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Septide and neurokinin A are high-affinity ligands on the NK-1 receptor: evidence from homologous versus heterologous binding analysis

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Abstract The three main tachykinins, substance P, neurokinin A (NKA), and neurokinin B, are believed to be selective ligands for respectively the NK-1, NK-2 and NK-3 receptors. However, NKA also has actions which cannot be mediated through its normal NK-2 receptor and the synthetic peptide [pGlu^6,Pro^9]-Substance P$^{9-11}$ — called septide — is known to have tachykinin-like actions despite its apparent lack of binding to any known tachykinin receptor. In the cloned NK-1 receptor expressed in COS-7 cells NKA and septide as expected were poor competitors for radiolabeled substance P. However, by using radiolabeled NKA and septide directly, it was found that both peptides in homologous binding assays as well as in competition against each other in fact bound to the NK-1 receptor with high affinity: K_d values of 0.51 ± 0.15 nM (NKA) and 0.55 ± 0.03 nM (septide). It is concluded that NKA and septide are high-affinity ligands for the NK-1 receptor but that they are poor competitors for substance P, which in contrast competes very well for binding with both NKA and septide.

Key words: Substance P; Neurokinin A; Septide; 7TM receptor; Neuropeptide; Ligand binding

1. Introduction

Tachykinin peptides are important neuropeptides in both the central and peripheral nervous system involved in, for example, nociception and control of smooth muscle activity [1]. In mammalian species three homologous tachykinin peptides are believed to be the natural endogenous ligands for three homologous receptors: substance P (SP) on the NK-1 receptor, neurokinin A (NKA) on the NK-2 receptor, and neurokinin B (NKB) on the NK-3 receptor. These receptors which are about 60–70% identical are generally considered to be selective for each of the peptides. For example, the affinity for SP on the NK-1 receptor is approximately 100-fold higher than that of NKA and NKB [1]. The three tachykinin receptors were cloned in the late 1980s mainly by Nakanishi and coworkers and since then no new receptors have been identified in this family [2–4]. However, it has been a well-recognized pharmacological enigma that the three known tachykinin receptors could not account for all the actions which were found for tachykinin peptides. Already 10 years ago it was recognized that certain SP analogs, as for example [pGlu^6,Pro^9]Substance P$^{9-11}$ (septide) acted as potent SP-like compounds; importantly, without being able to bind with high affinity to SP receptors [5–10]. On this basis, the possible existence of a distinct 'septide receptor' is still being discussed [11]. Also, several biological effects of NKA could not be explained by an action through the NK-2 receptor and, since NKA apparently could not bind to any other known tachykinin receptor, it was suggested that another-NKA preferring receptor might exist [8,9].

The expected pharmacological profile of the tachykinin receptors including the lack of binding of septide was confirmed when the cloned receptors were expressed in cell lines [2–4,12–15]. Nevertheless, it was recently found that septide and also NKA in fact were able to activate the cloned NK-1 receptor at low concentrations — despite the fact that they were unable to compete for binding with radiolabeled SP or radiolabeled non-peptide antagonists in the same system [14,15]. Interestingly a non-peptide antagonist, RP67,580 acted as a classical competitive antagonist when the NK-1 receptor was stimulated with SP but as an insurmountable antagonist against septide [14–16]. Moreover, whereas SP stimulated phosphatidylinositol turnover and adenylate cyclase with about the same potency, septide and NKA were relatively selective in stimulating only the phopholipase C pathway [15]. All these studies indicate that both septide and NKA in fact can act as agonists on the NK-1 receptor — although they are an 'atypical' agonist. Nevertheless, the problem still remains that these peptides do not show any corresponding binding to this receptor.

In mutant receptors we have previously observed that the affinity of an agonist can be up to 1000-fold lower when determined in heterologous binding assay than when determined in homologous binding assays [17,18]. Thus, we decided to investigate whether a similar phenomenon could account for the apparent lack of affinity of septide and NKA for the NK-1 receptor by using radiolabeled septide and NKA directly instead of studying only the indirect effect of these peptides on the binding of SP or on the binding of a non-peptide antagonist in heterologous assays.

2. Materials and methods

2.1. Materials

SP, NKA, and septide (Fig. 1) were purchased from Peninsula. ^{125}I-Bolton-Hunter (^{125}I-BH) labeled SP was prepared and purified as previously reported [19]; ^{125}I-NKA (labeled at His^1) was from Amersham; and [4,5-^{3}H-Leu^5]Septide (^{3}H-septide) was purchased from Cambridge Research Biochemicals (Cheshire, UK). All tissue culture materials were from Life Technologies and all other chemicals from Sigma.

2.2. Binding assays

COS-7 cells were transfected with the cDNA coding for the human NK-1 receptor in the eukaryotic expression vector pTEJ8 using the calcium phosphate precipitation method as described [19]. Cells were transferred to 12-well plates on the second day and competition binding assays were performed on whole cells for 3 h at 4°C on the third

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day after transfection [19]. Initial experiments showed that steady-state binding was reached with all three radioligands under these conditions.

2.3. Calculations

Competition binding data were analyzed and IC_{50} values were determined by non-linear regression analysis and dissociation and inhibition constants (K_d and K_i) and B_{max} values were calculated using the Prism 4.0 software (GrafPad Software, San Diego, CA).

3. Results

3.1. Competition binding with ^{125}I-BH–substance P

The competition binding curve for SP on the NK-1 receptor transiently expressed in COS-7 cells using ^{125}I-BH-SP was best fitted to a two-component curve identifying a very-high-affinity state (IC_{50} = 0.068 nM; 32%) as well as a normal high-affinity state (IC_{50} = 0.51 nM; 68%). As expected, NKA and septide both showed low affinity in the assay using ^{125}I-BH-SP (Fig. 2). However, for these peptides the Hill coefficients were also < 1.0 and for NKA a high-affinity site of 2.8 nM (48%) and a low-affinity site of 83 nM (52%) could be identified. For septide two affinity states could be calculated with affinities of respectively 17 nM (41%) and > 1000 nM (59%).

3.2. Competition binding with ^{125}I-NKA

The radiolabeled NKA bound surprisingly well to the NK-1 receptor and in homologous competition binding NKA displayed a high affinity (K_d = 0.51 ± 0.15 nM) and a Hill coefficient of −0.99. Even more surprisingly, in competition with radiolabeled NKA septide also appeared as a high-affinity ligand on the NK-1 receptor having a K_i value of 0.90 ± 0.35 nM (Fig. 3, upper panel). For SP only the very high-affinity binding state with a K_i value of 0.05 ± 0.01 nM and a Hill coefficient of −1.30 could be detected in competition against ^{125}I-NKA (Fig. 3, upper panel).

3.3. Competition binding with ^{3}H-septide

In the homologous competition binding assay septide showed the same high affinity as against radiolabeled NKA (K_d = 0.55 ± 0.03 nM; Hill coefficient = −0.94 (Fig. 3, lower panel). Just like septide was able to compete with high affinity for NKA binding so was NKA able to compete with high affinity for septide binding on the NK-1 receptor (K_i = 0.21 ± 0.01 nM; Hill coefficient = −1.06). For SP only the very-high-affinity state could be detected in competition against ^{3}H-septide (K_i = 0.06 ± 0.01 nM) and with a Hill coefficient of −1.50 (Fig. 3, lower panel).

The B_{max} value for ^{125}I-BH–SP binding to the NK-1 receptor expressed in these COS-7 cells was 80.3 ± 15.5 fmol/10^5 cells whereas for ^{125}I-NKA and ^{3}H-septide B_{max} values were calculated to be 23.8 ± 3.5 and 11.5 ± 1.1 fmol/10^5 cells, respectively.

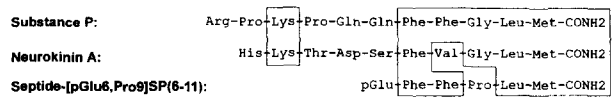


Fig. 1. Amino acid sequence of the employed tachykinin peptides, SP, NKA and septide, [pGlu^6,Pro^9]Substance$^{9-11}$. Residues which are conserved from SP to NKA and septide are boxed.

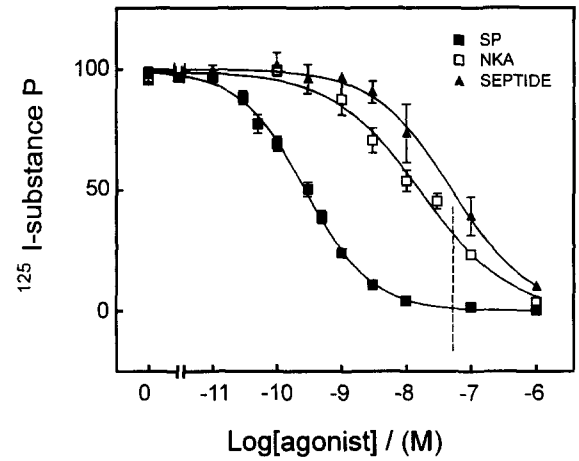


Fig. 2. Competition binding experiments using ^{125}I-BH–SP in the NK-1 receptor transiently expressed in COS-7 cells. Binding experiments were performed on whole cells as described in the text with unlabeled SP (■–■, n = 18), NKA (□–□, n = 7), and septide (▲–▲, n = 3).

4. Discussion

4.1. The NK-1 receptor as a general tachykinin peptide receptor

In the present study it was found that the tachykinin peptides NKA and septide, i.e. [pGlu^6,Pro^9]Substance P$^{9-11}$, both bind with surprisingly high affinity to the cloned NK-1 receptor having K_d values of 0.5 nM. SP is still the preferred ligand for the NK-1 receptor displaying two high-affinity binding states of which one is a very-high-affinity state with a K_d value of 50 pM. The other binding state for SP has a 10-fold lower affinity but is still a high-affinity state with a K_d value of 0.5 nM corresponding to the affinity determined for NKA and septide. SP is able to compete with very high affinity for binding with either radiolabeled NKA or septide but in both cases with a Hill coefficient which is greater than 1.0. This could indicate either the occurrence of positive cooperativity in the binding process or at least that the binding mode is complex. Interestingly, NKA and septide can only compete with low affinity with the radiolabeled SP tracer — which presumably will bind preferentially to its very-high-affinity state. This phenomenon has been the basis for the fact that the high-affinity binding of NKA and septide to the NK-1 receptor has been overlooked for several years. The observed low affinity of these peptides as determined in competition with the 'normal' ligand, SP, has probably discouraged people from applying radiolabeled NKA and septide to the NK-1 receptor. However, the observation of high-affinity binding of NKA and septide to the NK-1 receptor is obviously in good agreement with the previous reports showing that even low concentrations of NKA and septide can activate phosphatidylinositol turnover through the NK-1 receptor expressed in cell lines [14,15] (and data not shown). Thus, it appears that the NK-1 receptor can function as a general tachykinin receptor, which binds subnanomolar concentrations of both of the two naturally occurring, cosynthesized neuropeptides, SP and NKA, as well as the synthetic tachykinin peptide, septide. In light of these observations, there may not be a need for a special 'septide receptor' or an extra, elusive NKA binding receptor [11].

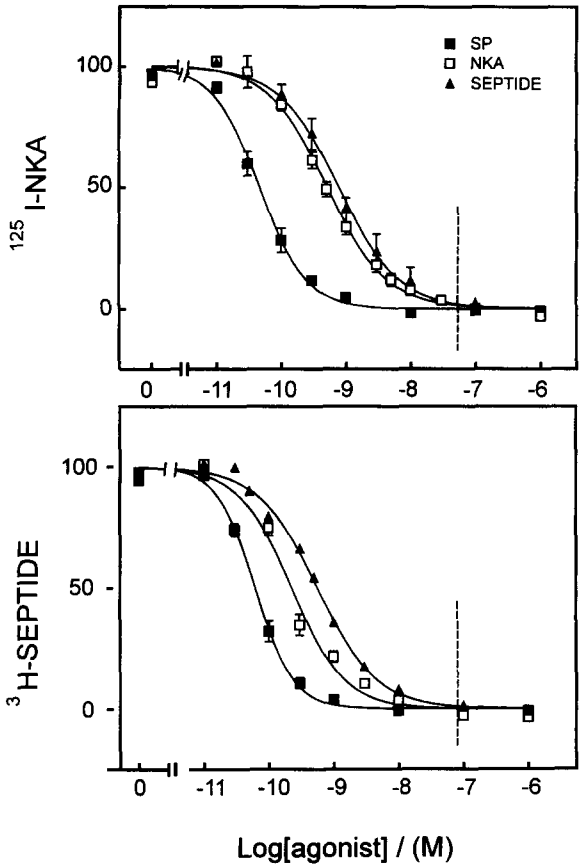


Fig. 3. Competition binding experiments in the NK-1 receptor transiently expressed in COS-7 cells. Upper panel: ^{125}I-NKA was used as radioligand in competition with unlabeled SP (■–■, $n=4$), NKA (□–□, $n=16$), and septide (▲–▲, $n=3$). Lower panel: ^{3}H-septide was used as radioligand in competition with unlabeled SP (■–■, $n=3$), NKA (□–□, $n=3$), and septide (▲–▲, $n=5$). The vertical line indicates the IC_{50} for NKA and septide as determined in competition with radiolabeled SP (see Fig. 2).

4.2. *Affinity measured by homologous versus heterologous binding*

The observation that the affinity of an agonist can be significantly (> 1000-fold) higher when determined in homologous binding assay than in competition with a radiolabeled antagonist was originally performed in a series of NK-1 receptors with point substitutions down along the inner, hydrophilic face of TM-2 [17]. In these mutant receptors it had initially been shown that SP had a very low (10^{-6} M) affinity as determined in heterologous binding [20]. However, both homologous binding [17] and signal transduction assays [20] indicated that SP in fact bound with nanomolar affinity to these receptors. The interpretation was that the mutations had impaired the ability of the receptor to interconvert between conformations which each bound either the agonist, SP, or the radiolabeled antagonist with high affinity and that they had not affected the actual high-affinity binding site for SP [17]. Similar results have recently been obtained in the κ-opioid receptor in a series of constructs with the corresponding point-substitutions on the inner face of TM-II as well as in a series of chimeric μκ-constructs [18]. In the opioid system the affinity of peptide as well as non-peptide agonists was underestimated by several orders of magnitude as determined in heterologous competition with radiolabeled non-peptide antagonist [18].

Thus, the actual binding affinity is only faithfully measured in homologous binding assays. The apparent affinity determined in heterologous binding assays may be influenced by some kinetic and/or energetic factor related to conformational interchange. In this context it may also be questioned whether certain mutations which apparently *increase* the affinity for ligands such as NKA and other tachykinin peptides as determined in heterologous binding experiments [20,21] in fact only reveal the fact that these peptides always have had a high affinity for the wild-type receptor as shown in the present study. Thus, in fact, such mutations may have made it easier for the agonist peptides to compete with the employed radioligand in the heterologous binding assays. This point could be clarified by performing homologous binding assays in these mutant receptors.

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