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Agonist and antagonist modulation of [^{35}S]-GTPγS binding in transfected CHO cells expressing the neurotensin receptor

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1 The functional interaction of the cloned rat neurotensin receptor with intracellular G-proteins was investigated by studying the binding of the radiolabelled guanylyl nucleotide analogue [^{35}S]-GTPγS induced by neurotensin to membranes prepared from transfected Chinese hamster ovary (CHO) cells.

2 The agonist-induced binding of [^{35}S]-GTPγS was only detected in the presence of NaCl in the incubation buffer. However, it was also demonstrated that the binding of [^{3}H]-neurotensin to its receptor was inhibited by NaCl. In the presence of 50 mM NaCl, the binding of the labelled nucleotide was about 2 fold increased by stimulation with saturating concentrations of neurotensin (EC_{50} value of 2.3 ± 0.9 nM).

3 The stimulation of [^{35}S]-GTPγS binding by neurotensin was mimicked by the stable analogue of neurotensin, JMV-449 (EC_{50} value of 1.7 ± 0.4 nM) and the neurotensin related peptide neuromedin N (EC_{50} value of 21 ± 6 nM).

4 The NT-induced [^{35}S]-GTPγS binding was competitively inhibited by SR48692 (pA_2 value of 9.55 ± 0.28), a non-peptide neurotensin receptor antagonist. SR48692 alone had no effect on the specific binding of [^{35}S]-GTPγS.

5 The response to neurotensin was found to be inhibited by the aminosteroid U-73122, a putative inhibitor of phospholipase C-dependent processes, indicating that this drug may act at the G-protein level.

6 Taken together, these results constitute the first characterization of the exchange of guanylyl nucleotides at the G-protein level that is induced by the neuropeptide neurotensin after binding to its receptor.

Keywords: Neurotensin; SR 48692; U-73122; [^{35}S]-GTPγS; G-protein

Introduction

It is now well established that most of the biological effects mediated by the neuropeptide neurotensin result from the interaction of this peptide with G-protein coupled receptors that are present at the surface of the responding cells. The stimulation of the high affinity neurotensin receptors is known to promote the activation of an intracellular biochemical cascade that involves a molecular exchange of guanylyl nucleotides at the active site of the Gα subunit of the G proteins (Gilman, 1987; Taylor, 1990). The limiting step in the enzymatic hydrolysis of guanosine 5′-triphosphate (GTP) into guanosine 5′-diphosphate (GDP) by G-proteins is the dissociation of GDP from the active site. The major consequence of the coupling of an agonist-receptor complex with the G-protein is the facilitation of this GDP release that allows the binding of GTP to the Gα subunit (Florio & Sterweis, 1989). After binding of GTP, the Gα subunit dissociates from the βγ subunit and is able to activate the intracellular effectors involved in the biochemical response. The dissociation of GTP from the G-protein is very slow, but due to the GTPase activity associated with the Gα subunit, GTP is rapidly hydrolyzed and GDP is regenerated.

On the basis of the critical role of GTP in the process, synthetic non-hydrolyzable analogues of this nucleotide (guanosine-5′-*O*-(γ-thiotriphosphate (GTPγS) and GppNHp) have been widely used to induce the activation the intracellular effectors by switching the cascade to the active state (Gilman, 1987). More recently, the radiolabelled guanylyl nucleotide analogue [^{35}S]-GTPγS has been used as a high affinity ligand, in order to measure the activation of receptors linked to G-proteins (Hilf *et al*., 1989; Lorenzen *et al*., 1993; Nanoff *et al*., 1995).

The functional coupling of the neurotensin receptor with G-proteins has already been proposed on the basis of the putative molecular characteristics of the cloned receptor which share structural properties with all members of the G-protein coupled receptor family (Tanaka *et al*., 1990) In addition, the stimulation of the high-affinity neurotensin receptor induces the hydrolysis of phosphoinositide through the activation of phospholipase C, which is known to be activated by G-proteins (Goedert *et al*., 1984; Hermans *et al*., 1992). It has also been demonstrated that the affinity of neurotensin for the neurotensin receptor was modulated by guanylyl nucleotides, as is the case for most agonists of G-protein coupled receptors (Ahmad *et al*., 1987; Kitabgi *et al*., 1987; Bozou *et al*., 1989; Carraway *et al*., 1993). Finally, some biochemical effects of neurotensin were found to be dependent on the activity of G-proteins (Bozou *et al*., 1989; Fu *et al*., 1992; Wu & Wang, 1995).

As the exchange of GDP for GTP constitutes the first step in the cascade of intracellular signalling involving G-protein, the present study was undertaken to characterize neurotensin-induced [^{35}S]-GTPγS binding to homogenates of Chinese hamster ovary (CHO) cells expressing the neurotensin receptor. The same assay was used to characterize the biochemical properties of the non-peptide drug SR48692 at the neurotensin receptor. In addition, this assay was used to locate precisely the site of action of compound U-73122 which is known to inhibit phospholipase C-dependent cellular processes.

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Methods

Preparation of cell homogenates

Transfected CHO cells expressing the cloned rat high-affinity neurotensin receptor (B_{max} of 3.2 pmol mg^{-1} protein, Hermans *et al.*, 1992) were cultured in α-MEM containing 10% foetal calf serum in 10 cm plates. Confluent cells were washed with ice-cold phosphate buffered saline (PBS, 140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), scraped from the dishes and collected in PBS by low-speed centrifugation. The cell pellet was disrupted after 10–12 passages through a 26 gauge needle in 50 mM Tris-HCl pH 7.4, and the cell membranes were collected by centrifugation (49,000 *g*, 4°C). After 3 washing steps under the same conditions, the pellet was resuspended in 50 mM Tris-HCl, pH 7.4 and the protein level was measured. All the procedures were performed at 0–4°C.

Binding of [35S]-GTPγS and [3H]-neurotensin

The binding experiment was performed at 30°C in plastic tubes containing 25 or 50 μg protein resuspended in 1 ml (final volume) binding buffer. This buffer contained: 50 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 μM 1–10 phenanthroline, 0.1% bovine serum albumin, 0.03% bacitracin, 1 μM GDP (unless indicated) and 50 mM NaCl (unless indicated). As proposed by a number of authors, dithiothreitol (DTT) (1 mM) was added to the [35S]-GTPγS binding buffer (Florio & Sterweiss, 1989; Reithmann *et al.*, 1991; Lorenzen *et al.*, 1993). The binding was initiated by the addition of [35S]-GTPγS (0.05 nM final). The non-specific binding was measured in the presence of 0.1 mM Gpp(NH)p. Unless indicated, incubations were performed for 40 min and were terminated by addition of 3 ml washing buffer (50 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, 50 mM NaCl unless indicated). The suspension was immediately filtered through GF/C glass fibre filters and washed twice with washing buffer. Radioactivity trapped on the filter was estimated by scintillation counting. Binding of [3H]-neurotensin (1 nM) was performed under the same conditions, except that the incubation was performed at 25°C for 30 min and that glass fibre filters were presoaked for 1 h in 0.5% polyethyleneimine. All the binding data were analysed by non linear regression with the software GraphPad Prism, version 2.00.

Materials

[35S]-GTPγS (spec. act. 1000 Ci $mmol^{-1}$) was from Amersham. [3H]-neurotensin (Spec. act. 109 Ci $mmol^{-1}$) was from NEN-Dupont. Neurotensin, neuromedin N, dithiothreitol, bacitracin, bovine serum albumin, 1-10 phenanthroline, GDP, Gpp(NH)p and polyethylenimine were from Sigma. Compound U-73122 (1-[6-[[17β-3-methoxyestra-1-3-5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione) was from Calbiochem. SR 48692 (2-[1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl) pyrazol-3-yl)carbonylamino]tricyclo-(3.3.1.1.^{3,7})decan-2-carboxylic acid) was kindly provided by Dr D. Gully (Sanofi Recherche, France). Glass fibre filters were from Whatman.

Results

As the critical step in the activation of G-proteins is the release of GDP, the agonist-induced binding of [35S]-GTPγS on washed cell membranes is only observed when GDP is present in the incubation buffer. Accordingly, when homogenates of CHO cells expressing the neurotensin receptor were incubated in the absence or in the presence of a very low concentration of GDP (10 nM), the binding of [35S]-GTPγS (0.05 nM) was relatively high, but was not significantly modified after addition of a high concentration of neurotensin (Figure 1a). At high concentrations of GDP (>0.1 mM) the binding of [35S]-GTPγS was very low and in these conditions it was not possible to

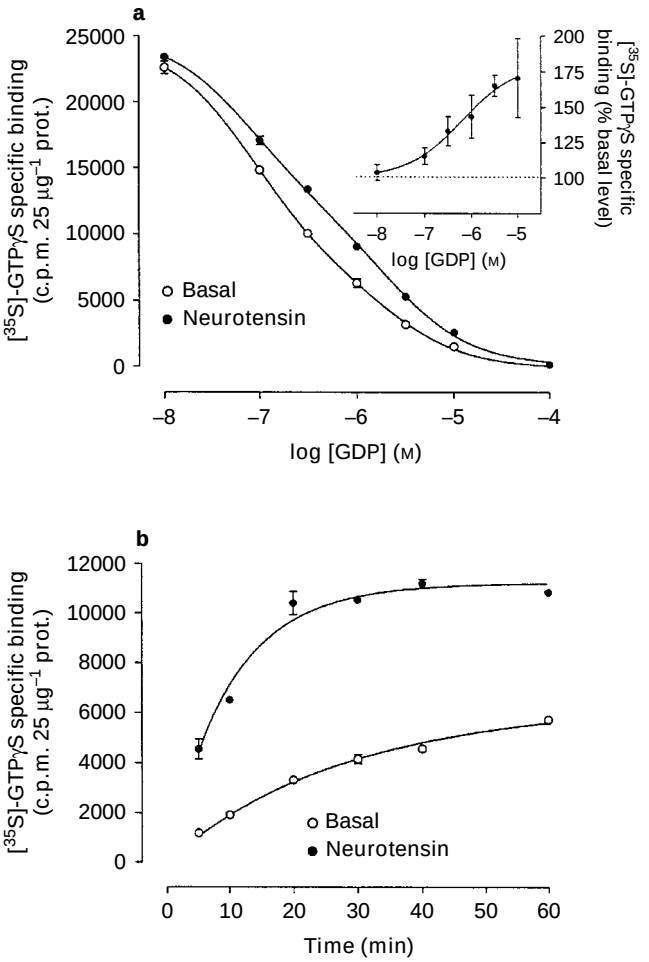

Figure 1 (a) Effect of increasing concentrations of GDP on the specific binding of [35S]-GTPγS to homogenates of transfected CHO cells in the presence and absence of neurotensin (100 nM). The specific binding was measured after a 40 min incubation period. Inset: relative stimulation of [35S]-GTPγS binding expressed as % basal (in the absence of agonist). (b) Time-course of basal and neurotensin (100 nM)-induced specific binding of [35S]-GTPγS to homogenates of transfected CHO cells. Data presented are means of triplicate determinations (experiment performed 3 times independently); vertical lines show s.d.

calculate the effect of neurotensin. The maximal effect of neurotensin was found to occur in the presence of 0.3–3 μM GDP and therefore, all experiments were performed in a buffer containing this nucleotide at a final concentration of 1 μM. At this concentration, the basal specific binding of [35S]-GTPγS (0.05 nM) was about 5000 c.p.m. 25 μg^{-1} protein and in these conditions, the non-specific binding of [35S]-GTPγS (0.05 nM), measured in the presence of Gpp(NH)p (0.1 mM), represented about 10–20% of the total binding (500–1000 c.p.m. 25 μg^{-1} protein).

Kinetic experiments indicated that both the basal [35S]-GTPγS binding to the cell homogenates and the binding induced by neurotensin progressively increased with time (Figure 1b). Experimental data best fitted with a monoexponential equation that indicated that neurotensin induces both an increase in the association rate (19.3 and 6.9 min half-time for association in the absence and presence of neurotensin, respectively) and an increase in the maximal binding (6,300 and 11,200 c.p.m. 25 μg^{-1} prot. in the absence and presence of neurotensin, respectively). The maximal ratio between basal and stimulated [35S]-GTPγS binding was observed after 40 min incubation and, therefore, in all experiments the response to the different stimulations was measured after a 40 min incubation period. In these conditions, the maximal [35S]-GTPγS

binding measured after stimulation with neurotensin was 150 to 200% of the basal level.

Agonist-induced binding of [35S]-GTPγS is usually measured in the presence of high concentrations of NaCl (100–150 mM). Accordingly, the neurotensin-induced binding of [35S]-GTPγS was found to be dependent on the concentration of this salt in the incubation buffer. Maximal increase in the binding of the nucleotide was observed in the presence of 150 mM NaCl, whereas almost no response was detected in NaCl-free buffer (Figure 2a). The analysis of the data presented in Figure 2a and b clearly indicate the two different effects of NaCl on the binding of [35S]-GTPγS in basal condition or after stimulation with neurotensin. NaCl decreases the basal binding of the nucleotide and thus allows the measure of the effect of the agonist on this binding (Figure 2a). However, NaCl is also found to decrease the potency of neurotensin in this assay in a concentration-dependent manner, as indicated by the progressive rightward shift of the dose-response curve to the agonist (Figure 2b).

In order to study whether the effect of NaCl on the potency of neurotensin was a consequence of an impaired binding of neurotensin on its receptor, the specific binding of [3H]-neurotensin on cell homogenates was measured in the presence of increasing concentrations of NaCl. As shown on Figure 3, the addition of NaCl in the binding buffer resulted in a concentration-dependent decrease in the [3H]-neurotensin binding (Figure 3), an effect that is thought to be related to a decrease in the affinity of neurotensin for its receptor, as previously shown in other studies (Ahmad *et al*., 1987; Bozou *et al*., 1989). For all these reasons, the characterization of the neurotensin-induced [35S]-GTPγS binding was performed in a buffer containing 50 mM NaCl, as a compromise between the binding of [35S]-GTPγS and the affinity of neurotensin for its receptor. It is important to note that the affinity of neurotensin for its receptor is known also to be modulated in the presence of guanylyl nucleotides or by magnesium ions at high concentrations (Bozou *et al*., 1986; Carraway *et al*., 1993; Labbé-Julié *et al*., 1994). In our study, incubation of the cell homogenates in the presence of GDP (1 μM) resulted in a limited reduction of the specific binding of [3H]-neurotensin (Figure 3). Similarly, it was demonstrated that the binding of [3H]-neurotensin was not significantly affected in the presence of dithiothreitol (1 mM) and Mg^{2+} ions (5 mM) which are also required for the measurement of the binding of guanylyl nucleotides to G-proteins (Figure 3).

The analysis of the concentration-response curve for neurotensin allowed the calculation of the potency of this peptide in this assay. In a buffer containing 50 mM NaCl, the half-maximal response was observed at 2.3±0.9 nM neurotensin (Hill slope of 0.96±0.13). The effect of neurotensin was mimicked by the peptidase-resistant analogue of neurotensin, compound JMV-449 (Dubuc *et al*., 1992) and by the neurotensin-related neuropeptide neuromedin N (Minamino *et al*., 1984), with half maximal responses at 1.7±0.4 nM (Hill slope of 0.78±0.12) and 21±6 nM (Hill slope of 0.84±0.10), respectively (Figure 4). When the experiment was performed in a buffer containing 150 mM NaCl, the amplitude of the maximal response was higher, but the EC_{50} value for neurotensin and JMV-449 were 13.2±0.4 and 8.6±0.8 nM, respectively (not shown).

Neurotensin-induced [35S]-GTPγS binding to the cell homogenates was found to be competitively inhibited by compound SR48692, a specific neurotensin receptor antagonist. In the presence of increasing concentrations of this antagonist, the sigmoidal concentration-response curve was progressively displaced to the right in an apparent parallel fashion (Figure 5). After analysis of the responses measured in the presence of different concentrations of antagonist, it was possible to estimate a pA_2 value for this antagonist at the neurotensin receptor of 9.55±0.28 (slope of 0.83±0.07). When SR48692 (1 μM) was added alone to the cell homogenates, no significant change in the basal [35S]-GTPγS binding was measured, indicating the absence of agonist properties of this drug. This also indicates the absence of constitutive activity of the neurotensin receptor in the transfected CHO cells. Such constitutive activity was previously shown when the neurotensin receptor was expressed at very high density in Sf9 cells (Boudin

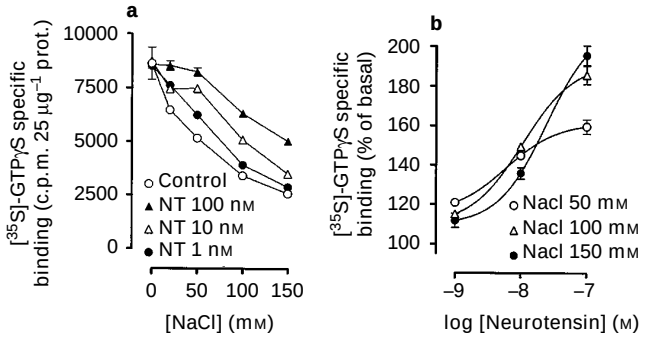

Figure 2 (a) Effect of increasing concentrations of NaCl on the specific binding of [35S]-GTPγS in the absence and presence of neurotensin (NT) 1, 10 and 100 nM. In (b) the results are expressed as percentage of the basal binding of the nucleotide measured in the same NaCl concentration. Only data for 50, 100 and 150 mM NaCl are presented. Means of triplicate determinations (experiment performed 2 times independently) are shown; vertical lines indicate s.d.

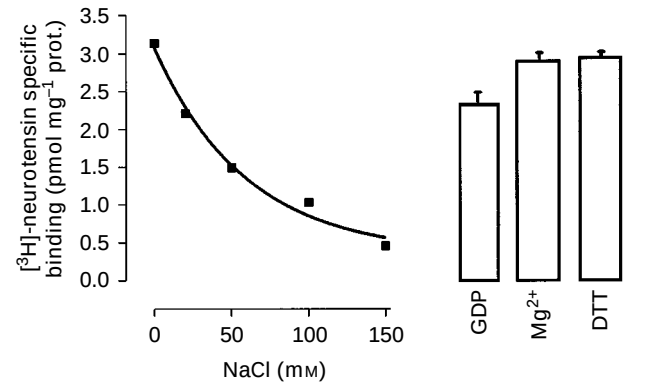

Figure 3 Effect of NaCl (increasing concentrations), GDP (10^{-6} M), $MgCl_2$ (5 mM) and dithiothreitol (DTT, 1 μM) on the specific binding of [3H]-neurotensin (1 nM). Means of triplicate determinations (experiment performed 2 times independently) are shown; vertical lines indicate s.d.

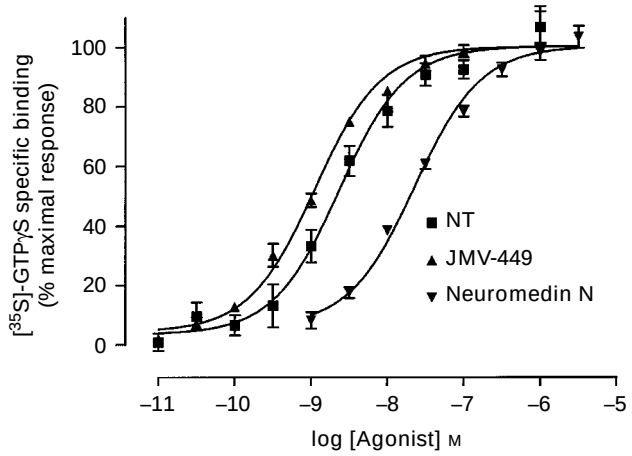

Figure 4 Dose-response curves for neurotensin (NT), neuromedin N- and JMV-449-induced [35S]-GTPγS specific binding to homogenates of transfected CHO cells. Results are expressed as % of maximal response. Means of triplicate determinations performed at least 3 times independently are shown; vertical lines indicate s.d.

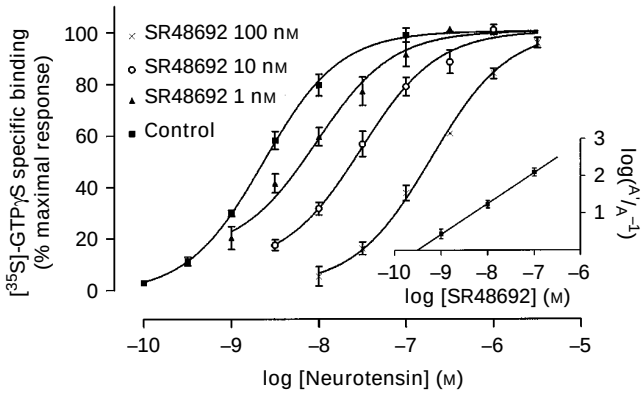

Figure 5 Dose-response curve for the neurotensin-induced [^{35}S]-GTPγS specific binding to homogenates of transfected CHO cells in the presence of increasing concentrations of compound SR48692. Results are expressed as % of maximal response and represent means of triplicate determinations performed 4 times independently; vertical lines show s.d. Inset: Schild plot for the inhibition allowing the determination of the pA_2 value. Data presented were obtained from the dose-ratios from 4 different experiments.

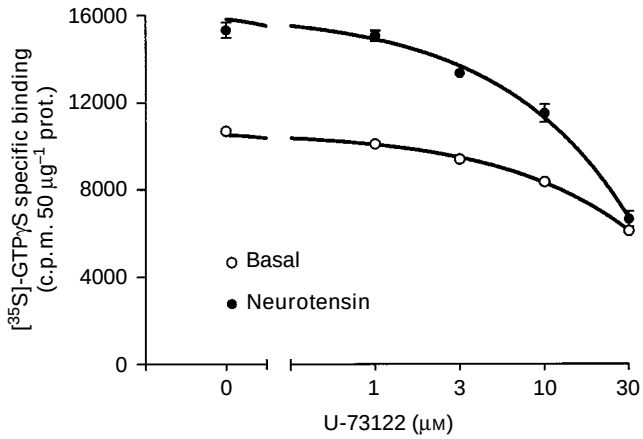

Figure 6 Effect of compound U-73122 on the basal and neurotensin (3 nM)-induced [^{35}S]-GTPγS specific binding to homogenates of transfected CHO cells. Means of triplicate determinations performed 3 times independently are shown; vertical lines show s.d.

et al., 1996) and this activity was found to be blocked by the neurotensin receptor antagonist SR48692.

Compound U-73122 is an aminosteroid that is known to block the phospholipase C-dependent processes efficiently (Smith *et al*., 1990). However, the precise site of action of this compound in the biochemical cascade that involves phospholipase C is still obscure. In order to clarify the mechanism of action of U-73122, the effect of this compound was measured on the neurotensin-induced [^{35}S]-GTPγS binding. Dimethyl sulphoxide (maximal final concentration, 0.8%) which was used for the solubilization of compound U-73122 had no effect on the binding of [^{35}S]-GTPγS. As shown in Figure 6, in the presence of U-73122 (10 or 30 μM), the basal [^{35}S]-GTPγS binding was found to be decreased as compared to control. In addition, when used at 30 μM, this compound was also found to inhibit about 80% of the neurotensin-induced binding of the nucleotide.

Discussion

In the present study, the neurotensin-induced [^{35}S]-GTPγS binding was measured in homogenates from CHO cells expressing the neurotensin receptor. This response was measured under conditions that decrease the spontaneous binding of [^{35}S]-GTPγS to G-proteins by addition of GDP and NaCl to the incubation buffer. Under these conditions, both neurotensin (NT) and its natural and synthetic analogues (neuromedin N (NN) and compound JMV-449) display agonist activities in the nanomolar range. The potencies of these drugs in the present assay were lower than their respective affinities usually found at the neurotensin binding site (<1 nM for NT and JMV449 and <10 nM for NN) (Dubuc *et al*., 1992; Chabry *et al*., 1994; Hermans *et al*., 1996). However, the rank of order of potencies was conserved: JMV-449 > neurotensin > neuromedin N. As already found for other receptors, the presence of GDP and sodium ions in the incubation buffer decreases the basal binding of [^{35}S]-GTPγS, but allows a better detection of the effect of agonists (Gierschik *et al*., 1991; Lorenzen *et al*., 1993). However, both GDP and Na^+ have been shown to inhibit significantly the specific binding of agonists to the neurotensin receptor (Bozou *et al*., 1986; 1989). It is noteworthy that sodium ions have opposite effects on the binding of neurotensin to its receptor and on the binding of [^{35}S]-GTPγS to G-proteins elicited by the peptide. Indeed, *in vivo*, these two events occur at distinct sites at the outer and inner faces of the cell membrane, in different ionic environments. However, the [^{35}S]-GTPγS binding assay requires direct access of agonists to the extracellular face of the receptor and of the nucleotides to the intracellular G-proteins and needs therefore, to be conducted on cell homogenates, under conditions that do not preserve the physiological characteristics that distinguish the intracellular and extracellular medium. It is intriguing to note that the physiological extracellular sodium concentration is close to 150 mM, which is considerably higher than that of the incubation buffer usually adopted for the characterization of neurotensin binding on cell homogenates which are completely devoid of sodium ions. This point was raised 10 years ago by Ahmad *et al*. (1987). However, on the basis of recent studies conducted with other G-protein coupled receptors, it is proposed that the effect of sodium ions on the binding of neurotensin does not result from a direct effect of these ions at the agonist binding site, but more likely results from an indirect effect that involves the action of sodium ions on the receptor-G-protein coupling (Lorenzen *et al*., 1993). Accordingly, the effect of sodium is only observed in binding experiments performed on homogenates. As a rule, high affinity neurotensin binding was measured on intact transfected CHO cells in culture medium containing 129 mM NaCl (K_d of 1.87 nM, Hermans *et al*., 1996). It was also found that higher concentrations of NaCl dramatically decreased the affinity of neurotensin for its receptor, in intact cells in acidic conditions (Mazella *et al*., 1991). However, in this case, it is possible that sodium ions have a direct effect on neurotensin binding. As described in the Results section, the accurate evaluation of agonist-induced nucleotide binding was only obtained in the presence of 50 mM NaCl. One may thus conclude that the relatively low potency of neurotensin to induce the response is a consequence of the experimental conditions required for the assay. For the vast majority of G-protein coupled receptors, the principal effect of sodium ions, like guanylyl nucleotides at high concentrations, is to convert the G-protein coupled receptor into its low affinity state for agonists, which is thought to represent a G-protein uncoupled state (Lorenzen *et al*., 1993; Wong *et al*., 1994). Therefore, the reason why most of the studies concerning the effect of diverse agonists on the [^{35}S]-GTPγS binding have been made in the presence of high concentrations of NaCl in the incubation buffer remains unresolved (Hilf *et al*., 1989; Reithmann *et al*., 1991; Nanoff *et al*., 1995).

In the present study, the maximal response was observed after 30 min incubation. This indicates that the response to agonists measured *in vitro*, under the experimental conditions described, is particularly slow as compared to the putative immediate activation of G-protein after stimulation of the receptor, even if a significant response was already obtained after

5 min stimulation with neurotensin. Such a slow agonist-induced [^{35}S]-GTPγS binding has also been observed in other studies (Hilf *et al*., 1989; Giershcick *et al*., 1991; Lorenzen *et al*., 1993; Traynor & Nahorski, 1995). Indeed, the response that is measured does not reveal only the speed of G-protein activation, but is also directly dependent on the binding of the agonist to the receptor, a process that is proposed to slowly (about 20 min) reach equilibrium (Sadoul *et al*., 1984; Kitabgi *et al*., 1984). In addition, due to its non-hydrolyzable property, the binding of [^{35}S]-GTPγS is almost irreversible and the response that is measured is the maximal association of the nucleotide with G-protein observed after prolonged activation, a situation that is probably never observed *in vivo*. In addition, it is important to note that the concentration of GTP in the cytoplasm of living cells is much higher than the concentration of the non-hydrolyzable analogue of GTP present in the present assay on homogenates.

The agonist-induced [^{35}S]-GTPγS binding assay was used to characterize the functional properties of the recently described non-peptide neurotensin antagonist, SR 48692 at the neurotensin receptor. As shown, this compound was found to inhibit competitively the neurotensin-induced binding of [^{35}S]-GTPγS with an activity in the nanomolar range. The antagonist properties of this compound against neurotensin have already been demonstrated in multiple studies, including experiments conducted at the second messenger level (Gully *et al*., 1993; Chabry *et al*., 1994; Hermans *et al*., 1995; Oury-Donat *et al*., 1995; Pugsley *et al*., 1995; Akunne *et al*., 1995; Schaeffer *et al*., 1995). As the exchange of guanylyl nucleotides at the active site of the G-protein constitutes the initial step in the intracellular signalling process, the [^{35}S]-GTPγS binding assay constitutes a more direct measure of the functional activation of the receptor or its inhibition than the measure of intracellular messengers that are located downstream in the biochemical cascade. The activity of the antagonist in this assay was significantly higher than that observed *in vitro*, in other second messenger assays (Chabry *et al*., 1994, IC_{50} of 10 nM; Oury-Donat *et al*., 1995, pA_2 of 8.7) or in binding experiments conducted on different tissue preparations from rat (Gully *et al*., 1993, IC_{50} of 4.0 nM; Azzi *et al*., 1994, K_i of 3.5 nM; Betancur *et al*., 1995, IC_{50} of 2.19 nM). However, it is generally proposed that the affinity of antagonists for G-protein coupled receptors is not affected by the presence of guanylyl nucleotides or sodium ions, even on cell homogenates (Birnbaumer *et al*., 1990). Accordingly, in the case of SR48692, a slight increase in its affinity for the neurotensin receptor in the presence of NaCl has been found (Labbé-Julié *et al*., 1994; 1995). This could explain the relatively greater activity of SR48692 in inhibiting the response to neurotensin measured in the presence of NaCl in the homogenates of transfected CHO cells. Furthermore, as shown in previous studies, the neurotensin antagonist was devoid of agonist activity at the neurotensin receptor (Gully *et al*., 1993; Azzi *et al*., 1994; Brouard *et al*., 1994; Nisato *et al*., 1994).

We have also studied the effect of compound U-73122 on neurotensin-induced [^{35}S]-GTPγS binding in homogenates of transfected CHO cells. Although most authors consider this compound to be an inhibitor of receptor-mediated phospholipase C activation, its site of action remains to be defined. This point is of importance since this compound was also found to inhibit the internalization of the neurotensin receptor in cultured cells, leading the authors to propose a direct link between the activation of phospholipase C and the receptor internalization process (Yamada *et al*., 1993). In the present study, when used at relatively high concentrations (30 μM), this compound was found to reduce dramatically the ability of neurotensin to promote the binding of the labelled nucleotide. Classically, U-73122 is known to inhibit the phospholipase C-dependent responses in a variety of models (Smith *et al*., 1990). At the G-protein level, an almost complete inhibition of the basal and agonist-induced GTPase activity by compound U-73122 has been shown previously (Thompson *et al*., 1991). However, as far as we know, the effect of this compound on the binding of guanylyl nucleotides has never been described. The concentration of U-73122 required to inhibit neurotensin-induced [^{35}S]-GTPγS binding was higher than that generally used to inhibit the hydrolysis of phosphoinositide. However, the potency and the efficacy of this compound vary from one model to another. Indeed, the specific activation of phospholipase C by neurotensin was previously found to be inhibited by U-73122, but at the highest concentration used, the maximal inhibition was only 60% (Yamada *et al*., 1993). On the basis of the present study, it is proposed that this drug acts at the G-protein level. Accordingly, the hypothesis that U-73122 acts on a component of the signal transduction pathway upstream of phospholipase C has been proposed (Yule & Williams, 1992; Babich *et al*., 1994). In addition, a specific disruption of receptor-G-protein coupling or a disruption of G-protein function by U-73122 has also been demonstrated previously (3.7 μM, Thompson *et al*., 1991). Taken together, these results and the previously published effect of U-73122 on the neurotensin receptor internalization support the possibility of a link between the activation of a G-protein and the internalization process.

As demonstrated in the present study, as well as in previously published studies, the technique of measuring agonist-induced [^{35}S]-GTPγS binding in optimal experimental conditions constitutes a simple assay that allows the determination of some pharmacological properties of agonists or antagonists at an early step in the biochemical cascade coupled to the receptor. The present study provided the first data on the specific activation of G-protein by the cloned neurotensin receptor, and the inhibition of this process by the non-peptide neurotensin receptor antagonist. The major second messenger cascade involved in the response of transfected CHO cells to neurotensin is the hydrolysis of phosphoinositide leading to the production of inositol phosphates and diacylglycerol (Watson *et al*., 1992; Hermans *et al*., 1992). However, in multiple models of tissues or cell lines, the involvement of other signalling cascades in the response to neurotensin has been proposed, including those leading to the modulation of cyclic nucleotides concentrations (Bozou *et al*., 1986; Kalivas, 1993; Slusher *et al*., 1994). Antibodies raised against the different Gα proteins are now available and a further step in the characterization of the biochemistry of the neurotensin receptor is to define precisely the involvement of the different G-proteins causing the intracellular response to neurotensin in cells and tissues.

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