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Characterization of G-Protein Coupled Receptor Kinase Interaction with the NK-1 Receptor Using BRET

Rasmus Jorgensen, Nicholas D. Holliday, Jakob L. Hansen, Milka Vrecl, Anders Heding, Thue W. Schwartz, and Christian E. Elling*.

7TM Pharma A/S, Fremtidsvej 3, DK-2970 Horsholm , Denmark (RJ, AH, TWS, and CEE); Institute of Cell Signaling, Queen's Medical Centre, Nottingham NG7 2UH, UK (NDH); Institute of Anatomy, Histology, and Embryology, Veterinary Faculty, University of Ljubljana, Slovenia (MV); Laboratory for Molecular Pharmacology, The Panum Institute 18.6, University of Copenhagen, Blegdamsvej 3, DK-2100, Denmark (JLH and TWS). Laboratory for Molecular Cardiology, The Danish National Research Foundation Centre for Cardiac Arrhythmia, The Heart Centre, Copenhagen University Hospital, Juliane Mariesvej 20, section 9302, DK-2100, Denmark (JLH).

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Running title: NK-1 receptor interaction with GRKs

*To whom correspondence should be addressed:

Christian E. Elling

7TM Pharma A/S

2970 Horsholm, Denmark

Tel: (+45) 3925 7777

Fax: (+45) 3925 7776

cee@7tm.com

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Abbreviations:

NK-1: Neurokinin-1, BRET: Bioluminescence Resonance Energy Transfer, βarr: beta-arrestin, 7TM: Seven Transmembrane, GRK: G-protein coupled receptor kinase, SP: Substance P.

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Abstract

To analyze the interaction between the Neurokinin-1 (NK-1) receptor and G-protein coupled receptor kinases (GRKs), we performed Bioluminescence Resonance Energy Transfer^2 (BRET^2) measurements between the family A NK-1 receptor and GRK2 and GRK5 as well as their respective kinase-inactive mutants. We observed agonist induced interaction of both GRK5 and GRK2 with the activated NK-1 receptor. In saturation experiments we observed GRK5 to interact with the activated receptor in a monophasic manner while GRK2 interacted in a biphasic manner with the low affinity phase corresponding to receptor affinity for GRK5. Agonist induced GRK5 interaction with the receptor was dependent on intact kinase-activity while the high affinity phase of GRK2 interaction was independent of kinase activity. Surprisingly, BRET^2 saturation experiments indicated that before receptor activation, the full length NK-1 receptor, but not a functional C-terminal tail truncated receptor, is pre-associated with GRK5 in a relatively low affinity state. We demonstrate that GRK5 can compete for agonist induced GRK2 interaction with the NK-1 receptor while GRK2 does not compete for receptor interaction with GRK5. We suggest that GRK5 is pre-associated with the NK-1 receptor and that GRK5, rather than GRK2, is a key player in competitive regulation of GRK subtype specific interaction with the NK-1 receptor.

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Introduction

Seven transmembrane (7TM) G-Protein Coupled Receptors (GPCRs) interact with heterotrimeric G-proteins upon activation. Receptor activation results in initiation of G-protein mediated signal transduction cascades leading to up- or downregulation of intracellular levels of secondary messengers such as cAMP, IP3, or Ca^{2+}. Termination of the receptor mediated signaling is regulated by second messenger regulated kinases and G-protein coupled receptor kinases (GRKs). GRK mediated phosphorylation of the receptor promotes receptor recruitment of the intracellular scaffolding proteins βarrestins (βarrs) from the cytosol thereby excluding the receptor from further G-protein interaction (Luttrell and Lefkowitz, 2002). In addition to uncoupling 7TM receptors from G-proteins, βarrs recruit the receptor to clathrin coated pits (Goodman, Jr. *et al.*, 1996) and initiates signaling pathways such as Mitogen Activated Protein Kinase cascades (Luttrell and Lefkowitz, 2002). Seven members of the mammalian GRK family have so far been described. These GRKs are classified in three subgroups based on sequence homology and function: the visual GRK1, and 7, nonvisual cytosolic GRK2, and 3, and nonvisual membrane associated GRK 4, 5, and 6. With GRK4 as the exception, non-visual GRKs are rather ubiquitously expressed in mammalian tissues. Although very little is known about GRK subtype specific phophorylation patterns of 7TM receptors, recent publications report GRK subtype specific initiation of cellular events (Kim *et al.*, 2005;Ren *et al.*, 2005). At present it is unknown how GRK subtype specific functional effects are obtained and details of the interaction pattern between 7TM receptors and GRK subtypes are largely undescribed. Classically, protein-protein interactions have been analyzed using co-immunoprecipitation techniques. For more detailed analysis, co-

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immunoprecipitation has several limitations including the necessity for lysis and detergent treatment of the cells as well as low time resolution, making it difficult to detect transient interactions. For the analysis of GRK2 and GRK5 interaction with the NK-1 receptor we used the Bioluminescence Resonance Energy Transfer (BRET) technique as it offers a relatively high resolution method for analysis of biological interactions in living cells (Gales *et al.*, 2005;Gales *et al.*, 2006;Perroy *et al.*, 2004). The BRET technique has previously been employed in the analysis of GRK2 but not GRK5 interaction with 7TM receptors (Hasbi *et al.*, 2004;Jorgensen *et al.*, 2007).

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Materials and methods

Molecular biology - Human βarr2 N-terminally tagged with GFP^2 (GFP^2-βarr2) and the Renilla Luciferase (RLuc) cDNA were purchased from PerkinElmer, Wellesley, MA. Human GRK2, and GRK5 cDNA were cloned from a pancreatic cDNA library (Clontech, Palo Alto, CA). GFP^2 or RLuc fusions were generated using standard methods. GRK2-GFP^2-Membrane was made by inserting the cDNA sequence encoding the C-terminal 17 amino acid residues from human K-ras (KDGKKKKKKSKTKCVIMS) followed by a stop codon in a 3' position of the GRK2-GFP^2 cDNA lacking the stop codon. Mutations were made using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All cDNA clones were verified by sequencing. Substance P (SP) peptide was obtained from Bachem, Bubendorf, Switzerland.

Transfections and tissue culture – cDNAs were transiently expressed in HEK293 cells transfected by calcium phosphate precipitation according to previously reported methods (Elling and Schwartz, 1996). HEK293 cells were obtained from the European Collection of Animal Cell Cultures. The cells were routinely maintained and passaged as recommended by the supplier.

Inositol phosphate assay. Agonist induced IP accumulation was measured as previously described (Liu *et al.*, 2003).

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BRET^2 assays - BRET^2 measurement was done using a Mithras LB 940 plate reader (Berthold Technologies, Bad Wildbad, Germany) as earlier described (Jorgensen *et al.*, 2007). All measurements were made at room temperature. The reading time was five minutes after agonist addition for dose response curves. For kinetic studies variable reading times were used and agonist was added with an injector. For BRET^2 saturation experiments a range of transfections were made with a stable amount of NK1R-RLuc cDNA and increasing amounts of cDNA encoding the GFP^2 fused protein. Reading times were as indicated in the figures. The background signal from Rluc was determined by co-expressing the Rluc construct with empty vector, and the BRET^2 ratio generated from this transfection was subtracted from all other BRET^2 ratios. Expression levels of Rluc- and GFP^2-tagged constructs for each BRET^2 saturation experiment were monitored by luminescence and fluorescence measurements. Luminescence was measured on the Mithras LB 940 plate reader two seconds after Deep Blue C addition. For fluorescence measurements, cells from the same transfections were plated in a black clear-bottom microplate (ViewPlate; PackardBioScience, Inc., Montreal, Canada) at a density of ~5 × 10^4 cells per well. After 1 h incubation in darkness, the total fluorescence was measured using the NovoStar microplate reader (BMG LabTech, Offenburg, Germany), with an excitation line at 485nm and an emission filter at 520 nm. Background values obtained with cells transfected with the relevant RLuc construct but no GFP^2 construct were subtracted and the means of quadruplicate wells/sample were then calculated.

Confocal fluorescence microscopy – HEK293T cells were grown to 50 % confluence on poly-L-lysine coated 22 mm diameter glass coverslips, and transiently transfected

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(Lipofectamine, Invitrogen, Paisley U.K.) with the FLAG-NK1 receptor and GRK5-GFP^2, GRK2-GFP^2, or GRK2-GFP^2-Membrane cDNAs (0.5 µg + 0.5 µg per well). After 24 h, the medium was exchanged for serum free OPTIMEM (Invitrogen, Paisley U.K.) including 1 % bovine serum albumin (BSA; 30 min, 37°C), and the cells were then incubated for 30 min at 37°C in OPTIMEM / 1 % BSA including M2 anti FLAG antibody (2.5 µg/ml, 150 µl per coverslip; Sigma, Poole, U.K.). Vehicle (50 µl medium) or SP (10 nM final concentration) were added for 5 min, and cells were then washed in phosphate buffered saline (PBS), fixed in 3 % paraformaldehyde in PBS (15 min, 21°C) and permeabilized with 0.075 % Triton X-100 for 5 min. Bound M2 antibody was detected by goat anti-mouse IgG conjugated to Rhodamine Red X (Invitrogen, Paisley U.K., 1:1000). Cells were post-fixed in 3 % paraformaldehyde and coverslips were mounted in 1:1 PBS:glycerol, sealed with nail varnish. Confocal microscopy was performed on a Zeiss LSM 510M laser scanning microscope with a Plan-Apochromat 63x NA 1.4 oil objective. Images were acquired in multitrack mode using an Ar 488 nm laser line and 505 – 550 nm bandpass emission filter (GRK-GFP^2), and HeNe 543 nm excitation and longpass 560 nm filter (Rhodamine Red X secondary antibody). 10 – 15 representative cells were scanned for each data group, using gain and offset adjustments to obtain a full range of image intensities without saturation. Channel acquisition parameters were similar in the control and SP-treated examples given in Figure 5.

Calcium measurements - Calcium measurements were made using the NovoStar microplate reader (BMG LabTech, Offenburg, Germany) as previously described (Kubale *et al.*, 2007).

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Receptor internalization – NK-1 receptor internalization was measured using the acid wash method with ^{125}I-SP tracer (Amersham Biosciences, Little Chalfont, UK) as previously described (Kubale *et al.*, 2007).

Data analysis –Nonlinear regression analysis was made using Prism (GraphPad Software, Inc., San Diego, CA). BRET50 values were determined by one phase exponential association or two phase exponential association using Prism.

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Results

The NK-1 receptor, a prototypical family A 7TM receptor, has been demonstrated to undergo GRK2 and GRK5 mediated phosphorylation *in vitro* (Nishimura *et al.*, 1998;Walker *et al.*, 1999). However, the interaction pattern of the receptor and the GRKs in living cells has not been described. To analyze the interactions between the NK-1 receptor and GRK2 and GRK5 respectively, we employed a BRET assay in transfected HEK293 cells. The BRET assay is a proximity based assay that measures energy transfer from a Renilla Luciferase (Rluc)-tagged protein to a Green Fluorescent Protein (GFP)-tagged protein upon addition of the Rluc substrate deep blue C (Mercier *et al.*, 2002;Milligan, 2004). The BRET signal is highly distance dependent and is obtained if the Rluc energy donor and the GFP acceptor are within approximately 100 Å or less of each other. We measured BRET^2 by using the GFP^2 blue-shifted variant of GFP. We tagged the NK-1 receptor C-terminally with RLuc (NK1R-RLuc) and the GRKs C-terminally with GFP^2 (GRK2-GFP^2 and GRK5-GFP^2). Functional expression of the NK1R-RLuc construct has formerly been demonstrated (Vrecl *et al.*, 2004) and other reports utilize GRK2 and GRK5 with C-terminal tags (Jorgensen *et al.*, 2007;Hasbi *et al.*, 2004;Li *et al.*, 2003;Schulz *et al.*, 2002;Thiyagarajan *et al.*, 2004).

To further validate the functionality of GFP^2-tagged GRK constructs and to analyze the ability of GRKs to regulate NK-1 receptor signaling we measured the ability of the NK-1 receptor to mediate agonist induced inositol phosphate accumulation after co-expression with either vector, GRK2, GRK2-GFP^2, GRK2(K220R), GRK2(K220R)-GFP^2, GRK5,

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GRK5-GFP^2, GRK5(K215R), or GRK5(K215R)-GFP^2 . The GRK2(K220R) and GRK5(K215R) mutants have been demonstrated to be kinase-inactive and are in some reports described to be dominant negative (Pronin and Benovic, 1997;Tiruppathi *et al.*, 2000;Diviani *et al.*, 1996;Kong *et al.*, 1994). We observed that co-expression of the NK-1 receptor with either GRK2 construct caused a substantial attenuation of agonist induced inositol phosphate accumulation (figure 1A) demonstrating that GRK2 can regulate NK-1 receptor signaling and that the GFP^2 fused GRK2 is functional. Furthermore, the ability of the kinase-inactive GRK2 mutants to attenuate NK-1 receptor mediated signaling indicates that GRK2 can exert a phosphorylation independent effect on receptor signaling. When measuring receptor mediated inositol phosphate accumulation in the presence of co-expressed GRK5 constructs we found that both GRK5 and GRK5-GFP^2 coexpression attenuated receptor signaling to a similar level suggesting that GRK5 can regulate NK-1 receptor signaling and that GFP^2-fused GRK5 remains functional (figure 1B). Co-expression of the kinase-inactive GRK5(K215) mutant appeared to cause a relatively modest attenuation of NK-1 receptor mediated inositol phosphate accumulation (figure 1B). However, the effect was not significant (students t-test) and was not observed with GFP^2-fused GRK5(K215R) construct suggesting that GRK5 does not exert phosphorylation independent effects on NK-1 receptor signaling.

To further distinguish phosphorylation dependent and independent effects of GRK2 and GRK5 on NK-1 receptor signaling we employed a naturally occurring splice variant of the NK-1 receptor in which all of the receptor tail C-terminal to the palmitoylated Cys is lacking (Richardson *et al.*, 2003) The NK1R(1-324) variant (numbers referring to

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remaining amino acid residues) has been reported not to be phosphorylated and to have reduced ability to interact with βarrs (Oakley *et al.*, 2001;Richardson *et al.*, 2003). We measured the ability of the tail truncated NK-1R(1-324) construct to mediate agonist induced inositol phosphate accumulation when co-expressed with either vector, GRK5, GRK5-GFP^2, GRK2, or GRK2-GFP^2 (figure 1C). Co-expression of GRK5 or GRK5-GFP^2 did not influence the signaling of the NK-1R(1-324) construct while GRK2 and GRK2-GFP^2 co-expression attenuated NK-1R(1-324) mediated inositol phosphate accumulation, although to a lesser degree than observed with the wild type receptor (max signal with GRK2 co-expression relative to control was 2% and 28% for NK1R(wt) and NK1(1-324), respectively). Thus, the data suggests that the GRK5 effect on NK-1 receptor signaling is dependent on intact GRK5 kinase activity and on the presence of the receptor C-terminal tail, while GRK2 appears to have both phosphorylation-dependent and phosphorylation-independent effects on NK-1 receptor signaling.

To ensure that the NK-1 receptor is not constitutively desensitized when co-expressed with GRK2 or GRK5 we analyzed whether the receptor can generate a calcium signal the initial 50 seconds after agonist addition. As seen in figure 2 both wild type NK-1 receptor and NK1R-RLuc co-expressed with either vector, GRK2, or GRK5 mediates a calcium signal immediately following agonist addition. Thus, GRK co-expression does not cause constitutive desensitization of the NK-1 receptor although a suppression of the max signal is observed in the presence of GRK co-expression (figure 2). As the time-dependent decrease of the peak in agonist induced calcium signal reflects receptor desensitization as well as post-receptor mechanisms we normalized the signals to percent

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of max signal for optimal comparison (figure 2 insert) (Kubale *et al.*, 2007). Co-expression of GRK2 or GRK5 caused the calcium signal to decrease with faster kinetics relative to co-expression with vector indicating that the receptor desensitizes faster when GRKs are co-expressed (figure 2 insert). Furthermore, the data suggests that the NK1R-RLuc fusion is comparable to NK-1R(wt) in the ability to generate an agonist induced calcium signal (figure 2).

We next assessed the interaction between NK1R-RLuc and GRK5-GFP^2 in transfected HEK293 cells by analyzing the BRET^2 signal at progressive time points after addition of 10 nM SP – an agonist concentration which we found to have full efficacy in inducing recruitment of βarr2 to the NK-1 receptor (supplementary figure 1). When measuring BRET^2 between NK1R-RLuc and GRK5-GFP^2 we observed a relatively high basal signal that increased to reach a transient peak at ~20 seconds after agonist addition and returning to basal after ~1 minute (figure 3A). To analyze the interaction between the NK-1 receptor and GRK5 in more detail we performed BRET^2 saturation experiments. In these experiments the amount of the GFP^2-fused protein is gradually increased relative to a steady amount of the RLuc-fused protein (Gales *et al.*, 2006;Mercier *et al.*, 2002). In case of a specific interaction between the tagged proteins the BRET^2 signal should display a hyperbolic curve as a function of the GFP^2/RLuc expression ratio (Gales *et al.*, 2006;Mercier *et al.*, 2002). In contrast, non-specific background arising from random collisions of the tagged proteins ("bystander BRET") will result in a BRET^2 signal that depends linearly on the GFP^2/RLuc expression ratio (Gales *et al.*, 2006;Mercier *et al.*, 2002). Interestingly, we observed a hyperbolic curve between GRK5-GFP^2 and the

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NK1R-RLuc indicating that there is a basal association of GRK5 with the resting (i.e. receptor before agonist exposure) NK-1 receptor (figure 3B). The GFP/RLuc ratio resulting in half maximal saturation is referred to as the BRET50 value and is a measure of the affinity in arbitrary units (Gales *et al.*, 2006;Mercier *et al.*, 2002). Hence, $BRET^2$ saturation experiments are suitable for comparing affinities on a relative scale. Twenty seconds of receptor stimulation with 10 nM SP (corresponding to the time point of the peak in the $BRET^2$ signal between GRK5-GFP^2 and NK1R-RLuc) caused the curve to saturate with a lower BRET50 value reflecting increased affinity of GRK5 for the receptor (BRET50 values of 0.19 and 0.48 with and without agonist addition, respectively). Thus, the $BRET^2$ saturation data are consistent with the time course experiments and demonstrate that GRK5 is recruited during receptor activation to form a more close complex with the NK-1 receptor ~20 seconds after receptor activation. As some random interaction will occur between any proteins it was important to control that the apparent specific interaction between GRK5 and the resting receptor did not simply arise from bystander $BRET^2$ between two membrane localized proteins. The tail truncated NK-1(1-324) which, in the inositol phosphate signaling assay, was observed to be signaling competent but not regulated by GRK5 was C-terminally RLuc tagged for the experiments (NK1R(1-324)-RLuc). The NK1R(1-324)-RLuc construct mediated agonist induced inositol phosphate accumulation virtually similar to the full length NK1R-RLuc, demonstrating that also the RLuc fused tail truncated receptor construct was expressed and signaling competent (supplementary figure 2). When performing $BRET^2$ saturation experiments with the truncated NK1R(1-324)-RLuc construct and GRK5-GFP^2 we observed a linear curve as a function of the GFP^2/RLuc ratio both before and after agonist

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addition, suggesting that the NK1R(1-324)-RLuc construct did not interact with GRK5 (figure 3B). Importantly, it furthermore suggested that the BRET^2 signal between NK1R-RLuc and GRK5-GFP^2 is not simply caused by both proteins being localized to the membrane. We further proceeded to characterize the interaction pattern of the kinase inactive GRK5(K215R) mutant with the NK-1 receptor. Surprisingly, when performing time course experiments with NK1R-RLuc and GRK5(K215R)-GFP^2, we did not observe an agonist induced increase in the BRET^2 signal but rather a time-dependent decrease in the signal reaching a plateau ~1 minute after agonist addition (figure 3C). Furthermore, the basal BRET^2 signal was higher than observed with the NK1R-RLuc / GRK5-GFP^2 combination (figure 3). When performing BRET^2 saturation experiments with the NK1R-RLuc and GRK5(K215R)-GFP^2 we observed the BRET^2 signal, without agonist addition, to describe a slightly hyperbolic curve as a function of the GRK5(K215R)-GFP^2 / NK1R-RLuc ratio (figure 3D). Compared to GRK5-GFP^2 interaction with NK1R-RLuc the basal BRET^2 signal for GRK5(K215R)-GFP^2 was higher over a range of expression ratios but it did not reflect a higher affinity phenotype (figure 3 B and D). Twenty seconds after addition of the agonist SP the maximum BRET^2 signal decreased without concomitant changes in the shape of the curve (figure 3D). Thus, the BRET^2 data suggest that the GRK5(K215R) mutant is organized in a low affinity association with the resting NK-1 receptor and that the mutant is not further recruited to the receptor after receptor activation.

We proceeded by characterizing the interaction between the NK-1 receptor and GRK2. When performing BRET^2 time course experiments with NK1R-RLuc and GRK2-GFP^2

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we observed a rapid recruitment of GRK2-GFP^2 to the NK1R-RLuc (figure 4A). The signal peaked transiently at 10-20 seconds after agonist addition and then stabilized at a lower level (figure 4A). When performing BRET^2 saturation experiments, the BRET^2 signal after agonist addition described a hyperbolic curve as a function of GRK2-GFP^2 / NK1R-RLuc that was best fitted with a two phase exponential association model (BRET50 values of 0.015 and 0.17 for the high affinity and low affinity phase respectively, measured 5 minutes after agonist addition) (figure 4B). Interestingly, the curve after 20 seconds of agonist addition (corresponding to the peak in the BRET^2 signal in the time course measurements) was similar to the curve after 5 minutes of agonist addition, suggesting that the transient peak does not describe a higher affinity phenotype of the GRK2 / NK-1 receptor complex relative to the signal plateau (figure 4B). As a control we performed BRET^2 saturation experiments with GRK2-GFP^2 and the receptor tail truncated NK1R(1-324)-RLuc. Without agonist addition the BRET^2 signal displayed a linear curve as a function of the GRK2-GFP^2 / NK1R(1-324)-RLuc (figure 4B). Twenty seconds of agonist addition caused a slight increase in BRET^2 signal which may suggest that there is a low level of agonist dependent GRK2 recruitment to the NK1R(1-324) (figure 4B). To analyze the dependency of GRK2 kinase activity for the observed interaction with the NK-1 receptor we performed BRET^2 time course experiments of NK1R-RLuc interaction with the kinase inactive GRK2 mutant GRK2(K220R)-GFP^2 after addition of 10 nM SP. In contrast to the results obtained with GRK5, the kinase inactive GRK2(K220R) mutant was recruited to the activated NK-1 receptor in a pattern similar to the wild type GRK2 (figure 4C). We proceeded by performing BRET^2 saturation experiments with GRK2(K220R)-GFP^2 and NK1R-RLuc. Interestingly, we

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observed GRK2(K220R) interaction with the NK-1 receptor to saturate in a monophasic manner with a BRET50 value of 0.019 corresponding to the high affinity phase of GRK2 interaction with the NK-1 receptor (figure 4D). Thus, the biphasic nature of the $BRET^2$ saturation curve for GRK2 interaction with the NK-1 receptor appears to consist of a kinase-independent and a kinase-dependent phase.

To analyze whether the difference in phenotype of GRK2 and GRK5 interaction with the NK-1 receptor depends on the difference in cellular localization of the two GRKs we made a membrane localized GRK2-GFP^2 (GRK2-GFP^2-Membrane) by fusing the C-terminal 17 amino acid residues from human K-ras to the C-terminus of GRK2-GFP^2. This sequence contains a polybasic signal and a prenylation sequence that in combination ensures localization to the plasma membrane (Hancock *et al.*, 1991). We observed co-expression with GRK2-GFP^2-Membrane to suppress NK-1 receptor mediated inositol phosphate accumulation to less than one percent of control, demonstrating that the construct was functional (data not shown). The $BRET^2$ signal was observed to saturate in a monophasic manner as a function of GRK2-GFP^2-Membrane / NK1R-RLuc (figure 4E). After agonist addition the saturation curve was best fitted with a two phase exponential association model ($p=0.0005$) with the additional high affinity phase as the minor phase (figure 4E insert). Thus, when GRK2 is artificially membrane localized it displays basal interaction with the NK-1 receptor but some agonist induced recruitment can still be observed.

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As the BRET^2 data do not reveal where in the cell an interaction occurs we performed confocal microcopy with a FLAG-tagged NK-1 receptor and either GRK5-GFP^2 or GRK2-GFP^2 to see whether the proteins dissociate after receptor internalization. We observed the unstimulated NK-1 receptor to be localized to the plasma membrane in a clustered pattern (figure 5 B and C). As expected, GRK5-GFP^2 was also membrane localized and some clustering was observed in a pattern overlapping NK-1 receptor localization (figure 5 A and C). After five minutes of stimulation with 10 nM SP, extensive NK-1 receptor internalization was observed without concomitant internalization of GRK5 (figure 5 D-F). Thus, GRK5 co-localize with the NK-1 receptor in membrane clusters but it does not co-internalize with the receptor. When FLAG-NK1R was co-expressed with GRK2-GFP^2 we again observed extensive receptor internalization (supplementary figure 3). Although GRK2-GFP^2 did not appear to co-internalize with the receptor, the cytoplasmatic localization of GRK2-GFP^2 made it difficult to conclude on this issue (supplementary figure 3). Therefore, we took advantage of the membrane localization of the GRK2-GFP^2-Membrane construct to analyze the internalization pattern. We observed GRK2-GFP^2-Membrane to be localized to the plasma membrane as expected (figure 5 G). Although surface localization of FLAG-NK1R before agonist addition was still observed when co-expressed with GRK2-GFP^2-Membrane there was relatively more constitutively internalized receptor compared to co-expression with GRK5-GFP^2 or GRK2-GFP^2 (compare figure 5 panel B and H and supplementary figure 3 panel B). Following 5 minutes of agonist exposure the receptor internalized without any apparent concomitant internalization of GRK2-GFP^2-Membrane (figure 5 J-L). Hence,

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GRK2-GFP^2-Membrane appears to increase constitutive internalization of the NK-1 but does not co-internalize with the receptor.

To analyze the influence of receptor internalization on the observed pattern of GRK2 and GRK5 interaction with the NK-1 receptor we performed BRET^2 time course experiments in the presence 450 mM sucrose to inhibit receptor internalization (Heuser and Anderson, 1989). The BRET^2 assay was not affected by addition of sucrose to the assay buffer (data not shown). In a control experiment we found 450 mM sucrose to significantly inhibit the kinetics of agonist induced NK-1 receptor internalization and to inhibit the maximal internalization (supplementary figure 4). Five minutes after agonist addition hypertonic sucrose caused ~65% inhibition of receptor internalization relative to control (supplementary figure 4). Both when measuring GRK5-GFP^2 and GRK2-GFP^2 recruitment to NK1R-RLuc (figure 6A and 6B, respectively) we found the addition of sucrose in the buffer to cause a considerable increase in the basal BRET^2 signal. However, the pattern of the agonist induced BRET^2 signal as a function of time did not differ between the experiments with or without addition of sucrose to the buffer. Thus, the observed decrease of the peak BRET^2 signal between the NK-1 receptor and GRK5 or GRK2 in time course experiments appears to be independent of receptor internalization.

As it is unknown whether GRK subtypes compete directly for the receptor substrate we performed BRET^2 competition experiments. In these experiments an untagged protein is co-expressed with the pair of tagged proteins to analyze for competition (Marullo and Bouvier, 2007). To analyze for competition of GRK5 recruitment to the NK-1 receptor

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we co-expressed NK1R-RLuc with GRK5-GFP^2 and either vector, GRK2(wt), GRK2(K220R), GRK5(wt), or GRK5(K215R) (figure 7A). Interestingly, only GRK5(wt) competed for agonist induced GRK5-GFP^2 interaction with NK1R-RLuc (figure 7A). The ratio of GFP^2/RLuc did not significantly differ between the transfection conditions and were within the saturated range (range of GFP^2/RLuc was 0.61-0.86) (supplementary figure 5A). When analyzing the ability of untagged proteins to compete for agonist induced recruitment of GRK2-GFP^2 to NK1R-RLuc we found both GRK2(wt), GRK2(K220R), GRK5(wt), and GRK5(K215R) to compete (figure 7B). Compared to GRK2(wt), GRK2(K220R), and GRK5(wt), untagged GRK5(K215R) was far less efficacious in the ability to compete for agonist induced recruitment of GRK2-GFP^2 to NK1R-RLuc and the competition only reached significance at time points 20 and 45 seconds after agonist addition (figure 7B). The range of GFP^2/RLuc was 0.83-1.39 (plus vector = 1.1) and thus within the saturated area (supplementary figure 5B).

Discussion

We here characterize the interaction pattern of GRK2 and GRK5 with the NK-1 receptor. Although the role of GRKs in phosphorylating activated 7TM receptors thereby promoting binding of βarrs is well established (Luttrell and Lefkowitz, 2002;Pao and Benovic, 2002), details of the interaction pattern of GRK subtypes with a 7TM receptor have never been compared.

In the analysis of GRK effect on NK-1 receptor inositol phosphate signaling we observed both GRK2 and GRK5 to attenuate NK-1 receptor signaling. Interestingly, GRK2

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appeared to have a kinase-independent effect on NK-1 receptor signaling. In contrast, the observed effect of GRK5 on NK-1 receptor signaling appeared to be kinase-dependent. A kinase-independent effect of GRK2 is not unprecedented as GRK2 and GRK3, in several instances, have been reported to exert phosphorylation-independent roles in 7TM receptor desensitization (Pao and Benovic, 2002). GRK2 and 3 (but not GRK5) can bind G-protein Gαq/11 and Gβγ subunits and the phosphorylation-independent effect on receptor desensitization is thought to originate from sequestering of these G-protein subunits and stimulation of G-protein GTPase activity (Carman *et al.*, 1999b;Pao and Benovic, 2002;Pitcher *et al.*, 1992;Kunapuli and Benovic, 1993). In fact, GRK2 and 3 are reported to depend on interaction with liberated Gβγ subunits for recruitment to activated 7TM receptors (Pitcher *et al.*, 1992). In a BRET^2 assay we observed limited agonist induced interaction between a tail truncated NK-1R(1-324) construct and GRK2 which indicates that the effect of GRK2 on G-protein signaling of this receptor construct is not based on receptor interaction *per se*. This is consistent with the paradigm that kinase-independent effects of GRK2 is mediated by interaction with G-protein subunits rather than direct modulation of the receptor. Furthermore, the results suggest that the NK-1 receptor C-terminal tail is necessary for quantitative GRK2 interaction.

When analyzing GRK interaction with the NK-1 receptor we observed several interesting differences in the GRK subtype specific interaction patterns. Agonist induced interaction of GRK5 with the NK-1 receptor was kinase dependent and saturated in a monophasic manner while GRK2 saturated in a biphasic manner with the high affinity phase being independent of kinase activity. It is possible that the high affinity phase of GRK2

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interaction with the activated NK-1 receptor represents initial GRK2 recruitment while the lower affinity phase represents a conformational change promoted by phosphorylation. It is interesting to note that the monophasic interaction of GRK5 with the activated receptor appears to correspond to the kinase-dependent phase of GRK2 interaction with the receptor (BRET50 values of 0.19 and 0.17 respectively). Thus, our data suggests a difference in recruitment modes for GRK2 and GRK5 to the activated NK-1 receptor.

$BRET^2$ saturation experiments showed the basal $BRET^2$ signal for NK-1 receptor interaction to be higher, at the same expression ratios, for GRK5(K215R) compared to GRK5. As inhibition of receptor internalization with hypertonic sucrose was observed to increase the basal $BRET^2$ signal it is possible that GRK5(wt), but not GRK5(K215R), may promote constitutive phosphorylation with subsequent receptor internalization for a subpopulation of receptors, thereby decreasing the basal GRK5 / NK-1 receptor $BRET^2$ signal. The high basal $BRET^2$ signal declined after receptor activation indicating that GRK5(K215R), like GRK5(wt), is localized close to the resting receptor and that dissociation/removal occurs after activation. The lack of agonist induced receptor recruitment of GRK5(K215R) was surprising and clearly distinguishes it from the kinase-inactive GRK2(K220R) mutant. However, in saturation experiments, GRK5 lacks the high affinity phase of receptor interaction which is seen for GRK2 and retained in GRK2(K220R).

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Interestingly, our BRET^2 saturation data indicates that GRK5 is pre-associated with the resting NK-1 receptor and that receptor activation causes recruitment of GRK5 to a higher affinity complex with the receptor. The results do not allow us to distinguish between true translocation of GRK5 to the activated receptor from a nearby position, in for example a microdomain structure, or activation dependent reorganization of a preexisting GRK5/NK-1R complex. However, the BRET^2 sensitive range of ~100Å corresponds to the diameter of only 2-3 7TM receptors in the membrane plane suggesting relatively close proximity (Palczewski *et al.*, 2000). The notion that GRK5 may associate with resting 7TM receptors is further supported by reports which describe that overexpression of GRK5 but not GRK2 can increase basal receptor phosphorylation (Premont *et al.*, 1994;Shetzline *et al.*, 1998;Shetzline *et al.*, 2002;Diviani *et al.*, 1996). Additionally, GRK5 sedimentation experiments with phospholipids versus rhodopsin containing membranes have indicated that GRK5 can bind directly to receptors in the membrane (Pronin and Benovic, 1997). When analyzing the interaction between the NK-1 receptor and a membrane-localized GRK2 construct we also observed an agonist independent monophasic hyperbolic saturation curve suggesting basal interaction. This indicates that the ability to pre-associate with the NK-1 receptor depends on subcellular localization rather than properties inherent to specific GRK subtypes. However, several differences in the phenotype of GRK5 and membrane attached GRK2 was observed. After receptor activation the interaction of the NK-1 receptor with GRK2-GFP^2-Membrane was best fitted with a two phase association model which corresponds to the phenotype of GRK2. Furthermore, co-expression of the NK-1 receptor and GRK2-GFP^2-Membrane was in confocal microscopy experiments observed to cause an increase in

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constitutive receptor internalization that was not as apparent with GRK2-GFP^2 or GRK5-GFP^2 co-expression indicating that GRK2-GFP^2-Membrane have constitutive effects on the NK-1 receptor.

When performing confocal microscopy we observed co-localization of GRK5 and the resting NK-1 receptor to the plasma membrane (including some clusters) and GRK5 did not co-internalize with the NK-1 receptor, thereby demonstrating that the proteins dissociate before or during receptor internalization. Similarly, we did not observe co-internalization of GRK2 (or membrane associcated GRK2) and the NK-1 receptor, suggesting that the plateau in BRET^2 signal, observed in time course experiments for GRK2 interaction with the NK-1 receptor, reflects a steady state rather than quantitative co-internalization. Following receptor internalization into cytoplasmatic vesicles, increased random interaction with the cytosolic GRK2 may occur which would cause the background BRET^2 signal to increase. Inhibition of receptor internalization with hypertonic sucrose did not prevent or delay the time dependent decrease in BRET^2 signals for agonist dependent receptor interaction with either GRK2 or GRK5, suggesting that dissociation of GRK/NK-1R complexes occurs before receptor internalization.

BRET^2 competition experiments suggested that GRK5 can compete for agonist induced GRK2 recruitment to the NK-1 receptor while GRK2 does not compete for GRK5 recruitment to the activated receptor. This demonstrates that GRK subtypes do compete for the receptor substrate and suggests that the competition is orchestrated by more than simply relative amounts of GRK subtypes or relative affinities for the activated receptor.

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Indeed, the competition data would support a hypothesis that GRK5 pre-association with the NK-1 receptor, causing a local high concentration of GRK5, gives an advantage of proximity that outweighs the higher affinity of GRK2 for the activated receptor. The competition between GRK subtypes could be both steric and functional. Steric competition between large proteins that interact with the same domain of the receptor would be expected and GRK5 mediated receptor phosphorylation and subsequent uncoupling would prevent further GRK2 recruitment as GRK2 depends on G-protein activation for translocation (Pitcher *et al.*, 1992).

The untagged GRK2(K220R) mutant was observed to compete for GRK2-GFP^2 recruitment to the NK-1 receptor, thereby further supporting reports that the GRK2(K220R) mutant is dominant negative (Diviani *et al.*, 1996;Kong *et al.*, 1994). However, as untagged GRK5(K215R), did not compete for GRK5-GFP^2 recruitment to the activated NK-1 receptor, our results do not support the use of the GRK5(K215R) mutant as a dominant negative tool. It remains possible that GRK5(K215) has a competitive effect on basal GRK5 pre-association with the resting receptor that we do not see in our assay. As the GRK constructs may have an effect on constitutive receptor internalization which the basal BRET^2 signal appears to be highly sensitive to, we can not determine whether effects of overexpressed untagged GRKs on the basal BRET^2 signal reflects competition or changes in the ratio of surface receptor/total receptor.

Our data suggest that GRK5 rather than GRK2 is a key player in the regulation of GRK subtype specific interaction with the NK-1 receptor. As the ability of GRK5 to regulate

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GRK2 interaction with the receptor appears to be largely dependent on kinase activity one could speculate that inhibition of GRK5 kinase activity would not only prevent GRK5 mediated receptor phosphorylation but furthermore allow GRK2 mediated phosphorylation. In this context it is interesting to note that GRK5 kinase activity can be inhibited by calcium/calmodulin and structural proteins such as actin, α-actinin and caveolin (Carman *et al.*, 1999a;Freeman *et al.*, 1998;Freeman *et al.*, 2000;Pronin *et al.*, 1997).

In conclusion, we demonstrate differences in affinity, kinase dependency, and level of basal interaction for GRK2 and GRK5 interaction with the NK-1 receptor. We suggest that, rather than relative amounts of GRK subtypes, GRK5 pre-association with the receptor is involved in the orchestration of subtype specific GRK regulation of receptor signaling. It will be interesting to see future research delineate in more detail how the complex interaction pattern between receptors and GRKs, as well as other accessory proteins, is biologically regulated and how this contribute to the regulation of receptor signaling.

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Figure legends

Figure 1: Agonist induced inositol phosphate accumulation.

Dose response curves for agonist induced inositol phosphate accumulation in HEK293 cells transiently transfected with cDNA encoding the NK-1 receptor and the indicated cDNAs. A, SP induced inositol phosphate accumulation in cells with NK-1 receptor co-expressed with either vector, GRK2, GRK2-GFP^2, GRK2(K220R), or GRK2(K220R)-GFP^2. B, SP induced inositol phosphate accumulation in cells with NK-1 receptor co-expressed with either vector, GRK5, GRK5-GFP^2, GRK5(K215R), or GRK5(K215R)-GFP^2. C, SP induced inositol phosphate accumulation in cells with tail truncated NK-1(1-324) receptor co-expressed with either vector, GRK5, GRK5-GFP^2, GRK2, or GRK2-GFP^2. The response is expressed as percent of the max response for the receptor co-transfected with empty vector. The curve for NK-1R+vector in A and B is identical. Results are the means ± SEM of duplicate determinations in three independent experiments.

Figure 2: Agonist induced calcium signal.

Kinetics of agonist (10 nM SP) induced calcium signal in transiently transfected HEK293 cells. The cells were either transfected with cDNAs encoding NK1R(wt)+ vector or NK1R-RLuc co-transfected with either vector, GRK2-GFP^2, or GRK5-GFP^2 as indicated (cDNA ratio of 1:2). The inserted figure shows the signals normalized to percent of maximum generated calcium signal for each transfection condition. Data represents the

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mean ±SEM for a single experiment carried out in hexaplicate determinations and are representative of a total of three independent experiments.

Figure 3: $BRET^2$ measurements with the NK-1 receptor and GRK5 constructs.
A, time course of GRK5-GFP^2 interaction with NK1R-RLuc after the addition of 10 nM SP. B, $BRET^2$ saturation experiments with increasing amounts of GRK5-GFP^2 relative to either NK1R-RLuc or NK1R(1-324)-RLuc. Measurements were made without receptor stimulation (buffer) or stimulation with 10 nM SP for 20 seconds. C, time course of GRK5(K215R)-GFP^2 interaction with NK1R-RLuc after the addition of 10 nM SP. D, $BRET^2$ saturation experiments with increasing amounts of GRK5(K215R)-GFP^2 relative to NK1R-RLuc. Measurements were made without receptor stimulation (buffer) or stimulation with 10 nM SP for 20 seconds. A and C, results are the means ± SEM of duplicate determinations in at least four independent experiments. B and D, data are pooled from at least three independent experiments.

Figure 4: $BRET^2$ measurements with the NK-1 receptor and GRK2 constructs.
A, time course of GRK2-GFP^2 interaction with NK1R-RLuc after the addition of 10 nM SP. B, $BRET^2$ saturation experiments with increasing amounts of GRK2-GFP^2 relative to either NK1R-RLuc or NK1R(1-324)-RLuc. Measurements were made without receptor stimulation (buffer) or stimulation with 10 nM SP for 20 seconds or 5 minutes. C, time course of GRK2(K220R)-GFP^2 interaction with NK1R-RLuc after the addition of 10 nM SP. D, $BRET^2$ saturation experiments with increasing amounts of GRK2(K220R)-GFP^2 relative to NK1R-RLuc. Measurements were made without receptor stimulation (buffer)

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or stimulation with 10 nM SP for 20 seconds. E, $BRET^2$ saturation experiments with increasing amounts of GRK2-GFP^2-Membrane relative to NK1R-RLuc. Measurements were made without receptor stimulation (buffer) or stimulation with 10 nM SP for 20 seconds. Insert in (E) shows amplification of the areas of the curve marked with a dotted box. A and C, Results are the means ± SEM of duplicate determinations in at least three independent experiments. B, D, and E, data are pooled from at least three independent experiments.

Figure 5: Confocal microscopy in HEK293 cells co-expressing FLAG-NK-1 receptor and either GRK5-GFP^2 (A-F) or GRK2-GFP^2-Membrane (G-L). Visualization of GRK5-GFP^2 (A and D) or FLAG-NK-1 receptor (B and E) in co-expressing cells that have been stimulated with either vehicle (A-C) or 10 nM SP (D-F) for five minutes at 37º C. C and F are overlays of A plus B and D plus E, respectively. Visualization of GRK2-GFP^2-Membrane (G and J) or FLAG-NK-1 receptor (H and K) in co-expressing cells that have been stimulated with either vehicle (G-I) or 10 nM SP (J-L) for five minutes at 37º C. I and L are overlays of G plus H and J plus K, respectively. Panels to the right of C and I represents amplifications of the areas marked with a white box in the respective panels. White bars corresponds to a 10 µm scale.

Figure 6: Effect on $BRET^2$ signals of inhibition of receptor internalization by hypertonic sucrose.

A, time course of GRK5-GFP^2 interaction with NK1R-RLuc after the addition of 10 nM SP with or without addition of 450 mM sucrose to the assay buffer as indicated. B, time

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course of GRK2-GFP^2 interaction with NK1R-RLuc after the addition of 10 nM SP with or without addition of 450 mM sucrose to the assay buffer as indicated. Results are the means ± SEM of duplicate determinations in three independent experiments.

Figure 7: BRET^2 competition experiments.

Time course of GRK5-GFP^2 (A) or GRK2-GFP^2 (B) interaction with NK1R-RLuc after the addition of 10 nM SP. The Cells were co-transfected with the indicated cDNAs to analyze for the ability to compete with the GFP^2-fused GRKs for NK1R-Luc interaction. Results are the means ± SEM of duplicate determinations in five (A) or four (B) independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ versus values for co-transfection with vector (unpaired students t-test).

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Fig. 1

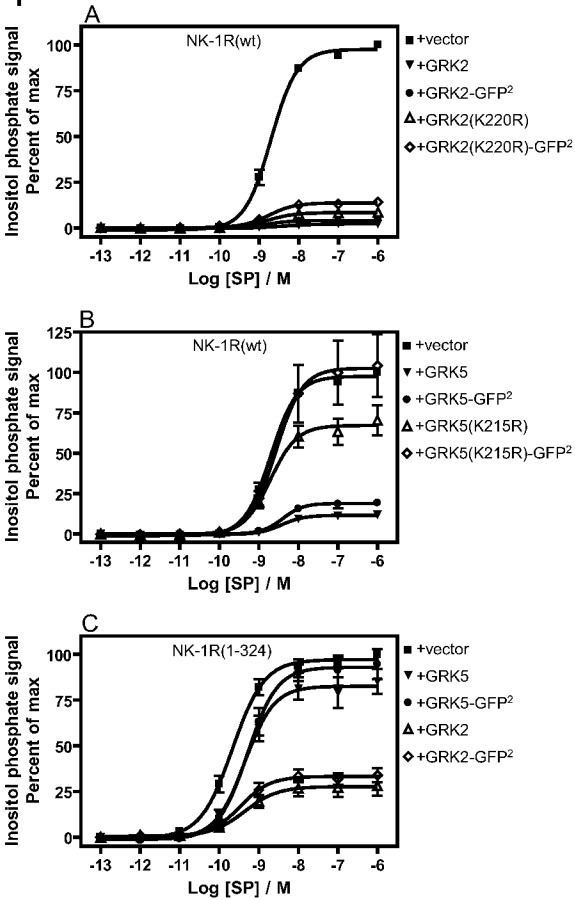
A
NK-1R(wt)
Inositol phosphate signal
Percent of max
100
75
50
25
0
-13 -12 -11 -10 -9 -8 -7 -6
Log [SP] / M
+vector
+GRK2
+GRK2-GFP2
+GRK2(K220R)
+GRK2(K220R)-GFP2
B
NK-1R(wt)
Inositol phosphate signal
Percent of max
125
100
75
50
25
0
-13 -12 -11 -10 -9 -8 -7 -6
Log [SP] / M
+vector
+GRK5
+GRK5-GFP2
+GRK5(K215R)
+GRK5(K215R)-GFP2
C
NK-1R(1-324)
Inositol phosphate signal
Percent of max
100
75
50
25
0
-13 -12 -11 -10 -9 -8 -7 -6
Log [SP] / M
+vector
+GRK5
+GRK5-GFP2
+GRK2
+GRK2-GFP2

Fig. 2

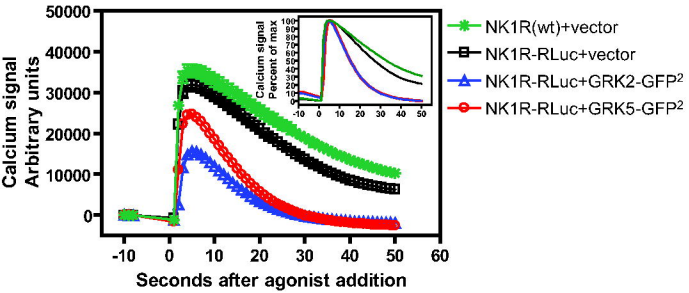
Calcium signal
Arbitrary units
50000
40000
30000
20000
10000
0
-10
0
10
20
30
40
50
60
Seconds after agonist addition
Calcium signal
Percent of max
NK1R(wt)+vector
NK1R-RLuc+vector
NK1R-RLuc+GRK2-GFP2
NK1R-RLuc+GRK5-GFP2

Fig. 3

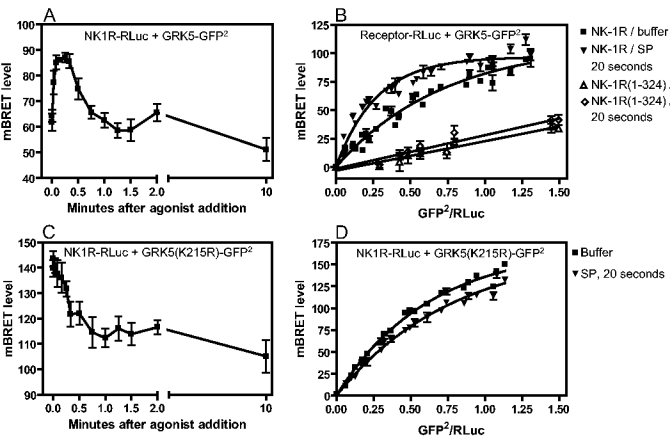
A
NK1R-RLuc + GRK5-GFP2
mBRET level
Minutes after agonist addition
B
Receptor-RLuc + GRK5-GFP2
mBRET level
GFP2/RLuc
NK-1R / buffer
NK-1R / SP
20 seconds
NK-1R(1-324) / buffer
NK-1R(1-324) / SP
20 seconds
C
NK1R-RLuc + GRK5(K215R)-GFP2
mBRET level
Minutes after agonist addition
D
NK1R-RLuc + GRK5(K215R)-GFP2
mBRET level
GFP2/RLuc
Buffer
SP, 20 seconds

Fig. 4

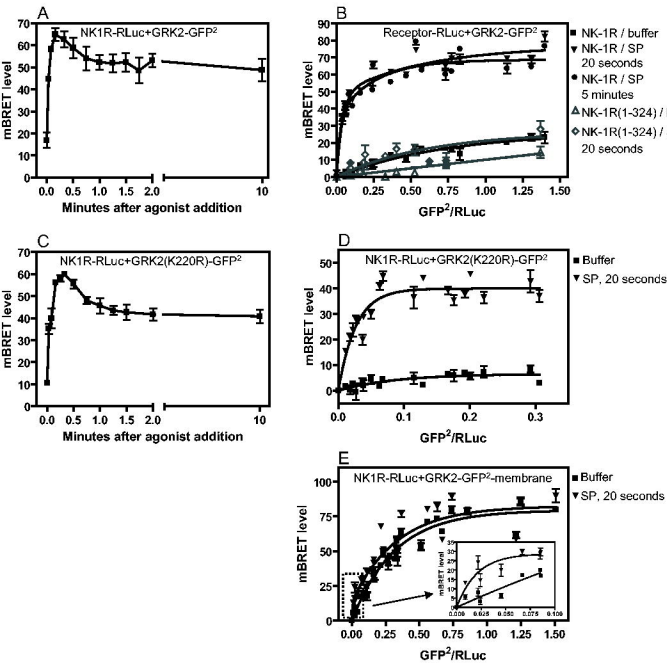
A
NK1R-RLuc+GRK2-GFP2
mBRET level
Minutes after agonist addition
B
Receptor-RLuc+GRK2-GFP2
mBRET level
GFP2/RLuc
NK-1R / buffer
NK-1R / SP 20 seconds
NK-1R / SP 5 minutes
NK-1R(1-324) / buffer
NK-1R(1-324) / SP 20 seconds
C
NK1R-RLuc+GRK2(K220R)-GFP2
mBRET level
Minutes after agonist addition
D
NK1R-RLuc+GRK2(K220R)-GFP2
mBRET level
GFP2/RLuc
Buffer
SP, 20 seconds
E
NK1R-RLuc+GRK2-GFP2-membrane
mBRET level
GFP2/RLuc
Buffer
SP, 20 seconds

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Fig. 5

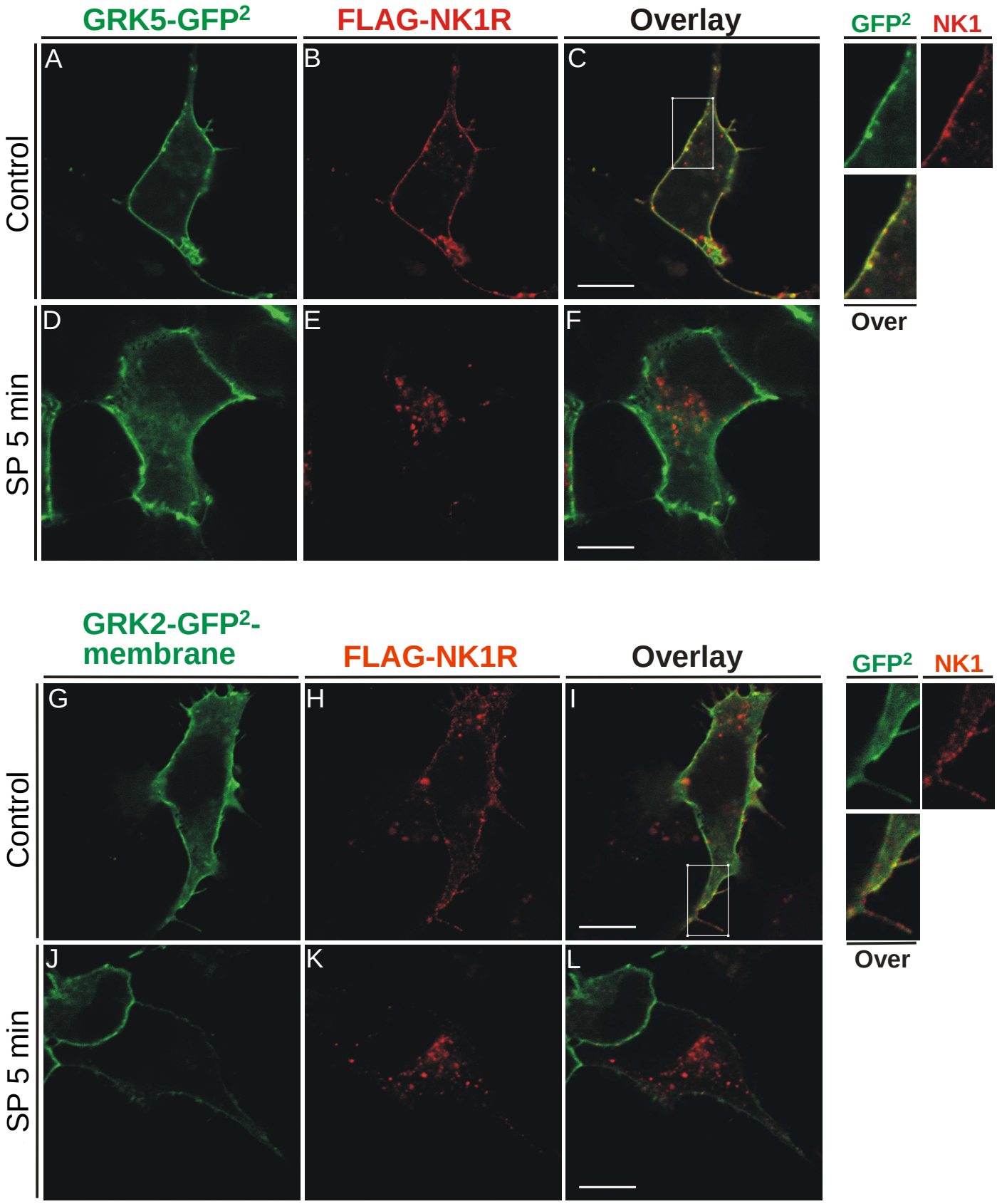

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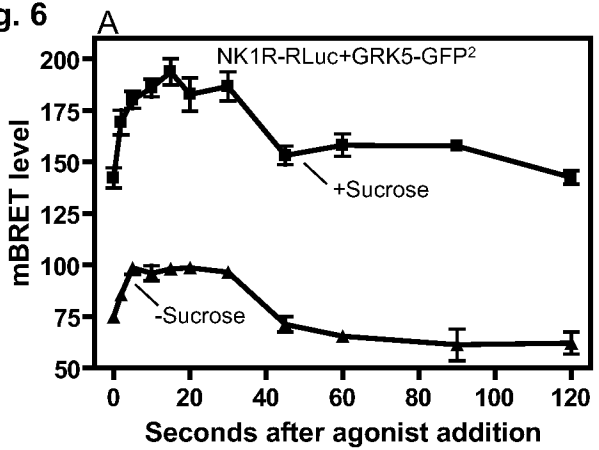
Fig. 6


A
NK1R-RLuc+GRK5-GFP2
+Sucrose
-Sucrose
mBRET level
200
175
150
125
100
75
50
0
20
40
60
80
100
120
Seconds after agonist addition

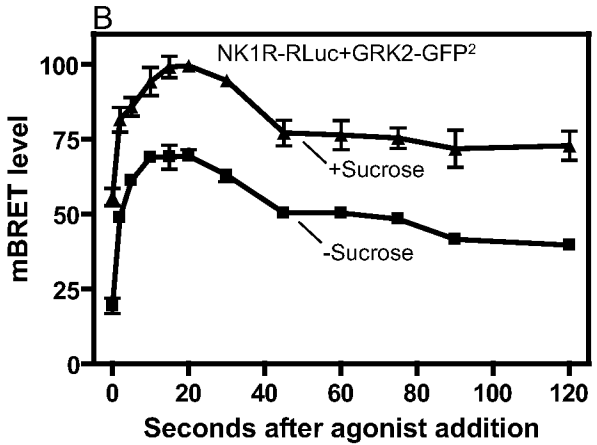
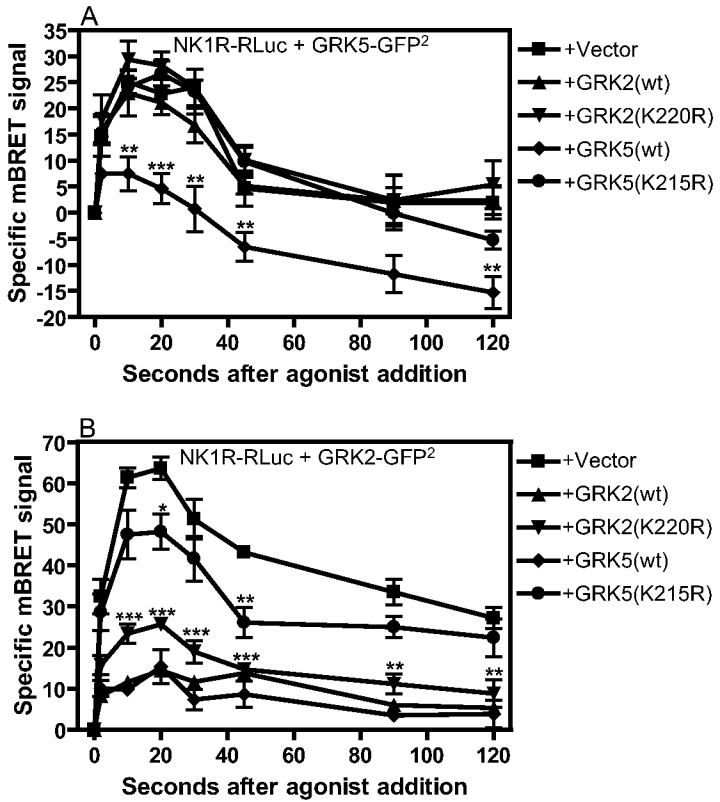

B
NK1R-RLuc+GRK2-GFP2
+Sucrose
-Sucrose
mBRET level
100
75
50
25
0
0
20
40
60
80
100
120
Seconds after agonist addition

Fig. 7


A
NK1R-RLuc + GRK5-GFP2
Specific mBRET signal
Seconds after agonist addition
+Vector
+GRK2(wt)
+GRK2(K220R)
+GRK5(wt)
+GRK5(K215R)
B
NK1R-RLuc + GRK2-GFP2
Specific mBRET signal
Seconds after agonist addition
+Vector
+GRK2(wt)
+GRK2(K220R)
+GRK5(wt)
+GRK5(K215R)