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THE NONPEPTIDE NK-2 ANTAGONIST SR 48968 IS ALSO A NK-3 ANTAGONIST IN THE GUINEA PIG BUT NOT IN THE RAT

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SR 48968 was first described as a NK-2 nonpeptide receptor antagonist; we report here that SR 48968 interacts also with guinea pig but not rat NK-3 cortical binding sites. Furthermore, SR 48968 is shown to inhibit the senktide- (a NK-3 selective agonist) evoked stimulation of phosphoinositide turnover in guinea pig ileum slices. The species difference observed for the NK-3 receptor with SR 48968 was confirmed by the determination of the affinities of NK-3 peptide agonists. [Pro^7]neurokinin B particularly was found to have a greater affinity for cortical NK-3 binding sites in the rat than in the guinea pig.

Important progress has been made during the last few years in the development of new agonists and antagonists for the receptors of the tachykinin family. Indeed, selective agonists have already been described for the NK-1, NK-2 and NK-3 tachykinin receptors whose endogenous ligands are substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) respectively (1,2). For example, [Pro^9]SP is a selective NK-1 agonist, [Lys^5,MeLeu^9,Nle^{10}]NKA(4-10) is specific for the NK-2 receptor and senktide is an exclusive NK-3 agonist (3). More recently, nonpeptide antagonists for the NK-1 receptor have been discovered (4,5,6). CP-96,345 and RP 67580 have been shown to have very potent antagonist properties in different NK-1 bioassays. In addition, the use of these two compounds has revealed species differences since for instance CP-96,345 is a more potent antagonist in the guinea pig while RP 67580 is more efficient in the rat (7,8,9). A nonpeptide antagonist for the NK-2 receptor has also been recently described; SR 48968 antagonizes with great efficacy peripheral and central biological responses induced by NKA or selective NK-2 agonists (10,11,12). SR 48968 inhibits the binding of [^{125}I]NKA to receptors on rat duodenal membranes but not the binding of [^{125}I]SP or [^{125}I]Eledoisin to membranes from the rat cerebral cortex (11). Additional indications for the selectivity of SR 48968 for NK-2 receptors was demonstrated using several isolated smooth muscle preparations, this compound acts as an antagonist in the rabbit pulmonary artery (a typical NK-2 bioassay) but not in the rabbit vena cava (a preparation enriched in NK-1 receptors), or the rat portal vein (a preparation enriched

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in NK-3 receptors) (10). Species differences in the pharmacological properties of NK-2 receptors have also been reported using peptide antagonists such as MEN 10,376, R 396 or L-659,877 (13,14), but NK-2 receptors from various species could not be distinguished with SR 48968 (10).

In the present experiments, the specificity of SR 48968 was further explored by comparing its effects in different NK-3 assays from the rat and the guinea pig. It will be particularly shown that SR 48968 is not only a NK-2 antagonist since it acts also as a NK-3 antagonist in the guinea pig ileum revealing species differences for NK-3 receptors.

METHODS

Binding assays.

Rat or guinea pig cortical membranes were used with [^{3}H]senktide as the selective ligand to estimate the affinities of SR 48968 and NK-3 agonists for NK-3 binding sites (15). Male Spague-Dawley rats (200-300 g) or Hartley guinea pigs (300-400 g) (Charles River, Cléon, France) were sacrificed by decapitation and their brains removed rapidly. Cerebral cortices were dissected away from the rest of the brains homogenized (10 s) in Tris-HCl (50 mM; pH 7.4) at 4°C (10 ml per gram of tissue) with a polytron apparatus and centrifuged at 15,000 x g for 20 min at 4°C with a sigma 202MK centrifuge. The pellet was resuspended in cold buffer and centrifuged again under the same conditions. Membranes were finally resuspended (2.5 ml per gram of initial tissue) in the incubation medium consisting of Tris-HCl (50 mM; pH 7.4) containing $MnCl_2$ (3 mM), bovine serum albumin (BSA) (0.4 mg/ml), bacitracin (0.08 mg/ml), chymostatin (0.004 mg/ml), leupeptin (0.004 mg/l) and thiorphan (1 μM).

Rat or guinea pig cortical synaptosomes, prepared as previously described (16) were also used with [^{125}I]Bolton Hunter Eledoisin ([^{125}I]BHE) as the ligand for estimating the affinities of SR 48968 and NK-3 agonists for NK-3 binding sites.

Twenty μl suspension of the membranes or synaptosomes (approximately 400 μg of protein for cortical membranes and 200 μg of protein for synaptosomes) were added to the incubation medium in a final volume of 200 μl and incubated at 22°C for either 60 min with [^{3}H]senktide (~ 1.5 nM) (Specific Activity: 46.8 Ci/mmol, N.E.N) or 15 min with [^{125}I]BHE (~ 40 pM) (Specific Activity: 2000 Ci/mmol). Incubations were stopped either by filtration through two Whatman GF/C filters, pretreated for 3 to 4 hrs with polyethyleneimine (0.1%), using a JSI Multividor apparatus ([^{3}H]senktide binding on cortical membranes) or (centrifugation for 30 s in an Eppendorf Microfuge ([^{125}I]BHE binding on cortical synaptosomes). All assays were run in triplicate and nonspecific binding was defined as the amount of labelled ligand bound in the presence of 1 μM of the unlabelled compound.

Phosphoinositides breakdown in the guinea pig ileum.

Male Hartley guinea pigs (300-500 g) (Charles River, France) were decapitated and their ileums rapidly removed. The mucus of each ileum was scraped with a blunted scalpel and the resulting muscle cross chopped with a Mac Ilwain tissue chopper (350 μm x 350 μm x 350 μm). Slices were dispersed with a Pasteur pipette in 10 ml of freshly gased (95% O_2 : 5% CO_2) Krebs solution containing NaCl (118 mM), KCl (4.7 mM), $CaCl_2$ (1.25 mM), $MgSO_4$ (1.2 mM), $NaHCO_3$ (25 mM), KH_2PO_4 (1.2 mM), glucose (11 mM), bacitracin (0.03 mg/ml) and BSA (1 mg/ml) (pH 7.4). Slices were then washed for 30 min at 37°C, the continuously gased Krebs solution being changed at 15 min. Guinea pig ileum slices were then preincubated for 90 min at 37°C in 12 ml Krebs buffer containing 96 μCi of [^{3}H]-*myo*-inositol (Myo-[2-^{3}H]inositol, Specific Activity: 19 Ci/mmol; Amersham, France) under an atmosphere of 95% O_2 : 5% $C0_2$. Prelabelled slices were subsequently washed 6 times with 15 ml Krebs buffer. Final incubation of samples for 30 min at 37°C under a gased atmosphere was performed in polypropylene tubes containing 100 μl of pre-gased Krebs buffer, LiCl (10 nM final), 125 μl of the ileum slices suspension (~ 1.3 mg of protein) and the agonist in a volume

of 25 µl. In experiments in which SR 48968 was used, tissues were incubated with this drug 15 min before the exposure to senktide. Incubations were terminated by the addition of 1 ml chloroform/methanol (1:2; vol/vol), and 0.5 ml chloroform and 0.5 ml water were added 15 min later. Samples were then vortexed and centrifuged at 1000 x g for 5 min; 1 ml of the aqueous phase was applied to a column containing 1.5 ml of Dowex AG 1-X8 resin and 5 ml water. The eluant was collected in scintillation vials and unchanged [^{3}H]-*myo*-inositol counted with 8 ml of Aquasol-2 (N.E.N). [^{3}H]glycerophosphoinositol was removed by an elution (8 ml) with disodium tetraborate (5 mM) and ammonium formate (60 mM). [^{3}H]inositol monophosphates were eluted with 8 ml of ammonium formate (200 mM) and formic acid (100 mM) and counted in scintillation vials as described above.

RESULTS

Affinities of SR 48968 for guinea pig but not rat NK-3 cortical binding sites.

SR 48968 had no affinity for NK-3 binding sites from the rat cerebral cortex labelled by either [^{125}I]BHE or [^{3}H]senktide (IC_{50} always over 10 000 nM).

However, binding assays performed with [^{125}I]BHE on cortical synaptosomes from the guinea pig revealed an affinity of SR 48968 for NK-3 binding sites in this species with an IC_{50} = 3.3 ± 1.7 x 10^{-7} M. Similar results were obtained using [^{3}H]senktide and cortical membranes from the guinea pig, SR 48968 having an IC_{50} of 3.2 ± 0.4 x 10^{-7} M (Fig. 1, Table 1). In all cases the competition curve was monophasic and n_H close to unity.

Antagonist properties of SR 48968 on the guinea pig NK-3 receptor.

The activity of SR 48968 on guinea pig NK-3 receptors was determined by measuring phosphoinositide hydrolysis in ileum slices. SR 48968 had no agonist properties by itself but antagonized with great efficacy the stimulatory effect of the selective NK-3 agonist senktide on phospholipase C activity, its pK_B being 7.08 (Figure 2). Interestingly, [Trp^7,βAla^8]NKA(4-10) a compound which has been described to act as a NK-3 antagonist in the rat portal vein (17) was without effect in the guinea pig ileum which is in agreement with our binding data (Table 1).

Affinities of various peptides for rat and guinea pig NK-3 binding sites.

As just indicated, the nonpeptide compound SR 48968 previously described as an NK-2 antagonist (11) clearly allows us to distinguish the differences between rat and guinea pig NK-3 receptors. In contrast, species differences in NK-3 binding sites could not be demonstrated with two other NK-2 peptide antagonists MEN 10,376 and R 396 (Neosystem, Strasbourg, France) (Table 1).

Different pharmacological properties of NK-3 receptors between the rat and the guinea pig were also observed with NK-3 peptide agonists using cortical membranes and [^{3}H]senktide as a ligand. Indeed, IC_{50} values for NKB and [Arg^0]NKB were 1.6 and 3.9 nM respectively in rat cortical membranes while they reached 14 and 11 nM in guinea pig cortical membranes indicating that both NK-3 agonists have a greater affinity for rat rather than guinea pig NK-3 binding sites (Table 1). However, this species

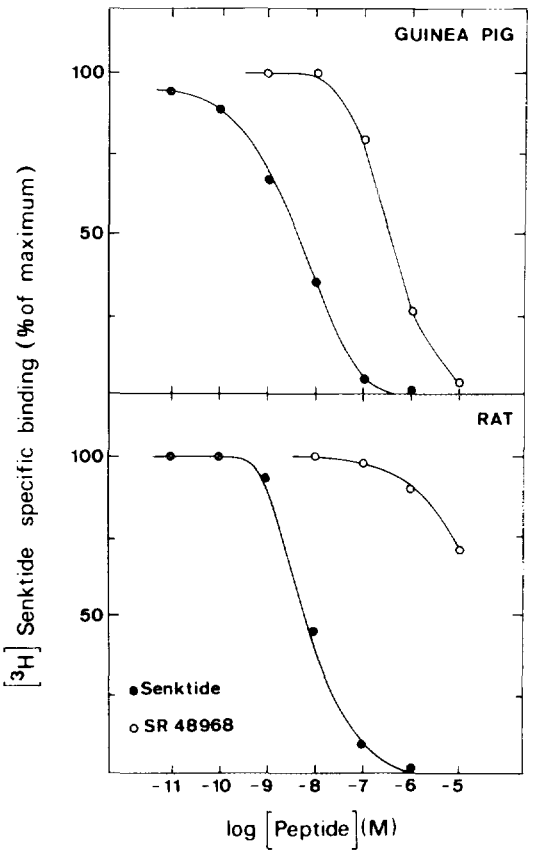

Figure 1 . Competitive inhibition by senktide and SR 48968 of [^{3}H]senktide binding to guinea pig and rat cortical membranes.

Competitive inhibition of [^{3}H]senktide binding by senktide (black symbols) and SR 48968 (open symbols) to guinea pig (upper panel) and rat (bottom panel) cortical membranes. Membranes were incubated with [^{3}H]senktide (~1.5 nM) and increasing concentrations of the peptide agonist or the nonpeptide antagonist. Nonspecific binding was determined with senktide (1 μM). Each point is the mean of data obtained in two to four experiments, each value being determined in triplicate. Results are expressed as a percentage of the control [^{3}H]senktide specific binding.

difference was not observed when [^{125}I]BHE was used as the ligand. A much more dramatic difference, which confirms species specificity, was seen with [Pro^7]NKB but not the other NK-3 agonist senktide. Indeed, [Pro^7]NKB was much more potent in the rat than in the guinea pig using either [^{125}I]-BHE (IC_{50} = 14 and 700 nM respectively) or [^{3}H]senktide (IC_{50} = 160 and 1600 nM respectively) as ligands (Table 1).

DISCUSSION

Two subtypes of NK-2 receptors nammed NK-2_A and NK-2_B have been identified thanks to several NK-2 peptide antagonists including MEN 10,376, R 396 and

Table 1. Affinities of some tachykinin analogs for cortical NK-3 binding sites

	[^{125}I]BHE		[^{3}H]senktide	
	Rat	Guinea pig	Rat	Guinea pig
	IC_{50} values (nM)			
[Pro^{9}]SP	> 10 000	> 10 000	> 10 000	> 10 000
[Lys^{5},MeLeu^{9},Nle^{10}]NKA(4-10)	> 10 000	> 10 000	> 10 000	> 10 000
MEN 10,376	> 10 000	> 10 000	> 10 000	> 10 000
R 396	> 10 000	> 10 000	> 10 000	> 10 000
SR 48968	13 000	330	28 000	320
Senktide	6.9	6.9	5.3	4.6
NKB	1.9	1.8	1.6	14
[Arg^{0}]NKB	1.4	1.4	3.9	11
[Pro^{7}]NKB	14	700	160	1 600
[Trp^{7}ßAla^{8}]NKA(4-10)	> 10 000	> 10 000	5 300	> 10 000

IC_{50} values (nM) (mean of two to five independant experiments) were determined on cortical membranes ([^{3}H]senktide binding) or synaptosomes ([^{125}I]BHE). The standard deviation did not exceed 70 % of the mean. Some data concerning [^{125}I]BHE binding on rat cortical synaptosomes have been published previously in references 1 and 16. Specific binding of [^{3}H]senktide on rat and guinea pig cortical membranes represented 42% and 69% of total binding, respectively, while specific binding of [^{125}I]BHE on rat and guinea pig cortical synaptosomes represented 68% and 70% of total binding, respectively, in agreement with results obtained in previous studies (15,16,21).

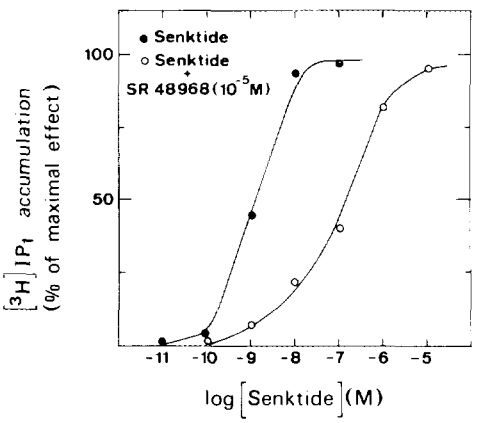

Figure 2 . Antagonism by SR 48968 of the senktide-induced activation of phospholipase C in guinea pig ileum slices.

SR 48968 was added into the incubation medium 15 min before the selective NK-3 agonist (senktide) used at various concentrations and the accumulation of [^{3}H]inositol monophosphates determined at the end of the 30 min incubation as described in materials and methods. Results corresponding to the accumulation of [^{3}H]inositol monophosphates are expressed as percent of control values. Each point is the mean of data obtained in three to five experiments, each value being determined in triplicate. Senktide stimulated phospholipase C with a great efficacy (EC_{50} = 1.5 ± 0.2 nM), SR 48968 inhibited the senktide-induced [^{3}H]inositol monophosphates accumulation with a pK_B = 7.08. pK_B was calculated according to the formula: pK_B = log(dose ratio - 1) - log[B] where [B] is the molar concentration of the antagonist.

L-659,877. The NK-2_A subtype is found in the guinea pig , cow, rabbit and human while the NK-2_B subtype is present in the hamster and rat. To our knowledge, there is no evidence yet indicating that these two subtypes of the NK-2 receptor are simultaneously present in the same species. The nonpeptide compound SR 48968 is a very potent NK-2 antagonist in all preparations acting equally well on NK-2_A and NK-2_B subtypes (10). However, as indicated by this study SR 48968 has allowed us to demonstrate a species difference in the pharmacological properties of NK-3 receptors since this NK-2 antagonist recognizes also NK-3 receptors in the guinea pig but not in the rat.

The first indication for an action of SR 48968 on NK-3 receptors of the guinea pig was obtained by comparative binding studies performed on guinea pig and rat membranes or synaptosomes from the cerebral cortex (a brain area particularly rich in NK-3 binding sites) using two NK-3 ligands: [^{125}I]BHE and [^{3}H]senktide (15,16,18). Indeed, SR 48968 exhibited a good affinity for NK-3 binding sites in the guinea pig (IC_{50} around 300 nM) while in agreement with previous findings it had no affinity for rat NK-3 binding sites. Senktide is a highly selective NK-3 agonist (19) which has been shown to stimulate the turnover of phosphoinositides in guinea pig ileum slices providing further evidence for the presence of NK-3 receptors in this tissue (20). Supporting further this statement, we have observed that few ^{3}H-NKA binding sites (NK-2 receptors) are present in this tissue and that senktide has no affinity for these binding sites (unpublished observations). Our results indicate that SR 48968 which is devoided of agonist properties inhibits the senktide-induced accumulation of [^{3}H]inositol monophosphates with a pK_B of 7.08. This confirms that SR 48968 is interacting with NK-3 receptors in the guinea pig. However, according to results obtained by Advenier et al. (10) SR 48968 is still more potent on NK-2 receptors in this species (guinea pig trachea pA_2: 10.5).

Binding studies performed with NK-3 agonists provided further support for the existence of species difference in the pharmacological properties of NK-3 receptors. This was particularly striking with [Pro^7]NKB since this selective NK-3 agonist (1) exhibited a 50 fold higher affinity for rat cortical NK-3 binding sites than for those of the guinea pig. Interestingly enough, the other potent and selective NK-3 agonist senktide recognized equally guinea pig and rat NK-3 binding sites. Therefore, [Pro^7]NKB is the first tachykinin agonist which allows phamacological separation of one type of tachykinin receptor (NK-3 receptors in the present case) in two species. On the other hand, results obtained with NKB and [Arg^0]NKB are in good agreement with those of Renzetti et al. (21) from a binding study performed with [^{3}H]senktide on guinea pig and rat brain cortical membranes. However, the higher affinity of these two NK-3 agonists for rat rather than guinea pig NK-3 receptors could not be confirmed using [^{125}I]BHE as another NK-3 radioligand. Although this difference will have to be demonstrated in further studies, it could be due to the existence of NK-3 subtypes within a single species differently labelled by either [^{125}I]BHE or [^{3}H]senktide .

In conclusion, our study indicates that the species difference previously established for NK-1 and NK-2 receptors can be now extended to NK-3 receptors. It is

particularly amazing that the rat and the guinea pig exhibit different pharmacological patterns for the three tachykinin receptors. In all cases, antagonists were found to be the key compounds for distinguishing the different types of receptors. Interestingly enough NK-3 receptors of the guinea pig were recognized by a non peptide antagonist SR 48968 known for its potent antagonist activity on NK-2 receptors. The discovery of the NK-3 antagonist activity of SR 48968 in the guinea pig will certainly rapidly lead to the synthesis of compounds acting both on rat and guinea pig NK-3 receptors but without effect on other tachykinin receptors. As already shown, thanks to the cloning of corresponding mRNAs, structure differences have been shown among some species for NK-1, NK-2 as well NK-3 receptors (22,23,24). Very likely these structure variations are responsible for the different pharmacological properties noted within each class of tachykinin receptors from one species to another.

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