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Contribution of tachykinin receptor subtypes to micturition reflex in guinea pigs

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Abstract

The aim of the present study was to determine the role of tachykinin in the micturition reflex in guinea pigs. We investigated the effects of tachykinin NK_1 receptor antagonists, GR205171 ([2-methoxy-5-(5-trifluoromethyl-tetrazol-1-yl)-benzyl]-(2*S*-phenyl-piperidin-3*S*-yl)-amine), CP99994 ((+), (2*R*, 3*R*)-3-(2-methoxybenzyl-amino)-2-phenylpiperidine) and FK888 (N^2-[(4*R*)-4-hydroxy-1-(1-methyl-1*H*-indol-3-yl) carbonyl-L-prolyl]-*N*-methyl-*N*-phenylmethyl-3-(2-naphthyl)-L-alaninamide), the tachykinin NK_2 receptor antagonist, SR48968 ((+)-*N*-methyl-[4-(4-acetylamino-4-phenyl piperidino)-2-(3, 4-dichloro-phenyl)butyl] benzamide), and the tachykinin NK_3 receptor antagonist, SB223412 ((*S*)-(−)-*N*-(α-ethylbenzyl)-3-hydroxy-2-phenylquinoline-4-carboxamide) on rhythmic bladder contraction. GR205171 and CP99994 but not SR48968 or SB223412 reduced bladder contraction frequency. FK888 inhibited the frequency very slightly at the highest dose tested. The distribution of tachykinin NK_1 receptor antagonists to the central nervous system after intravenous administration was examined using an ex vivo binding assay. GR205171 was distributed to the brain and spinal cord, but the tachykinin NK_1 receptor antagonist, FK888, was not. These results suggest that tachykinin NK_1 receptors, which are located in the central nervous system, play an important role in micturition in guinea pigs.

Keywords: Tachykinin; Micturition; Urinary bladder; Central nervous system

1. Introduction

Tachykinins are peptides sharing the common C-terminal amino acid sequence, Phe–X–Gly–Leu–Met–NH_2 (Saria, 1999). Endogenous mammalian tachykinins have been designated as substance P, neurokinin A and neurokinin B (Pritchard and Phillip, 1995). Receptors are referred to as tachykinin NK_1, NK_2 and NK_3 receptors (Maggi, 1995). Substance P is the most potent tachykinin for the NK_1 receptor, whereas neurokinin A exhibits the highest affinity for the tachykinin NK_2 receptor and neurokinin B for the tachykinin NK_3 receptor.

An increasing number of pharmacological studies using selective tachykinin receptor antagonists have demonstrated that tachykinins play an important role in the sensory control of bladder contraction (Ishizuka et al., 1995; Maggi et al., 1993; de Groat et al., 1993; Kawatani et al., 1993; Kamo et al., 2000) due to their localization in afferent pathways of the bladder and the spinal cord (Burcher et al., 2000; Birder et al., 1999). Palea et al. (1996) reported that the tachykinin NK_2 receptor appears to mediate bladder smooth muscle contraction in isolated urinary bladder, and that spinal tachykinin NK_1 receptors were involved in the micturition reflex (Palea and Pietra, 1999). Lecci et al. (1993) reported that intrathecal administration of tachykinin NK_1 receptor antagonists increased bladder capacity in anaesthetized rats without changing voiding pressure, whereas tachykinin NK_2 receptor antagonists were ineffective. However, the relationship between distribution of tachykinin antagonists and pharmacological action is not yet fully understood.

The first aim of this study was to elucidate the role of tachykinin subtypes in the micturition reflex. For this purpose, we compared effects of tachykinin NK_1, NK_2 and NK_3 receptor antagonists under the same conditions using a distention-induced rhythmic bladder contraction

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model. The second aim of this study was to clarify the site of action of the tachykinin NK_1 receptor antagonist. For this purpose, we studied NK_1 binding activity in the brain and spinal cord after intravenous administration of two kinds of tachykinin NK_1 receptor antagonists, GR205171 ([2-methoxy-5-(5-trifluoromethyl-tetrazol-1-yl)-benzyl]-(2*S*-phenyl-piperidin-3*S*-yl)-amine) and FK888 (N^2-[(4*R*)-4-hydroxy-1-(1-methyl-1*H*-indol-3-yl) carbonyl-L-prolyl]-*N*-methyl-*N*-phenylmethyl-3-(2-naphthyl)-L-alaninamide).

2. Materials and methods

2.1. Animals

Male Hartley guinea pigs (Japan SLC, Hamamatsu) were used. The animals were housed under conditions of temperature 23 ± 2 °C, 55 ± 5% humidity and 12-h light cycle (0700–1900) before the start of experiments. They were fed standard laboratory chow with water ad libitum. All animal experimental procedures were performed under the guidelines of the Animal Experiment Committee of Fujisawa Pharmaceutical.

2.2. Distention-induced rhythmic bladder contraction

Guinea pigs, weighing 358 to 408 g, were anesthetized with urethane (1.2 g/kg, i.p.), and the lower abdomen was opened along the midline to expose the urinary bladder. After performing a small dissection at the apex of the bladder, a small balloon was inserted into the bladder. The balloon was connected through a three-way stopcock attached to a pressure transducer for measurement of intravesical pressure. A tube for drug administration was inserted into the jugular vein.

To obtain a high amplitude of bladder contractions due to the micturition reflex, 1–2 ml of water was applied from the other end of the three-way stopcock into the balloon. The stimulus was adjusted according to the response obtained. Intravesical pressure was recorded on a thermal pen-type recorder via an amplifier (AP-621G, Nihon Kohden, Tokyo, Japan). The test compound was administered intravenously when bladder responses were stabilized. Amplitude and frequency of contraction were measured every 15 min.

2.3. Distribution of tachykinin NK_1 receptor antagonists to the central nervous system (ex vivo binding)

Male Hartley guinea pigs, weighing 331 to 810 g, received intravenously 1 mg/kg of GR205171 or FK888. The guinea pigs were anesthetized with ether, and blood was collected from the abdominal aorta with a heparinized syringe 5 min after administration. After the animals were perfused with 50 ml of saline, the brain and spinal cord were isolated and washed with saline. The collected blood was centrifuged at 1000 × *g* (CF7D, Hitachi) for 10 min at 4 °C to obtain plasma samples. Tissues were weighed and homogenized with a Polytron (setting 7, 30 s, Kinematica) in 4 volumes of distilled water and stored at −20 °C until use. One hundred microliters of plasma or 500 μl of the homogenate was mixed with 100 μl of methanol, 500 μl of 0.1 N NaOH and 4 ml of ethyl acetate. After shaking for 10 min, the mixture was centrifuged at 1000 × *g* for 10 min. The organic layer (2.5 ml) was evaporated to dryness in a

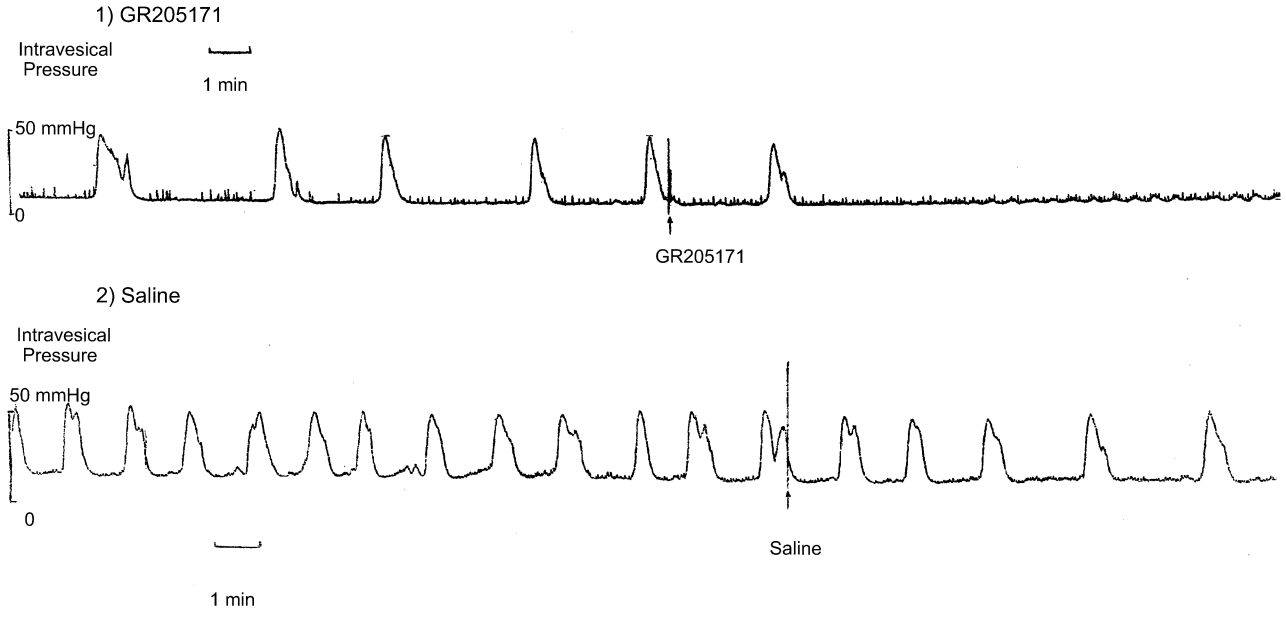

Fig. 1. Typical tracings showing intravesical pressure in guinea pigs. (1) Effect of GR205171, (2) Saline (control) on rhythmic bladder contraction.

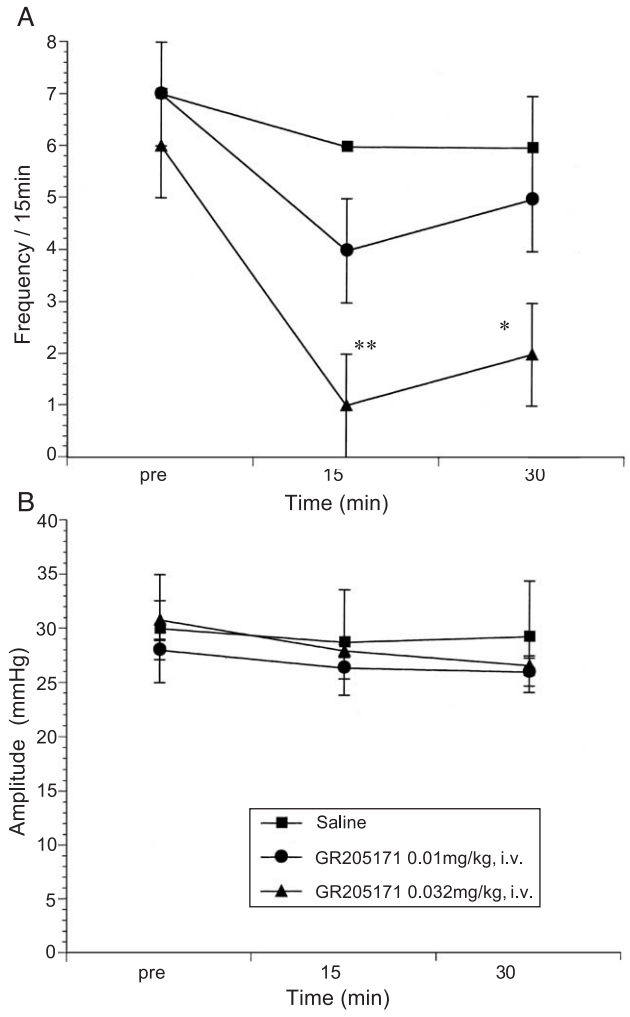

Fig. 2. Effect of GR205171, a tachykinin NK_1 receptor antagonist, on frequency (A) and amplitude (B) in guinea pigs. Saline (closed squares) or GR205171 was administered intravenously at 0.01 (closed circles) and 0.032 (closed triangles) mg/kg. Values are means ± S.E.M. for five animals. $*P<0.05$, $**P<0.01$, significant difference from the value before administration (Dunnett's multiple comparison).

vacuum and then the residue was dissolved in 0.1 ml of dimethylsulfoxide. The recovery of several compounds with this extraction protocol was more than 86%.

2.4. Preparation of cell membrane fraction of Chinese hamster ovary (CHO) cells transfected with human tachykinin NK_1 receptor cDNA

A CHO cell line permanently expressing human tachykinin NK_1 receptors was established by cDNA transfection (Aramori et al., 1994). The cells were grown in α-minimum essential medium Eagle (MEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, collected and homogenized in 25 mM Tris–HCl buffer (pH 7.4) containing 250 mM sucrose, 10 mM $MgCl_2$, 1 mM EDTA and 5 μg/ml (*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride (A-PMSF). The homogenate was centrifuged (RL-500SP, TOMY; 500 × *g*, 4 °C, 10 min) and the supernatant was collected. The pellet was homogenized again, centrifuged, and the supernatant was collected. The supernatant was centrifuged (CP100α, HITACHI, with a P45AT rotor; 100,000 × *g*, 4 °C, 60 min) and the pellet was suspended in 25 mM Tris–HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, 1 mM EDTA and 5 μg/ml A-PMSF. The suspension was stored at −80 °C until use. The amount of protein was determined using BIO-RAD PROTEIN ASSAY Reagent, with bovine serum albumin as standard.

2.5. Radioligand binding assay

Fifty microliters of various concentrations of drug solution diluted with the binding buffer (50 mM Tris–HCl

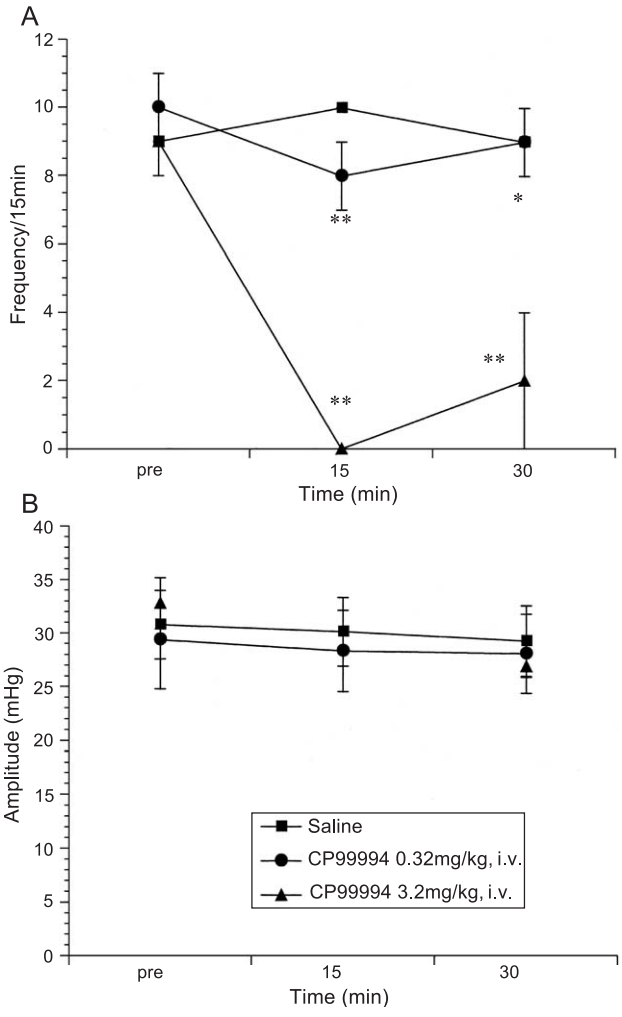

Fig. 3. Effect of CP99994, a tachykinin NK_1 receptor antagonist, on frequency (A) and amplitude (B) in guinea pigs. Saline (closed squares) or CP99994 was administered intravenously at 0.32 (closed circles) and 3.2 (closed triangles) mg/kg. Values are means ± S.E.M. for five animals. $*P<0.05$, $**P<0.01$, significant difference from the value before administration (Dunnett's multiple comparison).

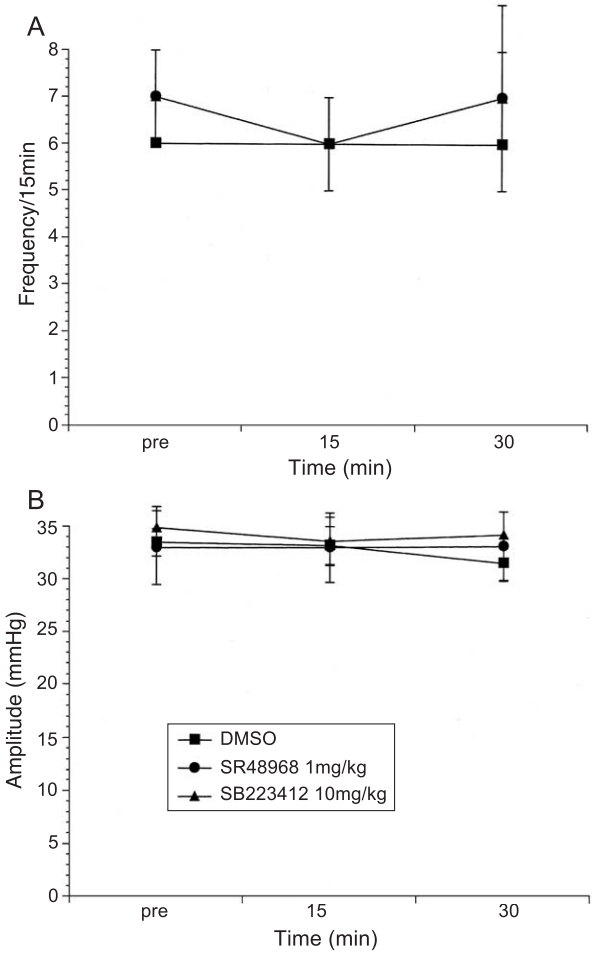

Fig. 4. Effect of SR48968 (a tachykinin NK_2 receptor antagonist) and SB223412 (a tachykinin NK_3 receptor antagonist) on frequency (A) and amplitude (B) in guinea pigs. DMSO (closed squares), SR48968 (1 mg/kg, closed circles) or SB223412 (10 mg/kg, closed triangles) was administered intravenously. Values are means ± S.E.M. for six (DMSO) to five (SR48968, SB223412) animals.

buffer, pH 7.4) containing 5 mM $MnCl_2$, 200 μg/ml bovine serum albumin, 5 μg/ml A-PMSF, 4 μg/ml leupeptin, 40 μg/ml bacitracin, 20 μg/ml chymostatin, and 10 mM phosphoramidone or binding buffer alone were added to a 96-well plate. For estimation of nonspecific binding, 50 μl of cold substance P solution (15 μM) was added. Then, 100 μl of [^{125}I]Bolton–Hunter substance P solution (final concentration of 0.1 nM, NEN Life Science Products) was added. The binding reaction was initiated by adding 100 μl of the membrane preparation. The mixture was incubated for 90 min at room temperature and harvested on a glass filter (Filtermats, plain, 1.5 μm, Molecular Devices) pretreated with 0.1% polyethyleneimine by a cell harvester. The filters were washed with 50 mM Tris–HCl buffer (pH 7.4) containing 5 mM $MnCl_2$ and dried, then the radioactivity was measured. Specific binding was calculated by subtracting nonspecific binding from total binding. The data were expressed as percentages of specific binding determined in vehicle-treated animals.

2.6. Chemicals

GR205171, CP99994 ((+), (2*R*, 3*R*)-3-(2-methoxybenzyl-amino)-2-phenylpiperidine), SR48968 ((+)-*N*-methyl-[4-(4-acetylamino-4-phenyl piperidino)-2-(3, 4-dichloro-phenyl)butyl] benzamide), SB223412 ((*S*)-(−)-*N*-(α-ethylbenzyl)-3-hydroxy-2-phenylquinoline-4-carboxamide) and FK888 were synthesized by Fujisawa Pharmaceutical, GR205171 and CP99994 was dissolved in saline. SR48968, SB223412 and FK888 were dissolved in dimethylsulfoxide.

2.7. Statistical analyses

The values are expressed as means ± S.E.M. of three to five experiments. Statistical analysis was performed using the analysis of variance, based on a randomized block design, followed by Dunnett's multiple comparisons. Differ-

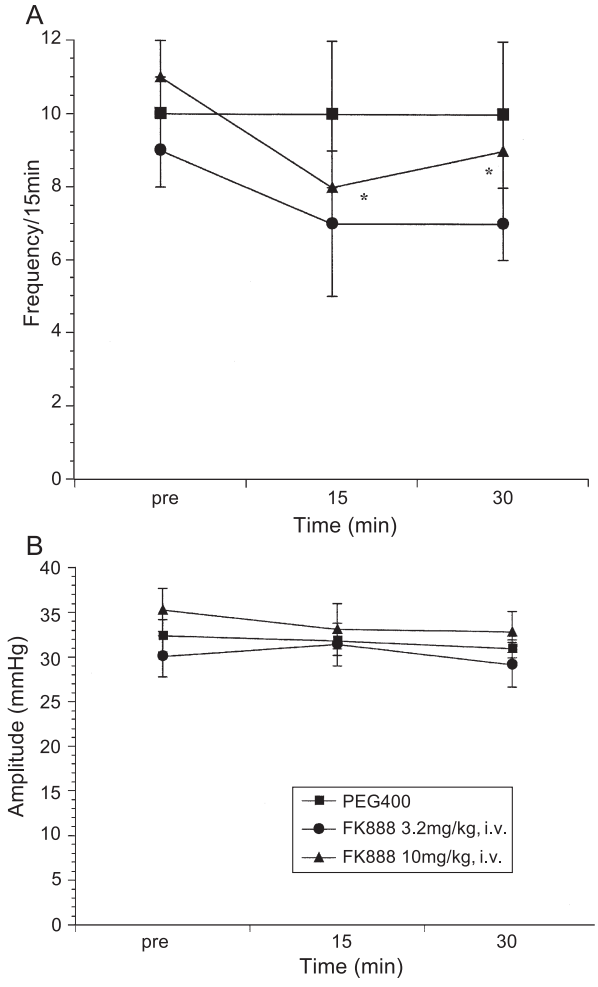

Fig. 5. Effect of FK888, a tachykinin NK_1 receptor antagonist, on frequency (A) and amplitude (B) in guinea pigs. PEG400 (closed squares) or FK888 was administered intravenously at 3.2 (closed circles) and 10 (closed triangles) mg/kg. Values are means ± S.E.M. for five animals. *$P < 0.05$, significant difference from the value before administration (Dunnett's multiple comparison).

ences with a P value less than 0.05 were considered to be significantly different.

3. Results

3.1. Effects of selective tachykinin receptor antagonists on distention-induced rhythmic bladder contraction

Infusion of saline into the urinary bladder induced rhythmic bladder contraction, as shown in Fig. 1. The effects of antagonists were quantitated in terms of the frequency and amplitude of the rhythmic bladder contractions for 15 min. Intravenous injection of a tachykinin NK_1 receptor-selective antagonist, GR205171, dose-dependently reduced the frequency of the rhythmic bladder contraction (Fig. 2). When the contractions appeared again, the amplitude of contraction was not affected. CP99994 also reduced the frequency of the rhythmic bladder contraction without affecting the amplitude of contraction (Fig. 3).

Intravenous injection of a tachykinin NK_2 receptor-selective antagonist, SR48968, or a tachykinin NK_3 receptor-selective antagonist, SB223412, did not reduce the frequency or amplitude of contraction (Fig. 4). In addition, a tachykinin NK_1 receptor antagonist, FK888, slightly decreased the frequency after 10 mg/kg injection (Fig. 5). FK888 did not significantly decrease the amplitude of contraction.

3.2. Distribution of tachykinin NK_1 antagonists to the spinal cord and the brain

Extracts from brain and spinal cord, as well as plasma after GR205171 injection showed almost complete inhibition of [^{125}I]Bolton–Hunter substance P binding to tachykinin NK_1 receptors (Fig. 6). In contrast, extracts from brain and spinal cord after FK888 injection did not show inhibition of [^{125}I]Bolton–Hunter substance P binding to tachykinin NK_1 receptors, while the extract from plasma inhibited the binding.

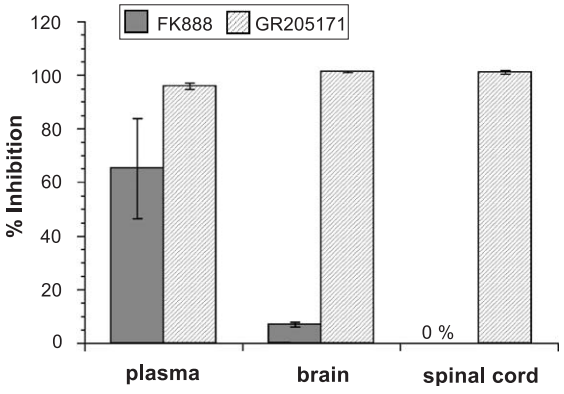

Fig. 6. Inhibition of the ex vivo binding of [^{125}I]Bolton–Hunter substance P to tachykinin NK_1 receptors by tissue extracts after FK888 and FR205171 administration in guinea pigs. Ex vivo measurements were made 5 min after i.v. injection (1 mg/kg). Results are expressed as % inhibition of specific [^{125}I]Bolton–Hunter substance P binding measured in vehicle-treated animals. Values are means ± S.E.M. for three experiments.

4. Discussion

There have been many reports that acetylcholine is the excitatory neurotransmitter involved in bladder contraction (Andersson, 1993; Eglen et al., 1996; Somogyi et al., 1998; Oyasu et al., 1994; Yamamoto et al., 1995a,b). Recent findings related to the sensory side of the micturition reflex have created new interest in the role of tachykinin receptors.

Some selective antagonists have been found recently. GR205171 potently inhibits [^{3}H] substance P binding to human tachykinin NK_1 receptors expressed in CHO cells or rat cortex (pK_i value of 10.6 or 9.5, respectively; Gardner et al., 1996), while it has lower affinity for tachykinin NK_2 receptors in rat colon ($pIC_{50} < 5$) and tachykinin NK_3 receptors in guinea pig cortex ($pIC_{50} < 5$). This compound showed potent inhibition of both acute and delayed in vivo emetic responses to cisplatine in piglets receiving a tachykinin NK_1 receptor antagonist (Grélot et al., 1998). CP99994 potently inhibits [^{3}H]substance P binding to human tachykinin NK_1 receptors expressed in CHO cells (K_i value of 0.42 nM), whereas for tachykinin NK_2 and NK_3 receptors, the K_i values were 17,000 and >10,000 nM, respectively (Sarau et al., 1997). CP99994 inhibited coughing in the guinea pig and cat by a central site of action (Bolser et al., 1997). Sarau et al. (1997) reported that SR48968 potently inhibits human tachykinin NK_2 receptor binding ($K_i = 0.23$ nM), while it has lower affinity for NK_1 ($K_i = 454$ nM) and NK_3 ($K_i = 707$ nM) receptor binding. Santucci et al. (1993) reported that SR48968 inhibits in vivo thalamic responses evoked by thermal nociception. SB223412 inhibited the binding of [^{125}I][MePhe^7]neurokinin B to CHO hNK_3 cell membranes with a K_i value of 1.0 nM, whereas for tachykinin NK_1 and NK_2 receptors, the K_i values were >10,000 and 144 nM, respectively (Sarau et al., 1997). In mice, SB223412 produced dose-dependent inhibition of behavioral responses induced by a tachykinin NK_3 receptor agonist. Therefore, we chose GR205171 (and CP99994), SR48968 and SB223412 as tachykinin NK_1, NK_2 and NK_3 receptor antagonists, respectively, and investigated the effects of these antagonists on the micturition reflex.

GR205171 and CP99994 inhibited the micturition reflex, but SR48968 and SB223412 did not. These results indicate that receptors of the tachykinin NK_1 type, but not NK_2 or NK_3 type, are involved in the micturition response in guinea pigs. Radioligand binding indicates that both tachykinin NK_1 and NK_2 receptors are located on guinea pig bladder (Banasiak and Burcher, 1994). Likewise, bladder contraction can be elicited in vitro through either tachykinin NK_1 or NK_2 receptors on smooth muscle (Longmore and Hill, 1992). However, our present results suggest that such

peripheral tachykinin NK_2 receptors do not mediate the distention-induced bladder contraction. Palea and Pietra (1999) demonstrated involvement of spinal tachykinin NK_1 receptors in modulating the inhibitory effect of capsaicin on the micturition reflex in guinea pigs. Doi et al. (2000) reported that a tachykinin NK_1 receptor antagonist, TAK-637, decreased the number of distention-induced bladder contractions. Our findings are in accordance with their results. It has been reported that stimulation by a tachykinin NK_3 receptor agonist had no effect on the urinary bladder, whereas a tachykinin NK_2 receptor agonist elicited contraction of the strips (Mussap et al., 1996; Nsa Allogho et al., 1997; Tramontana et al., 1998). These reports and our present study indicate that tachykinin NK_3 receptors are not located in the urinary bladder.

Among tachykinin NK_1 receptor antagonists, GR205171 inhibited the micturition reflex while FK888 did not. The ability of the two tachykinin NK_1 receptor antagonists to bind to tachykinin NK_1 receptors in the central nervous system (CNS), following peripheral administration, was investigated using ex vivo binding. We used an early time point (5 min) as the effects of the antagonists on the micturition reflex were seen immediately after its administration. The results clearly show that, in guinea pigs, GR205171 gained rapid access to the CNS following intravenous administration. In contrast, FK888 could not gain access to the CNS. Extracts from plasma after FK888 injection showed about 65% inhibition of [^{125}I]Bolton–Hunter substance P binding to tachykinin NK_1 receptors, while extracts from brain and spinal cord showed only about 5% and almost no inhibition, respectively. FK888 is a potent peptide tachykinin NK_1 receptor antagonist and it has been shown that inhalation of the drug inhibits irritant-induced cough in guinea pigs (Sekizawa et al., 1995; Ujiie et al., 1993). Our present results indicate that peripheral tachykinin NK_1 receptors do not play an important role in distention-induced bladder contraction. Abundant experimental data indicate that tachykinins make a significant contribution as sensory transmitters during nociceptive stimulation (Yoshimura and de Groat, 1997; Lecci and Maggi, 2001). Substance P-positive nerve fibers have been detected in proximity to spinal dorsal root ganglion neurons projecting to the bladder (Wang et al., 1998; Birder et al., 1999). Based on these findings, central tachykinin NK_1 receptors, especially receptors in the spinal cord, should play an important role in mediating the micturition reflex in guinea pigs. Although we have not examined the CNS permeability of the tachykinin NK_2 receptor antagonist SR48968, or of the tachykinin NK_3 receptor antagonist, SB223412, several studies have demonstrated that they access the CNS. SR48968 had antidepressant-like activity after 0.3–10 mg/kg, i.p. injection in guinea pigs (Steinberg et al., 2001), inhibited thalamic responses after 0.5 mg/kg, i.v. injection in rats (Longmore and Hill, 1992) and showed anxiolytic activity after 0.05–5 μg/kg, i.v. injection in mice (Stratton et al., 1993). For SB223412, direct evidence for central nervous system access was provided by disposition studies, involving infusion of the compound in rats (Sarau et al., 1997). Although the CNS effect was not reported for guinea pigs, the potentiation by [MePhe^7]neurokinin B of histamine-induced microvascular leakage was abolished by SB223412 after 1 mg/kg, i.v. injection in guinea pig airways, suggesting that SB223412 also shows antagonistic activity in guinea pigs at this dose (Daoui et al., 2001).

In conclusion, our findings strongly suggest that tachykinin NK_1 receptors, but not NK_2 or NK_3 receptors, play an important role in mediating the micturition reflex in the central nervous system, presumably in the spinal cord.

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