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Characterization of the effect of SR48692 on inositol monophosphate, cyclic GMP and cyclic AMP responses linked to neurotensin receptor activation in neuronal and non-neuronal cells

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1 Neurotensin stimulated inositol monophosphate (IP1) formation in both human colonic carcinoma HT29 cells and in mouse neuroblastoma N1E115 cells with EC_{50} values of 3.5 ± 0.5 nM ($n=4$) and 0.46 ± 0.02 nM ($n=3$), respectively. Neurotensin also stimulated cyclic GMP production with an EC_{50} of 0.47 ± 1.2 nM and inhibited cyclic AMP accumulation induced by forskolin (0.5 μM) with an IC_{50} of 1.33 ± 1.5 nM ($n=3$) on the N1E115 cell line.

2 The competitive antagonism by the non-peptide neurotensin receptor antagonist, SR48692 of neurotensin-induced IP1 formation revealed pA_2 values of 8.7 ± 0.2 ($n=3$) for HT29 and 10.1 ± 0.2 ($n=3$) for N1E115 cells. SR48692 also antagonized the cyclic GMP and cyclic AMP responses induced by neurotensin in the N1E115 cell line with pA_2 values of 10.7 ± 0.7 ($n=3$) and 9.8 ± 0.3 ($n=3$), respectively.

3 In CHO cells transfected with the rat neurotensin receptor, neurotensin stimulated IP1 and cyclic AMP formation with EC_{50} values of 3.0 ± 0.5 nM ($n=3$) and 72.2 ± 20.7 nM ($n=3$), respectively. Both effects were antagonized by SR48692, giving pA_2 values of 8.4 ± 0.1 ($n=3$) for IP1 and 7.2 ± 0.4 ($n=3$) for cyclic AMP responses.

4 Radioligand binding experiments, performed with [^{125}I]-neurotensin (0.2 nM), yielded IC_{50} values of 15.3 nM ($n=2$) and 20.4 nM ($n=2$) for SR48692 versus neurotensin receptor binding sites labelled in HT29 and N1E115 cells, respectively.

5 In conclusion, SR48692 appears to be a potent, species-independent antagonist of the signal transduction events triggered by neurotensin receptor activation in both neuronal and non-neuronal cell systems.

Keywords: Neurotensin; SR48692; inositol monophosphate; cyclic GMP; cyclic AMP; HT29 cells; N1E115 cells

Introduction

Neurotensin is a tridecapeptide originally discovered in bovine hypothalamus (Carraway & Leeman, 1973). Subsequently, the presence of high concentrations of neurotensin has been detected in numerous areas of the central nervous system (CNS) (Carraway & Leeman, 1976; Emson *et al.*, 1985) and in gastrointestinal tissues (Rosell & Rökaeus, 1981). Recently, the molecular cloning of rat (Tanaka *et al.*, 1990) and human (Vita *et al.*, 1993) neurotensin receptors was reported and indicated that this receptor belongs to the family of G protein-coupled receptors, consisting of seven putative transmembrane spanning regions. Activation of neurotensin receptors has been shown to be associated with an enhanced phosphatidyl inositol turnover in different regions of the CNS (Erwin & Radcliffe, 1993), this stimulatory action being weakly effective and only observed in the presence of high concentrations of neurotensin (Goedert *et al.*, 1984; Erwin & Radcliffe, 1993). Several cellular models have been used to investigate further the second messengers affected by neurotensin. In the human colonic carcinoma cell line, HT29, the binding of neurotensin to its receptor leads to an increase in phosphatidyl inositol turnover without affecting cyclic nucleotide levels (Amar *et al.*, 1986). In contrast, in the electrically excitable mouse neuroblastoma N1E115, neurotensin was found to increase inositol phosphates (Snider *et al.*, 1986) and intracellular concentrations of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Gilbert *et al.*, 1986) and to decrease adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels (Bozou *et al.*, 1986). Furthermore, in Chinese hamster ovary cells transfected with the rat neurotensin receptor (rNTRCHO), neurotensin was reported to stimulate the production of inositol phosphates (Hermans *et al.*, 1992; Watson *et al.*, 1992) as well as the formation of cyclic AMP (Yamada *et al.*, 1993). The variety of coupling mechanisms leading to the generation of different second messengers following neurotensin receptor activation may suggest linkage to multiple effectors by a single receptor type or alternatively may add support to the notion of neurotensin receptor heterogeneity already suggested on the basis or *in vitro* and *in vivo* structure activity studies (Al-Rodhan *et al.*, 1991; Labbé-Jullié *et al.*, 1994). Attempts to examine this alternative hypothesis have been limited as yet by the lack of availability of potent and selective neurotensin receptor antagonists.

Recently, SR48692 has been described as a non-peptide antagonist of neurotensin receptors (Gully *et al.*, 1993) possessing nanomolar affinity for different tissues and cells from various species. This compound has been reported to reverse various functional events linked to neurotensin receptor activation such as intracellular $[Ca^{2+}]_i$ mobilisation in HT29 cells, facilitation by neurotensin of the K^+-evoked release of [^{3}H]-dopamine in rat striatal slices and the turning behaviour induced by intrastriatal injection of neurotensin in mice (Gully *et al.*, 1993). However, SR48692 only partially prevents the retrograde axonal transport of neurotensin in the rat nigrostrial system (Steinberg *et al.*, 1994), does not block the excitatory response of rat substantia nigra neurones to neurotensin (Pinnock & Woodruff, 1994) and does not counteract neuro-

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tensin-induced hypothermia in rat and mice (Dubuc *et al.*, 1994). Although, species differences in SR48692 affinity cannot be excluded, these observations may support the concept of neurotensin receptor subtypes that SR48692 could help to discriminate.

By use of cellular models from different origins possessing various coupling mechanisms leading to distinct neurotensin-mediated cellular events, the purpose of the present work was to provide a further biochemical characterization of SR48692, with the aim of discriminating putative neurotensin receptor subtypes.

Methods

Cell cultures

The HT29 cell line was obtained from American Type Culture Collection (ATCC) (Rockville, MD, U.S.A.). Cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with 10% foetal calf serum, 4 mM glutamine, 200 iu ml^{-1} penicillin and 200 μg ml^{-1} streptomycin in a humidified incubator under 5% CO_2/95% air.

Non-differentiated neuroblastoma N1E115 cells (obtained from J. Fournier, Sanofi Recherche Toulouse, France) were cultured as described above for HT29 cells and induced to differentiate for 48 h in an DMEM-F12 medium containing 0.5% foetal calf serum, 2 mM glutamine and 1.5% dimethylsulphoxide. Differentiated cells were reincubated for 4 h in normal culture medium before use for second messenger measurement (Poustis *et al.*, 1984).

CHO cells (from ATCC) were transfected with the cDNA coding for the rat neurotensin receptor. The transfected cells (CHO-NTR) were cultured as described above for HT29 and N1E115 cells in an α-modified Eagle's medium (α-MEM) without nucleosides, containing 10% foetal calf serum, 4 mM glutamine and 300 μg ml^{-1} Geneticin (G418).

Measurement of inositol phosphate levels

Cells cultured on six-well plates were labelled for 24 h with 5 μCi ml^{-1} [^{3}H]-inositol. LiCl (20 mM) was added 15 min before addition of neurotensin alone or in the presence of various concentrations of SR48692. After 30 min stimulation, the reaction was stopped by aspiration of the medium and rapid addition of 1 ml cold methanol/0.1 M HCl (50:50, vol/vol). The aqueous phase was removed and applied to 1 ml Dowex columns, and inositol monophosphate (IP1) was eluted with 0.2 M ammonium formate/0.1 M formic acid. Radioactivity was quantified by liquid scintillation counting.

Measurement of cyclic GMP levels

N1E115 cells cultured in 24 well-plates were incubated for 4 h in normal culture medium and 30 min in 25 mM HEPES/Tris (pH 7.5), containing NaCl 140 mM, KCl 5 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 0.9 mM, 0.2% bovine serum albumin (BSA) and 4.5 g l^{-1} glucose. Cells were then incubated for 5 min in the presence or absence of SR48692 and stimulated for 20 s with neurotensin. The reaction was terminated by aspiration of the medium and rapid addition of 1 ml ethanol. Extracts were centrifuged at 3000 *g* for 10 min and supernatants were evaporated in a speed vacuum concentrator and cyclic GMP content was measured with the radioimmunoassay kit from Amersham (Les Ulis, France).

Measurement of cyclic AMP levels

N1E115 cells were cultured as described previously for cyclic GMP; cyclic AMP was measured according to the protocol of Amar *et al.* (1987). In brief, differentiated cells preincubated for 30 min at 37°C in incubation buffer were treated with or without SR48692 for 5 min before addition of 1 mM IBMX and 30 s later, forskolin alone or forskolin in the presence of neurotensin were added for another 30 s. The reaction was stopped by aspiration of the medium and rapid addition of 1 ml ethanol. After extraction, cyclic AMP content was measured with cyclic AMP assay kits from Amersham.

Ligand binding assay

Cell homogenates from confluent HT29 and N1E115 cells were prepared as described by Bozou *et al.* (1989). Binding assays were performed at 20°C for 20 min in 50 mM Tris-HCl buffer (pH 7.5) containing 0.2% bovine serum albumin, 1 mM phenanthroline and 1 mM EDTA in the presence of [^{125}I]-neurotensin (0.2 nM) and variable concentrations (0.1 nM to 1 μM) of SR48692. Non-specific binding was determined with 1 μM unlabelled neurotensin.

Drugs

Neurotensin was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Stock solutions of peptide were made in distilled water and stored as frozen aliquots. Mono [^{125}I]-iodo [Tyr^3]-neurotensin (2000 Ci $mmol^{-1}$) and [^{3}H]-inositol (80 Ci $mmol^{-1}$) were obtained from Amersham (Les Ulis, France). LiCl was purchased from Osi (Oulchy le Château, France) and Dowex-1 AG1-X8 (formate form) was from Biorad (Richmond, CA, U.S.A.). Forskolin, 1,10 phenanthroline and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma (St Louis, MO, U.S.A.).

All cell culture-related chemicals were obtained from GIBCO (Cergy Pontoise, France). SR48692, {2-(1-(7-chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazol-3-carbonyl]-amino]-adamantane-2-carboxylic acid} (Figure 1) was synthesized at Sanofi Recherche (Montpellier, France) and solubilized in dimethylsulphoxide for stock solutions. The proportion of dimethylsulphoxide did not exceed 0.01% (vol/vol), which was shown to have no effect on basal or neurotensin-evoked responses.

Data analysis

Inhibitory concentration-effect relationships were constructed by fitting the combined weighted data using SigmaPlot software (Jandel Scientific, Corte Madera, CA, U.S.A.) according to the logistic equation: $Y = Y_{max}/(1 + ([SR]/IC_{50})^n)$, where Y_{max} is the response observed in the absence of antagonist, [SR] is the variable concentration of antagonist, IC_{50} is the value of [SR] which inhibits 50% of the response and n is a factor describing the steepness of the curve. Similarly, the concentration-response curves for activation of responses by neurotensin were fitted according to the equation: $Y = Y_{max} - (Y_{max}/(1 + ([A]/EC_{50})^n))$, where Y_{max} is the maximal response observed, [A] is the variable concentration of neurotensin, EC_{50} is the value of [A] which evokes 50% of the maximal response observed and n is a factor describing the steepness of

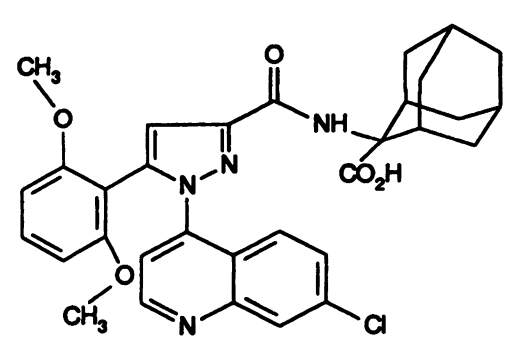

Figure 1 Structure of SR48692 (2-{[1-(7-chloroquinolin-4-yl)-5-(2,6-dimethoxyphenyl)-1H-pyrazole-3-carbonyl]amino}-adamantane-2-carboxylic acid).

the curve. The 3 parameters (Y_{max}, IC_{50} or EC_{50} and n) were determined by nonlinear curve fitting (Marquardt-Levenberg least squares procedure) and given with the corresponding standard deviations.

K_b values were calculated according to the Cheng-Prusoff relationship (Cheng & Prusoff, 1973; Craig, 1993) $K_b = IC_{50}/[1 + ([A]/EC_{50})]$, where IC_{50}, EC_{50} and [A] are the parameters described previously.

Results

Effect of neurotensin on IP1 formation in HT29 cells

As previously reported, incubation of HT29 cells with neurotensin in the presence of lithium produced a rapid and transient stimulation of IP3 and IP2 levels, and a more persistent increase of IP1 concentration (Amar *et al.*, 1986). Thus, because of the transient nature of the IP3 response and the fact that at short incubation times it can be expected that the agonist will not be in equilibrium with the receptor, we did not attempt to perform functional studies using this response, but rather used the formation of the IP1 for comparison with binding data.

The concentration-response curve for the effect of neurotensin on IP1 formation in HT29 cells is presented in Figure 2a. Application of neurotensin for 30 min stimulated IP1 formation in a concentration-dependent manner between 0.1 nM and 1 μM with a half-maximal stimulation (EC_{50}) of 3.5±0.52 nM ($n=4$) and a maximal activation occurring at 1 μM (basal level = 31.5±2.4 pmol/10^6 cells; maximal level = 80.3±7.6 pmol/10^6 cells).

Effect of SR48692 on IP1 formation induced by neurotensin in HT29 cells

Figure 2b shows the effect of SR48692 on inositol phosphate accumulation induced by various concentrations of neurotensin. SR48692, which has no effect on basal IP1 formation up to 1 μM (data not shown), potently antagonized the neurotensin-induced responses with IC_{50} of 6.7±0.6 nM, 28.7±4.1 nM, 194±53.3 nM, 910±158 nM for 3, 10, 100 and 1000 nM of neurotensin, respectively ($n=3$ for each determination). The Schild plot analysis shown in insert of Figure 2b, indicates a competitive antagonism for SR48692. The slope of the Schild plot was 0.9±0.1 and the pA_2 values was 8.7±0.2.

Effect of neurotensin on IP1 formation in N1E115 cells

As previously shown by Amar *et al.* (1987), incubation of differentiated N1E115 cells for 30 min in the presence of lithium with various concentrations of neurotensin, produced a concentration-response curve for the stimulatory effect of neurotensin on the intracellular level of IP1. Figure 3a illustrates that neurotensin stimulated IP1 formation with a half-maximal stimulation (EC_{50}) of 0.46±0.02 nM ($n=3$), a maximal activation occurring at 10 nM (basal level = 25.0±2.1 pmol/10^6 cells; maximal level = 131.3±19.1 pmol/10^6 cells).

Effect of SR48692 on IP1 formation induced by neurotensin in N1E115 cells

As already reported for the HT29 cell line (Figure 2b), SR48692, which has no effect on basal IP1 formation up to 1 μM (data not shown), potently antagonized the IP1 accumulation induced by various concentrations of neurotensin (Figure 3b) with IC_{50} values of 0.1±0.03 nM, 2.1±0.7 nM, 15±0.1 nM, 28±9.0 nM, 500±19.7 nM for 0.1, 0.3, 1.0, 3.0 and 10 nM, respectively ($n=3$ for each determination). The Schild plot analysis shown in insert of Figure 3b illustrates a competitive antagonism for SR48692, with a slope of 1.1±0.1 and a pA_2 value of 10.1±0.2.

Effect of neurotensin on cyclic GMP accumulation in N1E115 cells

The ability of neurotensin to stimulate cyclic GMP production is illustrated in Figure 4a. Application of neurotensin for 20 s enhanced cyclic GMP accumulation in a concentration-dependent manner, with a half-maximal stimulation (EC_{50}) of 0.47±3.5 nM ($n=3$), which was closely correlated with its potency in stimulating IP1 formation, the maximal activation occurred at 10 nM (basal level = 11.1±1.8 pmol/10^6 cells; maximal level = 249.6±19.2 pmol/10^6 cells).

Effect of SR48692 on cyclic GMP accumulation induced by neurotensin in N1E115 cells

As already reported for IP1 formation in N1E115 cells (Figure 3b), SR 48692 had no effect on basal cyclic GMP production up to 1 μM (data not shown), but potently antagonized the

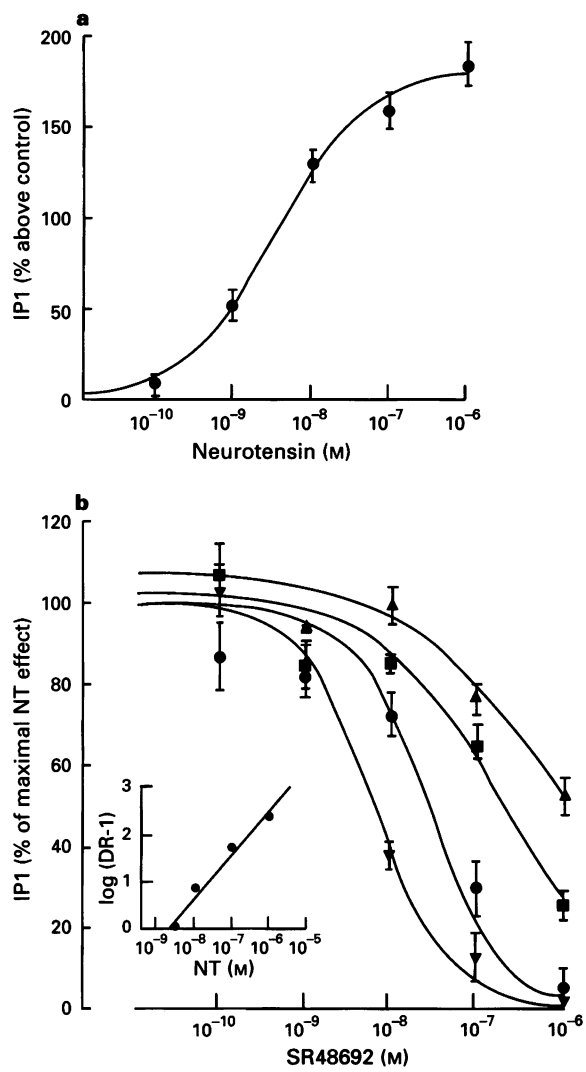

Figure 2 (a) Concentration-response curve for the stimulation of inositol monophosphate (IP1) levels induced by neurotensin in the HT29 cell line. Results are expressed as a percentage of stimulation above control. Each point represents the mean±s.e.mean of triplicate determinations from three independent experiments. (b) Antagonism by SR48692 of the neurotensin-induced IP1 formation in HT29 cells. Concentration-response curves for 3 (▼), 10 (●), 100 (■) and 1000 nM (▲) neurotensin. The results are expressed as a percentage of maximal neurotensin response. Each point represents the mean± s.e.mean of three separate experiments performed in triplicate. Insert: corresponding Schild plot for the antagonistic effect of SR48692 in HT29 cells. DR–1 = dose ratio minus 1, derived from EC_{50} and IC_{50} values obtained from the curves shown in (a) and (b) respectively.

cyclic GMP formation induced by various concentrations of neurotensin (Figure 4b) with IC_{50} values of 4.8 ± 0.2 nM, 25.0 ± 6.0 nM, 86.6 ± 1.2 nM, 1190 ± 16.7 nM for 0.3, 1.0, 10, and 100 nM, respectively ($n = 3$ for each determination). The Schild plot analysis shown in insert of Figure 4b illustrates the competitive antagonism for SR48692 on the cyclic GMP production with a slope of 0.9 ± 0.1 and a pA_2 value of 10.7 ± 0.3.

Effect of neurotensin on cyclic AMP production on N1E115 cells

Bozou *et al.* (1986), previously reported that neurotensin receptor activation was negatively linked to cyclic AMP accumulation. Figure 5a demonstrates that neurotensin inhibited in a concentration-dependent manner the formation of cyclic AMP induced by 0.5 μM forskolin with a half-maximal inhibition (IC_{50}) of 1.33 ± 1.5 nM ($n = 3$) and a maximal inhibition occurring at 1 μM (basal level = 2.2 ± 0.1 pmol/10^6 cells; maximal level = 6.6 ± 0.2 pmol/10^6 cells).

Effect of SR48692 on cyclic AMP formation induced by neurotensin in N1E115 cells

As already shown for IP1 and cyclic GMP production, SR48692 also potently antagonized the inhibitory effect of various concentrations of neurotensin on cyclic AMP formation (Figure 5b), with IC_{50} values of 2.8 ± 1.8 nM, 18.2 ± 15.3 nM, 50.8 ± 22.6 nM, 850 ± 300 nM for 0.3, 1.0, 10 and 100 nM respectively ($n = 3$ for each determination). The

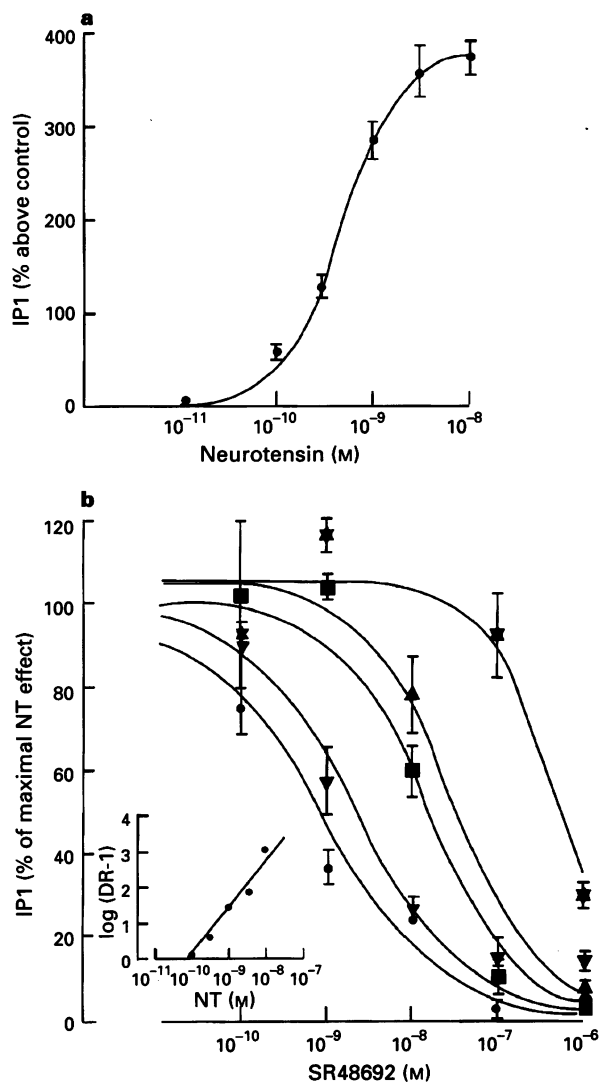

Figure 3 (a) Concentration-response curve for the stimulation of inositol monophosphate (IP1) levels induced by neurotensin in N1E115 cell line. Results are expressed as a percentage of stimulation above control. Each point represents the mean ± s.e.mean of triplicate determinations from three independent experiments. (b) Antagonism by SR48692 of the neurotensin-induced IP1 formation in N1E115 cells. Concentration-response curves for 0.1 (●), 0.3 (▼), 1 (■), 3 (▲) and 10 nM (★) neurotensin. The results are expressed as a percentage of maximal neurotensin response. Each point represents the mean ± s.e.mean of three separate experiments performed in triplicate. Insert: corresponding Schild plot for the antagonistic effect of SR48692 in N1E115 cells. DR–1 = dose ratio minus 1, derived from EC_{50} and IC_{50} values obtained from the curves shown in (a) and (b) respectively.

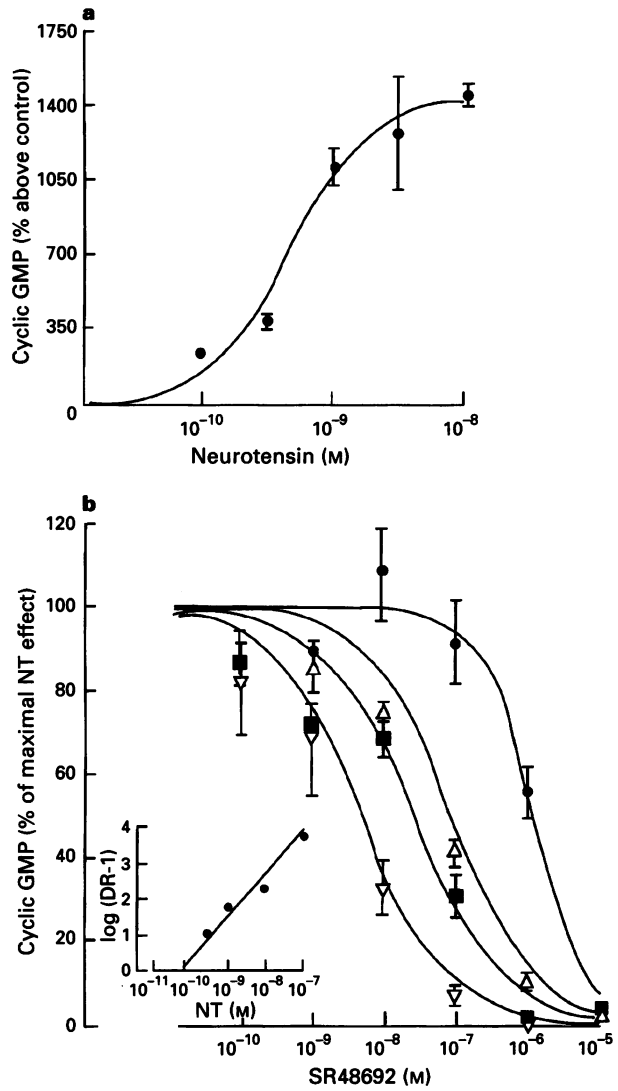

Figure 4 (a) Concentration-response curve for the stimulation of cyclic GMP levels induced by neurotensin in the N1E115 cell line. Results are expressed as a percentage of stimulation above control. Each point represents the mean ± s.e.mean of triplicate determinations from three independent experiments. (b) Antagonism by SR48692 of the neurotensin-induced cyclic GMP production in N1E115 cells. Concentration-response curves for 0.3 (▽), 1 (■), 10 (△) and 100 nM (●) neurotensin. The results are expressed as a percentage of maximal neurotensin response. Each point represents the mean ± s.e.mean of three separate experiments performed in triplicate. Insert: corresponding Schild plot for the antagonistic effect of SR48692 in N1E115 cells. DR–1 = dose ratio minus 1, derived from EC_{50} and IC_{50} values obtained from the curves shown in (a) and (b), respectively.

Schild plot analysis shown in insert of Figure 5b illustrates the competitive antagonism of SR48692 on the cyclic AMP response, with a slope of 1.0 ± 0.2 and a pA_2 value of 9.8 ± 0.3.

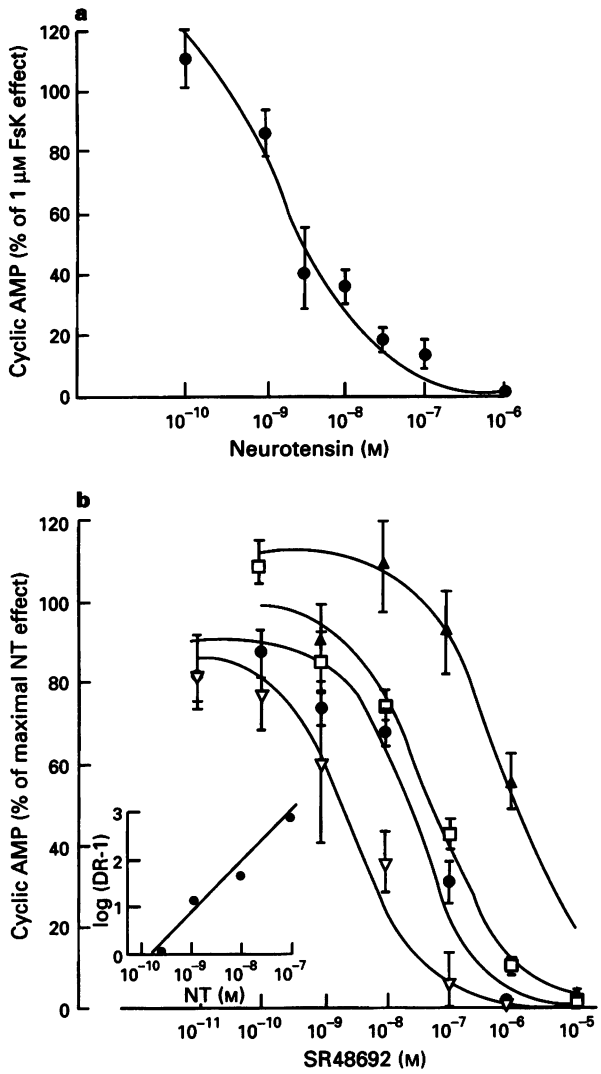

Figure 5 (a) Concentration-response curve for the neurotensin-induced inhibition of cyclic AMP production in the presence of 0.5 μM forskolin. Results are expressed as a percentage of maximal forskolin (Fsk) effect. Each point represents the mean ± s.e.mean of triplicate determinations from three independent experiments. (b) Antagonism by SR48692 of the neurotensin-induced cyclic AMP inhibition in N1E115 cells. Concentration-response curves for 0.3 (▽), 1 (●), 10 (□) and 100 nM (▲) neurotensin. The results are expressed as a percentage of maximal neurotensin response. Each point represents the mean ± s.e.mean of three separate experiments performed in triplicate. Insert: corresponding Schild plot for the antagonistic effect of SR48692 in N1E115 cells. DR–1 = dose ratio minus 1, derived from EC_{50} and IC_{50} values obtained from the curves shown in (a) and (b) respectively.

Effect of neurotensin and SR48692 on IP1 and cyclic AMP production on CHO-NTR cells

As shown in Table 1, neurotensin stimulated cyclic AMP accumulation in transfected CHO cells with a fifty fold lower efficacy than for the cyclic AMP response in N1E115 cells (EC_{50} of 72 nM vs 1.3 nM). The antagonistic effect of SR48692 exhibited a loss of potency revealed by a pA_2 value of 7.2 vs 9.8. However, neurotensin stimulated IP1 formation on these cells with an EC_{50} of 3.0 ± 0.2 nM ($n = 3$) which was very close to that obtained on HT29 cells ($EC_{50} = 3.5$ nM). The pA_2 value obtained for the antagonistic effect of SR48692 on the IP1 response was 8.4 ± 0.03 (vs 8.7 for HT29).

Binding studies on HT29 and N1E115 cells

The nonpeptide antagonist, SR4862 inhibited in a competitive and concentration-dependent manner the specific binding of [^{125}I]-neurotensin (0.2 nM) to HT29 and N1E115 cell membranes. Hill coefficients for SR48692 were in the two cell lines close to unity and IC_{50} values were 15.3 ± 8.6 nM for HT29 and 20.4 ± 11.6 nM for N1E115 cell membranes (mean ± s.d., $n = 2$). In comparison, the IC_{50} for neurotensin was 1.4 nM for HT29 and 1.2 nM for N1E115 cells ($n = 2$).

Discussion

The present work was undertaken to provide a further characterization of the non-peptide neurotensin receptor antagonist, SR48692 using distinct neurotensin-mediated cellular events and to discriminate putative neurotensin receptor subtypes. The earliest step in neurotensin action is an increase in phosphatidyl inositol turnover. In the human HT29 and murine N1E115 cell lines, we have demonstrated that neurotensin potently stimulated IP1 formation with EC_{50} values of 3.5 nM and 0.46 nM, respectively. As previously shown (Amar *et al.*, 1986; 1987), we also observed that the stimulatory effect of neurotensin on IP1 formation is followed by a decrease at higher concentrations of neurotensin in N1E115 cells (data not shown). This effect, generally interpreted as a desensitization of neurotensin receptors, is reported not to occur in HT29 cells (Yamada *et al.*, 1993). Furthermore, in HT29 cells, the G protein involved in phospholipase C activation is insensitive to pertussis toxin (Bozou *et al.*, 1989), whereas in N1E115 cells, the neurotensin effect on inositol phosphate production was reduced by the toxin (Amar *et al.*, 1987). Taken together, these differences in neurotensin action (EC_{50} values, desensitization, pertussis toxin sensitivity) could argue in favour of distinct neurotensin receptors in these two cell lines.

SR48692 behaved as a competitive antagonist on the IP1 response in HT29 and N1E115 cells with a concentration-dependent antagonism, giving pA_2 values in the low nanomolar range and slopes near unity. Interestingly, we found a significant difference in pA_2 values (8.7 ± 0.2 for HT29 and 10.1 ± 0.2 for N1E115 cells) which could also be compatible with the possibility of neurotensin receptor heterogeneity between the two cell lines.

Table 1 Summary of EC_{50} and pA_2 values obtained for neurotensin and SR48692, respectively, on the different intracellular second messengers investigated

	HT29		*N1E115*		*CHO-NTR*	
	EC_{50} (nM)	pA_2	EC_{50} (nM)	pA_2	EC_{50} (nM)	pA_2
IP1	3.5	8.7	0.46	10.1	3.0	8.4
Cyclic GMP	ND	ND	0.47	10.7	ND	ND
Cyclic AMP	ND	ND	1.33	9.8	72.2	7.2

ND: not detectable

However, this hypothesis is hard to reconcile with the fact that the capacity of SR48692 to displace [^{125}I]-neurotensin was identical in each cell line, cells for which the reported K_d values for [^{125}I]-neurotensin are similar (~1 nM) (Amar *et al.*, 1985; Bozou *et al.*, 1989). Furthermore possibly as a result of differences in coupling mechanisms between the two cell lines, the EC_{50} for IP1 formation showed one log difference which may explain part of the difference observed in pA_2 values. Indeed, when the K_b values were calculated according to the Cheng-Prusoff relationship (Craig, 1993), we found similar K_b values for HT29 (K_b = 3.6 nM) and N1E115 (K_b = 1.3 nM) cells.

In the N1E115 cell line, neurotensin was also able to induce an increase in cyclic GMP production whereas neurotensin did not produce this effect in HT29 cells. As previously demonstrated (Amar *et al.*, 1987), we show in the present study that neurotensin stimulated cyclic GMP accumulation with a potency (EC_{50} = 0.47 nM) similar to that found for IP1 formation. SR48692 antagonized the neurotensin-induced cyclic GMP production with a pA_2 value of 10.7, identical to that observed for the antagonism of IP1 formation (pA_2 = 10.1). These results, suggesting that the formation of these two second messengers is mediated by the same neurotensin receptor, strengthen the notion that the production of inositol 1,4,5 trisphosphate from PIP2 could activate the mobilization of Ca^{2+} from intracellular stores, leading in turn to a stimulation of cyclic GMP levels.

The third intracellular messenger which was modified after neurotensin receptor activation in the N1E115 cell line was cyclic AMP. In our study, we show that neurotensin inhibited cyclic AMP levels stimulated by forskolin with an EC_{50} of 1.3 nM. This result is in agreement with previous work using prostaglandin E_1 to stimulate cyclic AMP production (Bozou *et al.*, 1986), but contrasts with the failure to report an effect of neurotensin on this nucleotide response (Goedert *et al.*, 1984; Gilbert *et al.*, 1986). The reasons for these discrepancies are still obscure. However, we demonstrate that the inhibitory effect of neurotensin on the forskolin-induced cyclic AMP production was antagonized by SR48692 in a concentration-dependent manner, thus suggesting the involvement of high affinity neurotensin receptors. As already suggested (Bozou *et al.*, 1986) we can propose, in view of the lower efficacy of neurotensin to affect cyclic AMP levels (EC_{50} = 1.3 nM), versus cyclic GMP or IP1 levels (EC_{50} = 0.46 and 0.47 nM, respectively), that different neurotensin receptors and/or G-proteins may be involved in these responses. However, the potency of SR48692 in reversing the effect of neurotensin on the cyclic AMP response was very close to that found in antagonism studies on neurotensin-induced IP1 and cyclic GMP responses. These results are thus more in favour of the involvement of neurotensin receptors coupled to different G-proteins rather than of different neurotensin receptor subtypes in the physiological responses of N1E115 cells. In order to investigate further the neurotensin receptor coupling mechanism, we studied the effect of neurotensin and SR48692 on CHO-NTR cells. In this cellular model, neurotensin was found to stimulate cyclic AMP production with an EC_{50} of 72 nM. The pA_2 value for SR48692 on this response was 7.2, demonstrating that in CHO-NTR cells, SR48692 behaved also as a potent antagonist. In their transfected CHO cells, Yamada *et al.* (1993) also reported a positive coupling to adenylyl cyclase and showed a similar EC_{50} of 34 nM for neurotensin in stimulating cyclic AMP production. As previously described (Watson *et al.*, 1992) in this cellular model, neurotensin was able to induce an increase in IP1 formation with an EC_{50} of 3.0 nM. The antagonistic effect of SR48692 on the IP1 response gave a pA_2 value of 8.4, which is close to the pA_2 value (8.7) obtained in HT29 cells. Furthermore, we found a K_b value of 3.4 nM, which was very similar to those obtained for HT29 (3.6 nM) and N1E115 (1.3 nM) cells. Taken together, these results do not suggest species difference in affinity and/or efficacy for SR48692 and cannot explain the lack of antagonism reported by others with this compound (Steinberg *et al.*, 1994; Pinnock & Woodruff, 1994).

In conclusion, in human peripheral, mouse neuronal and rat NTRCHO cell lines, our results clearly demonstrate that the non-peptide neurotensin receptor antagonist SR48692, at concentrations equimolar to that of neurotensin (see Table 1), fully and competitively antagonized the intracellular events induced by neurotensin. Thus, these series of experiments do not allow a discrimination of putative neurotensin receptor subtypes suggested by *in vivo* (Dubec *et al.*, 1994; Steinberg *et al.*, 1994) and *in vitro* (Labbé-Jullié *et al.*, 1994; Pinnock & Woodruff, 1994) studies.

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