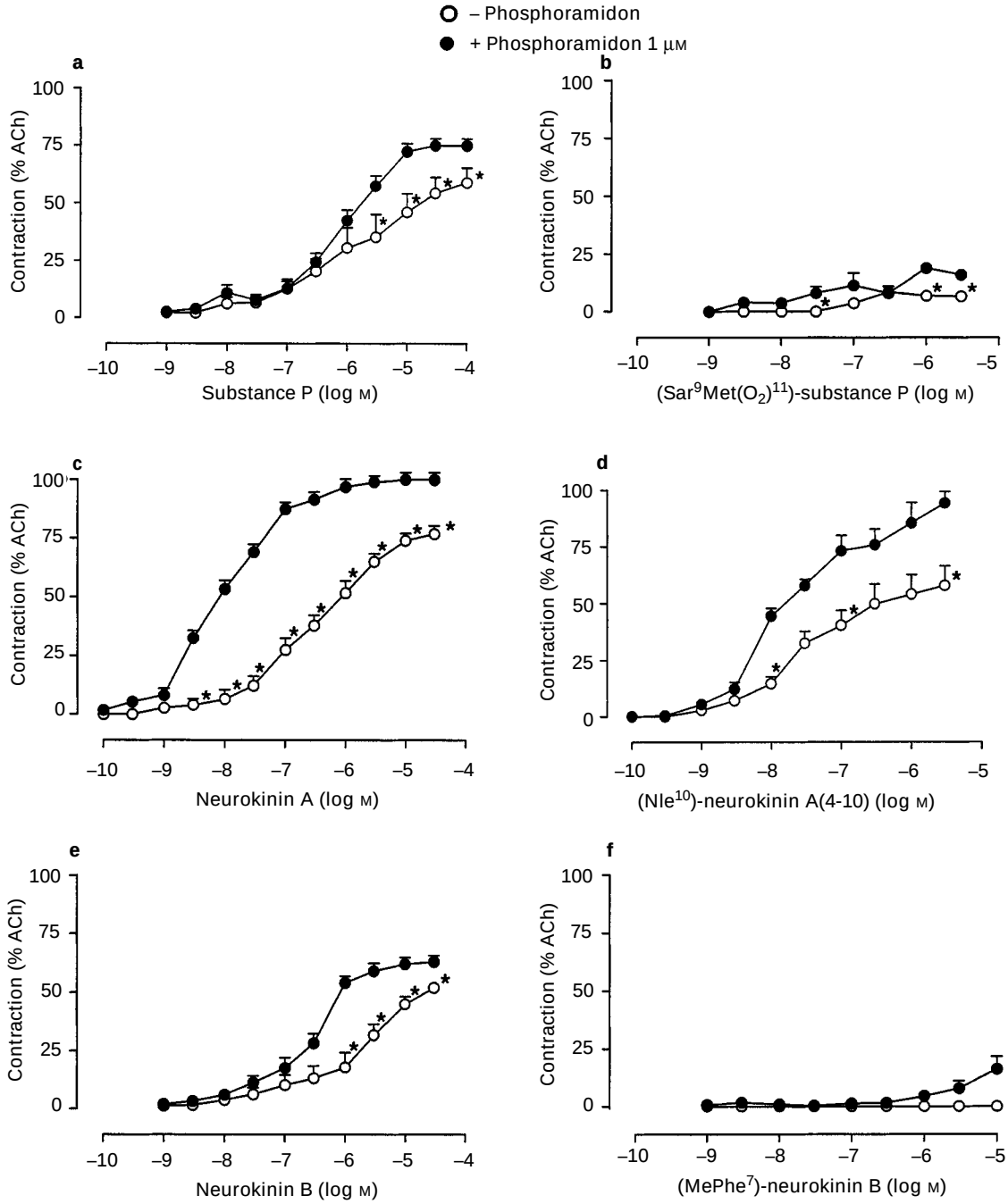

Figure 1 Concentration-response curves for substance P (a), [$Sar^9Met(O_2)^{11}$]-SP (b), neurokinin A (c), [Nle^{10}]-NKA(4-10) (d), neurokinin B (e) and [$MePhe^7$]-NKB (f) in the oestrogen-primed rat uterus. Curves were constructed in the absence and in the presence of the neutral endopeptidase inhibitor phosphoramidon (1 μM), added to the bath 20 min before the application of an agonist and replaced after each concentration was washed out. Data points represent areas under the force time curve, expressed as a percentage of that to ACh (1 mM) during a 5 min period. Values are means for 5 to 18 experiments, with s.e.mean shown by vertical lines. *Significant difference from responses in the presence of phosphoramidon: $P<0.01$, unpaired t test.

7.14±0.12% ($n=6$) and 24.32±4.22% ($n=10$) of the response to ACh 1 mM in the absence and in the presence of phosphoramidon, respectively ($P<0.05$ vs the corresponding values in 1.9 mM Ca^{2+}-containing solution). [$Sar^9Met(O_2)^{11}$]-SP (1 nM–3 μM) failed to induce a significant contractile response in uterine strips without pretreatment with phosphoramidon ($n=3$) and produced small transient contractions in the presence of the neutral endopeptidase inhibitor ($n=6$, not shown).

Table 2 pD_2 values of SP, NKA, NKB and [Nle^{10}]NKA (4–10) in the oestrogen-primed rat uterus

	Control		*With phosphoramidon*	
	n	pD_2	n	pD_2
Substance P	7	5.95±0.19	17	6.12±0.10
Neurokinin A	8	6.73±0.21	14	8.04±0.07**
	3	6.92±0.16‡	3	7.90±0.12**‡
Neurokinin B	13	5.76±0.21	18	6.59±0.07**
	3	5.43±0.29‡	3	6.67±0.19*‡
(Nle^{10})neurokinin A (4–10)	6	7.53±0.12	6	7.89±0.03

Values (means±s.e.mean) are calculated from areas under the force-time curve. *n*: number of experiments in *n* different animals. ‡pD_2 values obtained for NKA or NKB in second non-cumulative log concentration-response curves, constructed 60 min after the first curve. Significant differences from the control, *$P<0.05$, **$P<0.01$.

Effect of antagonists

The log concentration-response curves to SP, NKA and NKB in the absence (control tissues) and in the presence of selective antagonists of tachykinin receptors were constructed in uterine strips pretreated with phosphoramidon (1 μM). The effect of the selective tachykinin NK_2 receptor antagonist SR 48968 on the log concentration-response curves to NKA is shown in Figure 2a. The application of increasing antagonist concentrations led to a rightward shift of the log concentration-response curve without any significant decrease in the maximal contractile effect. The inhibition appeared competitive as the slope of the Schild plot obtained from these data was not significantly different from unity (1.28±0.24, $n=6$, $P>0.05$), with a correlation coefficient (r) of 0.98. The mean pK_B value for SR 48968 (calculated at all antagonist concentrations) was 9.16±0.08 ($n=24$, 6 different animals). SR 48968 (0.1 μM) also shifted to the right the log concentration-response curve to SP (Figure 2c), with an apparent pK_B value of 7.63±0.13 ($n=10$). At 0.1 μM, SR 48968 produced a non-parallel shift and a flattening of the log concentration-response curve to NKB ($n=6$, Figure 2d) and abolished the log concentration-response curve to [Nle^{10}]-NKA(4-10) ($n=4$, not shown) but failed to

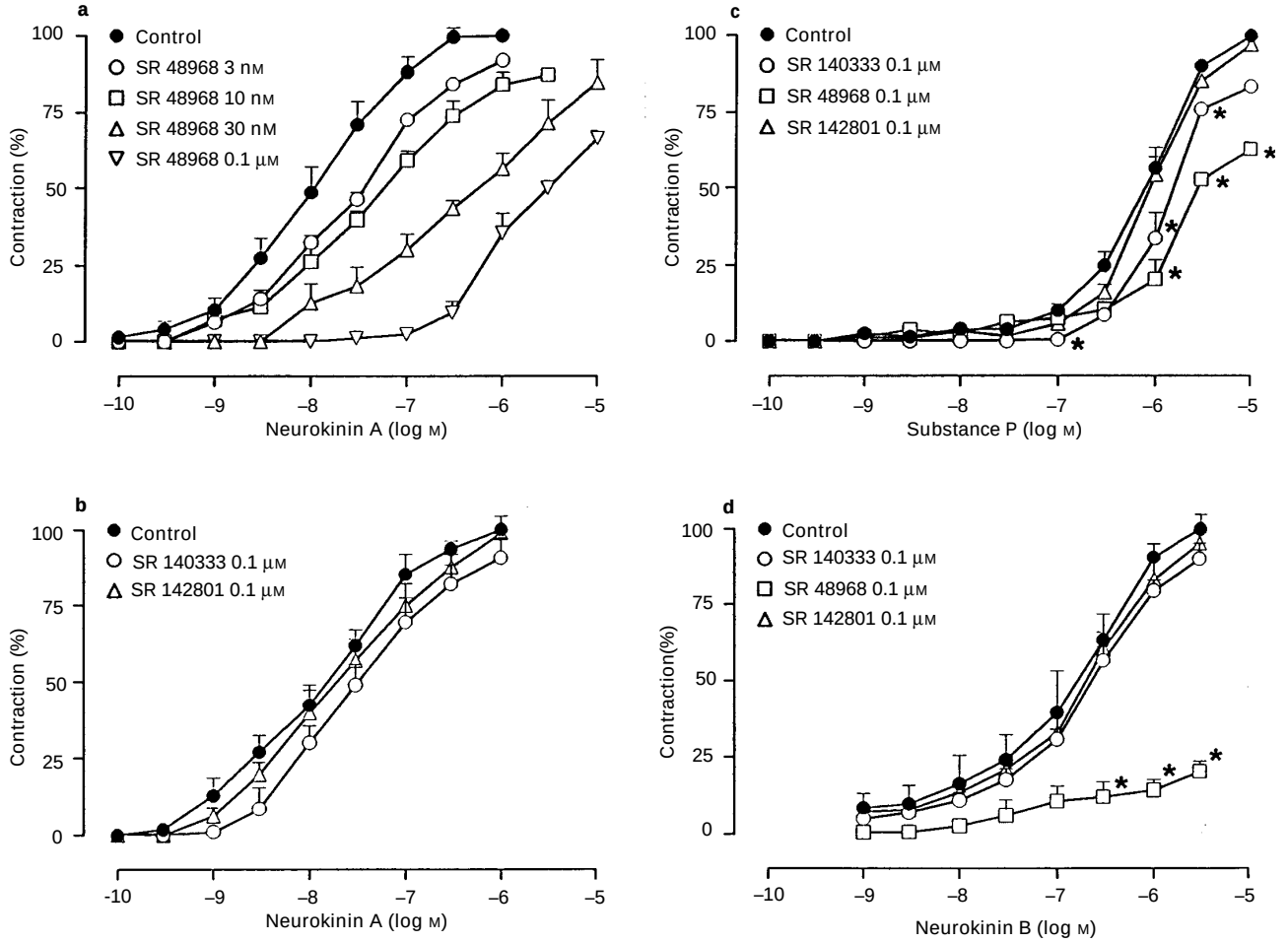

Figure 2 Effect of the tachykinin NK_1 receptor antagonist SR 140333, the tachykinin NK_2 receptor antagonist SR 48968 and the tachykinin NK_3 receptor antagonist SR 142801 on the log concentration-response curves of neurokinin A (a,b), substance P (c) and neurokinin B (d) in rat isolated uterine strips incubated with phosphoramidon (1 μM). Data points represent areas under the force-time curve, expressed as a percentage of the maximal response (area) to the agonist, and are means for 6 to 10 experiments with s.e.mean shown by vertical lines. *Significant difference from control responses: $P<0.01$, paired t test.

inhibit the contraction induced by [Sar^{9}Met(O$_2$)^{11}]-SP (0.1 μM) ($n=6$, Figure 3c).

The selective tachykinin NK_1 receptor antagonist SR 140333 (0.1 μM) shifted the log concentration-response curve of SP to the right (Figure 2c) with an apparent pK_B value of 7.19 ± 0.22 ($n=8$). SR 140333 (0.1 μM) did not significantly modify ($P>0.05$) the concentration-response curves of NKA (Figure 2b), NKB (Figure 2d) and [Nle^{10}]-NKA(4-10) ($n=3$, not shown) but inhibited the contraction elicited by [Sar^{9}Met(O$_2$)^{11}]-SP (0.1 μM) ($n=6$, Figure 3b).

The selective tachykinin NK_3 receptor antagonist SR 142801 (0.1 μM) did not modify either the log concentration-response curves to SP (Figure 2c), NKA (Figure 2b) NKB (Figure 2d) or [Nle^{10}]-NKA(4-10) ($n=3$, not shown) or the contraction induced by [Sar^{9}Met(O$_2$)^{11}]-SP (0.1 μM) ($n=6$, Figure 3d).

Phosphoramidon and the three non-peptide tachykinin receptor antagonists had no effect on the log concentration-response curves to oxytocin (0.1 nM – 1 μM). The E_{max} (areas, expressed as a percentage of the maximal response to oxytocin in control tissues) and pD_2 values obtained were: $E_{max}=100\%$ and $pD_2=8.40\pm0.22$ in control tissues; $E_{max}=94.96\pm3.29$ and $pD_2=8.22\pm0.11$ in the presence of SR 140333 (0.1 μM); $E_{max}=102.90\pm2.36$ and $pD_2=8.32\pm0.08$ in the presence of SR 48968 (0.1 μM); $E_{max}=95.39\pm6.50$ and $pD_2=8.14\pm0.34$ in the presence of SR 142801 (0.1 μM) and $E_{max}=104.66\pm4.03$ and $pD_2=8.33\pm0.05$ in the presence of phosphoramidon (1 μM) ($n=3$ for each group of experiments, 3 different animals, $P>0.05$). Similarly, the response to ACh (1 mM) was unaffected by phosphoramidon or the presence of the tachykinin antagonists.

Expression of tachykinin receptors in the rat uterus

PCR amplification of equal amounts of cDNA from oestrogen-primed rat uteri, as determined from the amplification of the β-actin sequence, revealed single transcripts corresponding to the expected product sizes encoding cDNA for the tachykinin NK_1 receptor (587 bp) and the tachykinin NK_2 receptor (491 bp) ($n=6$ different animals). mRNA encoding the 325 bp product expected for the tachykinin NK_3 receptor was undetected. To ascertain that NK_3 receptor expression could be detected by RT-PCR with the NK_3-specific primers used in the present study, we analysed the expression of tachykinin receptors in the rat cerebral cortex. A single band for cDNA encoding the tachykinin NK_3 receptor was visualized in this positive control by amplification of an equal amount of cerebral cortex cDNA to that used in the experiments with uterine cDNA. This brain region also expressed mRNA for tachykinin NK_1 receptors, whereas we failed to detect significant levels of the tachykinin NK_2-receptor mRNA. Figure 4 illustrates a typical example of RT-PCR products from rat uterus and cerebral cortex, showing the presence of single transcripts for NK_1 and NK_2 receptors (uterus) or for NK_1 and NK_3 receptors (cerebral cortex), present at the expected position. Amplification of 3 μl aliquots of undiluted RT solutions from oestrogen-treated rat uterus, corresponding to 1 μg to total RNA, permitted us to visualize a very low abundance transcript for the tachykinin NK_3 receptor (not shown). In all experiments, the two negative controls yielded no detectable products, indicating that (i) all reagents were free of target sequence contamination and (ii) the RT-PCR products do not come from contaminating genomic DNA.

The identity of PCR products was confirmed by restriction enzyme analysis. All three products were digested and shown to contain the predicted *Hinf*I and *Rsa*I restriction sites at the correct locations (not shown), according to the previously published sequences for the rat tachykinin NK_1, NK_2 and NK_3 receptors (Sasai & Nakanishi, 1989; Shigemoto *et al*., 1990; Hershey *et al*., 1991).

Discussion

The two major findings in the present study were: (i) the oestrogen-primed rat uterus expresses tachykinin receptors of the NK_1, NK_2 and NK_3 types, the last one being present only in trace amounts, and (ii) tachykinin NK_2, and to a lesser extent, NK_1, but not NK_3 receptors, are involved in the contractile responses elicited by tachykinins in this tissue.

The results we obtained in functional studies with SP, NKA, NKB and several of their analogues with well defined selectivities at the known tachykinin NK_1, NK_2 and NK_3 receptor types (Regoli *et al*., 1994) confirm previous findings suggesting the presence and the predominance of tachykinin NK_2 receptors in the oestrogen-primed rat uterus (Pennefather *et al*., 1993; Fisher *et al*., 1993). NKA and the selective NK_2 receptor agonist [Nle^{10}]-NKA(4 – 10) produced concentration-dependent contractile responses in the submicromolar range

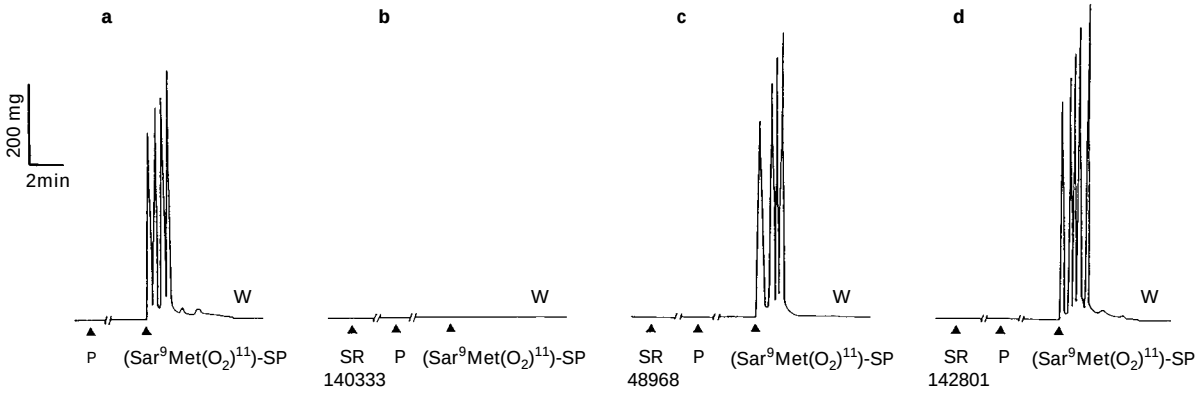

Figure 3 Typical tracings showing the effect of [Sar^{9}Met(O$_2$)^{11}]-SP (0.1 μM) on the mechanical activity of the oestrogen-primed rat uterus. Responses were obtained in the presence of phosphoramidon (P, 1 μM) and in the absence (control, a) or in the presence of SR 140333 (0.1 μM, b), SR 48968 (0.1 μM, c), and SR 142801 (0.1 μM, d). Traces are representative of typical results of 6 experiments. W = washing.

and maximum responses to both agonists were similar to that elicited by a maximally effective concentration of ACh (1 mM). The tachykinin receptor agonists [$Sar^9Met(O_2)^{11}$]-SP (NK_1-selective) and [$MePhe^7$]-NKB (NK_3-selective) acted as weak uterine stimulants and produced small contractile responses, compared with the other agonist. In the presence of phosphoramidon, the order of potency of natural tachykinins was NKA > NKB > SP, which is consistent with a predominance of tachykinin NK_2 receptors in this tissue (Regoli *et al*., 1994). A comparison of pD_2 values for SP, NKA and NKB in the absence and in the presence of phosphoramidon indicates the important role played by the neutral endopeptidase 24.11 in inactivating tachykinins in the uterus (Pennefather *et al*., 1993; Fisher *et al*., 1993), and, as observed in other tissues (Sekizawa *et al*., 1987), responses to NKA and NKB were more susceptible to this enzyme than SP. The involvement of

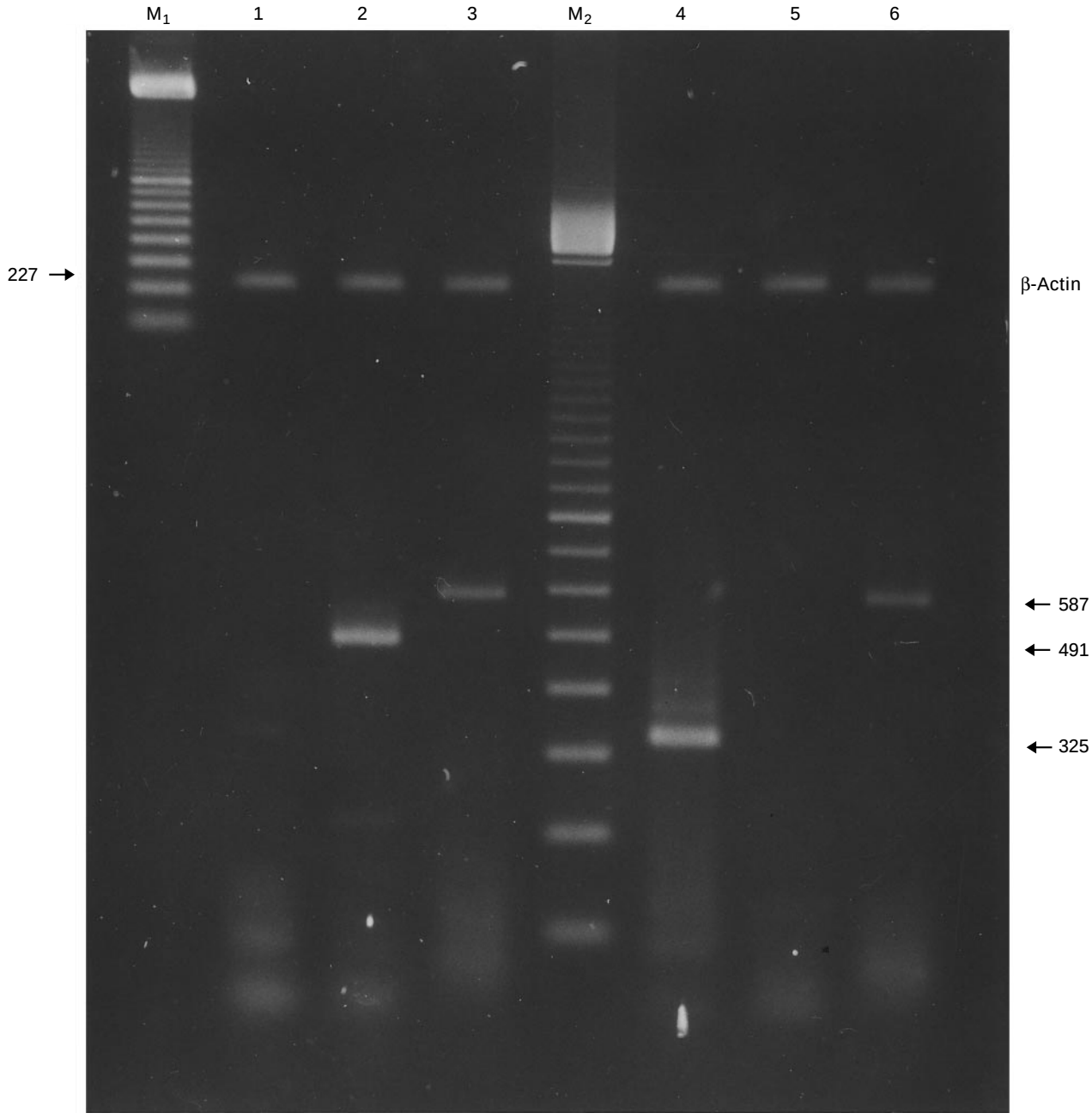

Figure 4 Agarose gel showing products of reverse-transcriptase-polymerase chain reaction (RT-PCR) assay for cDNA from oestrogen-primed rat uterus (lanes 1, 2 and 3) and cerebral cortex (lanes 4, 5 and 6). Equal amounts of uterine and cerebral cDNA, as determined from the previous amplification of the β-actin sequence, were amplified with β-actin-specific primers and with tachykinin NK_1-, NK_2- and NK_3-receptor-specific primers. β-Actin RT-PCR products and molecular size standard (M_1) were loaded in the agarose gel 50 min later than those for tachykinin receptors and the correspondent molecular size standard (M_2). Lanes 3 and 6 show the presence of a single transcript corresponding to the size predicted for the tachykinin NK_1 receptor (587 base pairs) for the uterus and the brain cortex, respectively. The specific band corresponding to the size predicted for tachykinin NK_2 receptors (491 base pairs) was detected in the uterus (lane 2) but not in the cerebral cortex (lane 5). The specific band corresponding to the size predicted for tachykinin NK_3 receptors (325 base pairs) was detected in the cerebral cortex (lane 4) but not in the uterus (lane 1).

NK_2 receptors is further substantiated by the fact that SR 48968, a potent non-peptide tachykinin NK_2 receptor selective antagonist (Emonds-Alt *et al* 1992; Advenier *et al*., 1992) produced a rightward and parallel shift of the concentration-response curve to NKA. The antagonism appeared competitive as the slope of the resultant Schild plot was not significantly different from 1. The calculated pK_B value (9.16) was within the range of pK_B values obtained for this antagonist on tachykinin NK_2 receptors in rat tissues (Advenier *et al*., 1992). SR 48968 (0.1 μM) also antagonized responses to NKB, suggesting that this agonist is primarily activating NK_2 receptors in the oestrogen-primed rat uterus.

This study also presents evidence that a functionally efficient population of tachykinin NK_1 receptors may be present in the oestrogen-primed rat uterus. This was indicated by the finding that SP in a low concentration range (1 nM – 0.1 μM) and the selective tachykinin NK_1 receptor agonist [$Sar^9Met(O_2)^{11}$]-SP produced small contractile responses (maximal effect 15 – 33% of the peak increase in force induced by ACh 1 mM), which were abolished by the selective NK_1 receptor antagonist SR 140333 but not affected by the selective NK_2 receptor antagonist SR 48968. These responses were markedly reduced in a low Ca^{2+} (0.54 mM Ca^{2+}) solution and could not be reproduced in the same preparation suggesting that NK_1 receptor desensitization may occur on repeated administration in the rat uterus. Rapid desensitization of this tachykinin receptor by SP has been previously described in electrophysiological studies of tachykinin NK_1 receptors expressed in *Xenopus* oocytes (Nakanishi, 1991) or in studies on cardiovascular and behavioural effects of SP in rats (Culman *et al*., 1993). SR 140333 inhibited responses to SP with an apparent pK_B value of 7.19. A similar pK_B value (7.4) was found for this selective tachykinin receptor antagonist in the rat urinary bladder, a tissue containing NK_1 and NK_2 receptors (Regoli *et al*., 1994). The selective tachykinin NK_2 receptor antagonist SR 48968 shifted the responses of SP to the right with an apparent pK_B of 7.63, a value which is lower than that obtained for SR 48968 with NKA (9.16). Taken together, these data suggest that uterine responses to high concentrations of SP (0.1 μM – 0.1 mM) involve activation of both NK_1 and NK_2 receptors.

Finally, the present functional studies demonstrate that tachykinin NK_3 receptors are not involved in the mechanical responses evoked by tachykinins in uterine smooth muscle. This conclusion was supported by the following observations: (i) the selective agonist for the tachykinin NK_3 receptor [$MePhe^7$]-NKB acted as a very weak uterine stimulant; (ii) the response to NKB was inhibited in the presence of SR 48698 and (iii) SR 142801, a potent non-peptide antagonist of the tachykinin NK_3 receptor (Emonds-Alt *et al*., 1995; Chung *et al*., 1995) failed to modify tachykinin-mediated contraction. As observed with other tachykinin antagonists, SR 142801 exhibits species-related selectivity, being significantly more potent on the human and guinea-pig NK_3 receptor than on the rat homologue (Emonds-Alt *et al*., 1995). Nevertheless, recent studies have shown that the rat NK_3 receptor still binds to SR 142801 with high affinity (Chung *et al*., 1995; Beaujouan *et al*., 1997), demonstrating that, at the concentration used (0.1 μM), SR 142801 selectively blocks tachykinin NK_3 receptors in our preparation.

The population of tachykinin receptors in the oestrogen-primed rat uterus was finally investigated by analysing the presence of NK_1, NK_2 and NK_3 receptor transcripts in uterine mRNA. Single bands for cDNA encoding NK_1, NK_2 and NK_3 receptors were detected. The conclusion that the observed RT-PCR products were generated from the tachykinin receptors mRNA was based on the following experimental evidence: (i) the generation of single transcripts in each case, using NK_1-, NK_2- and NK_3-specific primers; (ii) the size correspondence of those products to the targeted regions of the three rat tachykinin receptors mRNA and (iii) restriction enzyme analysis, which demonstrated the presence and correct location of the corresponding restriction sites in the three products. Recent pharmacological studies have suggested the existence of more than one molecular form of the tachykinin NK_1 and NK_2 receptors differentially distributed in certain tissues (Patacchini *et al*., 1991; Meini *et al*., 1994) and a novel human tachykinin NK_3 receptor homologue has been characterized (Donaldson *et al*., 1996; Krause *et al*., 1997). This NK_3 receptor homologue has not been cloned in the rat. We have found (Pinto *et al*., 1997) that the sequence of the fragment amplified for the tachykinin NK_3 receptor is identical to that previously published for the rat brain tachykinin NK_3 receptor (nucleotide positions 918 – 1242 base pairs, Shigemoto *et al*., 1990). From the present results we are unable to clarify whether tachykinin receptors other than the three well-known cloned ones exist in the rat uterus, due to the lack of additional bands with the tachykinin receptor specific primers used in our assay.

Despite the expression of mRNA for tachykinin NK_1, NK_2 and NK_3 receptors, the oestrogen-primed rat uterus expresses predominantly the NK_1 and NK_2 types. The NK_3 receptor mRNA was present in a trace amount only detected by PCR amplification of large quantities of uterine cDNA. The results of functional studies demonstrate that NK_3 receptors did not play a significant role in mediating contractile responses to tachykinins in the oestrogen-primed rat uterus. However, binding studies by Barr *et al*. (1991) identified a tachykinin NK_3 receptor in uteri from rats in the dioestrous stage of the oestrous cycle. These data are in agreement with our previous finding showing that oestrogens down-regulate tachykinin NK_3 receptor mRNA expression levels in uteri from ovariectomized rats (Pinto *et al*., 1997). This is interesting because it could suggest a hormonal regulation of the uterine population of tachykinin receptors. Further studies to assess the influence of the hormonal state in the expression of tachykinin receptors are currently underway.

The precise physiological or pathophysiological role of tachykinins in the female genital tract remain unclear. Along with CGRP, SP and NKA are located in nerves, mostly of sensory origin, that innervate the female reproductive tract in virtually all mammalian species examined (Ottesen & Fahrenkrug, 1990; Papka & Shew, 1994). In the rat, nerve terminals are associated with the myometrial and vascular smooth muscle and are distributed throughout the endocervix (Traurig *et al*., 1984; 1988; Shew *et al*,. 1991). SP relaxes the uterine vasculature (Ottesen *et al*., 1983) and SP, NKA and NKB stimulate myometrial contractility (present data, Pennefather *et al*., 1993). In addition, our study shows that functionally active transducing receptors for tachykinins are expressed in the rat uterus. It thus appears likely that tachykinin receptor activation may represent an important intervening factor in the cascade of events occurring in the reproductive tract of the female rat during the oestrous cycle and/or during pregnancy.

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