Evidence for the dual coupling of the rat neurotensin receptor with pertussis toxin-sensitive and insensitive G-proteins

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Abstract We previously demonstrated the functional coupling of the rat neurotensin receptor NTS1 with G-proteins on transfected CHO cell homogenates by showing modulation of agonist affinity by guanylyl nucleotides and agonist-mediated stimulation of [^{35}S]GTPγS binding. In the present study, we observed that $G_{i/o}$-type G-protein inactivation by pertussis toxin (PTx) resulted in a dramatic reduction of the NT-induced [^{35}S]GTPγS binding whereas the effect of guanylyl nucleotide was almost not affected. As expected, NT-mediated phosphoinositide hydrolysis and intracellular calcium mobilization were not altered after PTx treatment. This suggests the existence of multiple signaling cascades activated by NT. Accordingly, using PTx and the PLC inhibitor U-73122, we showed that both signaling pathways contribute to the NT-mediated production of arachidonic acid. These results support evidence for a dual coupling of the NTS1 with PTx-sensitive and insensitive G-proteins.

Key words: Neurotensin; Pertussis toxin; Arachidonic acid; G-protein; GTPγS; Phospholipase

1. Introduction

The effects of the neuropeptide neurotensin (NT) are thought to result from its high affinity interaction with specific membrane receptors. Early binding studies conducted on brain tissues and cell lines led many authors to propose the existence of multiple NT binding sites. Three NT receptor subtypes have now been cloned and named: high affinity neurotensin receptor (NTS1), nts2 and nts3. NTS1 and nts2 belong to the large family of G-protein-coupled receptors [1,2] whereas nts3 corresponds to the previously characterized gp95/sortilin containing a single transmembrane domain [3] (for review, see [4]). In contrast to nts2 and nts3, that were only clearly identified in the late 1990s, NTS1 has received much more attention and was extensively studied in a large variety of cell lines, tissues and recombinant systems for more than a decade. The coupling of this receptor with G-proteins is demonstrated by the ability of guanylyl nucleotides and Na^+ ions to modulate the binding affinity of NT agonists [5–7]. Although both positive and negative controls of adenylyl cyclase by NT were reported in some cultured cells expressing NTS1 [5,8], most of the effects of NT have been attributed to the activation of PLC (for review, see [4,9]). This suggests the involvement of $G_{q/11}$-type G-proteins and accordingly, many responses to NT are unaffected by the inactivation of $G_{i/o}$-type G-proteins by pertussis toxin (PTx) or *N*-ethylmaleimide [10–13]. However, this is not the rule and a number of authors have also proposed a possible coupling of NT receptors with other signaling pathways that are proposed to involve PTx-sensitive G-proteins [12,14–17].

Using transfected Chinese hamster ovary (CHO) cells expressing NTS1 (CHO-NTR), we have previously reported on the NT-induced accumulation of inositol phosphates (InsP) [10], production of arachidonic acid [18], mobilization of intracellular calcium [19] and increase in [^{35}S]GTPγS binding [20]. On the basis of further biochemical analysis, we now provide evidence that these functional responses are dependent on the dual coupling of the receptor with both PTx-sensitive and insensitive G-proteins. In addition, selective inhibition of these signaling pathways revealed that both couplings contribute to the NT-induced production of arachidonic acid.

2. Materials and methods

2.1. Materials

Fura-2 AM was obtained from Molecular Probes, guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) (specific activity 1000 Ci/mmol) [^{3}H]arachidonic acid (100 Ci/mmol) and [^{3}H]NT (101 Ci/mmol) were from NEN-Dupont, *myo*-[2-^{3}H]inositol (70–120 Ci/mmol) from Amersham. PTx, NT, ionomycin, dithiothreitol, 1,10-phenanthroline, GDP, 5′-guanylylimidodiphosphate (Gpp(NH)p) and polyethylenimine were from Sigma Chemical Co, compound U-73122 was from RBI. Glass fiber filters were from Whatman and Brandel.

2.2. Cell culture

Transfected CHO cells expressing the rat NTS1 [10] were grown in MEM alpha medium supplemented with 10% fetal calf serum, 85 μg/ml streptomycin and 85 U/ml penicillin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2–95% air. For $[Ca^{2+}]_i$, measurements, cells were cultured on glass coverslips. When indicated, 100 ng/ml PTx was added in the culture medium 20 h before use.

2.3. Binding of [^{35}S]GTPγS and [^{3}H]NT

The specific binding of [^{35}S]GTPγS and [^{3}H]NT were measured on cell homogenates as previously described [20]. Briefly, cell homogenates (25–50 μg) were resuspended in binding buffer (50 mM Tris–HCl pH 7.4 containing 5 mM $MgCl_2$, 1 μM 1,10-phenanthroline,

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Abbreviations: NT, neurotensin; NTS1, high affinity neurotensin receptor; InsP, inositol phosphates; CHO, Chinese hamster ovary; Gpp(NH)p, guanylylimidodiphosphate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; PTx, pertussis toxin; CHO-NTR, transfected CHO cells expressing the rat NTS1

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0.1% BSA). For [^{35}S]GTPγS binding studies, this buffer was supplemented with 1 μM GDP, 150 mM NaCl and 1 mM dithiothreitol. The homogenate was incubated for 30 min in the presence of either 0.05 nM [^{35}S]GTPγS or 0.5 nM [^{3}H]NT at 37 or 30°C, respectively. The non-specific binding of [^{35}S]GTPγS and [^{3}H]NT were measured in the presence of 0.1 mM Gpp(NH)p or 0.1 μM NT, respectively. Incubation was terminated by addition of 3 ml ice-cold binding buffer and the suspension was immediately filtered through GF/B glass fiber filters (pre-soaked for 1 h in 0.5% polyethylenimine for [^{3}H]NT binding experiments) and washed twice with the same buffer using a 24 channels Brandel Harvester (Semat). Radioactivity trapped on the filter was estimated by scintillation counting. All the binding data were analyzed by non-linear regression using the software GraphPad Prism, version 2.00.

2.4. Measurement of [^{3}H]InsP

For assessment of [^{3}H]InsP, cells grown in 24 multiwell plates were labeled with 1 μCi/ml, *myo*-[2-^{3}H]inositol for 48 h in culture medium. Thereafter, cells were washed three times with 0.5 ml of Ham-F12 medium supplemented with 10 mM LiCl and 0.1% BSA and 1 μM 1,10-phenanthroline, and incubated in this buffer for 15 min before the addition of the NT. Experiments were performed at 37°C in a final volume of 0.5 ml/well and were terminated by the addition of 0.5 ml ice-cold 1 M trichloroacetic acid. After extraction on ice for 20 min, samples (0.8 ml) were collected from the well, mixed with 200 μl EDTA (10 mM, pH 7.0) and extracted with 1 ml of a 1:1 (v/v) mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane. An 800 μl sample of the aqueous extract was mixed with 50 μl $NaHCO_3$ (62.5 mM) and the [^{3}H]InsP fraction was recovered by ion-exchange chromatography as previously described [21].

2.5. [Ca^{2+}]$_i$ measurements

Cells were loaded with 5 μM Fura-2 AM for 60 min at room temperature. Rinsed coverslips were then mounted in a thermostatized (20°C) and perfused microscope chamber (1 ml). Fura-2-loaded cells were excited alternatively at 340 and 380 nm and fluorescence emission was monitored at 510 nm using a Deltascan spectrofluorimeter (Photon Technology international) coupled to an inverted microscope (Nikon Diaphot, oil immersion objective 100× N.A. 1.3). Fluorescence intensities from single cells excited at the two wavelengths were recorded and [Ca^{2+}]$_i$ was estimated using an intracellular calibration procedure, after cell permeabilization with 5 μM ionomycin at the end of the experiment [22].

2.6. Measurement of arachidonic acid release

Cells grown in 6-well dishes were incubated with 0.5 pCi/well [^{3}H]arachidonic acid for 24 h at 37°C allowing a progressive incorporation of labeled arachidonic acid into membrane phospholipids. After washing, the cells were stimulated with NT for 5 min in Krebs buffer medium containing fatty acid free BSA (0.2%) and [^{3}H]arachidonic acid released in the medium was quantified by liquid scintillation counting [18].

3. Results

3.1. Functional coupling with G-proteins

The functional coupling of the NTS1 expressed in CHO-NTR cells was evaluated by measuring the ability of NT to induce [^{35}S]GTPγS binding and by the ability of Gpp(NH)p to decrease the specific binding of [^{3}H]NT to cell homogenates. As previously described, in the presence of GDP (1 μM), NT was found to considerably enhance (150% above basal) the specific binding of [^{35}S]GTPγS on CHO-NTR homogenates (Fig. 1A). The relative low potency of NT found in these experiments is a consequence of the high concentration of NaCl (150 mM) required in this functional assay that decreases the affinity of NT for its receptor. On cells previously treated with PTx, the functional response to NT was dramatically reduced (maximal 13% increase in nucleotide binding above basal). However, the potency of NT was not affected (pEC_{50} values of 7.98 ± 0.12 and 7.93 ± 0.14 on homogenates from control and PTx-treated cells, respectively). The effect of PTx treatment on the interaction of the NTS1 with G-protein was further investigated by measuring the effects of Gpp(NH)p and NaCl on the binding of [^{3}H]NT. Pretreating the cells with PTx resulted in a significant decrease in the specific binding of (0.5 nM) [^{3}H]NT measured on cell homogenates. However, on both control and PTx-treated cells, Gpp(NH)p was found to decrease the specific binding of [^{3}H]NT in a similar extent and with a similar potency (pEC_{50} values of 6.95 ± 0.12 and 7.13 ± 0.17 on homogenates from control and PTx-treated cells, respectively). Addition of NaCl (150 mM) in the assay also decreased the specific binding of [^{3}H]NT. As usually reported [7,23], the effect of Na^{+} and Gpp(NH)p were additive and after their co-application, the binding of [^{3}H]NT was almost undetectable. Pretreatment of the cells with PTx did not prevent the effect of NaCl on the binding of [^{3}H]NT.

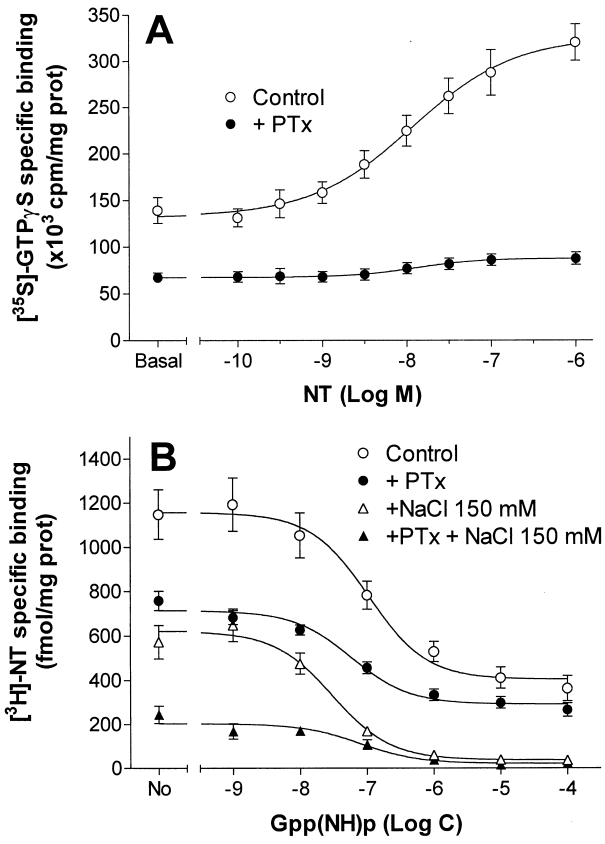

Fig. 1. (A) NT-induced [^{35}S]GTPγS binding on homogenates from CHO-NTR cells previously treated in the presence (closed symbols) or in the absence (open symbols) of PTx (100 μg/ml, 20 h). (B) Effect of Gpp(NH)p (increasing concentrations) and NaCl (control, circles; 150 mM NaCl, triangles) on the specific binding of [^{3}H]NT to homogenates from CHO-NTR cells previously treated in the presence (closed symbols) in the absence (open symbols) of PTx (100 μg/ml, 20 h). Data shown are mean ± S.D. from five (A) and three (B) different experiments performed in duplicate, respectively.

3.2. NT-induced [^{3}H]InsP production and [Ca^{2+}]$_i$ mobilization

We previously showed that NTS1 expressed in transfected CHO cells is functionally coupled to PLC [10]. The G-proteins involved in this coupling were studied by measuring the effect of PTx treatment on NT-induced phosphoinositide hydrolysis,

evaluated by the accumulation of [^{3}H]InsP (measured after 30 min stimulation with NT in the presence of LiCl). NT induced a nearly fourfold increase in [^{3}H]InsP accumulation with a potency in accordance with its affinity for the NTS1 receptor [24] (pEC_{50} value of 8.89 ± 0.23, $n = 5$), (Fig. 2A). Neither the efficacy, nor the potency of NT were affected by PTx treatment (pEC_{50} value of 9.16 ± 0.17, $n = 5$).

CHO-NTR cells respond to NT stimulation with a large release of Ca^{2+} from the endoplasmic reticulum followed by sustained entry of Ca^{2+} from the extracellular space [19]. This response is triggered by $InsP_3$ as shown by its sensitivity to micro-injected heparin [22]. In the present study, we focused our attention on the initial phase which is better characterized in the absence of extracellular calcium (0.2 mM EGTA). This response was maximal at a concentration of 1 nM NT, consisting in a $[Ca^{2+}]_i$ transient that reached about 1μM and faded away in about 1 min (Fig. 2B). The $[Ca^{2+}]_i$ transient induced by 10 nM NT was not affected by PTx treatment indicating that the initial response to NT was not dependent on the coupling of the receptor with PTx-sensitive G-protein. Accordingly, complete inhibition was obtained by addition (15 min prior NT) of 5 μM U-73122, an inhibitor of PLC, whose activation is proposed to mainly involve PTx-insensitive G-protein. At 1 μM, this effect was only partial (peak reaching 180 nM, data not shown), in agreement with the micromolar potency of this compound reported in the literature [8,25].

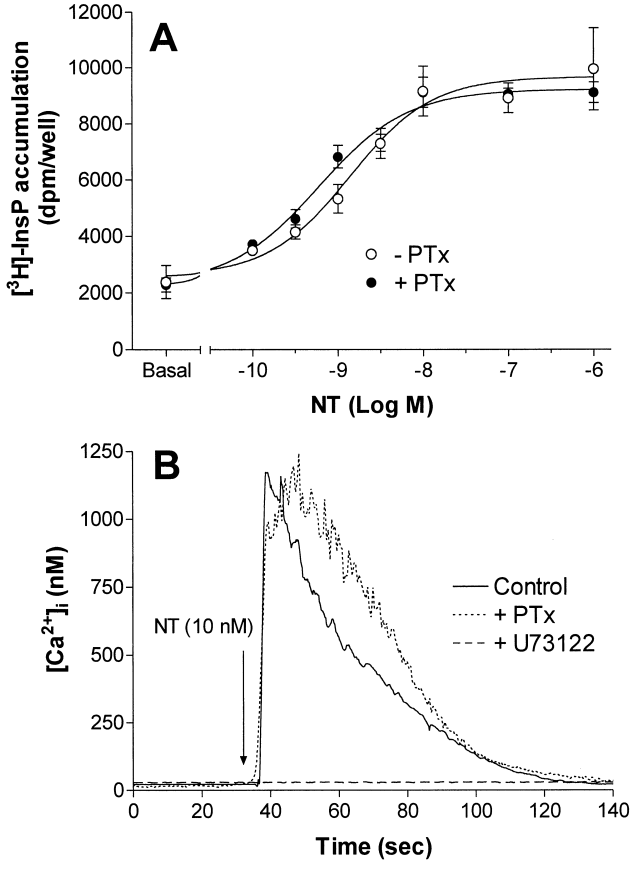

Fig. 2. (A) NT-induced [^{3}H]InsP accumulation in CHO-NTR cells previously treated in the presence (closed symbols) or in the absence (open symbols) of PTx. Data shown are mean ± S.D. from five different experiments performed in triplicate. (B) Effect of PTx (100 μg/ml, 20 h) and U-73122 (5 μM, 15 min) on the NT (10 nM)-induced $[Ca^{2+}]_i$ mobilization in single CHO-NTR cells in the absence of extracellular Ca^{2+}. Control cells, solid line; PTx treated cells, dotted line; U-73122 treated cells, dashed line. Data shown are representative of at least five determinations.

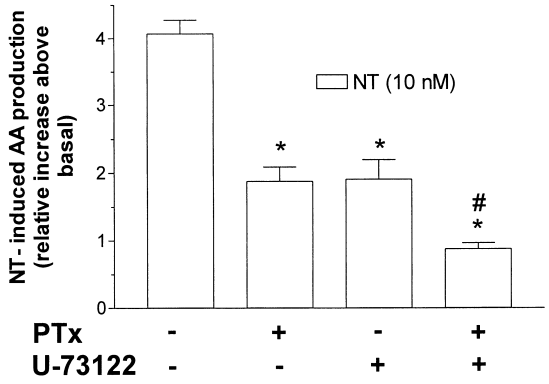

Fig. 3. Effect of PTx (100 μg/ml, 20 h) and U-73122 (5 μM, 15 min) on the NT (10 nM)-induced arachidonic acid production. Data shown are mean ± S.D. from 9 to 19 different determinations performed in duplicate. $^*P < 0.05$ as compared to control; $^\#P < 0.05$ as compared to either PTx or U-73122 alone (ANOVA followed by Student-Newman-Keuls test).

3.3. NT-induced [^{3}H]arachidonic acid release

NTS1 was also found to be coupled to the activation of PLA_2 [18,26] and such coupling was shown to partially depend on Ca^{2+} mobilized from the reticulum. Fig. 3 shows that NT-induced arachidonic acid production was decreased after PTx treatment (53% of inhibition). In addition, partial inhibition (52%) of this response was also obtained in the presence of 5 μM U-73122 (added 15 min prior NT). These effects were additive, suggesting that NT-induced production of arachidonic acid involves both a PTx-sensitive G-protein and a PLC-related PTx-insensitive pathway.

4. Discussion

In the vast majority of cellular and tissular models where NTS1 has been studied, NT-mediated hydrolysis of phosphoinositides and mobilization of Ca^{2+} have been observed. Accordingly, stimulation of NTS1 is uniformly proposed to activate PLC through a preferential coupling with $G_{q/11}$-type G-proteins. In a previous work performed on CHO-NTR cells, NT was shown to activate G-proteins, as revealed by a robust increase in the specific binding of [^{35}S]GTPγS binding [20]. The detection of nucleotide exchange induced by NT was considered to reveal this predominant functional coupling. However, an extensive review of the recent specialized literature indicates that unless sophisticated approaches involving immunoprecipitation of G-proteins are used, the analysis of [^{35}S]GTPγS binding (on cell homogenates or by autoradiography) is almost only applicable to receptors coupled to $G_{i/o}$-type G-proteins [27–30]. On the basis of our previous studies on the activation of PLC and PLA_2 by NT [10,18], these considerations prompted us to investigate the nature of the G-proteins specifically activated by NT in CHO-NTR cells. In this purpose, discrimination between $G_{q/11}$- and $G_{i/o}$-type G-proteins was performed by measuring the effect of PTx treatment on the NT signaling at both G-protein and effector levels.

The key finding of the present study is the evidence for the simultaneous coupling of NTS1 with both PTx-sensitive and insensitive G-proteins. Thus, the nucleotide exchange induced by NT was almost completely abolished on PTx-treated cells, whereas as expected, such treatment was without effect on the activation of PLC. The weak detection of the coupling with $G_{q/11}$-type G-proteins, inherent to this [^{35}S]GTPγS binding methods (see above), especially when simultaneous coupling with another G-protein is observed, precludes any quantitative comparison on the relative coupling to either PTx-sensitive and insensitive signaling pathways. In addition, despite the small amplitude of the response measured on PTx-treated cells, analysis of NT-induced [^{35}S]GTPγS binding revealed a potency for NT that was almost identical to that found on control cells. In both cases, the low potency of NT has to be attributed to the relatively high concentration of NaCl required in this assay that dramatically decreases the affinity of agonists for the receptors [31]. Although some hypothesis have been proposed to explain these differences in the measurements of nucleotide exchange at distinct G-proteins [32], our attempts to enhance the NT-induced [^{35}S]GTPγS binding on PTx-treated cells (by changing the GDP or NaCl concentrations, the incubation time and the temperature) were not conclusive.

In complement to [^{35}S]GTPγS binding studies, the coupling of the NTS1 with G-proteins was investigated by evaluating the ability of the non-hydrolyzable guanylyl nucleotide Gpp(NH)p to decrease the affinity of NT for its receptor on cell homogenates. Binding of agonists to both $G_{q/11}$- and $G_{i/o}$-type G-proteins linked receptors are known to be effectively affected by guanylyl nucleotides. Surprisingly, PTx treatment did not affect the coupling of NTS1 to G-proteins in this indirect functional assay. Such discrepancy between the results obtained using these two complementary approaches suggests that although the coupling with $G_{i/o}$-type G-protein is clearly demonstrated, the interaction with PTx-insensitive G-proteins is predominant. The link between the ability of Na^+ ions to modulate the binding of agonist and the functional coupling to G-proteins is still controversial for many receptors [33]. However, in the case of NTS1, it was clearly demonstrated that the mutation of a conserved aspartate residue within the second transmembrane domain conferring this Na^+ ion sensitivity was affecting the functional activation of G-proteins [7]. In our hands, the effect of Na^+ ions was still observed after PTx treatment. Based on these observations and on the fact that the activation of PLC is also resistant to PTx, one may propose that the modulation of NT binding by both Na^+ and Gpp(NH)p on CHO-NTR cells derives from the predominant functional coupling with $G_{q/11}$-type G-protein.

NTS1 has been shown to induce arachidonic acid release through the activation of PLA_2 [18,26]. This activation was shown to require calcium [18,34]. However contrasting with the well established activation of PLC by $G\alpha_{q/11}$, the G-proteins and intracellular pathways involved in PLA_2 activation seem more varied and/or controversial. Calcium directly activates the enzyme by binding to its amino-terminal C2 domain (μM range), triggering its translocation from the cytosol to the nuclear envelope and the reticulum [35–37]. An indirect activation by calcium has also been shown, involving the calcium-calmodulin dependent protein kinase II, the PKC and/or the MAP kinase [18,34,38–40]. Finally, G_i-type G-proteins are also implicated in the regulation of PLA_2 [41] possibly through the activation of MAP kinases by $G\alpha_{i2}$ [42], by the βγ subunits released upon G-protein activation [43] or through an influx of calcium [44]. In this study, both PTx and the PLC inhibitor U-73122 decreased the arachidonic acid production induced by NT. However, at a concentration that completely inhibits $[Ca^{2+}]_i$ transients induced by NT stimulation, U-73122 only decreased by half the amount of arachidonic acid produced. Similarly, the inhibition was also partial after a PTx treatment expected to maximally abolish the $G_{i/o}$ activation. The maximal effects of U-73122 and PTx treatments were additive suggesting that activation of PLA_2 by NTS1 not only results from the 'G_q-PLC – InsP3 – calcium' cascade but also from a direct coupling with $G_{i/o}$ type G-proteins.

Simultaneous coupling of membrane receptor with different G-proteins has frequently been reported, especially when using recombinant models in which high levels of receptor expression may facilitate the detection of either promiscuous or specific but low-efficient coupling. A variety of responses induced by NT in vivo were shown to be dependent on $G\alpha_{i/o}$-proteins and/or inhibited by PTx [15,17,25]. However, the nature of the NT receptor subtype(s) expressed in these models was not identified. Moreover, in some of these studies, the responses tested are those frequently proposed not to depend on the activation of NTS1 (analgesia, hypothermia). In contrast, the present study provides a clear-cut evidence for an independent functional coupling of the NTS1 to both PTx-sensitive and PTx-insensitive G-proteins. Such functional coupling of NTS1 with both $G\alpha_q$- and $G\alpha_{i1}$-proteins was observed in a model of *Escherichia coli* expressing NTS1-Gα fusion proteins (R. Grisshammer and E. Hermans, unpublished results). The existence of such multiple coupling is likely to have physiological and pharmacological consequences, leading to rather complex responses to agonists [45]. For instance, it would be of interest to show whether the regulation profile of these distinct signaling pathways differ, as suggested for other G-protein-coupled receptors [45].

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