Time is of the essence - investigating kinetic interactions between drug, endogenous neuropeptides and receptor

Nederpelt, I.

Chapter 5

Kinetic binding and activation profiles of endogenous tachykinins targeting the NK1 receptor

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Abstract

Ligand-receptor binding kinetics (i.e. association and dissociation rates) are emerging as important parameters for drug efficacy *in vivo*. Awareness of the kinetic behavior of endogenous ligands is pivotal, as drugs often have to compete with those. The binding kinetics of neurokinin 1 (NK1) receptor antagonists have been widely investigated while binding kinetics of endogenous tachykinins have hardly been reported, if at all. Therefore, the aim of this research was to investigate the binding kinetics of endogenous tachykinins and derivatives thereof and their role in the activation of the NK1 receptor.

We determined the binding kinetics of seven tachykinins targeting the NK1 receptor. Dissociation rate constants (k_{off}) ranged from 0.026 ± 0.0029 min^{-1} (Sar^9, $Met(O_2)^{11}$-SP) to 0.21 ± 0.015 min^{-1} (septide). Association rate constants (k_{on}) were more diverse: substance P (SP) associated the fastest with a k_{on} value of 0.24 ± 0.046 nM^{-1} min^{-1} while neurokinin A (NKA) had the slowest association rate constant of 0.001 ± 0.0002 nM^{-1} min^{-1}. Kinetic binding parameters were highly correlated with potency and maximal response values determined in label-free impedance-based experiments on U-251 MG cells.

Our research demonstrates large variations in binding kinetics of tachykinins which correlate to receptor activation. These findings provide new insights in the ligand-receptor interactions of tachykinins and underline the importance of measuring binding kinetics of both drug candidates and competing endogenous ligands.

Introduction

Ligand-receptor binding kinetics are reflected by the association and dissociation rates of a ligand to and from its receptor. These kinetic parameters are increasingly acknowledged as a key player in drug-target interactions and functional effects *in vivo* [1-5]. Understanding of desired binding kinetics of a drug for the target of interest is crucial for efficient and efficacious drug development. For example, for the muscarinic M_3 receptor a slow drug-target dissociation rate is desirable to achieve prolonged *in vivo* efficacy and better patient compliance [6]. In contrast, for the dopamine D_2 receptor a fast dissociation rate is desired to minimize on-target side effects [7]. Notably, the majority of successful drugs achieve their potency by competing with endogenous ligands for the same orthosteric binding site. Therefore, knowledge of the pharmacological behaviour of endogenous ligands could benefit the understanding of desirable binding kinetics of competing drugs for the target of interest.

The tachykinin receptor family consists of three neuropeptide G protein-coupled receptors (GPCRs), the neurokinin 1 receptor (NK_1R), neurokinin 2 receptor (NK_2R) and neurokinin 3 receptor (NK_3R). The endogenous ligands for these receptors are substance P (SP), neurokinin A (NKA), neurokinin B (NKB). Each endogenous tachykinin has a specific rank order to activate tachykinin receptors with regards to potency and affinity, namely SP>NKA>NKB for the NK_1 receptor, NKA>NKB>SP for the NK_2 receptor and NKB>NKA>SP for the NK_3 receptor. In 2000, a fourth endogenous tachykinin was discovered, namely hemokinin-1 [8]. Tachykinin receptors and their endogenous ligands are distributed throughout the central and peripheral nervous system and play an important role in e.g. nociception, cell proliferation, smooth muscle contraction and inflammation [9-11].

The neurokinin 1 receptor couples predominantly through the $G\alpha_q$ protein signaling pathway, but can also induce $G\alpha_s$ protein and β-arrestin signaling [12, 13]. Although a plethora of literature is available on G protein signaling of NK1 peptide agonists [14-16] and binding kinetics of NK1 antagonists [17-19], the kinetic binding parameters of the most well-known endogenous tachykinins (i.e. SP, NKA, NKB and Hemokinin-1) and their synthetic or truncated derivatives (i.e. septide, Pro^9-SP, SP(4-11) and Sar^9,$Met(O_2)^{11}$-SP) have rarely been reported, if at all. Therefore, our aim was to determine the binding kinetics of the afore mentioned tachykinins (Figure 1) by using a radiolabeled competition association assay. In an effort to correlate the binding kinetics to functional effects *in vitro* we used a label-free impedance-based assay to examine receptor activation in human astrocytoma U-251 MG cells endogenously expressing the NK1 receptor. This label-free system allows for the real-time monitoring of phenotypic receptor-mediated responses encompassing the entire

signaling cascade which makes it a very suitable system for this target that has been proven to activate multiple downstream signaling pathways [20, 21].

In summary, we have for the first time used a competition association assay to determine the binding kinetics of endogenous tachykinins and their most well-known derivatives. In addition, we were able to correlate the binding kinetics to functional effects *in vitro* using a whole-cell label-free technology. Our research illustrates the importance of knowledge of the association and dissociation rates of endogenous tachykinins and their role in receptor activation.

Figure 1: Amino acid sequences of the examined tachykinins. The differences between the peptides are expressed in green. Sar = methylated glycine, Met (O_2) = oxidized methionine and pGlu = pyro-glutamic acid.

Methods

Reagents and peptides

SP, hemokinin-1 and U-251 MG cells were purchased from Sigma-Aldrich (St. Louis, MO). NKA, NKB, septide, Pro^9-SP, SP (4-11), Sar^9,$Met(O_2)^{11}$-SP and antagonist SC203437 were obtained from Bio-Connect (Huissen, The Netherlands). Aprepitant was a kind gift from Roche Innovation Center Basel (Basel, Switzerland) and protease inhibitors (complete mini

cocktail) were purchased from Roche Diagnostics (Mannheim, Germany). [^{3}H][Sar^9,Met(O_2)^{11}]SP (specific activity 25-55 Ci/mmol) was obtained from Perkin Elmer (Boston, MA). Chinese Hamster Ovary (CHO) cells stably expressing the human neurokinin 1 receptor (CHOhNK1 cells) were kindly provided by AstraZeneca (Macclesfield, UK). xCELLigence E-plate 16 and 96 were purchased from Westburg (Leusden, the Netherlands). All other compounds and materials were obtained from standard commercial sources.

Cell culture

CHOhNK1 cells were cultured in Ham's F12 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 1 mg/ml G418 at 37 °C + 5% CO_2. U-251 MG cells were cultured in Earle's Minimal Essential Medium (EMEM) supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM glutamine, 1% non-essential amino acids (NEAA), 100 IU/ml penicillin and 100 µg/ml streptomycin at 37 °C + 5% CO_2. Membranes were prepared as described previously [22]. In short, CHOhNK1 cells were collected in 50 mM Tris HCl buffer (pH 7.4 at 25°C) supplemented with 2 mM $MgCl_2$ and subsequently centrifuged twice at 100 000x *g* in an Optima LE-80 K ultracentrifuge (Beckman Coulter, Fullterton, CA, USA) for 20 min at 4°C.

Radioligand equilibrium displacement assays

Displacement experiments were carried out using CHOhNK1 membrane aliquots containing 5-15 µg protein in a total volume of 100 µL assay buffer (50 mM Tris HCl, pH 7.4 at 25 °C, supplemented with 5 mM $MgCl_2$ and protease inhibitor cocktail (1 tablet/10 ml)) at 4 °C for 90 minutes. Ten concentrations of competing ligand were used in the presence of one concentration [^{3}H][Sar^9,Met(O_2)^{11}]SP (25 000 dpm, ~2.5 nM). This concentration ensured that total radioligand binding did not exceed 10% of that added to prevent ligand depletion. Non-specific binding was determined in the presence of an excess amount of SC-203437 (10 µM).

Homologous displacement assays were performed using CHOhNK1 membrane aliquots containing 5-15 µg protein, incubating at 4 °C for 90 minutes. Ten concentrations of [Sar^9,Met(O_2)^{11}]SP were used in the presence of four different concentrations [^{3}H][Sar^9,Met(O_2)^{11}]SP (i.e. 4 nM, 3 nM, 2 nM and 1 nM).

The reactions were terminated by the addition of 1 mL ice-cold wash buffer (50 mM Tris HCl, pH 7.4 at 25 °C, supplemented with 5 mM $MgCl_2$). Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/C filters saturated with 0.25% polyethylene imine (PEI) using a Brandel harvester. Filters were subsequently

washed three times with 2 mL ice-cold wash buffer. Filter bound radioactivity was determined using a liquid scintillation counter (Tri-Carb 2900 TR, PerkinElmer).

Radioligand kinetic association and dissociation assays

Association and dissociation experiments were performed similarly to Nederpelt *et al.* [22], using CHOhNK1 membrane aliquots containing 5-15 µg protein, incubating at 4 °C with one concentration [^{3}H][Sar^{9},Met(O_2)^{11}]SP (25 000 dpm, ~2.5 nM). Total incubation time of association experiments was 120 min with different time intervals. For dissociation experiments membrane aliquots were pre-incubated for 90 min and dissociation was initiated by addition of 10 µM SC-203437. The amount of radioligand still bound to the receptor was measured for 240 min at different time points.

Radioligand kinetic competition association assays

The binding kinetics of unlabeled peptides were quantified as described previously [22], using CHOhNK1 membrane aliquots containing 5-15 µg protein, incubating at 4 °C with one concentration [^{3}H][Sar^{9},Met(O_2)^{11}]SP (30 000 dpm, ~3 nM). Total incubation time of competition association experiments was 120 min with different time intervals. The assay was validated using three concentrations (0.3*IC_{50}, 1*IC_{50} and 3*IC_{50}) of [Sar^{9},Met(O_2)^{11}]SP while the binding kinetics of all remaining agonists were determined using a concentration where displacement of [^{3}H][Sar^{9},Met(O_2)^{11}]SP at 120 min was between 40 and 60%.

Label-free whole cell assays

Label-free whole-cell assays were performed using the xCELLigence a real-time cell analyzer (RTCA) system as described previously [23]. In short, this assay utilizes electrical impedance to measure changes in cell morphology. 20 000 U-251 MG cells/well were seeded in E-plates covered with golden electrodes on the bottom of each well, 18 hours prior to stimulation with increasing concentrations of agonist or vehicle control. For antagonistic assays, cells were incubated for 30 min with an excess of selective NK1 antagonist aprepitant (1 µM) prior to stimulation with submaximal (EC_{80}) concentrations of agonist.

Data analysis

All experimental data were analyzed using the nonlinear regression curve-fitting program GraphPad Prism v. 6.00 (GraphPad Software Inc., San Diego, CA). Radioligand binding assays were analyzed as described previously [22]. In short, association and dissociation rates of unlabeled ligands were calculated by fitting the data of the competition association assay using non-linear regression - kinetics of competitive binding [24] using equation 1.

Equation 1:

$$K_A = k_1[L]\cdot 10^{-9} + k_2$$

$$K_B = k_3[I]\cdot 10^{-9} + k_4$$

$$S = \sqrt{(K_A - K_B)^2 + 4\cdot k_1 \cdot k_3 \cdot L \cdot I \cdot 10^{-18}}$$

$$K_F = 0.5(K_A + K_B + S)$$

$$K_S = 0.5(K_A + K_B - S)$$

$$Q = \frac{B_{\max} \cdot k_1 \cdot L \cdot 10^{-9}}{K_F - K_S}$$

$$Y = Q \cdot \left(\frac{k_4 \cdot (K_F - K_S)}{K_F \cdot K_S} + \frac{k_4 - K_F}{K_F} e^{(-K_F \cdot X)} - \frac{k_4 - K_S}{K_S} e^{(-K_S \cdot X)}\right)$$

Where k_1 is the k_{on} of the radioligand ($M^{-1}min^{-1}$), k_2 is the k_{off} of the radioligand (min^{-1}), L is the radioligand concentration (nM), I is the concentration of the unlabeled competitor (nM), X is the time (min) and Y is the specific binding of the radioligand (DPM). These parameters are set during a competition association, obtaining k_1 from the control curve without competitor and k_2 from previously performed dissociation assays described under *2.4 Radioligand kinetic association and dissociation assays*. With that the k_3, k_4 and B_{max} can be calculated, where k_3 represents the k_{on} ($M^{-1}min^{-1}$) of the unlabeled ligand, k_4 stands for the k_{off} of the unlabeled ligand and B_{max} equals the total binding (DPM). All competition association data were globally fitted. Data were normalized to maximal specific binding of [^{3}H][Sar^9,Met(O_2)^{11}]SP in absence of competitor.

Data from xCELLigence experiments were exported from RTCA Software 1.2 (Roche, Germany) after normalizing the cell index (CI) traces to the time of agonist addition. Baseline was removed by subtracting vehicle or antagonist traces from corresponding normalized CI (NCI) traces. The maximal NCI response of each concentration (peak) was used for data analyses.

Maximal response (E_{max}) and potency (pEC_{50}) values for all agonists were analyzed with non-linear regression of peak analysis fitted by log(agonist) vs. response - Variable slope. Results were normalized to the maximal NCI response induced by SP. The peak of agonist-induced cellular responses in presence of antagonist were normalized to the peak obtained from treatment with submaximal concentrations of the corresponding agonist.

Results

Characterization of [^{3}H][Sar^9,Met(O_2)^{11}]SP

The kinetic binding parameters of [^{3}H][Sar^9,Met(O_2)^{11}]SP interacting with CHOhNK1 membranes were determined with traditional kinetic radioligand binding studies. Association and dissociation assays supplied k_{on} and k_{off} values of 0.17 ± 0.028 nM^{-1} min^{-1} and 0.016 ±

0.0015 min^{-1}, respectively (Figure 2A and 2B). The kinetic K_D (k_{off}/k_{on}) calculated from these experiments was 0.093 ± 0.018 nM. The dissociation constant was determined with homologous displacement experiments (Figure 2C) and yielded a K_D of 2.5 ± 0.7 and this value was used to convert IC_{50} values to K_i values in the equilibrium binding studies.

Binding affinity of hNK1R peptide agonists

The binding affinity of seven hNK1R peptide agonists was determined with equilibrium radioligand displacement studies. With the exception of NKB (no significant displacement at 10 µM, data not shown), all peptides were able to completely displace [^{3}H][Sar^9,Met(O$_2$)^{11}]SP in a concentration-dependent manner (Figure 3). Unlabeled Sar^9,Met(O$_2$)^{11}-SP, SP, Pro^9, hemokinin-1 and SP(4-11) all had nanomolar affinities, ranging from 2.1 nM for SP to 37 nM for SP(4-11). NKA and septide showed a much lower affinity in the micromolar range of 1933 nM and 2417 nM, respectively (Table 1).

Competition association assay optimization and validation with [^{3}H][Sar^9,Met(O$_2$)^{11}]SP

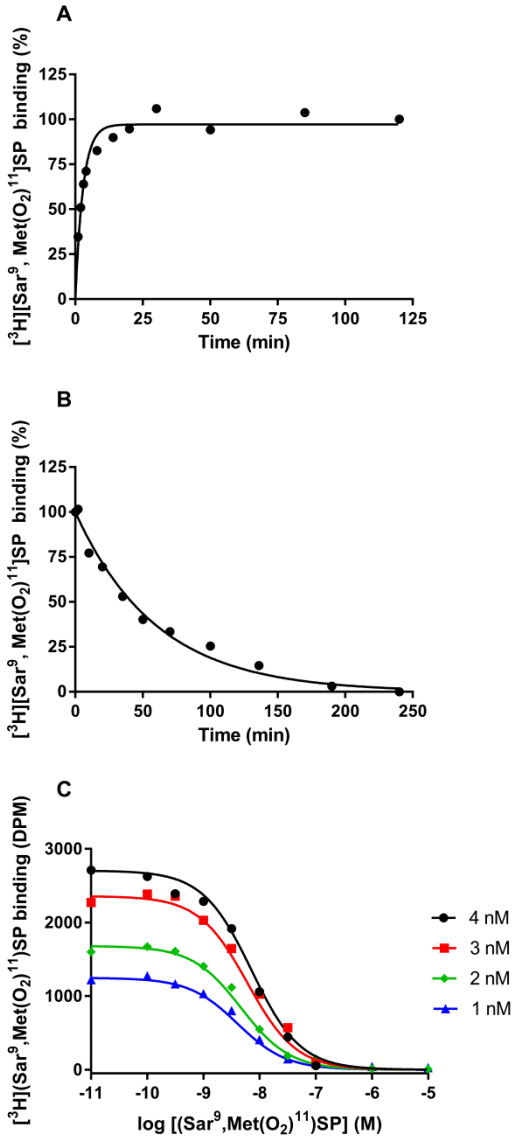

Figure 2: Association (A) and dissociation (B) kinetics of one concentration [^{3}H][Sar^9,Met(O$_2$)^{11}]SP (25 000 dpm, ~2.5 nM) and homologous displacement (C) of four concentrations [^{3}H][Sar^9,Met(O$_2$)^{11}]SP (i.e. 4 nM, 3 nM, 2 nM and 1 nM) by [Sar^9,Met(O$_2$)^{11}]SP on CHOhNK1 membranes at 4 °C. Representative graphs are shown from one experiment performed in duplicate.

The k_{on} (k_3) and k_{off} (k_4) values of unlabeled Sar^9,Met(O$_2$)^{11}-SP were quantified by fitting the k_{on} (k_1) and k_{off} (k_2) values of [^{3}H][Sar^9,Met(O$_2$)^{11}]SP into the model of 'kinetics of competitive binding' as described in Materials & Methods. Three different concentrations of Sar^9,Met(O$_2$)^{11}-SP, i.e. 0.3-fold, 1-fold and 3-fold its IC_{50} value, were tested and resulted in a shared k_{on} (k_3) and k_{off} (k_4) value of 0.094 ± 0.011 nM^{-1} min^{-1} and 0.026 ± 0.0029 min^{-1}, respectively (Figure 4A and Table 1). The association and dissociation rates obtained with this assay agreed fairly well with those obtained in traditional binding assays (Table 1). Kinetic dissociation constants and affinity values were also in good agreement, validating the competition association as a valuable tool for the determination of binding kinetics of unlabeled hNK1 ligands. To improve the throughput of this assay it was examined if using a single concentration (i.e. 1-fold their IC_{50} value) yielded similar binding

kinetics parameters. The k_{on} and k_{off} values of the single concentration method proved to be not significantly different, i.e. 0.078 ± 0.012 nM^{-1} min^{-1} and 0.024 ± 0.0038 min^{-1} respectively. Therefore, the remaining peptides were tested using this single concentration method.

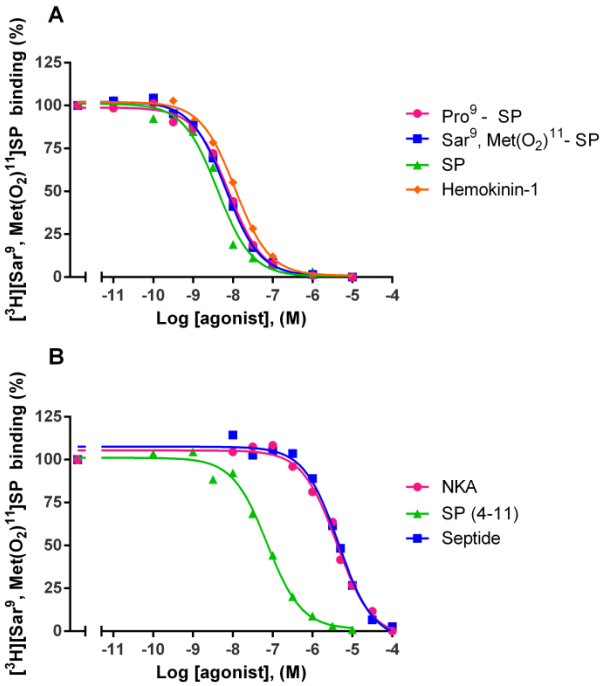

Figure 3: Displacement of one concentration [^{3}H][Sar^9,Met(O$_2$)^{11}]SP (25 000 dpm, ~2.5 nM) by NK1 peptide agonists on CHOhNK1 membranes at 4 °C. Representative graphs are shown from one experiment performed in duplicate (See Table 1 for affinity values).

Binding kinetics of hNK1R peptide agonists

The one-concentration competition association assay was used to determine the binding kinetics of the remaining agonists (Figure 4B and 4C). K_i values and kinetic K_D values of all peptides were highly correlated (r^2 = 0.99 and p<0.0001; data not shown), although kinetic K_D values were around 10-fold lower than K_i values. Dissociation rates varied approximately 9-fold, ranging from 0.026 ± 0.0029 min^{-1} for Sar^9,Met(O$_2$)^{11}-SP and 0.21 ± 0.015 min^{-1} for septide (Table 1). Interestingly, a 240-fold difference was observed in association rates for the peptide agonists. SP had the fastest association rate of 0.24 ± 0.046 nM^{-1} min^{-1} and NKA showed the slowest association rate of 0.001 ± 0.0002 nM^{-1} min^{-1} (Table 1).

NK1 receptor activation in human astrocytoma U-251 MG cells

To examine receptor activation we used a cell line that endogenously expresses the human NK1 receptor, i.e. astrocytoma U-251 MG cells. Stimulation of the cells with agonist resulted in a rapid transient (~2 min) negative effect on the impedance followed by a positive response for all agonists (Figure 5A, representative trace of SP). Potency values (EC_{50}) ranged from 0.04 ± 0.01 nM for Sar^9, $Met(O_2)^{11}$-SP to 3.9 ± 1.8 nM for NKA while only NKA showed a significant increase in E_{max} in comparison to SP (Figure 5B, 5C and Table 1). Pre-incubation with 1 µM aprepitant, a selective non-peptide NK1 receptor antagonist, completely abolished receptor signaling for all agonists (Figure 5D).

Correlation plots of binding and receptor activation parameters of hNK1R peptide agonists

Lastly, we investigated the correlation between the kinetic binding parameters (pk_{on} and pk_{off}) and the receptor activation parameters (pEC_{50} and E_{max}). The dissociation rate constants correlated well with association rate constants (R^2=0.73, P=0.014), potency (R^2=0.67, P=0.025), maximal response values (R^2=0.70, P=0.018) and a good correlation was obtained with affinity values (R^2=0.84, P=0.0039). In contrast, the association rate constants did not show any significant correlation with the *in vitro* maximal response (R^2=0.45, P=0.10). Finally, an excellent correlation was

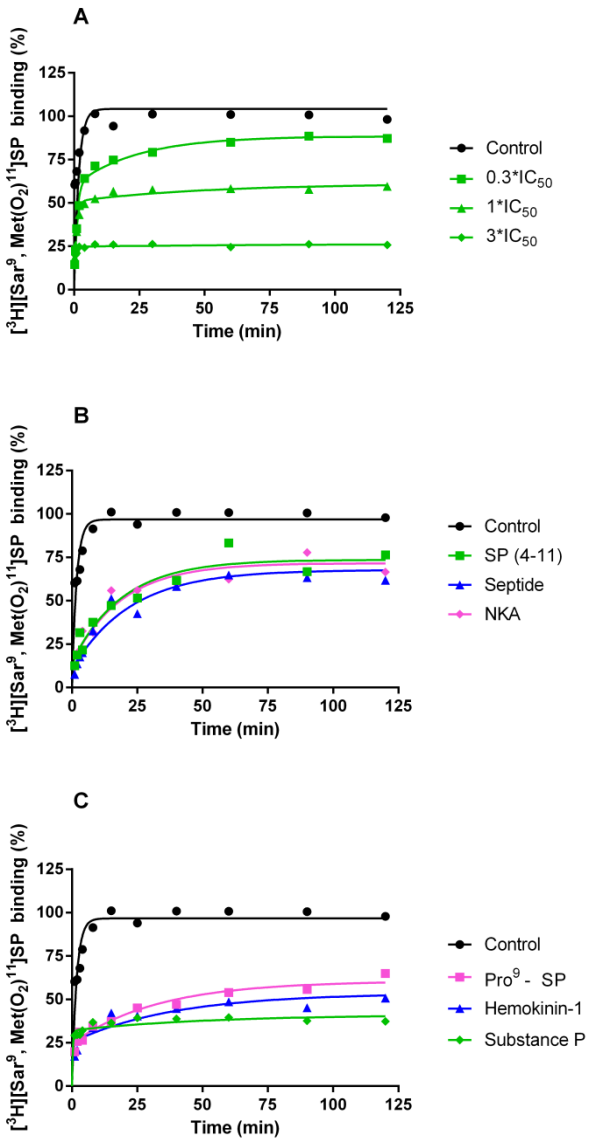

Figure 4: Competition association assay at 4 °C with one concentration [^{3}H][Sar^9,$Met(O_2)^{11}$]SP (30 000 dpm, ~3 nM) in the absence or presence of 0.3, 1 or 3*IC_{50} value of unlabeled [Sar^9,$Met(O_2)^{11}$]SP (A) or 1*IC_{50} value of SP (4-11), septide and NKA (B) or SP, Pro^9 and hemokinin-1 (C). Representative graphs are shown from one experiment performed in duplicate (See Table 1 for kinetic parameters).

observed between the association rate constants and the affinity (R^2=0.99, P<0.0001) and potency values (R^2=0.90, P=0.0012).

Discussion

Binding kinetics of endogenous tachykinins and their derivatives vary greatly

Ligand-receptor binding kinetics are defined by the association and dissociation rates of a ligand to and from its receptor. These parameters are increasingly recognized to be important in the understanding of a drugs mechanism of action [5, 25, 26]. Many successful drugs achieve their effects by competing with endogenous ligands, therefore insights into the kinetics of endogenous ligands could provide clues for the desired binding kinetics of potential drugs. In this study we focused on determining the binding kinetics of endogenous tachykinins and their derivatives targeting the NK1 receptor, including but not limited to Substance P, NKA and hemokinin-1 (Figure 1) using a competition association assay. This assay was first described in 1984 [24] and has to date been used to investigate the binding kinetics of ligands for quite a number of GPCRs [22, 27, 28]. Here, we validated the competition association assay as a reliable method to determine the binding kinetics of unlabeled NK1 peptide agonists. This was demonstrated by the similar k_{on} and k_{off} values in comparison to the traditional association and dissociation assays, as well as the excellent correlation between the affinity and kinetic K_D values. Notably, K_i values were consistently 10-fold higher in comparison to kinetic K_D values. It should be noted that there were some experimental differences between equilibrium displacement and competition association assays. For instance, equilibrium experiments were performed with 90 minutes incubation while the kinetic K_D from the competition association is not (or less) time dependent. Moreover, to improve the assay window for competition association assays the concentration radioligand and membranes was increased. Additionally, membrane batches differed between assays. These differences can add up to the 10-fold discrepancy between equilibrium K_i and kinetic K_D values reported in this study. Of note, literature K_i and K_D values of NK1 agonists are also quite variable and even differ up to 20-fold [16, 29-31]. Investigation of the correlations between the kinetic binding parameters (k_{on} and k_{off}) and the affinity values of all agonists showed a significant correlation between pK_i and pk_{off} values (R^2=0.84, P<0.005) and an excellent correlation between pK_i and pk_{on} values (R^2=0.99, P<0.0001). Since the association rate is often thought to be diffusion rate limited (10^8 ~ 10^9 M^{-1} s^{-1} [32]) and the affinity is calculated by dividing the dissociation rate by the association rate, it is commonly assumed that changes in affinity are directly derived from changes in dissociation rates.

Significant correlations between pK_i and pk_{off} values are often reported [5, 25, 33], while a correlation between the association rate and affinity is less prevalent. However, in this study it was demonstrated that for the most well-known tachykinins the association rates ranged 240-fold while dissociation rates only differed 8-fold. Takeda *et al.* also reported over 100-fold differences between the association rate of SP versus NKA while the dissociation rates only differed 3-fold [34]. Similar to synthetic ligands for the β2-adrenoceptor, orexin-2 receptor and Kv11.1 potassium channel [35-37], our findings illustrate that association rates are the main incentive that dictate the affinity of endogenous tachykinins targeting the NK1 receptor. It should be noted that in addition to binding kinetics, other parameters such as rebinding, ligand elimination, degradation and target vulnerability also play an important role in the mechanism of action of the ligand of interest [38].

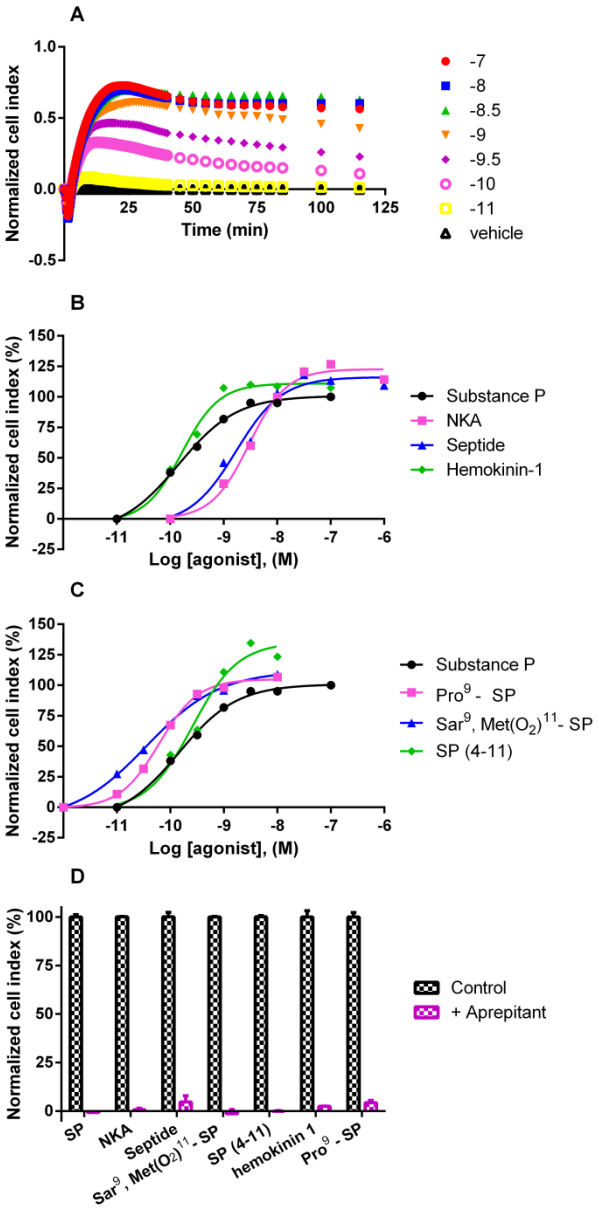

Figure 5: Concentration-dependent effects of NK1 agonists on U-251 MG cells. Representative xCELLigence traces of one experiment performed in duplicate of normalized cell index (NCI) after stimulation with increasing concentrations of SP (A) and representative concentration-effect curves of all agonists derived from peak analysis, data were normalized to maximal response after SP stimulation (B and C). Representative bar graph of one experiment performed in duplicate of peak analysis after stimulation with EC_{80} concentrations of agonist in presence or absence of 1 µM aprepitant (D).

Diversity in NK1 receptor potency but not activation profiles on label-free impedance-based system

In addition to the binding parameters we also investigated the receptor activation profiles of all tested tachykinins, except NKB. Most of the NK1 receptor signaling studies have been performed with G protein pathway-specific assays such as cAMP and IP turnover assays [14-16]. However, an assay that measures the whole cellular response over time overcomes the limitations of pathway-specific end-point assays and is thus more suitable to study NK1 receptor activation and its correlation to binding kinetics. Therefore we used a label-free impedance-based assay to study NK1 receptor activation in human astrocytoma U-251 MG cells that endogenously express the NK1 receptor. Addition of increasing concentrations of agonist resulted in a concentration-dependent increase in impedance for all tested tachykinins. These responses were selectively inhibited by the small molecule antagonist aprepitant, supporting a NK1 receptor specific response. All tachykinins had much higher potencies in comparison to literature values that used endpoint assays [16, 39], which is often observed in label-free assays that monitor cell morphology rather than one downstream signaling pathway [40, 41]. Of note, the cell index trace was identical for fastly and slowly associating agonists. It could be argued that numerous factors are involved in receptor activation kinetics, such as the rate of G protein-coupling and kinetics of production of second messengers. These factors could prove it difficult to selectively measure the effects of agonist-receptor association rates on receptor activation kinetics.

N-terminal domain of tachykinins is important for association rate and potency

The N-terminus of tachykinins is believed to be important for receptor affinity and selectivity, albeit indirectly by regulating peptide conformations that are crucial in peptide-receptor binding and activation [42, 43]. Valentin-Hansen *et al.* recently mapped SP binding sites on the NK1 receptor and reported that the first 6 C-terminal amino acids of SP are specifically organized fitting onto the surface of the receptor while the remaining N-terminal amino acids are forming a cone-shaped entity that could accommodate interactions with multiple residues of the N-terminal tail of the receptor [44]. Interestingly, in our study we found that tachykinins with shorter N-terminal domains such as NKA and septide not only had significantly reduced affinities and potencies but also had a more than 200-fold decrease in association rates (Table 1). It could be postulated that the slower association rates are due to a less optimal peptide conformation in consequence of a lack of interactions with the N-terminal tail of the receptor resulting in a decreased affinity and potency.

Binding kinetics correlate with in vitro activation profiles

A good correlation was observed between the dissociation rate and *in vitro* maximal response (Table 1). Correlations between *in vitro* maximal response and dissociation rates have been reported before. In example, Guo *et al.* [45] examined binding kinetics and efficacies of adenosine A_{2A} agonists and found that slower dissociation correlated with increased efficacies in a label-free assay. Moreover, a study investigating dissociation rates and functional effects of muscarinic M3 receptor agonists proved that high maximal response values were positively correlated with slow dissociation rates [46]. Collectively, these findings support our observation that receptor dissociation rates can be a good predictor of *in vitro* efficacies at the NK1 receptor. Moreover, a very good correlation was observed between the association rate and potency values. We found that tachykinins with faster dissociation rates and slower association rates (i.e. lower affinities) have lower potencies in comparison with SP. Interestingly, these agonists are known to only activate the $G\alpha_q$ pathway [16, 39, 47, 48]. A correlation between binding kinetics and biased signaling has been discussed before for the NK2 receptor. Specifically, an allosteric modulator for the NK2 receptor (i.e. LPI805), was investigated for its effects on the binding kinetics and signaling profile of NKA [14]. It was demonstrated that addition of LPI805 resulted in faster dissociation and slower association rates of NKA, as well as abolishing $G\alpha_s$ signaling.

In conclusion, the competition association assay was validated as a valuable tool to study the binding kinetics of tachykinins targeting the NK1 receptor. We have, for the first time, elucidated the binding kinetics of endogenous tachykinins and their most well-known derivatives and found small differences in their dissociation rates (8-fold difference) and large differences in their association rates (240-fold difference). Dissociation rates correlated well with *in vitro* efficacies, while association rates correlated highly with potency values obtained with a label-free assay. Taken together, these results indicate that diverging binding kinetics can play a significant role in differentially activating the NK1 receptor. Our research underlines the importance of knowledge of binding kinetics of endogenous ligands, as it was demonstrated that for endogenous tachykinins both dissociation and association rate constants differed significantly which in turn had differential effects in receptor activation. Moreover, these kinetic values should be considered when designing novel competing drugs targeting the NK1 receptor.

Table 1: Binding (affinity, association rate and dissociation rate) and activation (*in vitro* potency and maximal response) parameters of **the examined tachykinins.**

Ligand	**pK_i (and K_i in nM)**	**pK_D (and K_D in nM)**	**k_{on} ($nM^{-1}min^{-1}$)**	**k_{off} (min^{-1})**	**RT (min)**	**pEC_{50} # (and EC_{50} in nM)**	**E_{max} (%)##**
Substance P	8.7 ± 0.01 (2.1)	10 ± 0.09 (0.11)	0.24 ± 0.046	0.027 ± 0.0025	37 ± 3.4	9.9 ± 0.1 (0.07)	100 ± 3
Sar^9, $Met(O_2)^{11}$-SP #	8.5 ± 0.03 (3.5)	9.6 ± 0.07 (0.27)	0.094 ± 0.011	0.026 ± 0.0029	39 ± 4.4	10 ± 0.1 (0.04)	110 ± 1
Pro^9 - SP	8.4 ± 0.02 (3.8)	9.5 ± 0.05 (0.32)	0.18 ± 0.021	0.059 ± 0.0024	17 ± 0.67	10 ± 0.08 (0.06)	116 ± 8
Hemokinin-1	8.1 ± 0.08 (8.1)	9.1 ± 0.05 (0.79)	0.064 ± 0.013	0.048 ± 0.0065	21 ± 2.9	10 ± 0.1 (0.1)	135 ± 22
NKA	5.7 ± 0.04 (1933)	6.7 ± 0.1 (180)	0.0010 ± 0.00018	0.19 ± 0.036	5.3 ± 1.0	8.4 ± 0.16 (3.9)	145 ± 10*
SP(4-11)	7.4 ± 0.04 (37)	8.4 ± 0.06 (3.8)	0.037 ± 0.0025	0.14 ± 0.018	7.1 ± 0.89	9.9 ± 0.19 (0.25)	129 ± 10
Septide	5.6 ± 0.03 (2418)	6.8 ± 0.03 (171)	0.0012 ± 0.000042	0.21 ± 0.015	4.6 ± 0.4	8.7 ± 0.06 (1.8)	134 ± 11

Values are means ± SEM of three separate experiments performed in duplicate, #3-concentration competition association, # #Values were calculated with peak analysis and data were normalized to maximal response obtained for Substance P. Of note, NKB was unable to displace [^{3}H]-Sar^9, $Met(O_2)^{11}$-SP (-3% and -10% displacement at 10 µM). * $p < 0.05$, compared to E_{max} SP determined using one-way ANOVA with Dunnett's post-test.

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