FEBS 24329 FEBS Letters 486 (2000) 43–48

The common C-terminal sequences of substance P and neurokinin A contact the same region of the NK-1 receptor

Andrew A. Bremer, Susan E. Leeman, Norman D. Boyd*

Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, L-611, 80 E. Concord Street, Boston, MA 02118, USA

Received 18 August 2000; revised 10 October 2000; accepted 20 October 2000

First published online 16 November 2000

Edited by Richard Cogdell

Abstract Although neurokinin A (NKA), a tachykinin peptide with sequence homology to substance P (SP), is a weak competitor of radiolabeled SP binding to the NK-1 receptor (NK-1R), more recent direct binding studies using radiolabeled NKA have demonstrated an unexpected high-affinity interaction with this receptor. To document the site of interaction between NKA and the NK-1R, we have used a photoreactive analogue of NKA containing *p*-benzoyl-L-phenylalanine (Bpa) substituted in position 7 of the peptide. Peptide mapping studies of the receptor photolabeled by ^{125}I-iodohistidyl^{1}-Bpa^{7}NKA have established that the site of photoinsertion is located within a segment of the receptor extending from residues 178 to 190 (VVCMIEW-PEHPNR). We have previously shown that ^{125}I-BH-Bpa^{8}SP, a photoreactive analogue of SP, covalently attaches to M^{181} within this same receptor sequence. Importantly, both of these peptides (^{125}I-iodohistidyl^{1}-Bpa^{7}NKA and ^{125}I-BH-Bpa^{8}SP) have the photoreactive amino acid in an equivalent position within the conserved tachykinin carboxyl-terminal tail. In this report, we also show that site-directed mutagenesis of M^{181} to A^{181} in the NK-1R results in a complete loss of photolabeling of both peptides to this receptor site, indicating that the equivalent position of SP and NKA, when bound to the NK-1R, contact the same residue.

Key words: Substance P; Neurokinin A; NK-1 receptor; Photoaffinity labeling

1. Introduction

The tachykinins comprise a family of bioactive peptides which are structurally characterized by the conserved C-terminal sequence of -Phe-Xaa-Gly-Leu-Met-NH_2 [1–5][1]. Two mammalian tachykinins are substance P (SP) (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) and neurokinin A (NKA) (His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2) [4–6]. The mRNAs encoding both SP and NKA arise from tissue-specific differential RNA processing of the preprotachykinin-A gene [7–9]; post-translational modification by endoproteases and C-terminus amidation of protein precursors then yield the mature bioactive peptides.

The NK-1 receptor (NK-1R), the well-documented site of action for SP, is an integral membrane protein belonging to the rhodopsin-type family of G-protein coupled receptors [10–12]. Competition binding assays using radiolabeled SP and the NK-1R have shown that (i) SP binds with high affinity to the NK-1R and that (ii) NKA is a poor competitor for radiolabeled SP binding [13]. Since NKA displaces SP binding to the NK-1R only at high concentrations, the notion that NKA might functionally interact with the NK-1R was not immediately entertained. Recently, however, functional assays and direct binding studies using radiolabeled NKA have demonstrated that NKA can interact with the NK-1R with high affinity to elicit biological responses [14,15], despite its relative inability to compete for receptor binding with SP. The most straightforward explanation for this phenomenon is that the two peptides interact on the receptor at distinct sites. An alternative explanation, however, is the existence of multiple ligand–specific receptor conformations. We have addressed these issues directly by comparing the site of photoincorporation of a photoreactive analogue of NKA, ^{125}I-iodohistidyl^{1}-(*p*-benzoyl-L-phenylalanine)^{7}NKA (^{125}I-Bpa^{7}NKA), with that for the corresponding photoreactive analogue of SP, ^{125}I-(*N*-succinimidyl-3[4-hydroxyphenyl]propionate)^{3}-Bpa^{8}SP (^{125}I-BH-Bpa^{8}SP).

This study presents the first direct biochemical evidence for an NKA/NK-1R interaction, and moreover shows that the structurally conserved C-terminal sequences of both SP and NKA interact with a common region of the NK-1R.

2. Materials and methods

2.1. Materials

Purified SP and NKA were purchased from Sigma. Bpa^{8}SP was synthesized as described previously [16], and Bpa^{7}NKA was obtained from Jeff Kelly (Scripps Institute, San Diego, CA, USA). ^{125}I-Bolton Hunter (BH) reagent and ^{125}I (each with specific activities of 2200 Ci/mmol) were obtained from NEN Life Sciences. SP and Bpa^{8}SP were radioiodinated by coupling the Lys^{3} reside ε-NH_2 group to the ^{125}I-BH reagent as described previously [16], generating ^{125}I-BH-SP and ^{125}I-BH-Bpa^{8}SP, respectively. NKA and Bpa^{7}NKA were radioiodinated by coupling the His^{1} residue to the solid phase oxidant 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (IODO-GEN® iodination

*Corresponding author. Fax: (1)-617-638 4329.
E-mail: nboyd@bu.edu

Abbreviations: Bpa, *p*-benzoyl-L-phenylalanine; BH, Bolton Hunter (*N*-succinimidyl-3[4-hydroxyphenyl]propionate); CHO, Chinese hamster ovary; DTT, D,L-dithiothreitol; E2, NK-1R second extracellular loop; ^{125}I-Bpa^{7}NKA, ^{125}I-iodohistidyl^{1}-Bpa^{7}NKA; ^{125}I-BH-Bpa^{8}SP, ^{125}I-Bolton-Hunter^{3}-Bpa^{8}SP; MALDI, matrix-assisted laser desorption ionization; NKA, neurokinin A; NK-1R, NK-1 receptor; rNK-1R, rat NK-1 receptor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SP, substance P

[1] Three letter amino acid abbreviations denote amino acids of the corresponding ligand.

PII: S0014-5793(00)02228-6

Table 1
Average IC_{50} values for rNK-1R ligands

Competing ligand	^{125}I-BH-SP binding IC_{50} (nM ± S.E.M.)	^{125}I-NKA binding IC_{50} (nM ± S.E.M.)
SP	0.9 ± 0.3	0.09 ± 0.02
Bpa^{8}SP	1 ± 0.3	0.1 ± 0.03
NKA	46 ± 4	5 ± 2
Bpa^{7}NKA	48 ± 4	6 ± 2

reagent, Pierce) in the presence of ^{125}I, generating ^{125}I-iodohistidyl^1-NKA (^{125}I-NKA) and ^{125}I-Bpa^{7}NKA, respectively.

2.2. Cell culture and site-directed mutagenesis

Chinese hamster ovary (CHO) cells stably transfected with the cDNA encoding a geneticin resistance gene and (i) the full-length rat NK-1R (rNK-1R) [17], (ii) a site-directed rNK-1R mutant in which M^{174} was substituted by alanine[2] or (iii) a site-directed rNK-1R mutant in which M^{181} was substituted by alanine were kindly provided by Dr. J.E. Krause (Neurogen, Brandford, CT, USA). All transfected CHO cells were maintained as monolayer cultures in α-minimal essential medium (Gibco BRL Life Technologies) supplemented with 10% (v/v) Cool Calf™ 2 (Sigma) and 1 mg/ml geneticin (G-418 sulfate) (Gibco BRL Life Technologies), as described previously [18,19]. Cells were grown in an atmosphere of 95% air and 5% CO_2 at 37°C, and were harvested for experiments using phosphate-buffered saline based enzyme free dissociation buffer (Specialty Media).

2.3. Equilibrium displacement competition assay

Transfected CHO cells were harvested as described above and resuspended in ice-cold KRH buffer (20 mM HEPES, 1 mM $CaCl_2$, 2.2 mM $MgCl_2$, 5 mM KCl, 120 mM NaCl, pH 7.4) supplemented with 6 mg/ml glucose and 0.6 mg/ml bovine serum albumin (BSA). Cells were incubated for 2 h at 4°C with the radiolabeled ligands ^{125}I-BH-SP or ^{125}I-NKA, and binding was measured either alone or in the presence of increasing concentrations of unlabeled inhibitor. In all experiments, non-specific ^{125}I-BH-SP or ^{125}I-NKA binding was defined as binding in the presence of 1 μM unlabeled peptide. To separate bound ligand from free ligand, cells were filtered after incubation through Whatman GF/C filter paper (soaked > 2 h in 0.1% polyethylenimine) and washed three times in ice-cold KRH buffer (pH 7.4) with a Brandel Harvester apparatus. Bound radioactivity on the filters was quantified with γ-emission spectrometry. Competition assays were performed in triplicate and repeated at least three times.

2.4. Photoaffinity labeling of transfected CHO cells and identification of photolabeled receptors

Stably transfected CHO cells were photolabeled with ^{125}I-BH-Bpa^{8}SP or ^{125}I-Bpa^{7}NKA using the procedure described previously [18,19]. Transfected cells were harvested, pelleted and resuspended in ice-cold KRH buffer (pH 7.4) supplemented with 6 mg/ml glucose, 0.6 mg/ml BSA, 3 μg/ml chymostatin, 5 μg/ml leupeptin and 30 μg/ml bacitracin. The cell resuspensions were then incubated in the dark with ^{125}I-BH-Bpa^{8}SP (added to a final concentration of 0.5–1.0 nM) or ^{125}I-Bpa^{7}NKA (added to a final concentration of 3.0–3.5 nM) for 2 h at 4°C with gentle agitation. Competing peptides or non-peptide antagonists were added at the concentrations indicated. Following incubation, the mixtures were diluted 1:1 with ice-cold KRH buffer (pH 7.4) and irradiated at 365 nm by exposure to a 100 W long-wave UV lamp for 15 min at a distance of 6 cm. Cell membranes were then prepared as described previously [18,19]. ^{125}I-BH-Bpa^{8}SP- or ^{125}I-Bpa^{7}NKA-labeled membranes were next solubilized in sample buffer (0.125 M Tris, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.01% bromophenol blue, pH 6.8), heated at 55°C for 10 min, and then subjected to SDS–polyacrylamide gel electrophoresis (PAGE) as described by Laemmli [20].

The percent photoincorporation of ^{125}I-BH-Bpa^{8}SP or ^{125}I-Bpa^{7}NKA into the wildtype (WT), M174A and M181A rNK-1Rs was determined as described previously [16] by comparing the amount of photoligand incorporated into the receptors after UV exposure (as determined by SDS–PAGE resolution, autoradiography and γ-emission spectrometric analysis) to the amount of photoligand specifically bound to the receptors before UV exposure (as determined by equilibrium filtration binding assay).

2.5. Tryptic digestion of photolabeled receptors

^{125}I-BH-Bpa^{8}SP- or ^{125}I-Bpa^{7}NKA-labeled membranes were resuspended in 0.1% SDS, 50 mM Tris, 1 mM $CaCl_2$, pH 8.0, and digested at room temperature for 2 h with 0.02 mg/ml L-1-tosylamide-2-phenylethylchloromethyl ketone-treated bovine trypsin (Sigma). *N*α-*p*-tosyl-L-lysine chloromethyl ketone was added to the reaction mixtures in a 1:1000 dilution following incubation to terminate enzymatic activity. Sample buffer (± 30 mM D,L-dithiothreitol, DTT) was then added to the samples. The mixtures (± 30 mM DTT) were allowed to incubate for 1 h at room temperature before being heated for 10 min at 55°C, as described above. Tryptic cleavage fragments were separated and analyzed using the tricine-gel system of SDS–PAGE [21].

3. Results

3.1. Affinity of photoreactive tachykinin analogues for the NK-1R as measured by homologous and heterologous competition binding assays

To gain information on the bimolecular complexes formed between SP and the NK-1R as well as NKA and the NK-1R, a photoreactive analogue of each peptide was synthesized (Bpa^{8}SP and Bpa^{7}NKA, respectively) (Fig. 1). Importantly, the introduction of the photoreactive Bpa residue into either the eighth position of SP (normally a Phe) or the seventh position of NKA (normally a Val) does not significantly alter the receptor binding characteristics of these tachykinin analogues, making them useful for direct receptor photolabeling experiments. Both Bpa^{8}SP and Bpa^{7}NKA also elicit a calcium response from rNK-1R-expressing CHO cells (data not shown), consistent with their being functional agonists at this receptor. Therefore, since Bpa^{8}SP and Bpa^{7}NKA bind the NK-1R in a manner indistinguishable from that of SP and NKA, respectively, the introduction of the Bpa residue into the photoreactive tachykinin analogues does not significantly alter the functional properties of the ligands.

3.2. ^{125}I-Bpa^{7}NKA specifically photolabels the NK-1R

Our laboratory has successfully used Bpa^{8}SP as an efficient photoreactive ligand for the rNK-1R [18,22]; moreover, a radiolabeled derivative of Bpa^{8}SP, ^{125}I-BH-Bpa^{8}SP, has been used to identify M^{181} as a site of covalent attachment of SP to the rNK-1R [19]. In this report, we show that the

Bpa^{8}SP: Arg - Pro - Lys - Pro - Gln - Gln - *Phe* - ***Bpa***^{8} - *Gly* - *Leu* - *Met* - NH_2

Bpa^{7}NKA: His - Lys - Thr - Asp - Ser - *Phe* - ***Bpa***^{7} - *Gly* - *Leu* - *Met* - NH_2

Fig. 1. Amino acid sequences of Bpa^{8}SP and Bpa^{7}NKA. Note that the Bpa residue of both Bpa^{8}SP and Bpa^{7}NKA is located in an equivalent position within the conserved tachykinin C-terminal tail.

[2] Single letter amino acid abbreviations denote amino acids of the NK-1 receptor.

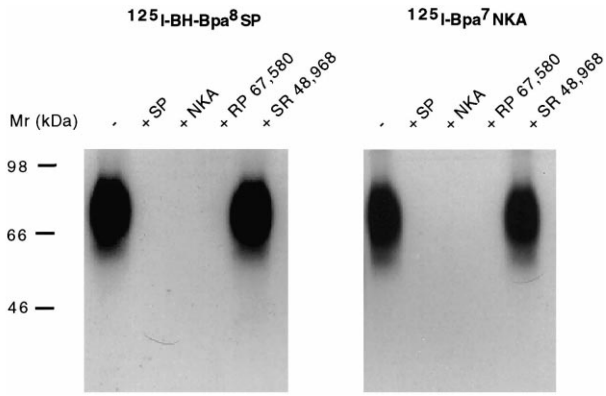

Fig. 2. Specific photolabeling of the rNK-1R with ^{125}I-BH-Bpa^{8}SP and ^{125}I-Bpa^{7}NKA. Both ^{125}I-BH-Bpa^{8}SP and ^{125}I-Bpa^{7}NKA specifically photolabel the same protein which migrates diffusely at $M_r \sim 80$ kDa on SDS–PAGE due to the heterogeneity of receptor glycosylation [37].

tachykinin derivative Bpa^{7}NKA is also a high-affinity ligand for the rNK-1R (Table 1), and that its radiolabeled derivative, ^{125}I-Bpa^{7}NKA, specifically photolabels the rNK-1R as well (Fig. 2). Pharmacological evidence of the specificity of the ^{125}I-BH-Bpa^{8}SP–rNK-1 and ^{125}I-Bpa^{7}NKA–rNK-1R interaction is provided by results showing that the addition of 1 μM SP, NKA or RP 67 580 (a rNK-1R non-peptide antagonist [23]) prevents rNK-1R labeling by both ^{125}I-BH-Bpa^{8}SP and ^{125}I-Bpa^{7}NKA, whereas the addition of 1 μM of SR 48 968 (a rNK-2R non-peptide antagonist [24]) has no effect on receptor photolabeling.

3.3. *^{125}I-Bpa^{7}NKA photolabels the same residue on the NK-1R as ^{125}I-BH-Bpa^{8}SP*

Since the seventh amino acid of NKA is in the equivalent position as the eighth amino acid of SP (Fig. 1), the use of Bpa^{7}NKA as a photoligand is strategic in that it allows direct comparison of ^{125}I-Bpa^{7}NKA–rNK-1R peptide mapping data with the published results of extensive peptide mapping studies performed on the ^{125}I-BH-Bpa^{8}SP-labeled rNK-1R [18,19,22]. In the absence of DTT, trypsinization of the ^{125}I-BH-Bpa^{8}SP-labeled rNK-1R results in the generation of two receptor fragments linked by a disulfide bond between C^{105} and C^{180}, with a combined $M_r \sim 8$ kDa. Reduction with DTT yields a smaller fragment ($M_r \sim 3$ kDa) that contains residues 178–190 of the NK-1R second extracellular (E2) loop. Large-scale isolation of this particular ~ 3 kDa rNK-1R fragment and characterization by matrix-assisted laser desorption ionization (MALDI) mass spectrometry has identified the residue within this sequence that serves as the site of covalent attachment of ^{125}I-BH-Bpa^{8}SP to the rNK-1R as M^{181} [19].

Peptide mapping studies performed on the ^{125}I-Bpa^{7}NKA-labeled rNK-1R show a remarkable similarity to those discussed above for the ^{125}I-BH-Bpa^{8}SP-labeled rNK-1R, i.e. the generation of a fragment at $M_r \sim 8$ kDa following trypsinization, which is reduced in size to $M_r \sim 3$ kDa following reduction with DTT (Fig. 3). These data suggest that the site of covalent attachment of ^{125}I-Bpa^{7}NKA to the rNK-1R is also to a residue in the $M_r \sim 3$ kDa fragment of the E2 loop extending from residues 178–190.

To test whether ^{125}I-Bpa^{7}NKA specifically photolabels rNK-1R residue M^{181}, photolabeling experiments using mutant rNK-1Rs were performed (Fig. 4). Two specific mutant rNK-1Rs, in which an alanine residue is substituted for either M^{174} (M174A) or M^{181} (M181A), have been characterized (Table 2) and are both functional. These two mutant receptors were constructed on the basis of M^{181} serving as the site of photoinsertion of ^{125}I-BH-Bpa^{8}SP to the rNK-1R [19], and M^{174} serving as the site of photoinsertion of another photoreactive SP analogue designed in our laboratory, ^{125}I-BH-Bpa^{4}SP, to the rNK-1R [25]. In this report, we show that when alanine is substituted for M^{181}, although ^{125}I-BH-Bpa^{8}SP binding is not significantly affected (IC_{50} values of 0.77 ± 0.12 nM for WT versus 0.82 ± 0.16 nM for M181A), the efficiency of photolabeling is significantly reduced (relative

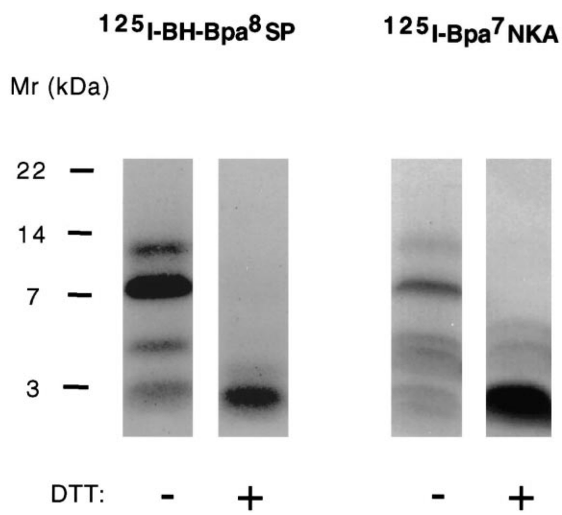

Fig. 3. Tryptic digestion pattern of ^{125}I-BH-Bpa^{8}SP- and ^{125}I-Bpa^{7}NKA-photolabeled rNK-1Rs. Tryptic digestion (0.02 mg/ml) of ^{125}I-BH-Bpa^{8}SP-labeled rNK-1R generates a major fragment at $M_r \sim 8$ kDa in the absence of 30 mM DTT (−), which is reduced in size to $M_r \sim 3$ kDa in the presence of 30 mM DTT (+). An identical tryptic digest pattern ±DTT is produced with ^{125}I-Bpa^{7}NKA-labeled rNK-1R.

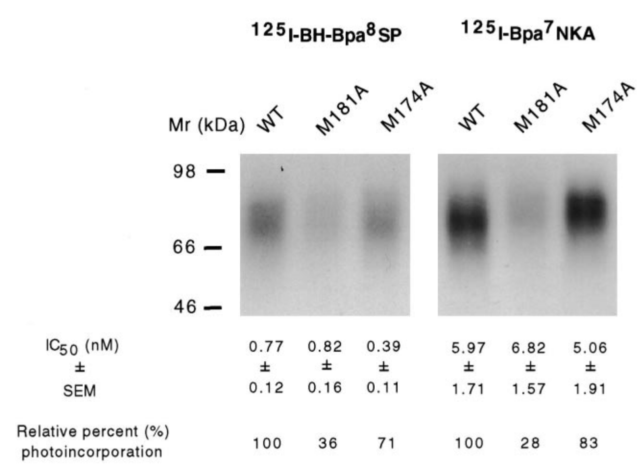

Fig. 4. Photoincorporation of ^{125}I-BH-Bpa^{8}SP and ^{125}I-Bpa^{7}NKA into WT and mutant rNK-1Rs. The photoincorporation of ^{125}I-BH-Bpa^{8}SP into the WT rNK-1R and M174A mutant rNK-1R is comparable; however, its photoincorporation into the M181A mutant rNK-1R is markedly reduced. A similar pattern of receptor photoincorporation efficiencies is reported when ^{125}I-Bpa^{7}NKA is the photoligand. Importantly, the M181A and M174A mutant rNK-1Rs display only slight differences in their binding affinity for the photoligands as compared to WT rNK-1R; IC_{50} values are shown.

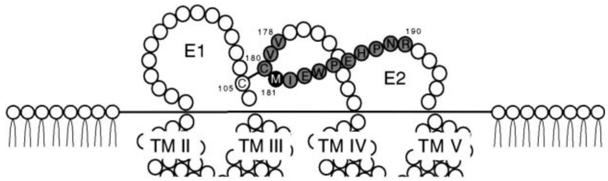

Fig. 5. Schematic diagram of the first (E1) and second (E2) extracellular loops of the rNK-1R. The residues shown in gray correspond to the $M_r \sim 3$ kDa trypsin-generated rNK-1R fragment (residues 178–190). A disulfide bond exists between C^{105} and C^{180}, linking E1 and E2 near the membrane surface. The E2 loop residue Met^{181}, the direct site of ^{125}I-BH-Bpa^{8}SP and ^{125}I-Bpa^{7}NKA photoinsertion to the rNK-1R, is shown in black. TM = transmembrane domain.

percent photoincorporation of 100% for WT versus 36% for M181A). However, when alanine is substituted for M^{174}, which is not the precise site of photoincorporation of ^{125}I-BH-Bpa^{8}SP, there is only a modest effect on binding affinity (IC_{50} values of 0.77 ± 0.12 nM for WT versus 0.39 ± 0.11 nM for M174A) and only a small decrease in photolabeling efficiency (relative percent photoincorporation of 100% for WT versus 71% for M174A).

These two mutant receptors were also photolabeled with ^{125}I-Bpa^{7}NKA (Fig. 4), and the binding and photolabeling results compared with those obtained with ^{125}I-BH-Bpa^{8}SP. In parallel with the results seen with ^{125}I-BH-Bpa^{8}SP, (i) a marked decrease in the efficiency of photoincorporation of ^{125}I-Bpa^{7}NKA into the M181A mutant was observed (relative percent photoincorporation of 100% for WT versus 28% for M181A) and (ii) a comparable efficiency of photoincorporation of ^{125}I-Bpa^{7}NKA into the M174A mutant was observed (relative percent photoincorporation of 100% for WT versus 83% for M174A). Again, paralleling the results seen with ^{125}I-BH-Bpa^{8}SP, the substitution of alanine for either M^{181} or M^{174} had no marked effect on ^{125}I-Bpa^{7}NKA binding affinity (Fig. 4). Taken together, these results provide strong evidence that the site of ^{125}I-Bpa^{7}NKA covalent attachment to the rNK-1R is the same as that of ^{125}I-BH-Bpa^{8}SP (i.e. E2 loop residue M^{181}) (Fig. 5). Interestingly, the mutant receptors in which alanine was substituted for M^{174} and M^{181} display only slight differences in their binding affinity for Bpa^{8}SP and Bpa^{7}NKA, indicating that neither M^{174} nor M^{181} per se contribute significantly to the total binding energy despite their location at the ligand–receptor interface. These findings suggest that several ligand–receptor contacts exist in the high-affinity binding complex, and are consistent with the view that multiple peptide–receptor interactions participate in peptide recognition and receptor activation.

4. Discussion

In this study, we (i) confirm published reports establishing that NKA binds to the NK-1R with high affinity [14,15] and (ii) present the first data directly identifying a site of interaction between NKA and the NK-1R at the bimolecular interface. First, we demonstrate that ^{125}I-Bpa^{7}NKA is an efficient and specific photoligand for the rNK-1R. Second, we restrict the site of photoinsertion of ^{125}I-Bpa^{7}NKA to an amino acid of the rNK-1R between residues 178 and 190 on the E2 loop. As previously reported, this same restricted segment of the rNK-1R contains M^{181}, the site of ^{125}I-BH-Bpa^{8}SP photoinsertion as documented by MALDI mass spectrometry [19]. (iii) We show that a comparable loss of photoinsertion efficiency results when either ^{125}I-BH-Bpa^{8}SP or ^{125}I-Bpa^{7}NKA is used to photolabel a mutant rNK-1R in which the side-chain of M^{181} has been removed. Taken together, these results indicate that in the high-affinity bound state, the equivalent positions within the common C-terminal sequences of SP and NKA are in close proximity to M^{181} on the E2 loop of the rNK-1R.

In our laboratory, we have demonstrated that when the side-chain of an identified contact residue of a photoligand on a receptor is removed, a marked loss of photoincorporation results in the absence of a significant affect on peptide binding [26]. Pertinent to this investigation, we show that when M^{181} of the rNK-1R is mutated to an alanine, the photolabeling efficiency of ^{125}I-BH-Bpa^{8}SP is dramatically reduced. Since we had restricted the site of ^{125}I-Bpa^{7}NKA photoinsertion to a region of the rNK-1R containing the site of ^{125}I-BH-Bpa^{8}SP covalent attachment (i.e. M^{181}), we found it plausible to speculate that both photoligands might have the same contact residue, particularly since the photoreactive amino acid of each ligand is in an equivalent position within the conserved tachykinin C-terminal tail. Paralleling the results obtained when ^{125}I-BH-Bpa^{8}SP is used to photolabel the M181A mutant receptor, we in fact demonstrate a significant loss of photoincorporation of ^{125}I-Bpa^{7}NKA into the M181A mutant receptor, validating its contact site to the rNK-1R as M^{181}. Thus, the strategy of using specifically mutated rNK-1Rs and photoaffinity labeling experiments to characterize the site of ^{125}I-Bpa^{7}NKA covalent attachment to the rNK-1R was both novel and successful, and eliminated the necessity of purifying the site of peptide–receptor interaction for analysis by mass spectrometry.

It is important to note that the observation of two structurally related peptides displaying differences in their binding affinity for a given receptor target depending on the assay used (i.e. homologous versus heterologous competition assays) is not limited to SP and NKA interacting with the NK-1R, but rather is emerging as a more general phenomenon for other structurally related peptide families [27]. For example, although vasoactive intestinal peptide (VIP) competes for the binding of the structurally similar peptide pituitary adenylate cyclase activating polypeptide (PACAP) to a receptor target that has been designated the PACAP receptor (PACAP-R) with low affinity, in direct binding experiments radiolabeled VIP is found to bind the PACAP-R with high affinity [28].

Table 2
Binding affinities of SP and NKA to WT, M174A and M181A rNK-1Rs

Receptor	^{125}I-BH-SP binding versus unlabeled SP IC_{50} (nM ± S.E.M.)	^{125}I-NKA binding versus unlabeled NKA IC_{50} (nM ± S.E.M.)
WT	0.9 ± 0.3	5.0 ± 2.0
M174A	1.4 ± 0.4	5.3 ± 2.1
M181A	1.5 ± 0.6	6.1 ± 2.4

The most straightforward explanation for the observation that two ligands can interact at the same receptor with high affinity but not readily compete is that they each interact with the receptor at distinct sites [27]. To address the issue as to whether SP and NKA interact with the NK-1R at the same or different sites, Wijkhuisen et al. recently followed a molecular recognition theory to identify regions of the NK-1R whose hydropathic profiles were opposed to that of the common tachykinin C-terminal sequence, and then performed binding experiments on receptors mutated in those particular regions [29]. They subsequently reported the identification of an NK-1R domain located at the distal end of the E2 loop that interacts with the C-terminus of NKA but not of SP, suggesting the existence of distinct binding sites on the receptor for the C-terminal sequences of these two peptides. In the current study, however, we have taken a direct approach to study the tachykinin/NK-1R complex by comparing the site of ^{125}I-Bpa^{7}NKA photoinsertion to the rNK-1R with that of the well-studied corresponding photoreactive analogue of SP, ^{125}I-BH-Bpa^{8}SP. By reporting that both ^{125}I-Bpa^{7}NKA and ^{125}I-BH-Bpa^{8}SP photolabel the same residue on the rNK-1R, we provide evidence against the existence of a distinct, NKA-preferring binding site, and conclude that the equivalent positions of SP and NKA within the conserved tachykinin C-terminal tail are in close proximity to M^{181} in the peptide/rNK-1R complex.

An alternative and somewhat more complex possibility to account for the apparent discrepancy in homologous versus heterologous competition assay results is that the NK-1R exists in multiple peptide-selective conformations that not only display differences in their ligand specificities [27], but also potentially in their G-protein and effector molecule interactions and evoked physiological responses [30]. We favor this multiple conformation theory. Particularly since SP and NKA arise from the same gene and are often co-expressed and co-released in regions of the central nervous system and periphery without detectable NK-2 receptors [31–33], that both peptides may stimulate peptide-specific effector responses through functional interaction with the NK-1R may have important physiological consequences.

Using the direct and efficient methods of photochemistry, combined with specific receptor residue site-directed mutagenesis, we have demonstrated that the common C-terminal sequences of SP and NKA contact the same region of the NK-1R. We furthermore predict that the divergent N-terminal regions of these two peptides will interact with different regions of the NK-1R. Extensive biochemical analysis of the high-affinity SP/rNK-1R complex has already been performed [18,19,22,34–37]. These studies directly document the importance of both the extracellular N-terminus and the E2 loop of the rNK-1R for peptide recognition, and support the viewpoint that several ligand–receptor interactions participate in binding and activation [38–40]. Just as multiple contacts exist between SP and the NK-1R in the bound state, so too will multiple interactions undoubtedly occur between NKA and the NK-1R. By directly evaluating and comparing the tachykinin/rNK-1R bimolecular interface using multiple photoreactive SP and NKA derivatives, we can use a photoaffinity-scanning approach to further investigate the existence of these non-distinct peptide-preferring receptor conformations, and determine which residue–residue contacts participate in the molecular processes of peptide recognition and receptor activation.

Acknowledgements: This work is sponsored by the Public Health Service grant from the National Institutes of Health: NS 3134.

References

[1] Erspamer, V. (1971) Annu. Rev. Pharmacol. 11, 327–350.
[2] Erspamer, V. (1981) Trends Neurosci. 4, 267–269.
[3] Pernow, B. (1983) Pharmacol. Rev. 35, 85–141.
[4] Nakanishi, S. (1987) Physiol. Rev. 67, 1117–1142.
[5] Maggio, J.E. (1988) Annu. Rev. Neurosci. 11, 13–28.
[6] Nakanishi, S. (1986) Trends Neurosci. 9, 41–44.
[7] Nawa, H., Kotani, H. and Nakanishi, S. (1984) Nature 312, 729–734.
[8] Kawaguchi, Y., Hoshimaru, M., Nawa, H. and Nakanishi, S. (1986) Biochem. Biophys. Res. Commun. 139, 1040–1046.
[9] Krause, J.E., Chirgwin, J.M., Carter, M.S., Xu, Z.S. and Hershey, A.D. (1987) Proc. Natl. Acad. Sci. USA 84, 881–885.
[10] Yokota, Y., Sasai, Y., Tanaka, K., Fujiwara, T., Tsuchida, K., Shigemoto, R., Kakizuka, A., Ohkubo, H. and Nakanishi, S. (1989) J. Biol. Chem. 264, 17649–17652.
[11] Hershey, A.D. and Krause, J.E. (1990) Science 247, 958–961.
[12] Takeda, Y., Chou, K.B., Takeda, J., Sachais, B.S. and Krause, J.E. (1991) Biochem. Biophys. Res. Commun. 179, 1232–1240.
[13] Ingi, T., Kitajima, Y., Minamitake, Y. and Nakanishi, S. (1991) J. Pharmacol. Exp. Ther. 259, 968–975.
[14] Hastrup, H. and Schwartz, T.W. (1996) FEBS Lett. 399, 264–266.
[15] Sagan, S., Beaujouan, J.-C., Torrens, Y., Saffroy, M., Chassaing, G., Glowinski, J. and Lavielle, S. (1997) Mol. Pharmacol. 52, 120–127.
[16] Boyd, N.D., White, C.F., Cerpa, R., Kaiser, E.T. and Leeman, S.E. (1991) Biochemistry 30, 336–342.
[17] Takeda, Y., Blount, P., Sachais, B.S., Hershey, A.D., Raddatz, R. and Krause, J.E. (1992) J. Neurochem. 59, 740–745.
[18] Boyd, N.D., Kage, R., Dumas, J.J., Krause, J.E. and Leeman, S.E. (1996) Proc. Natl. Acad. Sci. USA 93, 433–437.
[19] Kage, R., Leeman, S.E., Krause, J.E., Costello, C.E. and Boyd, N.D. (1996) J. Biol. Chem. 271 (42), 25797–25800.
[20] Laemmli, U.K. (1970) Nature (Lond.) 227, 680–685.
[21] Schagger, H. and Jagow, G.v. (1987) Anal. Biochem. 166, 368–379.
[22] Boyd, N.D. and Leeman, S.E. (1995) Ann. N. Y. Acad. Sci. 757, 405–409.
[23] Garret, C., Carruette, A., Fardin, V., Moussaoui, S., Peyronel, J.-F., Blanchard, J.-C. and Laduron, P.M. (1991) Proc. Natl. Acad. Sci. USA 88, 10208–10212.
[24] Emonds-Alt, X., Vilain, P., Goulaouic, P., Proietto, V., Broeck, D.V., Advenier, C., Naline, E., Neliat, G., Fur, G.L. and Breliere, J.C. (1992) Life Sci. 50, 101–106.
[25] Li, H. (1998) Ph.D. thesis, Boston University School of Medicine.
[26] Macdonald, D.M. (1998) Ph.D. thesis, Boston University School of Medicine.
[27] Maggi, C.A. and Schwartz, T.W. (1997) Trends Pharmacol. Sci. 18, 351–355.
[28] Hashimoto, H., Ogawa, N., Hagihara, N., Yamamoto, K., Imanishi, K., Nogi, H., Nishino, A., Fujita, T., Matsuda, T., Nagata, S. and Baba, A. (1997) Mol. Pharmacol. 52, 128–135.
[29] Wijkhuisen, A., Sagot, M.-A., Frobert, Y., Creminon, C., Grassi, J., Boquet, D. and Couraud, J.-Y. (1999) FEBS Lett. 447, 155–159.
[30] Sagan, S., Chassaing, G., Pradier, L. and Lavielle, S. (1996) J. Pharmacol. Exp. Ther. 276, 1039–1048.
[31] Sasai, Y. and Nakanishi, S. (1989) Biochem. Biophys. Res. Commun. 165, 695–702.
[32] Tsuchida, K., Shigemoto, R., Yokota, Y. and Nakanishi, S. (1990) Eur. J. Biochem. 193, 751–757.
[33] Otsuka, M. and Yoshioka, K. (1993) Physiol. Rev. 73, 229–308.
[34] Boyd, N.D., Macdonald, S.G., Kage, R., Luber-Narod, J. and Leeman, S.E. (1991) Ann. N. Y. Acad. Sci. 632, 79–93.

[35] Kage, R., Leeman, S.E. and Boyd, N.D. (1993) J. Neurochem. 60, 347–351.
[36] Boyd, N.D., Kage, R.K. and Leeman, S.E. (1994) in: The Tachykinin Receptors (Buck, S.H., Ed.), pp. 219–236, Humana Press, NJ.
[37] Kage, R., Hershey, A.D., Krause, J.E., Boyd, N.D. and Leeman, S.E. (1995) J. Neurochem. 64, 316–321.
[38] Fong, T.M., Huang, R.-R.C. and Strader, C.D. (1992) J. Biol. Chem. 267, 25664–25667.
[39] Yokota, Y., Akazawa, C., Ohkubo, H. and Nakanishi, S. (1992) EMBO J. 11, 3585–3591.
[40] Gether, U., Johansen, T.E. and Schwartz, T.W. (1993) J. Biol. Chem. 268, 7893–7898.