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[^{3}H][β-Ala^8]neurokinin A-(4–10): a novel, selective radioligand for the tachykinin NK_2 receptor

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Abstract

The binding characteristics of the novel radioligand [^{3}H][β-Ala^8]neurokinin A-(4–10) were assessed in hamster urinary bladder membranes. This labelled compound bound in a reversible, highly specific and concentration-dependent manner to a single class of high affinity binding sites with a K_d of 1.8 ± 0.2 nM and a B_{max} of 139 ± 21 fmol/mg of protein. Specific binding of [^{3}H][β-Ala^8]neurokinin A-(4–10) was displaced only by NK_2, but not by NK_1 or NK_3, tachykinin receptor agonists and antagonists. Neurokinin A, [β-Ala^8]neurokinin A-(4–10), L 659877 [cyclo(Leu-Met-Gln-Trp-Phe-Gly)], MEN 10376 (H-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2), MEN 10627 [cyclo(Met-Asp-Trp-Phe-Dap-Leu)cyclo(2β-5β)] and SR 48968 [(*S*)-*N*-methyl-*N*-[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide] displaced the binding with K_i values of 0.4 ± 0.1 nM, 1.9 ± 0.36 nM, 3.05 ± 0.1 nM, 7.9 ± 0.4 μM, 0.36 ± 0.02 nM and 2.5 ± 0.9 nM, respectively. Functional data, obtained in isolated hamster urinary bladder strips with the newly developed tachykinin NK_2 receptor antagonists (MEN 10627 and SR 48968), showed a good agreement with binding data. This novel radioligand could represent a new useful tool for the assessment of tachykinin NK_2 receptor antagonists.

Keywords: Urinary bladder, hamster; [^{3}H][β-Ala^8]neurokinin A-(4–10); Tachykinin, NK_2 receptor; Tachykinin; (Binding)

1. Introduction

Tachykinins are a family of peptides widely distributed both in the central and peripheral nervous system. Their release from peripheral nerve endings of capsaicin-sensitive primary afferents produces a variety of biological actions (collectively referred to as neurogenic inflammation), which, in most cases, depend upon activation of specific receptors expressed on target cells membranes (Maggi et al., 1993).

It is well established that at least three distinct receptors mediate the biological responses of tachykinins in mammalian tissues, that are NK_1, NK_2 and NK_3 receptors, at which substance P, neurokinin A and neurokinin B are the most potent natural ligands, respectively (Buck and Burcher, 1986; Lee et al., 1986). However, all natural tachykinins are capable of acting as full agonists at these three receptor types, although with different affinities (Regoli et al., 1987).

Therefore, the development of highly selective synthetic tachykinin analogues has been mandatory for a pharmacological characterization of these receptors. In this research effort, particular relevance has been paid to the identification of suitable and selective tracers to be used in radioligand binding studies. So far, [^{125}I]Bolton-Hunter[Sar^{9}Met(O_2)^{11}]substance P (Lew et al., 1990) and [^{3}H][Pro^9]substance P (Petitet et al., 1991) are available selective labelled tachykinin NK_1 agonists, whereas, [^{125}I]Bolton-Hunter scyliorhinin II (Mussap and Burcher, 1990) and [^{3}H]senktide (Guard et al., 1990) are the ligands highly specific for the tachykinin NK_3 receptor.

Until now, the tachykinin NK_2 receptor has been mainly characterised in binding studies using, as a tracer, [^{125}I]neurokinin A, that poorly discriminates among tachykinin receptors (Burcher et al., 1989; Geraghty et al., 1992). Therefore the synthesis of radioligands selective for tachykinin NK_2 receptors is an ongoing objective. Recently, Burcher et al. (1993) introduced the iodinated peptide [^{125}I][Lys^5,Tyr(I_2)^7,

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MeLeu[9],Nle[10]]neurokinin A-(4–10), which retains the same high selectivity for the tachykinin NK_2 receptor than the parent cold compound, [Lys[5],Tyr(I_2)[7], MeLeu[9],Nle[10]]neurokinin A-(4–10). In addition, also the tritiated form of a non-peptide selective tachykinin NK_2 receptor antagonist (SR 48968) has been recently introduced (Emonds-Alt et al., 1992).

In this paper we describe the binding characteristics of the tritiated form of a highly selective peptide agonist of tachykinin NK_2 receptors, [β-Ala[8]]neurokinin A-(4–10) (Rovero et al., 1989). We show that [^{3}H][β-Ala[8]]neurokinin A-(4–10) binds to NK_2 tachykinin receptors in hamster urinary bladder membranes with high affinity and remarkable selectivity.

Selection of this tissue was based on previous studies suggesting the hamster urinary bladder as a rather pure monoreceptorial NK_2 preparation (Burcher and Buck, 1986; Maggi et al., 1992). The competition profile of various tachykinins agonists and antagonists confirmed the excellent selectivity of this radiotracer for the tachykinin NK_2 receptors expressed in this preparation. Further, a rather close relationship was evident between binding and functional data obtained with two newly developed NK_2 receptor antagonists, MEN 10627 (Maggi et al., 1994) and SR 48968 (Emonds-Alt et al., 1992).

2. Materials and methods

2.1. Preparation of membranes from hamster urinary bladder

Hamster urinary bladder membranes were obtained as previously described (Burcher and Buck, 1986) with minor modifications. Briefly, animals were killed by cervical dislocation and the urinary bladders were rapidly removed and placed in ice-cold 50 mM Tris-HCl buffer, pH 7.4, containing (in mM) 120 NaCl and 10 KCl (buffer A). Minced tissues were homogenized using an Ultraturax (30 strokes, speed 7) and the homogenate was centrifuged at 30000 × g for 30 min at 4°C. The resultant supernatant was discarded and the pellet resuspended in Buffer B (50 mM Tris-HCl, pH 7.4, plus EDTA 10 mM and KCl 300 mM). This suspension was incubated on ice for 30 min and then centrifuged. The resulting pellet was washed twice with 50 mM Tris-HCl, pH 7.4, and finally resuspended in the same buffer and quickly frozen in dry-ice.

2.2. Binding assay

Aliquots of membrane preparation (100 μg) were incubated in triplicate in 0.5 ml assay buffer (50 mM Tris-HCl pH 7.4 containing $MnCl_2$ (2 mM), bovine serum albumin (0.1%), chymostatin (4 μM), bacitracin (40 μM), leupeptin (4 μM) and thiorphan (1 μM)) for 90 min at room temperature in the presence of several concentrations (0.1–30 nM) of [^{3}H][β-Ala[8]]neurokinin A-(4–10). Non-specific binding was determined in the presence of 1 μM nonradioactive [β-Ala[8]]neurokinin A-(4–10).

The binding reaction was terminated by rapid filtration through Whatman GF/B filters presoaked in 0.5% bovine serum albumin. Bound radioactivity was determined by scintillation counting.

For competition binding experiments, membranes were incubated in triplicate with 0.5 nM [^{3}H][β-Ala[8]]neurokinin A-(4–10) (the approximate K_d value, as determined in saturation binding experiments) and different concentration of each test compound to give full competition curves.

For kinetic studies, membranes were incubated with 0.5 nM [^{3}H][β-Ala[8]]neurokinin A-(4–10) in the presence and absence of [β-Ala[8]]neurokinin A-(4–10). The reaction was stopped at various interval times by rapid filtration.

For dissociation experiments, tubes containing the assay mixture were incubated for 1 h at 22°C until steady state was reached. At this point, dissociation was initiated adding an excess (1 μM) of unlabelled [β-Ala[8]]neurokinin A-(4–10). The reaction was terminated at time points between 1 and 120 min. Total and non-specific radioactivity were also measured at these additional times.

2.3. Binding assays in typical NK_1 and NK_3 preparation

Some binding experiments were performed in two classical preparations for NK_1 and NK_3 tachykinin receptors i.e. the human B lymphoblastoma cells (IM9) and the guinea pig cortical membranes, respectively. The methods were explained in details elsewhere (Goso et al., 1994; Renzetti et al., 1991). The possible displacement by cold [β-Ala[8]]neurokinin A-(4–10) (10 μM) of the binding of [^{3}H]substance P (to IM9 cells) or of [^{3}H]senktide (to guinea pig cortex) was assessed.

2.4. Functional studies

Male hamsters (Syrian golden hamsters, 100–120 g) were killed by cervical dislocation. The whole urinary bladder was rapidly removed, bladder strips were excised and placed in a 5 ml organ bath containing a standard Krebs solution gassed with 96% O_2 plus 4% CO_2, as described previously (Maggi et al., 1988). A resting load of 10 mN was applied to all tissues. Mechanical activity was measured by means of isometric transducers and recorded on a Basile 7050 Unirecord. After an equilibration period of 45 min, the prepara-

tions were repeatedly challenged with acetylcholine (1 mM) up to stabilization. After 20–30 min a cumulative concentration-response curve to [β-Ala^8]neurokinin A-(4–10) was constructed, the next concentration being added when the effect of the preceding one had reached a steady state. In all the experiments peptidase inhibitors (bestatin, captopril and thiorphan, 1 μM each) were added to the bath 15 min before the start of the concentration-response curve to the agonist.

The tachykinin NK_2 selective antagonists MEN 10627 or SR 48968 were tested against responses to [β-Ala^8]neurokinin A-(4–10); all concentrations of antagonist were added to the bath 15 min before the start of the dose-response curve to the agonist. In each preparation only one concentration of antagonist was studied.

Contractile responses are expressed as percentages of maximal response to [β-Ala^8]neurokinin A-(4–10) obtained before addition of the antagonist.

2.5. Data analysis

Binding data were analyzed using the collection of computer programs described by McPherson (1985). Scatchard and Hill transformations were performed by the Equilibrium Binding Data Analysis referred to as EBDA; data were further analyzed by the curvilinear regression program LIGAND (Munson and Rodbard, 1980). Data from association and dissociation experiments were analyzed by the computer program KINETIC.

Statistical analysis of functional data was performed by using Student's *t*-test for unpaired data or by analysis of variance, when applicable. The EC_{50} of the concentration response curves to [β-Ala^8]neurokinin A-(4–10) was calculated as described by Tallarida and Murray (1981), while the pA_2 were calculated according to Van Rossum (1963).

All data in the text are expressed as means ± S.E.M.

2.6. Materials

[β-Ala^8]neurokinin A-(4–10)[Methionine, L-methyl-^{3}H] was synthesized by New England Nuclear. It has a specific activity, determined by mass spectroscopy, of 87.5 Ci/mM and a radiochemical purity of approximately 89–90%. [^{3}H]Substance P (specific activity = 40 Ci/mmol) was purchased from Amersham International while [^{3}H]senktide (specific activit = 55.2 Ci/mmol) was obtained from New England Nuclear. [Sar^9,Met(O$_2$)^{11}]substance P, [MePhe^7]neurokinin B and neurokinin A were obtained from Peninsula Laboratories, Belmont, CA, USA; [β-Ala^8]neurokinin A-(4–10) and MEN 10376 (H-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2) were synthesized by Dr. L. Quartara, Chemistry Department of Menarini Pharmaceuticals, Florence, Italy. FK 888 (N^2-[(4*R*)-4-hydroxy-1-(1-methyl-1*H*-indol-3-yl)carbonyl-L-prolyl]-*N*-methyl-phenylmethyl-L-3-(2-napthyl)-alaninamide) was synthesized by Dr. E. Potier, Chemistry Department of Menarini Ricerche Sud, Pomezia, Italy. SR 48968 or (*S*)-*N*-methyl-*N*-[4-4-acetylamino-4-phenylpiperidino)-2-(3,4-dichiorophenyl)butyl]benzamide was kindly donated by Dr. X. Emonds-Alt, Sanofi Recherche, Montpellier Cedex, France. L 659877 or cyclo(Leu-Met-Gln-Trp-Phe-Gly) was from Cambridge Research Biochemicals. MEN 10627 or cyclo(Met-Asp-Trp-Phe-Dap-Leu)cyclo(2β-5β) was synthesized in the Chemistry Department of Menarini Pharmaceuticals, Florence,

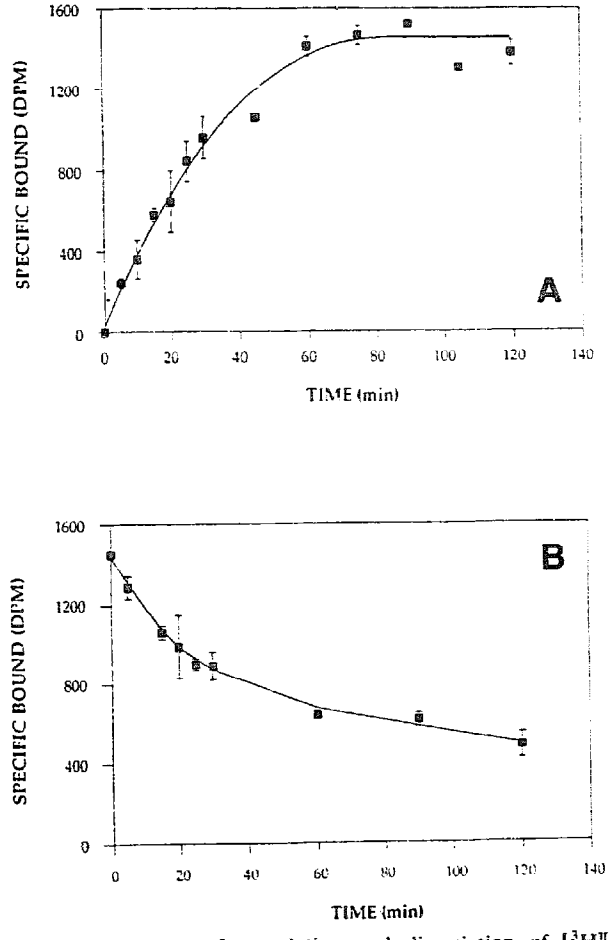

Fig. 1. Time course of association and dissociation of [^{3}H][β-Ala^8]neurokinin A-(4–10) to hamster urinary bladder membranes. (A) Membranes were incubated at 22°C in the presence of 0.5 nM [^{3}H][β-Ala^8]neurokinin A-(4–10) for the times shown. The reaction was stopped by filtration ($n = 3$). (B) After binding equilibrium was reached, 1 μM [β-Ala^8]neurokinin A-(4–10) was added to initiate dissociation of [^{3}H][β-Ala^8]neurokinin A-(4–10) ($n = 3$).

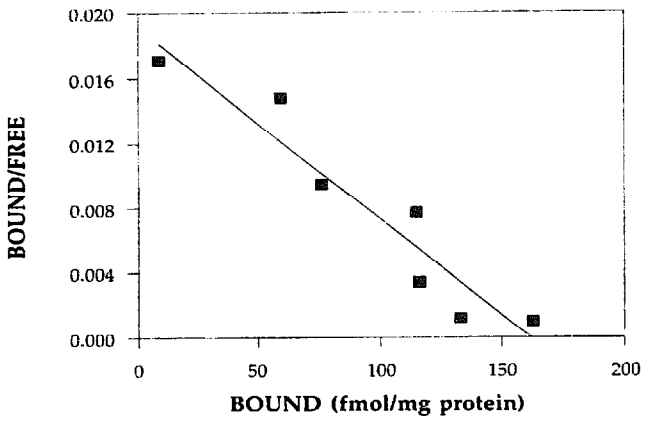

Fig. 2. Scatchard analysis of saturation curves of specific [^{3}H][β-Ala^8]neurokinin A-(4–10) binding to hamster urinary bladder membranes. Points represent mean values from a single experiment. The line was fitted using the LIGAND program. Five additional experiments gave similar results.

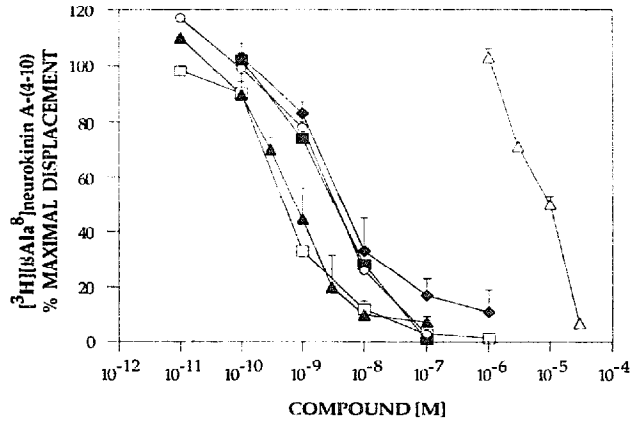

Fig. 3. Inhibition by non-radioactive [β-Ala^8]neurokinin A-(4–10) (○), neurokinin A (□), MEN 10627 (▲), SR 48968 (■), L 659877 (◆) and MEN 10376 (△) of specific [^{3}H][β-Ala^8]neurokinin A-(4–10) binding to hamster urinary bladder membranes. Points represent mean values from three independent determinations; error bars indicate ±S.E.M.

Italy. All the other chemicals were purchased from Sigma, St. Louis, MO, USA.

3. Results

3.1. Kinetic studies

When 0.5 nM of [^{3}H][β-Ala^8]neurokinin A-(4–10) was incubated with hamster urinary bladder membranes, under the described conditions, 3.2% of the labelled ligand was bound. 80% of this binding was specifically displaced by 1 μM [β-Ala^8]neurokinin A-(4–10). Kinetic studies showed that the association of [^{3}H][β-Ala^8]neurokinin A-(4–10) to hamster urinary bladder membranes was rapid and reversible: at 22°C, the specific binding of 0.5 nM [^{3}H][β-Ala^8]neurokinin A-(4–10) attained its peak by 60 min incubation, and then remained at an essentially constant level at least until 120 min (Fig. 1A). The association rate constant was $0.0468 \times 10^9 \pm 0.0128$ M^{-1} min^{-1} ($n = 3$). Dissociation experiments gave a dissociation rate constant of 0.00825 ± 0.00145 min^{-1} ($n = 3$, Fig. 1B), yielding a kinetic K_d value of 0.18 nM.

Based on these association and dissociation experiments, an incubation time of 90 min was used in all subsequent experiments.

3.2. Saturation studies

Scatchard analysis of specific [^{3}H][β-Ala^8]neurokinin A-(4–10) binding to hamster urinary bladder membranes was consistent with the existence of a single population of specific binding sites, yielding an affinity of 1.8 ± 0.2 nM and a maximal receptor density

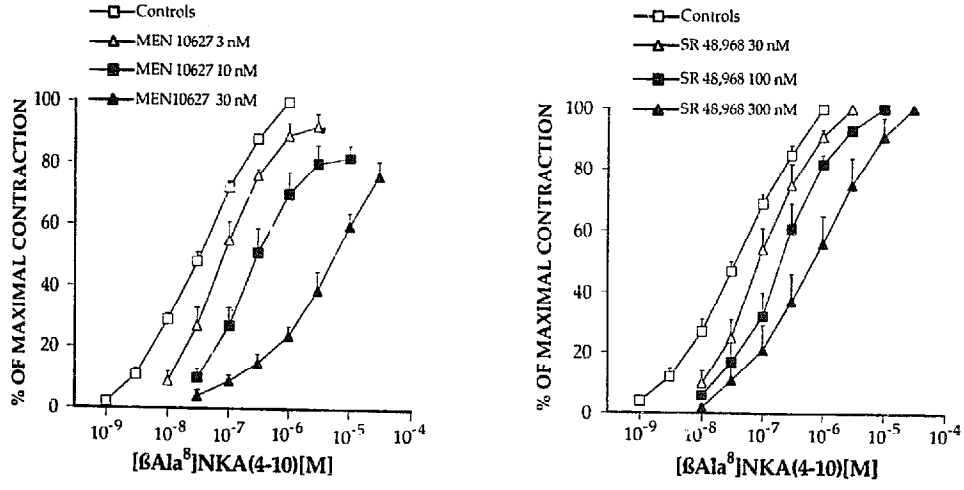

Fig. 4. Effect of increasing concentrations of MEN 10627 (left panel) and SR 48968, (right panel) on the concentration-response curves to [β-Ala^8]neurokinin A-(4–10) in hamster urinary bladder strips. Each value represents the mean ± S.E.M. of four to five experiments.

of 139 ± 21 fmol/mg of protein ($n = 6$; Fig. 2). The Hill coefficient was close to unity (0.99 ± 0.02).

This binding affinity was confirmed by competition experiments using non-radioactive [β-Ala^8]neurokinin A-(4–10): the cold ligand inhibited [^{3}H][β-Ala^8]neurokinin A-(4–10) binding to hamster urinary bladder membranes with a K_i value of 1.9 ± 0.36 nM ($n = 4$; Fig. 3).

3.3. Selectivity of the binding

The selective tachykinin NK_1 and NK_3 receptor ligands, [Sar^9]substance P sulfone and [MePhe^7]neurokinin B (Drapeau et al., 1987), failed to affect [^{3}H][β-Ala^8]neurokinin A-(4–10) binding to hamster urinary bladder membranes up to 1 and 0.1 μM, respectively ($n = 3$). Moreover the nonpeptide tachykinin NK_1 receptor antagonist, FK 888, was inactive up to 1 μM ($n = 3$). Neurokinin A inhibited [^{3}H][β-Ala^8]neurokinin A-(4–10) binding with a K_i value of 0.4 ± 0.1 nM ($n = 3$). Therefore specific [^{3}H][β-Ala^8]neurokinin A-(4–10) binding to hamster urinary bladder membranes most likely represents NK_2 receptors. Furthermore [β-Ala^8]neurokinin A-(4–10) at a concentration of 10 μM did not displace either the binding of [^{3}H]substance P to IM9 human lymphoblastoid cells (a typical NK_1 receptor binding assay) (Goso et al., 1994) or the binding of [^{3}H]senktide to guinea pig cortical membranes (a typical NK_3 tachykinin receptor binding assay) (Renzetti et al., 1991).

3.4. Competition studies

Specific binding of [^{3}H][β-Ala^8]neurokinin A-(4–10) in hamster urinary bladder was further characterized by using different tachykinin NK_2 receptor antagonists (Fig. 3). The cyclic peptide L 659877 inhibited [^{3}H][β-Ala^8]neurokinin A-(4–10) binding with a K_i of 3.05 ± 0.1 nM ($n = 3$), whereas the linear peptide MEN 10376 yielded a K_i of 7.9 ± 0.4 μM ($n = 3$), thus fitting the criteria of the NK_2 receptor subtype described in hamster smooth muscle preparations (Maggi et al., 1990). The non-peptide antagonist SR 48968 and the polycyclic peptide MEN 10627 displayed K_i values of 2.5 ± 0.9 nM ($n = 3$) and 0.36 ± 0.02 nM ($n = 3$), respectively.

3.5. Functional studies

Administration of [β-Ala^8]neurokinin A-(4–10) produced a concentration-related (1–1000 nM) contractions in isolated hamster urinary bladder strips with an EC_{50} (±95% c.l.) of 37 (31–43) nM and a pD_2 of 7.43 (7.36–7.5) ($n = 25$; Fig. 4), in good agreement with previously reported data (Advenier et al., 1992). MEN 10627 (3–30 nM) antagonized [β-Ala^8]neurokinin A-(4–10)-induced contractions with a pA_2 value of 9.06 ± 0.1 ($n = 12$). On the other hand, SR 48968 (30–300 nM) exerted a competitive antagonism with a pA_2 value of 7.82 ± 0.1 ($n = 10$) (Fig. 4).

4. Discussion

In this study we have gained evidence that [^{3}H][β-Ala^8]neurokinin A-(4–10) bound in a reversible, highly specific and concentration-dependent manner to a single class of high affinity binding sites in hamster urinary bladder membranes. Based on competition studies with tachykinin receptor agonists and antagonists, these binding sites most likely represent tachykinin NK_2 receptors. In fact, [^{3}H][β-Ala^8]neurokinin A-(4–10) binding was displaced by neurokinin A and by peptide (L 659877, MEN 10376 and MEN 10627) and non-peptide (SR 48968) tachykinin NK_2 receptor antagonists. On the other hand, tachykinin NK_1 receptor agonist ([Sar^9]substance P sulfone) and antagonist (FK 888), as well as a selective tachykinin NK_3 receptor agonist ([MePhe^7]neurokinin B), did not interfere with the radiotracer binding, even at high concentrations.

An heterogeneity in the tachykinin NK_2 receptor in various species and tissues has been described (Maggi et al., 1992). This suggestion mainly arises from a different rank order of potency of linear and cyclic peptide antagonists in various preparations. No particular difference is instead functionally detected by using agonists such as neurokinin A or [β-Ala^8]neurokinin A-(4–10) (Maggi et al., 1990). At this stage an accepted subclassification of the tachykinin NK_2 receptor is lacking and it is unclear whether or not real receptor subtypes or simple species-dependent variants exist. Notwithstanding our binding data in the hamster urinary bladder indicate a greater affinity of the cyclic peptide L 659877 as compared to the linear one MEN 10376, in agreement with previous functional data indicating this peculiar pharmacological profile of the tachykinin NK_2 receptor expressed in hamster smooth muscle preparations (Maggi et al., 1993).

Our binding data indicate the remarkable tachykinin NK_2 receptor affinity of the most recently developed peptide (MEN 10627) and non-peptide (SR 48968) tachykinin NK_2 receptor antagonists. An appreciable correlation exist between the ratio of their affinities in hamster urinary bladder obtained with a binding (pK_i of 9.4 ± 0.1 and 8.7 ± 0.3 for MEN 10627 and SR 48968, respectively) or a functional (pA_2 of 9.1 ± 0.1 and 7.8 ± 0.1 for MEN 10627 and SR 48968, respectively) approach.

Based on these data, we conclude that [^{3}H][β-Ala^8]neurokinin A-(4–10) is a suitable highly selective radioligand for the tachykinin NK_2 receptors. This tracer could have some advantages as compared to the

other available radioligands for the tachykinin NK_2 receptor ([^{125}I]neurokinin A, [^{125}I][Lys^5,Tyr(I_2)^7, MeLeu^9,Nle^{10}]neurokinin A-(4–10) and [^{3}H]SR 48968. Firstly, [^{3}H][β-Ala^8]neurokinin A-(4–10) is highly selective, while [^{125}I]neurokinin A has been demonstrated to bind also to tachykinin NK_1 receptors (Burcher et al., 1989; Geraghty et al., 1992).

Furthermore, an iodinated agonist, [^{125}I][Lys^5, Tyr(I_2)^7,MeLeu^9,Nle^{10}]neurokinin A-(4–10) has been recently developed (Burcher et al., 1993): also this compound is highly specific for the tachykinin NK_2 receptor. However, the radiolabelling with tritium could represent an advantage in different experimental situations (long half-life, short path length, low energy), being this radioisotope more easy to deal with than iodine.

Finally, extremely interesting has been the recent characterization of the tritiated analogue of the non-peptide tachykinin NK_2 receptor antagonist, SR 48968 (Emonds-Alt et al., 1993). Fong et al. (1992) demonstrated with a molecular approach that different epitopes within the tachykinin NK_1 receptor are recognized by substance P as compared to non-peptide antagonist. A similar discrepancy in the receptor domain(s) responsible for the binding of the natural ligand and non-peptide antagonist has been also suggested for the tachykinin NK_2 receptor (Gether et al., 1993). A conformational structural comparison of MEN 10627 and [β-Ala^8]neurokinin A-(4–10) indicated a strong overlapping of three key aminoacidic residues suggesting similarities in the recognition sites on the tachykinin NK_2 receptor for these two peptides (Giolitti and Maggi, 1994). On the other hand, Gether et al. (1993) showed that while SR 48968 binds to an epitope located in the *C*-terminal part of the receptor protein, neurokinin A binds to sites situated in the *N*-terminal region of the same receptor. Even if SR 48968 and MEN 10627 had at least partially different recognition sites in the tachykinin NK_2 receptor, our results indicate a similar ability to displace the binding of the tritiated peptide agonist. Recent functional findings with receptor-protection experiments indicate that coincubation of MEN 10627 and SR 48968 in guinea pig gallbladder and colon prevents the SR 48968 induced depression of maximal response suggesting that the two compounds share at least same mutually exclusive binding sites (Patacchini et al., 1994). Certainly, it deserves further investigations the tissue and species dependency of the binding profile and the antagonism of [^{3}H]SR 48968 and [^{3}H][β-Ala^8]neurokinin A-(4–10).

In conclusion, we have described the binding characteristic of [^{3}H][β-Ala^8]neurokinin A-(4–10) that could represent a new useful tool for the assessment of tachykinin NK_2 receptor antagonists and for investigations on the molecular mechanism of agonist and antagonist interaction with this receptor protein.

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