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► **To cite this version:**

Emeline Maillet, Nadia Pelligrini, Bernard Bucher, Marcel Hibert, Jean-Jacques Bourguignon, et al.. DUAL MODULATION OF NK2 TACHYKININ RECEPTOR SIGNALLING PATHWAYS BY AN ALLOSTERIC COMPOUND. Running Title: Dual allosteric modulation of NK2R. FASEB Journal, 2007. hal-02196081

**HAL Id: hal-02196081**

**<https://hal.science/hal-02196081>**

Submitted on 26 Jul 2019

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## DUAL MODULATION OF NK2 TACHYKININ RECEPTOR SIGNALLING PATHWAYS BY AN ALLOSTERIC COMPOUND

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Running Title: Dual allosteric modulation of NK2R

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**When stimulated by its natural agonist, neurokinin A, the tachykinin NK2 receptor undergoes stabilization in two active states that mediate distinct intracellular responses, a phospholipase C-mediated calcium response and an adenylyl cyclase-mediated cAMP production. Here, we show that the new compound LPI 805 modulates not only binding of agonists, but also differentially affects calcium and cAMP responses in opposite ways.**

**LPI 805 has no biological activity by itself. It accelerates dissociation kinetics of bound neurokinin A by altering the equilibrium between the two active states of the NK2 receptor. In co-application with agonists, LPI 805 potentiates calcium responses and simultaneously decreases, or even abolishes, cAMP-dependent responses of NK2 receptors. LPI 805 thus behaves as an allosteric modulator that exhibits dual positive/negative response regulation of the NK2 tachykinin receptor. These data not only strengthen to the notion of multiple active, and interconvertible conformations of G protein-coupled receptors, but also opens new ways to design pharmacological agents able to selectively modulate a subset of intracellular responses linked to members of this important receptor family.**

Tachykinins form a family of peptides released in many regions of the central and peripheral nervous system. They are involved in diverse functions such as nociception, neurogenic inflammation, smooth muscle contraction, vasodilation, chemotaxis and activation of immune cells (2,3). The tachykinins SP, NKA and NKB exhibit preferential binding to three G-protein coupled receptor (GPCR) subtypes: NK1R, NK2R and NK3R respectively, which are target to drugs

aimed at treating various disorders such as of asthma, emesis, irritable bowel syndrome, chronic pain (fibromyalgia, rheumatoid arthritis) as well as anxiety and depression (4,5).

A growing number of molecules interacting with GPCRs do not interact at the natural ligand (orthosteric) binding site. Rather, such compounds interact elsewhere on the receptor to modulate its activity: they are positive or negative allosteric ligands (6,7). Allosteric modulators are expected to exhibit several advantages over classic drugs such as decreased risks of clinical overdose (8) or activity constrained under physiological conditions. In addition, they potentially produce a larger variety of effects hence featuring a larger panel of pharmacological properties, arising from the combination of their intrinsic activity and their cooperative activity on orthosteric ligand binding and efficacy (for review, (9)).

Until recently, the most widely accepted model for GPCR activation was the extended ternary complex model (often referred to as the two-state model) (references listed in (10-12)) according to which i) receptor molecules interconvert between two conformations endowed with distinct structural, pharmacological and functional properties, and ii) receptors can spontaneously adopt an active conformation and couple to G proteins (references listed in (10)), thereby accounting for basal activity level.

Although this model aims at describing receptor properties within a minimal number of conformations, it is becoming increasingly clear that the two-state model no longer adequately mirrors the complexity of GPCR signaling. Hence, agonist properties (from partial to full agonist) would rely on both the number and the efficiency of the active receptor conformations they stabilize (1,13-15). Moreover, multiple G-

protein coupling pathways described for several GPCRs (16) suggest that GPCRs may adopt multiple active conformations that lead to different biological responses. For example, phosphorylation of a GPCR (17), interaction of the receptor with a partner protein such as RAMP or Calcyon (1,18,19), or with the agonist (references listed in (16)), lead(s) to the selection of particular signaling pathways out of multiple ones. In the case of GPCRs which elicit multiple intracellular responses, allosteric modulators are expected to be useful tools to investigate receptor coupling to each pathway and to modulate their balance under physiological conditions (9).

Previous reports (20-23), demonstrated that NK2 receptors can adopt at least two active conformations coupled to PLC/Ca<sup>2+</sup> or to AC/cAMP pathways, that can be selectively stabilized by different tachykinin agonists. We showed (21,22) that fluorescent neurokinin A rapidly stabilizes a PLC-coupled active state exhibiting intermediate affinity followed by slow stabilization of a higher affinity AC-coupled active state. In contrast, a modified and truncated fluorescent neurokinin A, Cys<sup>7</sup>NKA4-10, rapidly stabilizes the PLC-coupled active state but does not bind to the AC-coupled one, and thus does not promote cAMP production. Together with functional and binding data obtained on NK1 receptors (24-28), there are strong evidence supporting that multiple active states with distinct functional properties are accessible to tachykinin receptors.

In the present work, we characterize the first allosteric modulator of tachykinin receptors activity. This molecule, LPI 805, was optimized from a hit compound structure identified in a drug screening campaign carried out on the NK2 receptor (manuscript in preparation). Using a combination of equilibrium and kinetic binding experiments as well as physiological response measurements, we demonstrate that LPI 805 is able to modulate the relative proportion of each of the two active conformations of the receptor in opposite directions. Indeed, in the presence of agonist, LPI 805 causes a positive modulation of the PLC pathway of the tachykinin receptors and a negative modulation of the AC pathway. Thus, for the first time to our knowledge, we show that modulation of the NK2 receptor by LPI 805 not only relates to quantitative aspects of the response, as is described for enzymes or other receptors, but both quantitative and qualitative

properties of the NK2R function. This work in addition describes further experimental evidence for the existence of multiple active and interconvertible states of GPCRs. It also opens the way for the design of a new class of potentially powerful pharmacological tools to investigate and control tachykinins roles *in vivo*.

## Materials and Methods

*LPI805 compound* - LPI 805 was N,N-(2-methylnaphthyl-benzyl)-2-aminoacetonitrile.

*Synthesis of LPI805* - A mixture of 2-naphthaldehyde (10g, 64mmol) and benzylamine (6.86g, 64mmol) in methanol (0.3M) was heated at 60°C for 4 hours. After, the solution was cooled to 0°C, then, NaBH<sub>4</sub> was added and stirred at room temperature overnight. The solution was evaporated to dryness, and the residue was dissolved in ethylacetat. The organic layer was washed with NaHCO<sub>3</sub> sat. (x2) and NaCl sat. (x1). The organic solvent was dried and evaporated; the resulting oil was engaged without further purification. Yield: 91%. <sup>1</sup>HNMR: (200MHz, CDCl<sub>3</sub>): δ 7.89-7.82 (4H, m); 7.55-7.28 (8H, m); 4.02 (2H, s); 3.89 (2H, s). A mixture of secondary amine N-(2-naphthylmethyl)-benzylamine (1.24g,5mmol), chloroacetonitrile (380mg, 5.05mmol) and triethylamine (510mg, 5.1mmol). in dimethylformamide (0.3M) was heated at 60°C overnight. The solution was extracted with ethylacetat (10V) and washed with NaHCO<sub>3</sub> sat (x1), H<sub>2</sub>O (x1) and brine (x1). The solvent was dried and evaporated. The resulting oil was purified to flash chromatography on silica gel. Re-cristallisation in 2-propan-2-ol. Yield: 84%. <sup>1</sup>HNMR : (300MHz, CDCl<sub>3</sub>) : δ 7.90-7.80 (4H, m) ; 7.60-7.20 (8H, m) ; 3.92 (2H, s) ; 3.81 (2H, s) ; 3.40 (2H, s). <sup>13</sup>CNMR : (50MHz, CD<sub>3</sub>OD) : δ 136, 135, 132, 131, 130, 114, 60, 59, 41. CHN calculated : C= 83.88%, H= 6.34%, N= 9.77%. CHN found : C= 83.758%, H= 6.28%, N= 9.77%.

*Synthesis of fluorescent NKA analogs* - Two peptides, NKA(HKTDSFVGLM-NH<sub>2</sub>) and a cysteine-containing analogue of NKA4-10 (DSFCGLM-NH<sub>2</sub>), were derivatized one with iodoacetyl-Bodipy 530/550 to give NKAb0 and the other with iodoacetyl-C<sup>5</sup>-Texas Red to give TR<sup>7</sup>-C<sup>7</sup>NKA4-10 as described (21,29).

*Chemicals* - Synthetic peptides were obtained from Bachem. Protease inhibitors were obtained from Sigma and Calbiochem. Fluorescent labels

and ion chelators were from Molecular Probes. [<sup>3</sup>H]SR48968 and [<sup>3</sup>H]SR140333 were purchased from Amersham. SR48968 was kindly given by Dr. Emonds-Alt (46). Emerald Enhancer and CSPD were from Applied Biosystem. JetPEI was from Polyplus.

*NK2Rs and NK1R cDNA* - pCDNA3 expression vector (Invitrogen) encoding the human NK1R and human NK2R cDNAs were kindly given by Dr. Gerard (47). Rat NK2R cDNA in 5' frame fusion with EGFP cDNA was cloned in pCEP4 expression vector (Invitrogen) as described (29).

*Cell culture and Buffer* - HEK293 cells were stably transfected by calcium phosphate precipitation (29) and selected with 500 μg/ml Hygromycin B for pCep4 vector and with 600 μg/ml G418 for pcDNA3 vector. Unless otherwise stated, cells were assayed in Hepes-BSA buffer (137.5mM NaCl, 1.25mM MgCl<sub>2</sub>, 1.25mM CaCl<sub>2</sub>, 6mM KCl, 5.6mM glucose, 10mM Hepes, 0.4mM NaH<sub>2</sub>PO<sub>4</sub>, 1% Bovine Serum Albumin (w/v), pH 7.4) supplemented with protease inhibitors (22).

*Fluorescence binding* - Interaction of fluorescent peptides with chimeric EGFP-rNK2 was monitored on a Fluorolog 2 spectrofluorometer (SPEX) with excitation set at 470 nm (bandwidth=5nm), as a decrease of EGFP emission at 510 nm (bandwidth =10nm) reflecting fluorescence resonance energy transfer towards the acceptor group Bodipy as described (29). Cell (10<sup>6</sup>cells/ml) or membrane suspensions (100 μgprotein/ml) in Hepes-BSA buffer were placed at 20°C in a 1ml-quartz cuvette under constant stirring. Addition of fluorescent agonist to the suspension results in a decrease of EGFP fluorescence at 510 nm. The intensity of the fluorescence variation is proportional to receptor sites occupancy. Typically, saturation of receptor sites results in 40% decrease of total preparation fluorescence. Dissociation experiments are initiated by addition of an excess of unlabelled NKA (1 μM) and recorded as a recovery of EGFP fluorescence intensity as a function of time.

*Radioligand Binding* - Radioligand binding assays using [<sup>3</sup>H] SR48968 for NK2R and [<sup>3</sup>H] SR140333 for NK1R were carried out at 4°C on 50000 cells in Hepes-BSA buffer supplemented with 0.1 mM Phenylmethylsulfonyl fluoride (PMSF) as described (29). The number of receptors per cell was calculated from B<sub>MAX</sub>

values: hNK1R : 2.5 10<sup>5</sup>, hNK2R : 4 10<sup>4</sup>, EGFP-rNK2R : 3 10<sup>5</sup> molecules/cell.

*Intracellular calcium measurement* - Cells were harvested, loaded for 20 min in 1mL Hepes-BSA buffer supplemented with 3 μM Indo-1-AM, washed and diluted. Calcium measurements were performed at 20 °C using cell suspensions (5.10<sup>5</sup>/ml) in a spectrofluorimeter with excitation set at 338nm (bandwidth = 2nm). Indo-1 signal was detected at 405nm (bandwidth = 5nm).

*Cyclic AMP accumulation* - Receptor coupling to AC activation was determined on adherent cells plated in 24-well plates as described in (22). Agonist was added at the desired concentrations at 20°C during 30 min. Reaction was stopped by addition of one volume ice-cold 0.2M HCl. Pre-incubation with LPI805 or others drugs was carried out for 20 min.

*cAMP dependent reporter Gene Assay* - The Mercury<sup>TM</sup> SEAP System was used to quantify PKA activation in tachykinin receptor-expressing HEK293 cells (31). The SEAP assay was carried out as previously described (48) except that transfection was performed with JetPEI reagent on a single cell batch from which cells were dispatched in 96-well collagen-coated plates, the day after transfection. Various concentrations of agonist diluted in culture medium were applied on adherent cells at 37°C for 5 hours. Pre-incubation with LPI 805 or other drugs was performed for 20 min.

*Data analysis* - The GraphPad Prism<sup>TM</sup> software was used for all EC<sub>50</sub>, K<sub>D</sub>, K<sub>I</sub>, B<sub>MAX</sub> or E<sub>MAX</sub> determinations. The KaleidaGraph<sup>TM</sup> software was used for fitting NKABO binding traces. Bi-exponential dissociation was defined by equation : [F] = f(t) = A1\*exp(-V1\*t) + A2\*exp(-V2\*t). Where, t is the time in sec.; [F] is the FRET signal accounting for ligand bound to the receptor; V1 and V2 the off rate constants k<sub>off</sub> of respectively rapid and slow dissociation events in sec<sup>-1</sup>, A1 and A2 are the amplitudes of respectively rapid and slow dissociation.

## RESULTS

### LPI 805 is a modulator of orthosteric ligand binding to tachykinin NK2 receptors.

*Partial inhibition of fluorescent NKA binding to tachykinin NK2 receptor by LPI 805.*

LPI 805 (Scheme 1) was first characterized as a molecule altering the

interaction between an N-terminally EGFP-tagged rat NK2 receptor (EGFP-rNK2R) and bodipy-labeled neurokinin A (NKAb), as detected by fluorescence resonance energy transfer (29).

As shown in Fig. 1A, LPI 805 (50 $\mu$ M) slightly displaces NKAb saturation curve on EGFP-rNK2R from an apparent  $K_D$  of  $5.6 \pm 1$  nM to  $12.6 \pm 4$  nM and reduces maximal FRET amplitude by approximately 30% at saturating concentrations of NKAb ( $p > 0.05$ ). In a competition experiment carried out at sub-saturating concentration of NKAb (20 nM), increasing concentrations of LPI 805 reverse part (up to  $34 \pm 5$  %) of fluorescent ligand binding with an apparent  $IC_{50}$  of  $22 \pm 7$   $\mu$ M (Fig. 1B). In contrast, the well-known competitive antagonist SR48968 (29) fully inhibits NKAb binding ( $KI = 0.7 \pm 0.3$  nM) (Fig. 1B). Slight alteration of the degree of receptor sites occupancy at sub-saturating NKAb concentrations might reflect the action of a non-competitive allosteric ligand of the receptor which either modifies the ratio of high to low affinity receptor states or modifies NKA affinity for each individual receptor state -see (9)-. A typical experiment to differentiate an allosteric ligand from a poorly-competitive orthosteric ligand is to analyze its effect on dissociation rate constants of classical orthosteric ligands.

#### *Acceleration of NKAb dissociation rate by LPI 805.*

In order to monitor agonist binding and kinetics, cells expressing EGFP-rNK2R are mixed with the agonist NKAb and variation of EGFP fluorescence emission is recorded as a function of time (Fig. 2A). After association equilibrium has been reached, addition of an excess of non-fluorescent NKA (or of SR48968, data not shown) leads to complete dissociation of bound NKAb (Fig. 2A).

When LPI 805 (50 $\mu$ M) is added (time 600 sec) to the equilibrated NKAb-receptor complex (Fig. 2A), weak dissociation (15-20 %) of NKAb is detected and new equilibrium conditions are reached. This weak dissociation of NKAb by LPI 805 corresponds to the binding competition described in Fig. 2B. Its kinetic is however not determined due to a transient artifact that has been removed in the figure. When NKA (20  $\mu$ M) is further added (Fig. 2A), complete dissociation of NKAb is observed, yet with higher rate than without LPI 805. This effect of LPI 805 is reversible. Indeed,

when cells, pre-incubated with LPI 805 are washed, the kinetic of NKAb dissociation is restored to its initial value (data not shown). The acceleration of NKAb dissociation rate is also observed on membrane preparations. Thus appears to behave as an allosteric compound of the NK2R.

#### **Quantitative analysis of LPI 805 effect on NKAb dissociation kinetic .**

We previously reported that fluorescent NKA-Texas Red (NKA-TR) associates to several receptor conformations (21,22,29) and dissociates from two populations of EGFP-rNK2 receptor conformations with a rapid and a slow dissociation rate. NKA-TR in addition progressively stabilizes a high affinity (slow dissociation) conformation at the expense of a lower affinity (rapid dissociation) one (21). Comparably to NKA-TR, NKAb dissociation (initiated by the addition of 20 $\mu$ M NKA) follows a two step process with a rapid dissociation typically representing 30-40% of total dissociation and a slow dissociation accounting for 60-70% of the signal that are best described with average rate constants of  $0,04$   $s^{-1}$  and  $0,008$   $s^{-1}$  and (see triangles in Fig. 2B).

In the presence of LPI 805, the time course of NKAb dissociation speeds up with  $t_{1/2}$  dissociation times ranging from 68 sec (0 $\mu$ M LPI 805), to 57 sec (1 $\mu$ M), 46 sec (5 $\mu$ M) and 25 sec (50 $\mu$ M LPI 805) (Fig. 2B, right panel). All dissociation traces from figure 2B are fit with two rate constants ( $V1=0.04$   $sec^{-1}$  and  $V2=0.008$   $sec^{-1}$ ) identical to those used to fit control dissociation, and adjusted amplitudes of rapid and slow dissociation processes. LPI 805 increases the proportion of rapid dissociation (A1%) in a dose-dependent ( $EC_{50} = 8 \pm 1.5$   $\mu$ M) and saturable ( $81 \pm 3\%$ ) manner (Fig. 2C right panel). LPI 805 speeds up dissociation by increasing the contribution of the rapid dissociation process ( $EC_{50}$  rapid dissociation =  $2.5 \pm 0.8$   $\mu$ M) and by concomitantly decreasing that of the slow dissociation process ( $IC_{50}$  slow dissociation =  $9 \pm 1.3$   $\mu$ M) (Fig. 2C lower right panel).

In contrast, the competitive antagonist SR48968 (from 0 to 5nM) progressively reduces NKAb dissociation amplitude, due to competition between SR48968 and NKAb (Fig. 2B-left panel), without altering kinetics. All traces have similar dissociation  $t_{1/2}$  values: (45sec at 0nM, 44sec at 0.5nM, 49sec at 2nM, and 53sec at 5nM SR48968) and the ratio of

rapid to slow dissociation processes as well as the associated relative amplitudes remain constant ( $IC_{50} \sim 3nM$ ) (Fig. 2C, left panel).

The fluorescent NKA ligands, NKA-TR (21,22) and NKAb0 (this work) are agonists that bind, as described in (21), to at least three conformations of the receptor: R0, R1 and R2. Conformation R0 is defined as the resting state of the receptor while R1 and R2 are two active states that respectively trigger PLC pathway (R1) and AC pathway (R2) (Fig. 5). Slow dissociation has been assigned to the R2 conformation and a 5-10 fold faster dissociation rate constant has been assigned to dissociation from the R1 conformation. Accordingly, at a non-saturating concentration, NKAb0 (20 nM), predominantly stabilizes the R2 conformation (70%) while, in the presence of LPI 805, it predominantly stabilizes the R1 conformation. The functional correlation to such alteration of conformation stabilization by LPI 805 is that responses associated to R1 are expected to become larger, while those associated to R2 should be smaller. In order to validate the predicted allosteric effects of LPI 805 on the activity of NK2R, we measured responses associated to the NK2 receptor.

#### **LPI 805 potentiates tachykinin receptors-mediated intracellular calcium elevation.**

Fig. 3 shows that LPI 805 changes the time course of NK2 receptor-mediated calcium responses, and possibly their amplitudes depending on the agonist used.

Indeed, when the partial agonist TR<sup>7</sup>-C<sup>7</sup>NKA4-10 (21) is used to stimulate rat NK2R, LPI 805 potentiates the maximal amplitude of the calcium response (Fig. 3A, right panel). This effect accounts for about 50% increase of the peak response at all agonist concentrations. In addition to increasing the intensity of the calcium response, LPI 805 also prolongs elevation of the intracellular calcium concentration (Fig. 3A, right panel).

When the full agonist NKA is used instead, LPI 805 reproducibly increases the duration of intracellular calcium elevation (Fig. 3B, right panel) without significantly changing the maximal response amplitude. Semi-quantitative analysis of this effect (inset of Fig. 3B) reveals potentiation at all NKA concentrations tested.

In addition, LPI 805 potentiates calcium responses of wild type tachykinin receptors, as shown on human hNK1 and hNK2 receptor

subtypes activated by substance P (hNK1) or NKA (hNK2) (Fig. 3C).

LPI 805 does not affect Gq-dependent muscarinic M1 receptor calcium responses (data not shown), ruling out LPI 805 effects mediated downstream of tachykinin receptors.

Hence, LPI 805 behaves as a positive modulator of calcium signaling, not only on the rat NK2 fluorescent receptor, but also on wild-type human tachykinin NK1 and NK2 receptors.

#### **LPI 805 blocks EGFP-rNK2R mediated cAMP signaling.**

Several groups have described intracellular accumulation of cAMP mediated by tachykinin receptors in recombinant expression systems (21,23,30). We previously reported (21,22) that fluorescent and non-fluorescent NKA are able to stimulate cAMP formation in a dose-dependent manner.

We now measure cAMP accumulation by determining the enzymatic activity of secreted alkaline phosphatase (SEAP) expressed under the control of cAMP-responsive elements - CRE-SEAP assay (31)-. We find that NKA stimulates CRE-dependent alkaline phosphatase expression with an  $EC_{50}$  value ranging from 2 to 7 nM (Fig 5). All responses are fully abolished in the presence of the PKA inhibitor H89 (100 nM to 1  $\mu$ M), ruling out significant contribution of calcium-mediated activation of CREB (data not shown). In the presence of a conventional competitive antagonist, H8565 at 200 nM ( $KD$  value  $\approx$  20 nM, (32)), the  $EC_{50}$  of NKA decreases by 4.5-fold and no change in the maximal response amplitude is observed, as expected for competitive interaction of H8565 with the receptor (Fig. 4A).

In contrast, LPI 805 (at 10  $\mu$ M) reduces the amplitude of the response by 3-fold, or even abolishes it at higher concentration (40 $\mu$ M) (Fig. 4B), with no statistically significant alteration of the apparent affinity of NKA ( $EC_{50}$  value changes from  $7.4 \pm 1.2$  nM to  $23 \pm 15$  nM). Similar results are obtained when cAMP concentrations are determined using radio-immunoassay detection (data not shown). Maximal NKA response amplitude ( $E_{MAX}$ ) is dose-dependently reduced by LPI 805 with an  $IC_{50}$  ( $2.9 \pm 0.7\mu$ M) (Fig. 4C) comparable to the apparent affinity of LPI 805 for dissociation rate alterations ( $EC_{50} = 8 \pm 1\mu$ M).

To ascertain that the effect of LPI 805 on NK2R signaling AC pathway is not due to a non-specific inhibition of effectors of the

response, we have tested the effects of LPI 805 on the endogenously expressed  $\beta_2$ -adrenergic receptors activated by isoproterenol. We find that isoproterenol activates cAMP phosphatase alkaline production with an  $EC_{50} = 242 \pm 31$  nM, and maximal response intensity  $E_{max} = 7170 \pm 1100$  arbitrary luminescence units a.l.u. LPI 805 (20  $\mu$ M) changes neither the  $EC_{50}$  value of isoproterenol ( $EC_{50} = 269 \pm 33$  nM) nor its  $E_{max}$  ( $7370 \pm 1500$  a.u.), therefore ruling out direct inhibition of Gs-protein or of the adenylate cyclase cascade (data not shown).

The apparent effect of LPI 805 on AC pathway of NK2R is a decrease of the amount of receptors that are able to activate this pathway rather than an alteration of the efficacy/potency of the response. Thus, LPI 805 is a negative modulator of NK2R cAMP response.

## DISCUSSION

In their original description by Monod Wyman and Changeux in 1965, allosteric proteins interconvert between discrete conformations endowed with distinct structural, pharmacological and functional properties (33). This model, which was based on the resolution of hemoglobin structure (34), stated that regulatory proteins exist as oligomers and spontaneously adopt at least two conformations, thereby accounting for the regulation of biological activity of the protein and of its pharmacological properties. Alternatively, biological activity of regulatory proteins could be accounted for by variation of their quaternary structure, with for instance equilibrium taking place between inactive monomers and active oligomers (35).

Current knowledge on G protein-coupled receptors, places this family of regulatory proteins within such allosteric scheme. On one hand, the occurrence of spontaneous equilibrium between inactive and active conformations has received strong support from the measurement of basal second messenger production and its regulation by agonists, antagonists and inverse agonists (11,12). In addition, it is evident from recent work that GPCRs exist under various degrees of oligomerization, and that, depending on whether monomers assemble into homo- or hetero-oligomers, both pharmacological and functional properties are changed (18,19,36-38). Also, as for other allosteric proteins, co-activators or modulators of GPCR signaling have been identified for many G protein-coupled

receptors (6,8) and for some of them, characterization of their effects could be extended up to the whole animal level (39).

Recently, increasing experimental evidences report about GPCRs coupling to multiple intracellular signaling pathways (24,40-42). It also becomes accepted that GPCRs couple to different G proteins, not only as a result of over-expression, but also under more physiological conditions where they are endogenously expressed (43-45). The NK2R follows such scheme as it couples to two signaling pathways that are differentially sensitive to distinct agonists and which correspond to distinct conformations of the receptor (21,22). These conformations can be discriminated on the basis of their rates of interconversion, intrinsic ligand binding affinities, and capacity to lead to the production of two categories of intracellular second messengers (Fig. 5). Using a new molecular species that non competitively alters NKA binding to the NK2R, presumably by binding directly to the receptor, we show that the two signaling pathways are modulated in opposite ways, and that this alteration of cellular response pattern correlates with changes in agonist binding properties. As expected for a molecule that is able to modulate the relative proportions of receptor active conformations, we find that potentiation of calcium responses is associated to increased occupancy of the intermediate R1 conformation while reduction of cAMP responses coincides with reduced occupancy of the slowly stabilizing conformation which binds neurokinin A with high affinity. The effects of LPI 805 can thus be interpreted as a modulation of the equilibrium between the three conformations of the receptor that favors the R1 (and possibly R0) state at the expense of the R2 conformation. Since the effects of LPI 805 are readily reversed upon washing, we favor the hypothesis that the two active conformations of the NK2 receptor are interconvertible, rather than corresponding to independent, non-interconvertible, populations of receptors confined to distinct membrane areas (26).

Although we cannot provide firm demonstration that LPI 805 modulates NK2R upon interacting directly with the receptor protein, its absence of effect on the Gq-coupled muscarinic M1 receptor binding properties, as well as on the Gs-coupled beta-adrenergic receptor responses, supports the notion that LPI

805 i) exhibits binding selectivity and ii) is not acting on downstream proteins (e.g. G proteins or other intracellular effectors of the responses).

Finally, by orienting tachykinin action towards one particular signaling cascade, this allosteric modulator will be useful to elucidate

the role of each pathway into the generation of more integrated physiological responses such as those recorded at the cellular, organ or even whole animal level, and potentially to design new kind of compounds of therapeutic interest.

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## FOOTNOTES

This work was supported by the Association pour la Recherche contre le Cancer and the Centre National de la Recherche Scientifique (CNRS). E.M. was supported by grants from the CNRS and the Region Alsace. We thanks Drs. F. M. Decailot and D. Bertrand for helpful comments.

The abbreviations used are: SP, substance P; NKA, neurokinin A; NKB, Neurokinin B; NKRs, tachykinin receptors; AC, adenylate cyclase; PLC, phospholipase C; PKA, PKC, protein kinases A, C; bo, Bodipy; TR, Texas Red.

## FIGURES LEGENDS

**Scheme 1:** Structure of LPI 805 (N,N-(2-methylnaphthyl-benzyl)-2-aminoacetonitrile).

**Figure 1:** Effects of LPI 805 on the binding of the agonist NKAb<sub>o</sub> to EGFP-rNK2 receptors. Equilibrium binding experiments were performed on living cells expressing EGFP-rNK2 at 20°C. (A) Saturation binding curve of NKAb<sub>o</sub> in the absence (filled squares) or presence (open squares) of LPI 805 (50µM). Binding of NKAb<sub>o</sub> to the receptor leads to a decrease of EGFP fluorescence at 510nm. Binding is expressed as unrefined percent of fluorescence resonance energy transfer, FRET. Data points are mean ± SD of at least three experiments. (B) Competition curves using a sub-saturating concentration of NKAb<sub>o</sub> (20nM) and the antagonist SR48968 (open squares) or by LPI 805 (filled squares). Data points are mean ± SD or single value of at least two experiments.

**Figure 2:** Analysis of the dissociation of the agonist NKAb<sub>o</sub> in EFGP-rNK2 expressing cells at 20°C. (A) EGFP-NK2R fluorescence at 510nm is followed for 1600 sec. NKAb<sub>o</sub> (40nM) is added at time 20 sec. and association is followed during 600 sec. At 1200 sec, dissociation of bound NKAb<sub>o</sub> is initiated by addition of NKA (20µM). Fluorescence variations is recorded in the absence or in the presence of LPI 805 (50µM) added at time 600 sec. (B): NKAb<sub>o</sub> (20nM) dissociation experiments performed in the presence of increasing concentrations of LPI 805 (right) or competitive antagonist SR48968 (left). The lines are best fits of 600 sec recording dissociation curves obtained with two exponential adjustments containing a fast ( $k_{off} : v1 = 0.04 \text{ sec}^{-1}$ ) and a slow ( $k_{off} : v2 = 0.008 \text{ sec}^{-1}$ ) rate constants. Each 50<sup>th</sup> data points is shown in the traces. (C) Dissociation parameters obtained from two-exponentials fitting of NKAb<sub>o</sub> (20nM) dissociation curves with the same dissociation rates as in (B) in the presence of increasing concentrations of LPI 805 (right) or of the competitive antagonist SR48968 (left). Lower panels of rapid (A1) and slow (A2) dissociations. A1 and A2 are normalized amplitudes of respectively fast ( $v1=0.04\text{sec}^{-1}$ ) and slow ( $v2=0.008\text{sec}^{-1}$ ) dissociation steps of NKAb<sub>o</sub> (20nM).

Dotted lines show total amplitudes corresponding to the sum of A1 and A2. Upper panels represent the proportion of the fast dissociation rate amplitude A1 to the total amplitude dissociation at each concentration of LPI 805 or SR48968. Data points are mean  $\pm$  SD of at least two experiments.

**Figure 3:** Effects of LPI 805 on the calcium response of EGFP-rNK2, hNK1 and hNK2 receptors to various agonists. Indo-1 fluorescence (400nm) was followed over time at 20°C on whole cells loaded with 3 $\mu$ M Indo-1 pre-incubated during 20 minutes. Indo-1 signal, detected in the absence or in the presence of LPI 805 (20 $\mu$ M), was normalized to the basal value and to the maximal value induced by lysis of cells with digitonin (25 $\mu$ M). (A) Left, calcium peak dose-response relationship to the partial agonist TR<sup>7</sup>-C<sup>7</sup>NKA4-10 on EGFP-rNK2 expressing cells treated with or without LPI 805. Right, example traces from time-based calcium Indo-1 response of EGFP-rNK2R to TR<sup>7</sup>-C<sup>7</sup>NKA4-10 in the absence or presence of 20  $\mu$ M LPI 805. (B) Left, calcium peak dose-response relationship of the full agonist NKA on EGFP-rNK2 expressing cells treated with or without LPI 805. Right, example traces from time-based Indo-1 response from EGFP-rNK2R to NKA treated with or without LPI 805. Inset, area (signal-time) potentiation of calcium response induced by LPI 805 at each NKA concentration. (C) Example traces from time-based calcium responses of WT human NK1 and NK2 receptors to agonists SP (10 nM) and NKA (100 nM) treated with 20  $\mu$ M LPI 805 or not. (B) is a single representative experiment. (A) and (C) are single representative experiments done in duplicate (mean  $\pm$  SD).

**Figure 4:** Effects of LPI 805 on the cAMP response of EGFP-rNK2 to NKA. (A) CRE induced alkaline phosphatase (AP) activity dose-response relationship of NKA in the absence (filled squares) or in the presence of 200nM antagonist H8565 (open squares). Data points are mean  $\pm$  SD of at least two experiments done in triplicate. (B) CRE induced AP activity dose-response relationship of NKA in the absence (filled squares) or in the presence of 10 $\mu$ M LPI 805 (open squares) and 40 $\mu$ M LPI 805 (open triangles). Data points are mean  $\pm$  SD of two experiments done in triplicate. (C) LPI 805 Dose-response relationship on AP activity evoked by NKA. Maximal AP activity was quantified with an excess of NKA (20 $\mu$ M). Data points are mean  $\pm$  SD of three experiments done in triplicate.

**Figure 5:** Model of LPI 805 action on NK2 receptor. R1 and R2 are the two states of the receptor from which the agonist NKAb dissociates with slow (0.008 sec<sup>-1</sup>) and fast (0.04 sec<sup>-1</sup>) rates. L represents the agonist NKA or NKAb. The active conformation R1L is assigned to the PLC signalling pathway. The active conformation R2L is assigned to the AC signalling pathway. The two active states are in equilibrium and can interconvert. LPI 805 alters the balance between R1L and R2L states without changing intrinsic affinities of NKA for R1 and R2 receptor states. By increasing the number of receptors in R1L state and decreasing the number of receptors in R2L state, LPI 805 potentiates calcium responses to agonists and inhibits cAMP responses to agonists.

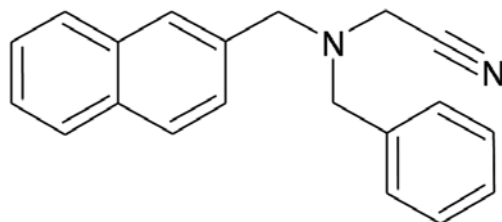
**Scheme 1**

Figure 1

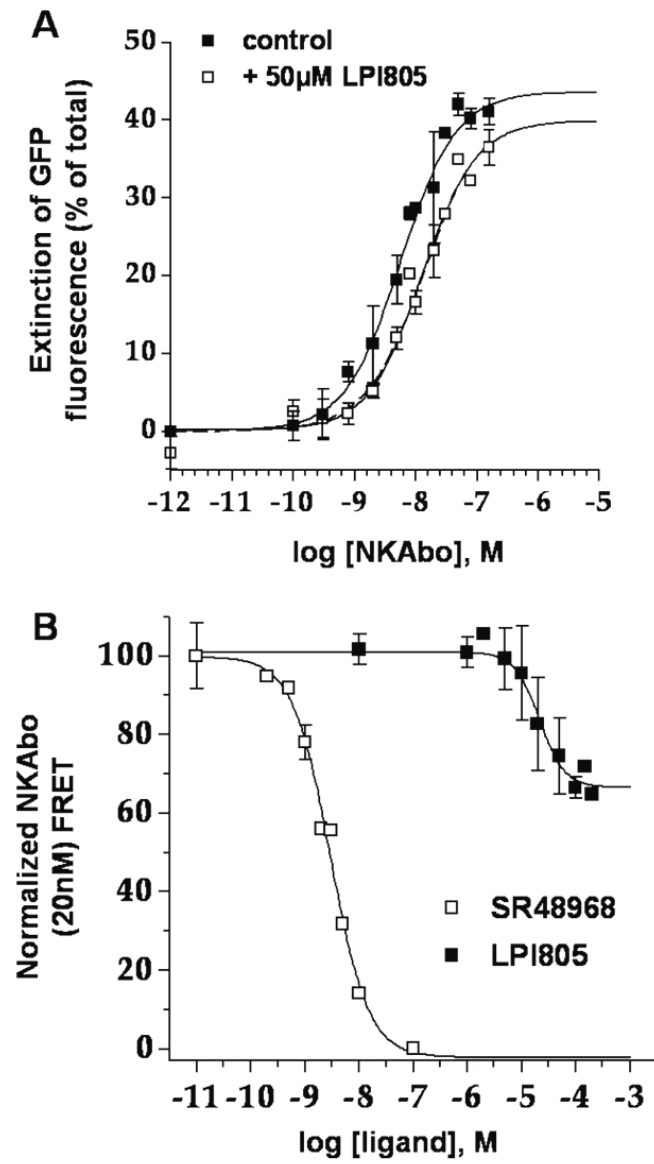


Figure 2

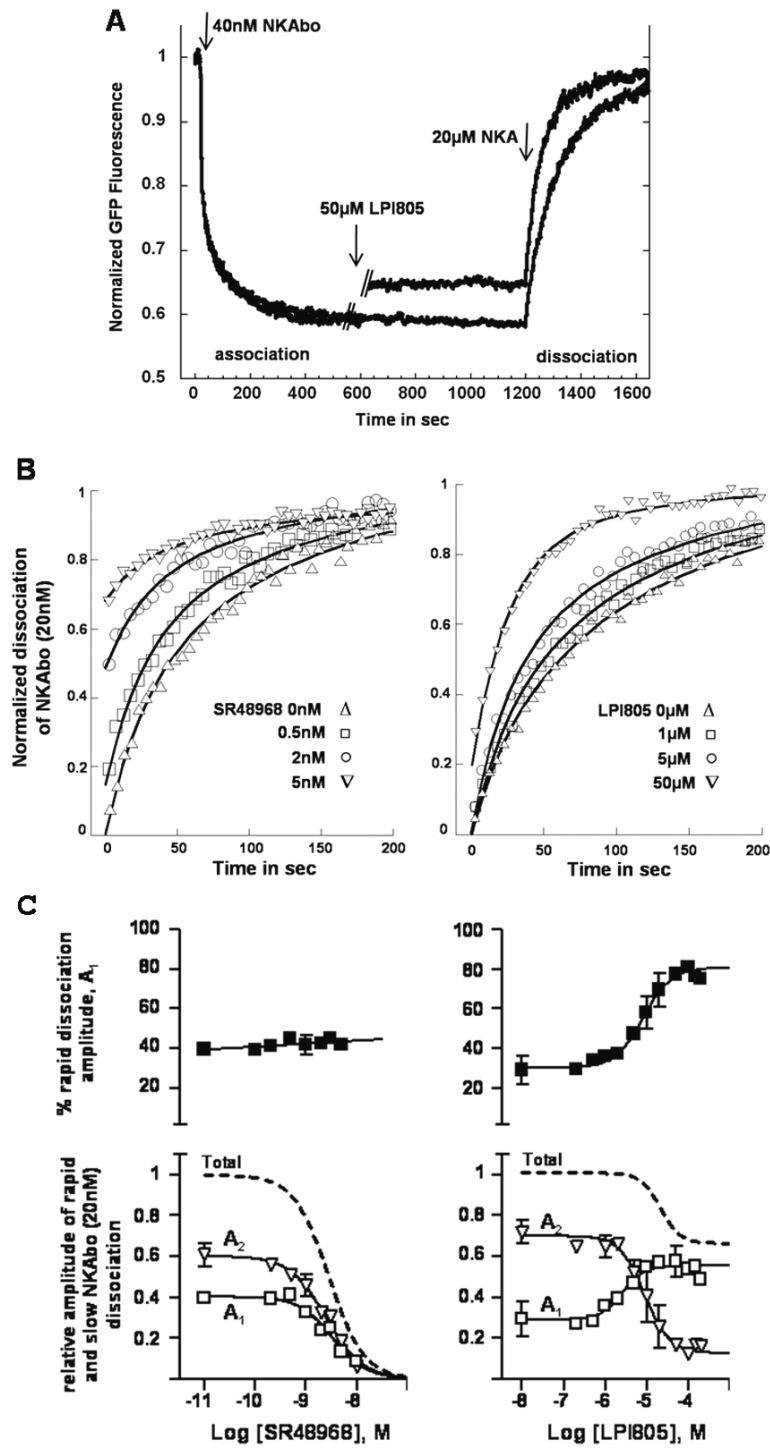


Figure 3

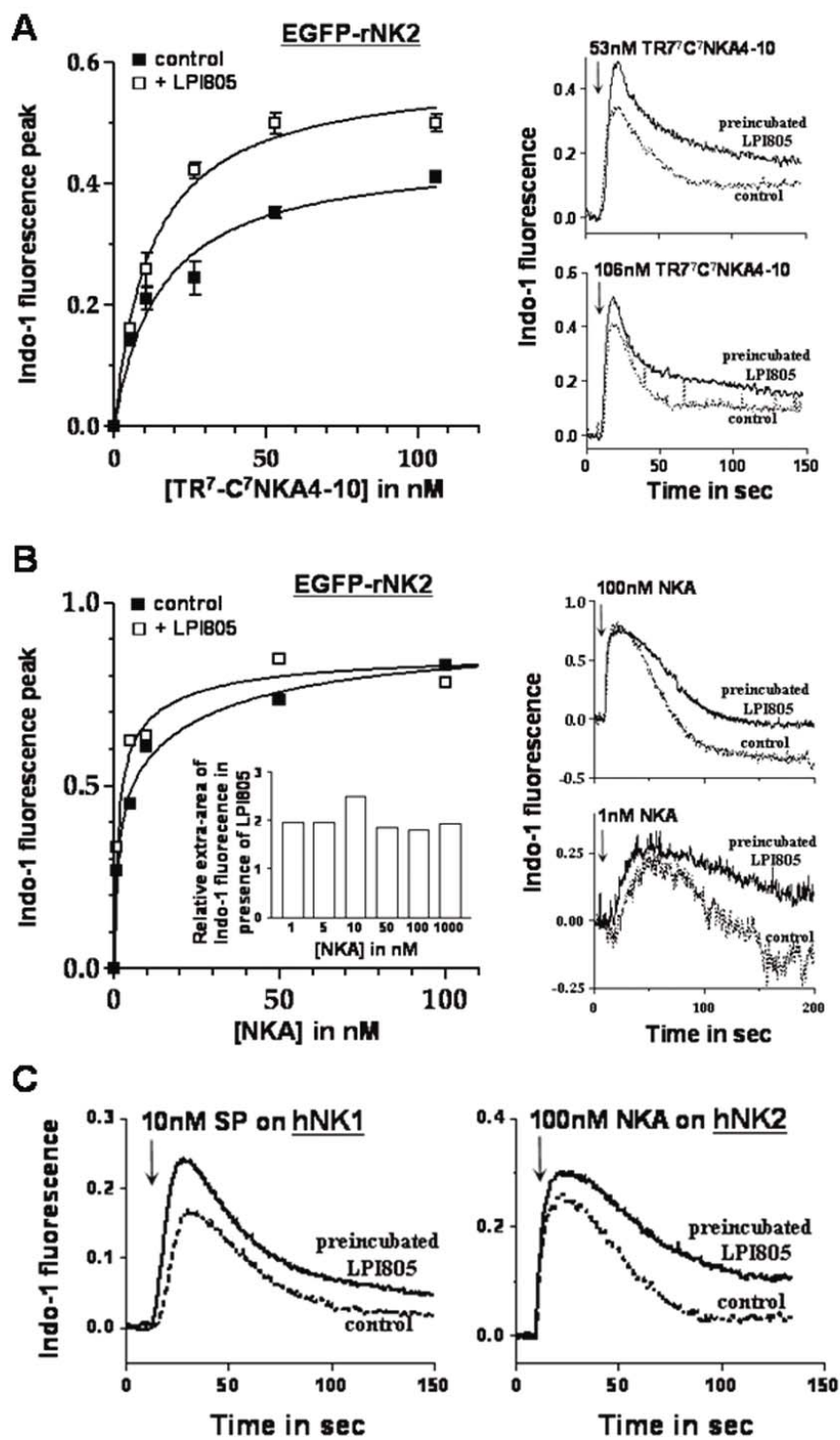


Figure 4

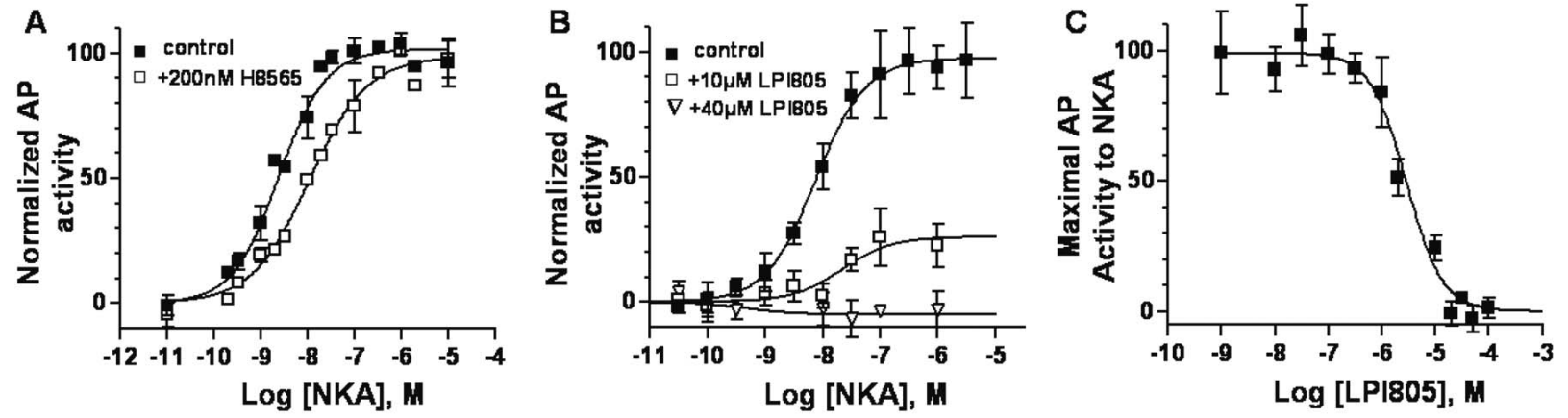


Figure 5

