Neurotensin stimulates inositol trisphosphate-mediated calcium mobilization but not protein kinase C activation in HT29 cells

Involvement of a G-protein

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It has previously been shown that neurotensin binds to high-affinity receptors in the adenocarcinoma HT29 cell line, and that receptor occupancy leads to inositol phosphate formation. The present study was designed to investigate further the effects of neurotensin on calcium mobilization and protein kinase C (PKC) activation in HT29 cells, and to assess the role of GTP-binding proteins (G-proteins) in the neurotensin response. Direct measurements of cytosolic Ca^{2+} variations using the fluorescent indicator quin 2 showed that neurotensin ($0.1\text{--}1\text{ }\mu\text{M}$) elicited Ca^{2+} transients in HT29 cells. These transients occurred after the neurotensin-stimulated formation of $\text{Ins}(1,4,5)\text{P}_3$, as measured by means of a specific radioreceptor assay. In addition, the peptide induced a decrease in the $^{45}\text{Ca}^{2+}$ content of cells previously equilibrated with this isotope. The peptide effect was rapid, long-lasting and concentration-dependent, with an EC_{50} of 2 nM . Phorbol 12-myristate 13-acetate (PMA) inhibited by 50 % the neurotensin effects on both intracellular Ca^{2+} and inositol phosphate levels. The inhibition by PMA was abolished in PKC-depleted cells. Pertussis toxin had no effect on either the Ca^{2+} or inositol phosphate responses to neurotensin. Epidermal growth factor (EGF) receptors which are present in HT29 cells have been shown to be down-regulated through phosphorylation by PKC in a variety of systems. Here, PMA markedly (70–80 %) inhibited EGF binding to HT29 cells. Scatchard analysis revealed that PMA abolished the high-affinity component of EGF binding, an effect that was totally reversed in PKC-depleted cells. In contrast, neurotensin slightly (10–20 %) inhibited EGF binding to HT29 cells, and its effect was only partly reversed by PKC depletion. Neurotensin had no detectable effect on *sn*-1,2-diacylglycerol levels in HT29 cells, as measured by a specific and sensitive enzymic assay. In membranes prepared from HT29 cells, monoiodo[^{125}I -Tyr 3]neurotensin bound to a single population of receptors with a dissociation constant of 0.27 nM . Sodium and GTP inhibited neurotensin binding in a concentration-dependent manner. Maximal inhibition reached 80 % with Na^+ and 35 % with GTP. IC_{50} values were 20 mM and $0.2\text{ }\mu\text{M}$ for Na^+ and GTP respectively. Li^+ and K^+ were less effective than Na^+ and the effects of GTP were shared by GDP and guanosine-5'-[$\beta\gamma$ -imido]triphosphate but not by ATP. Scatchard analysis of binding data indicated that Na^+ and GTP converted the high-affinity neurotensin-binding sites into lower affinity binding sites. The properties of the effects of Na^+ and GTP on neurotensin–receptor interactions are characteristic of those receptors which interact with G-proteins. Altogether, our data show that neurotensin receptors in HT29 cells activate phospholipase C through a pertussis-toxin-insensitive G-protein. This activation leads to an increase in inositol phosphate levels, and this in turn results in Ca^{2+} mobilization. The other branch of the bifurcating phospholipase C pathway, i.e. the activation of PKC by diacylglycerol, does not seem to be significantly operated by neurotensin. The physiological implications of these findings are discussed.

INTRODUCTION

There is now evidence that neurotensin acts as a neurotransmitter or neuromodulator in the central nervous system [1] and as a hormone in the gastrointestinal tract [2]. Although the properties and characteristics of neurotensin binding to its receptors on target cells have been well documented, relatively little is known concerning the cellular and molecular events subsequent to

receptor activation. There is however an increasing body of evidence that one of the earliest steps in neurotensin action is an increase in cellular inositol phosphate content. This has been demonstrated in slices of brain tissue [3], in pituitary cells [4], in the neuroblastoma N1E115 cell line [5] and in the human colonic adenocarcinoma HT29 cell line [6].

The stimulation of inositol phosphate production, which seems to be the common point of neurotensin

Abbreviations used: EGF, epidermal growth factor; G-protein, GTP-binding protein; [^{125}I]-neurotensin, monoiodo[^{125}I -Tyr 3]neurotensin; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; Gpp[NH]p, guanosine 5'-[$\beta\gamma$ -imido]triphosphate; [Ca^{2+}] $_i$, intracellular free Ca^{2+} concn.

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action in several systems, is due to the activation of phospholipase C. This receptor-activated enzyme hydrolyses the plasma membrane lipid $\text{PtdIns}(4,5)\text{P}_2$, leading to the production of InsP_3 and diacylglycerol [7]. It is now well established that these two molecules act as second messengers in the bifurcating pathway of cellular events. InsP_3 stimulates the release of intracellular Ca^{2+} from its endoplasmic reticulum pool, whereas diacylglycerol activates protein kinase C (PKC) [7,8]. Depending on the cell system, these two signalling pathways may contribute independently or synergistically to the final cellular response [8].

The present study was designed to investigate further the mechanisms and consequences of phospholipase C activation by neurotensin in HT29 cells. More specifically, three questions were addressed in this work. (1) Does neurotensin-induced inositol phosphate production lead to Ca^{2+} mobilization? This was studied both directly using the fluorescent probe quin 2 [9] and indirectly by measuring intracellular $^{45}\text{Ca}^{2+}$ content under equilibrium conditions [10,11]. (2) Is inositol phosphate production accompanied by an increase in diacylglycerol and an activation of PKC? Diacylglycerol levels were directly measured in HT29 cells by means of a sensitive and specific enzymic assay [12]. Furthermore, PKC activation was assessed by measuring epidermal growth factor (EGF) binding to HT29 cells, based on the fact that EGF receptors are present on these cells [13] and on the property of the kinase to desensitize EGF binding through EGF receptor phosphorylation [14]. (3) Is there a GTP-binding protein (G-protein) that couples the neurotensin receptor to phospholipase C activation? It is well established that agonist binding to receptors that are coupled to G-proteins is negatively modulated by guanyl nucleotides and monovalent cations [15]. Therefore the effects of these agents on the binding of neurotensin to HT29 cell membranes were investigated.

MATERIALS AND METHODS

Materials

Neurotensin was generously donated by C. Granier and J. van Rietschoten (Faculté de Médecine Nord, Marseille, France). Mouse EGF was purchased from Bethesda Research Laboratories. Monoiodo[^{125}I -Tyr 3]-neurotensin (referred to as ^{125}I -neurotensin) was prepared at a specific radioactivity of 2000 Ci/mmol and purified as previously described [16]. ^{125}I -EGF, at a specific activity of 1000 Ci/mmol, was purchased from New England Nuclear (Paris, France). $^{45}\text{Ca}^{2+}$ (10–40 mCi/mg), [^3H]inositol (10–20 Ci/mmol) and [^{32}P]ATP (3000 Ci/mmol) were from Amersham (Les Ulis, France). Quin 2 (tetra-acetoxymethyl ester) was from Calbiochem. PMA and unlabelled nucleotides were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Diacylglycerol kinase-containing membranes were purchased from Lipidex, Inc. (Middletown, WI, U.S.A.). All other reagents were of the best commercial grade available.

Cell culture

HT29 cells were obtained from Dr. J. Fogh (Sloan Kettering Institute for Cancer Research, Rye, NY, U.S.A.). Cells were cultured and propagated in 75 cm 2 Falcon culture flasks as described [6]. Cells were detached by trypsin treatment [6], washed in culture medium, and either frozen for binding experiments on cell homo-

genes or cultured for 3–5 days in 24-well Falcon plates before use in experiments with intact cells. For internal Ca^{2+} concentration measurements with the fluorescent probe quin 2, cells were plated in Falcon Petri dishes (100 mm), the bottoms of which were covered with rectangular glass coverslips.

Measurements of intracellular Ca^{2+} using the fluorescent probe quin 2

Variations of intracellular Ca^{2+} concentrations were measured by a modification of the method of Tsien *et al.* [9]. Briefly, cells grown on glass coverslips were incubated with 50 mM-quin 2 for 30 min at 37 °C. Then the cells were washed with Hepes-buffered saline (140 mM-NaCl, 5 mM-KCl, 0.9 mM-MgCl $_2$, 1.8 mM-CaCl $_2$ and 25 mM-Hepes, pH 7.5). The slides were placed in a thermostated quartz cuvette (1 cm $\times$ 1 cm). The fluorescence of quin 2-loaded cells was measured at an excitation wavelength of 339 nm and an emission wavelength of 492 nm using a Perkin Elmer 3000 spectrofluorimeter.

Measurement of cell content of $^{45}\text{Ca}^{2+}$ under equilibrium conditions

Cells were preincubated at 37 °C with $^{45}\text{Ca}^{2+}$ (4 mCi/ml) for 8 h in Dubelcco's modified Eagle's medium without serum. Then, cells were incubated with neurotensin or tested agents at the indicated concentrations for the indicated periods of time. At the end of the incubation, the medium was removed and the cells were washed four times with phosphate-buffered saline (140 mM-NaCl/10 mM-Na $_2$ HPO $_4$, pH 7.5) without Ca^{2+} . Intracellular $^{45}\text{Ca}^{2+}$ was extracted twice with 0.5 ml of 10 M-NaOH, and radioactivity was counted.

Measurement of intracellular inositol phosphate levels

Labelling of HT29 cells with [^3H]inositol and measurements of total inositol phosphate levels were performed exactly as previously described [6].

In experiments designed to specifically measure the active $\text{Ins}(1,4,5)\text{P}_3$ isomer, plated cells were preincubated with 20 mM-Li $^+$ for 30 min and then incubated with or without neurotensin (1 μM) for various times. The incubations were stopped by aspiration of the medium and addition of 0.75 ml of 7% (v/v) HClO $_4$. After centrifugation and neutralization with 10 M-KOH, portions of the supernatants were assayed for their $\text{Ins}(1,4,5)\text{P}_3$ content by means of a specific radioreceptor assay (TRK 1000, Amersham) according to the instructions of the manufacturer. $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ cross-react by only 0.22% and 1.0% respectively in this assay.

Quantitative assay for *sn*-1,2-diacylglycerol

Plated cells (10 6 cells/well) were incubated with or without neurotensin for various times. The incubations were stopped by aspiration of the medium and addition of 0.6 ml of methanol. The cells were scraped off the plates with a rubber policeman and transferred into centrifuge tubes containing 0.3 ml of chloroform for lipid extraction. After addition of 0.3 ml of chloroform and 0.3 ml of water, the organic and aqueous phases were separated by brief centrifugation. The chloroform phase was evaporated under N $_2$ and the dried lipid extracts were enzymically assayed for *sn*-1,2-diacylglycerol by means of diacylglycerol kinase from *Escherichia coli* exactly as described by Preiss *et al.* [12]. In this assay, *sn*-1,2-diacylglycerol is converted to

[^{32}P]phosphatidic acid in the presence of [^{32}P]ATP by the bacterial kinase, which is highly specific for glycerol acylated in positions 1 and 2.

^{125}I -EGF binding experiments

Cells were incubated at 37 °C for 30 min in 0.5 ml of Hepes-buffered saline (pH 7.5) with or without neurotensin or PMA at the indicated concentrations. The cells were then washed at 20 °C with Hepes-buffered saline containing 0.1 % bovine serum albumin. The equilibrium binding of ^{125}I -EGF to intact plated cells was measured at 20 °C exactly as previously described [13].

Neurotensin binding to HT29 cell membranes

HT29 cell membranes were prepared as follows. Frozen cell suspensions were thawed and centrifuged for 10 min at 10000 g and resuspended in 5 mM-Tris/HCl, pH 7.5. Cells were broken by 10 repeated passages through a syringe needle. The cell homogenate was centrifuged (10 min, 10000 g) and washed with 5 mM-Tris/HCl, pH 7.5. After another centrifugation, the crude cell membrane preparation was resuspended at the appropriate protein concentration in binding assay buffer (50 mM-Tris/HCl, pH 7.5/0.2 % bovine serum albumin). Protein concentrations were determined using the Bio-Rad protein assay reagent, using the procedure recommended by the manufacturer.

Binding assays were carried out at 25 °C for 20 min in 250 μl of binding assay buffer that contained ^{125}I -neurotensin, 0.05–0.1 mg of membrane protein and, when needed, monovalent cations, nucleotides, and unlabelled neurotensin. The binding assay buffer also contained 1 mM-1,10-phenanthroline which prevented the ligand from degrading [17]. Total, specific and non-specific binding were determined as previously described [16,18]. The dissociation constant, K_d , and maximal binding capacity, B_{max} , were derived from Scatchard analysis of either saturation experiments performed with increasing concentrations of ^{125}I -neurotensin, or competition experiments in which varying concentrations of unlabelled neurotensin were added to a fixed concentration (0.1 nM) of ^{125}I -neurotensin. The binding of 0.1 nM- ^{125}I -neurotensin reached equilibrium by 20–30 min (results not shown). Therefore, in all experiments binding was measured after 30 min of incubation. At 0.1 nM- ^{125}I -neurotensin, non-specific binding did not exceed 10 % of total binding. Curvilinear Scatchard plots were analysed by computer according to a two-sites model [19].

RESULTS

Effect of neurotensin on intracellular Ca^{2+} levels in HT29 cells

The addition of neurotensin to monolayered HT29 cells loaded with quin 2 caused a transient increase of fluorescence emission, reflecting a transitory increase of $[\text{Ca}^{2+}]_i$ (Fig. 1). This effect was rapid in onset and peaked after 1 min. This event was found to occur after the neurotensin-stimulated production of $\text{Ins}(1,4,5)\text{P}_3$, as measured by means of a specific radioreceptor assay (Table 1). Indeed, the formation of $\text{Ins}(1,4,5)\text{P}_3$ peaked after 10–15 s and then returned to basal levels over a 2 min period. When the same cell preparation was challenged with two successive neurotensin additions of increasing concentrations, the Ca^{2+} response to the

Fig. 1. Fluorescence records from quin 2-loaded HT29 cells

Quin 2 fluorescence was recorded from HT29 monolayers as described in the Materials and methods section. Neurotensin (NT) was added to the cuvette at the indicated concentrations. Maximal and minimal fluorescence values were obtained in the presence of digitonin (Digit.) and Mn^{2+} respectively. Similar results were observed in at least three other experiments.

second addition of peptide was markedly lower than the response to the first addition, thus indicating that the system became desensitized (Fig. 1b). Permeabilization of the cells with digitonin, which allows Ca^{2+} to enter the cell freely, induced the largest observable increase in fluorescence emission. As expected, an excess of Mn^{2+} quenched the quin 2-emitted fluorescence.

When 10^{-6} M-neurotensin was added to $^{45}\text{Ca}^{2+}$ -loaded cells, there was a rapid, time-dependent decrease in intracellular $^{45}\text{Ca}^{2+}$ content (Fig. 2, inset). The maximal decrease (24 %) was observed after 2 min and was followed by a slow refilling of the $^{45}\text{Ca}^{2+}$ intracellular pool. The peptide effect was concentration-dependent

Table 1. Time course of neurotensin-induced formation of $\text{Ins}(1,4,5)\text{P}_3$ formation in HT29 cells

HT29 cell monolayers were incubated with 1 μM -neurotensin for the indicated times, and their $\text{Ins}(1,4,5)\text{P}_3$ content was measured by means of a specific radioreceptor assay as described in the Materials and methods section. The data are the means $\pm$ S.E.M. of three determinations in one experiment.

Time (s)	$\text{Ins}(1,4,5)\text{P}_3$ (pmol/ 10^6 cells)
0	10.3 ± 0.9
10	102.4 ± 6.7
15	112.5 ± 7.8
20	76.8 ± 14.7
30	37.9 ± 4.7
40	21.6 ± 3.8
120	10.4 ± 0.6

Fig. 2. Time course and concentration-dependence of neurotensin effect on $^{45}\text{Ca}^{2+}$ content in HT29 cells

HT29 cells were preincubated with $^{45}\text{Ca}^{2+}$ for 8 h. Then, neurotensin was added in the presence of the isotope. At the end of the incubation, the radioactive medium was removed and the cells were rapidly washed four times with phosphate-buffered saline medium without Ca^{2+} at 0°C . The radioactivity left in the cells was extracted as described in the Materials and methods section. Inset: time course of neurotensin ($1\ \mu\text{M}$) effect. The results are expressed as the percentage of the cell radioactivity content in the absence of neurotensin. Data are the means $\pm$ S.E.M. from six experiments with duplicate determinations within each experiment. Main figure: concentration response curve for the effect of neurotensin. The cells were incubated for 4 min with varying concentrations of peptide. The results are expressed as percentage of maximum effect and are the means $\pm$ S.E.M. from four experiments with triplicate determinations within each experiment.

with an EC_{50} of 2 nM and a maximally effective concentration of 100 nM (Fig. 2).

Treatment of the cells for 15 min with 100 ng of PMA/ml inhibited by about 50% effects of neurotensin on both intracellular $^{45}\text{Ca}^{2+}$ levels and total inositol phosphate concentrations (Table 2). This inhibitory effect of short-term PMA treatment on Ca^{2+} and inositol phosphate responses was totally reversed by pretreatment of HT29 cells for 24 h with 100 ng of PMA/ml. Pertussis toxin had no effect on the neurotensin-induced Ca^{2+} and inositol phosphate responses (results not shown).

Effect of neurotensin on ^{125}I -EGF binding and diacylglycerol levels in HT29 cells

Scatchard analysis of ^{125}I -EGF binding to HT29 cells at equilibrium revealed the existence of two binding components (Fig. 3): a high-affinity, low-capacity component ($K_d = 31.7 \pm 4\ \text{pM}$, $B_{\text{max}} = 2.9 \pm 0.9\ \text{fmol}/10^6\ \text{cells}$), and a lower affinity high-capacity component

($K_d = 951 \pm 117\ \text{pM}$, $B_{\text{max}} = 24.1 \pm 3.6\ \text{fmol}/10^6\ \text{cells}$) (mean values $\pm$ S.E.M. of four experiments). The addition of $1\ \mu\text{M}$ -neurotensin to the binding assay resulted in a slight (10–20%) inhibition of EGF binding at all ligand concentrations tested (Fig. 3; Table 3). In contrast, PMA at 100 ng/ml produced a marked (70–80%) inhibition of EGF binding at low ligand concentrations (Fig. 3, Table 3). Indeed, in the presence of the phorbol ester, the high-affinity EGF-binding component was no longer detectable (Fig. 3). Scatchard analysis according to a one-site model yielded the following binding parameters: $K_d = 1.2\ \text{nM}$, $B_{\text{max}} = 16\ \text{fmol}/10^6\ \text{cells}$.

In HT29 cells depleted of PKC by a 24 h treatment with 100 ng of PMA/ml, the phorbol ester was no longer able to inhibit ^{125}I -EGF binding, whereas neurotensin still produced a small but significant inhibition of binding (Table 3). In these conditions, the inhibitory effect of neurotensin was reduced by about 50% as compared with the effect observed in control HT29 cells.

The Ca^{2+} ionophore ionomycin slightly inhibited the binding of EGF to HT29 cells (Table 3). This inhibitory effect was not affected by long-term treatment of the cells with PMA and was of the same magnitude as the inhibitory effect of neurotensin in PKC-depleted cells (Table 3).

The amount of *sn*-1,2-diacylglycerol detected in lipid extracts of HT29 cells was directly proportional to the number of extracted cells between 1×10^5 and 4×10^6 cells (results not shown). HT29 cells incubated with $1\ \mu\text{M}$ -neurotensin contained 1.07 ± 0.06 , 1.01 ± 0.06 , 1.06 ± 0.03 , 0.98 ± 0.09 and $1.04 \pm 0.10\ \text{nmol}/10^6\ \text{cells}$ of *sn*-1,2-diacylglycerol after 0, 0.5, 1, 3 and 5 min of incubation respectively.

Effects of monovalent cations and guanyl nucleotides on ^{125}I -neurotensin binding to HT29 cell homogenates

The binding of $0.1\ \text{nM}$ - ^{125}I -neurotensin to HT29 cell membranes was inhibited in a concentration-dependent manner by Na^+ and to a lesser extent by K^+ and Li^+ (Fig. 4). EC_{50} values were 40, 70 and 115 mM for Na^+ , K^+ and Li^+ respectively. Scatchard plots of neurotensin binding in the absence or in the presence of 33 and 100 mM- Na^+ are shown in Fig. 5. The binding parameters derived from these plots are shown in Table 4. In the absence of the cation, the Scatchard plot was linear, indicating the existence of one class of high-affinity neurotensin-binding sites in HT29 cell homogenates. In the presence of 33 mM- Na^+ , the Scatchard plot was curvilinear, and this resulted from the conversion of 80% of the binding sites into a lower affinity state. At 100 mM- Na^+ , all of the binding sites were in the low-affinity state (Fig. 5, Table 4).

The guanyl nucleotides GTP and GDP, and the nonhydrolysable GTP analogue guanosine 5'-[$\beta\gamma$ -imido]triphosphate (Gpp[NH]p) inhibited in a concentration-dependent manner the binding of $0.1\ \text{nM}$ - ^{125}I -neurotensin to cell homogenates (Fig. 6). EC_{50} values were 0.2, 0.4 and $1\ \mu\text{M}$ for GTP, Gpp[NH]p and GDP respectively. For all three nucleotides, maximum inhibition reached about 30% at $10\ \mu\text{M}$. At this concentration, ATP exerted no inhibitory effect on neurotensin binding (Fig. 6). Scatchard analysis of neurotensin binding to HT29 cell membranes in the presence of $0.1\ \text{mM}$ -GTP yielded a curvilinear plot and showed that 60% of the high-affinity binding sites were converted to low-affinity sites (Fig. 5, Table 4).

Table 2. Effect of PMA on neurotensin (NT)-induced Ca^{2+} and inositol phosphate responses in control and PKC-depleted cells

HT29 cell monolayers were incubated at 37 °C without (control cells) or with (PKC-depleted cells) 100 ng of PMA/ml for 24 h in the culture medium. Cells were loaded for 8 h with $^{45}\text{Ca}^{2+}$ or for 24 h with $[^3\text{H}]\text{inositol}$. Then, Ca^{2+} and inositol phosphate responses were measured as described in the Materials and methods section. The results are the means $\pm$ S.E.M. from triplicate determinations.

Additions	Ca^{2+} response		Inositol phosphate response	
	$[^{45}\text{Ca}^{2+}]$ (c.p.m./ 10^6 cells)	PMA effect (% of NT response)	$[^3\text{H}]\text{inositol phosphates}$ (c.p.m./ 10^6 cells)	PMA effect (% of NT response)
Control cells:				
None	9302 $\pm$ 370		2168 $\pm$ 187	
NT (0.1 μM)	7105 $\pm$ 90	100	4387 $\pm$ 103	100
PMA (100 ng/ml)	9033 $\pm$ 175		2255 $\pm$ 46	
NT (0.1 μM) + PMA (100 ng/ml)	7956 $\pm$ 104	50.5	3448 $\pm$ 17	51.7
PKC-depleted cells:				
None	8613 $\pm$ 98		2328 $\pm$ 14	
NT (0.1 μM)	7256 $\pm$ 219	100	3317 $\pm$ 164	100
PMA (100 ng/ml)	8867 $\pm$ 49		2139 $\pm$ 70	
NT (0.1 μM) + PMA (100 ng/ml)	7403 $\pm$ 80	104.8	3094 $\pm$ 312	105

DISCUSSION

Previous studies from this laboratory have shown that neurotensin binds to high-affinity receptors in HT29 cells [6,20] and that receptor occupancy leads to inositol phosphate formation [6]. The present work was designed

to investigate the effects of neurotensin on Ca^{2+} mobilization and PKC activation in HT29 cells.

Both direct and indirect pieces of evidence establish that neurotensin induces a rise in intracellular Ca^{2+} in HT29 cells. Direct measurements of cytosolic Ca^{2+} variations using the fluorescent indicator quin 2 [9] showed that neurotensin (0.1–1 μM) elicited Ca^{2+} transients in HT29 cells. These transients peaked at about 1 min after neurotensin addition. In a previous study, we showed that neurotensin-stimulated InsP_3 formation peaked at about 20 s [6]. However, the InsP_3 fraction was quantified by a method that does not separate $\text{Ins}(1,4,5)\text{P}_3$, the active Ca^{2+} -mobilizing agent, from its inactive isomer $\text{Ins}(1,3,4)\text{P}_3$ or from $\text{Ins}(1,3,4,5)\text{P}_4$. Here, by means of a specific radioreceptor assay, we have directly measured the neurotensin-stimulated formation of $\text{Ins}(1,4,5)\text{P}_3$ as a function of time, and show that it precedes the rise in intracellular Ca^{2+} .

Fig. 3. Scatchard analysis of ^{125}I -EGF binding to intact HT29 cells

Cell monolayers (10^6 cells/well) were incubated for 30 min in HEPES-buffered saline medium without (●) or with 1 μM -neurotensin (○) or 100 ng of TPA/ml (■). Then, binding experiments were performed as described in the Materials and methods section. The data are plotted according to Scatchard analysis and are the means of duplicate determinations in a typical experiment.

Table 3. Effect of neurotensin, PMA and ionomycin on ^{125}I -EGF binding to control or PKC-depleted HT29 cells

HT29 cell monolayers were incubated at 37 °C without (control cells) or with (PKC-depleted cells) 100 ng of PMA/ml for 24 h in the culture medium. Cells were washed with 2 ml of HEPES-buffered saline medium (pH 7.5), and neurotensin (NT), PMA or ionomycin were added for 15 min. Then the binding of 0.1 nM- ^{125}I -EGF was measured as described in the Materials and methods section. The data are the means $\pm$ S.E.M. of four experiments with triplicate determinations within each experiment.

Additions	EGF binding (% of control)	
	Control cells	PKC-depleted cells
None	100	100
NT (1 μM)	79.3 $\pm$ 2.3	91.1 $\pm$ 1.3
PMA (100 ng/ml)	27.3 $\pm$ 4.2	102.4 $\pm$ 2.2
Ionomycin (1 μM)	92.8 $\pm$ 1.1	91.5 $\pm$ 1.9

Fig. 4. Effects of increasing concentrations of monovalent cations on the binding of ^{125}I -neurotensin to HT29 cell membranes

Binding assays were carried out for 30 min at 25 °C as described in the Materials and methods section. The results are expressed as the percentage of 0.1 nM- ^{125}I -neurotensin binding in the absence of cations. The data are the means of duplicate determinations in a typical experiment. ●, Na^+ ; □, Li^+ ; ○, K^+ .

In our hands, the use of quin 2 to measure changes in $[\text{Ca}^{2+}]_i$ did not permit the establishment of the concentration dependency of the neurotensin effect with precision. This could in part be the consequence of the Ca^{2+} -buffering effect of quin 2 [21] which may have hindered the detection of small $[\text{Ca}^{2+}]_i$ variations, in particular at low neurotensin concentrations. This was also due to the response variability observed between different cell preparations, resulting from the use of cells attached to coverslips for fluorescence measurements. In

Fig. 5. Scatchard analysis of ^{125}I -neurotensin binding to HT29 cell membranes

Experiments were performed in the absence (●) or presence of 33 mM- Na^+ (□), 100 mM- Na^+ (■) or 0.1 mM-GTP (▲). The binding assay was carried out for 30 min at 25 °C as described in the Materials and methods section. The data are the means of duplicate determinations in a typical experiment.

addition, the same cell preparation could not be used for measuring the effect of more than one neurotensin concentration because of the strong desensitization that occurred in this case.

For all these reasons another technique was used to measure neurotensin-induced effects on $[\text{Ca}^{2+}]_i$ in HT29 cells. This indirect method was based on the fact that an increase in $[\text{Ca}^{2+}]_i$ activates a plasma membrane Ca^{2+} pump that extrudes the cation when its concentration rises above resting levels [10,11,22]. Thus it has been reported that Ca^{2+} mobilization by vasopressin and bombesin in Swiss 3T3 cells is accompanied by a rapid

Table 4. Effect of Na^+ and GTP on neurotensin binding parameters in HT29 cell membranes

Binding experiments were performed as described in the Materials and methods section. Binding parameters were derived from computer analysis of Scatchard plots such as that shown in Fig. 5. They represent the means $\pm$ S.E.M. from five control experiments and three experiments with Na^+ and GTP.

Binding parameter	Control	33 mM- Na^+	100 mM- Na^+	0.1 mM-GTP
K_d (h)* (nM)	0.27 ± 0.03	0.17 ± 0.03	—	0.22 ± 0.05
B_{max} (h) (fmol/mg)	115.5 ± 19.4	17.1 ± 9.1	—	27 ± 7
K_d (l)† (nM)	—	2.7 ± 0.8	1.53 ± 0.40	2.93 ± 0.13
B_{max} (l) (fmol/mg)	—	76 ± 13	92.8 ± 10.9	42 ± 10

* (h) Refers to high-affinity neurotensin-binding sites.

† (l) Refers to low-affinity neurotensin-binding sites.

Fig. 6. Effect of nucleotides on the binding of ^{125}I -neurotensin to HT29 cell membranes

Binding assays were carried out for 20 min at 25°C as described in the Materials and methods section. The results are expressed as the percentage inhibition of 0.1 nM ^{125}I -neurotensin binding compared with that in the absence of nucleotide. The data are the means of duplicate determinations in a typical experiment. ●, GTP; ○, Gpp[NH]p; □, GDP; △, ATP.

and long-lasting decrease in total cellular Ca^{2+} content consequent to the activation of a plasma membrane ATP-dependent Ca^{2+} pump [10,11]. Similar effects on Ca^{2+} content were observed here when neurotensin was incubated with HT29 cells.

The peptide response was concentration-dependent with an EC_{50} of 2 nM . This value is similar to the K_d (1.2 nM) of neurotensin binding to intact HT29 cells [6,20], but it is lower than the EC_{50} value (87 nM) for the peptide stimulation of inositol trisphosphate levels [6]. To be meaningful, however, comparison of K_d values with EC_{50} values needs to consider the incubation times at which these parameters were obtained. Binding experiments were performed at equilibrium (30 min incubation), whereas inositol trisphosphate and Ca^{2+} levels were measured after 20 s and 4 min respectively. Based on the binding parameters for the neurotensin-receptor interaction in the HT29 cell line [20], calculations that are not detailed here show that after 20 s and 4 min of incubation, half of the binding sites will be occupied for neurotensin concentrations of 80 nM and 7 nM respectively. The value of 80 nM is quite close to the EC_{50} for inositol trisphosphate stimulation (87 nM), thus indicating that neurotensin-induced inositol trisphosphate production is directly proportional to receptor occupancy. On the other hand, the value of 7 nM is somewhat higher than the EC_{50} for Ca^{2+} mobilization (2 nM), suggesting that there is amplification between neurotensin-stimulated inositol trisphosphate formation and the subsequent increase in $[\text{Ca}^{2+}]$ in HT29 cells.

It is well established that PKC exerts a negative

feedback control on phospholipase C-mediated formation of inositol phosphates [23]. In the present study, activation of PKC by PMA resulted in a similar decrease in neurotensin-induced inositol phosphates and Ca^{2+} responses. This decrease was markedly attenuated by HT29 cell depletion from PKC. Altogether, the present data strongly support the hypothesis that the effects of neurotensin on Ca^{2+} mobilization in HT29 cells is a direct consequence of the peptide-induced inositol trisphosphate formation.

Inositol trisphosphate is one of the products resulting from the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ by receptor-coupled phospholipase C. The other product is *sn*-1,2-diacylglycerol, which is the physiological activator of PKC [7,23]. It was therefore of interest to see whether neurotensin could activate this second messenger pathway. To this end, the effect of neurotensin on EGF binding to HT29 cells was investigated. Indeed, EGF receptors, which have previously been characterized in HT29 cells [13], can be desensitized to their own ligand through phosphorylation by direct activators of PKC such as the phorbol esters, and by receptor-mediated activation of the phospholipase C pathway by agents such as bombesin and vasopressin [14]. In the present study, PMA markedly (70–80 %) reduced EGF binding to high-affinity receptors in HT29 cells. The phorbol ester effect was abolished in PKC-depleted cells. These results are similar to those reported in other systems [14]. In contrast neurotensin only slightly (10–20 %) inhibited the binding of EGF to its receptors and this inhibition was partially insensitive to PKC depletion. The PKC-depletion-insensitive component of inhibition appeared to be the consequence of Ca^{2+} mobilization, as it was mimicked by the Ca^{2+} ionophore ionomycin. These results suggest that neurotensin only weakly, if at all, activated PKC in HT29 cells.

This led us to investigate directly the effects of neurotensin on diacylglycerol production in HT29 cells. To this end, a highly sensitive and specific enzymic diacylglycerol assay was used [12]. No effect of neurotensin could be observed on diacylglycerol levels in HT29 cells within the limit of detection of the assay. This could result from a rapid metabolism of diacylglycerol by diacylglycerol kinase and diacylglycerol lipases, as has been observed in platelets [24,25]. A rapid metabolism could explain the small effect of neurotensin on PKC-mediated inhibition of EGF binding in HT29 cells.

It is well documented that agonist-receptor binding and activation of inositol phosphate production through the phospholipase C pathway are coupled by G-protein(s). In some tissues, the G-protein that couples receptors to phospholipase C is sensitive to pertussis toxin, while in other tissues it is insensitive to the toxin [26]. The specificity and properties of neurotensin binding modulation by monovalent cations and guanyl nucleotides in HT29 cells, as reported here, are similar to what has been observed in other systems such as brain membranes [27] and the neuroblastoma cell line N1E115 [18], and are compatible with an interaction of the neurotensin receptor with a G-protein [15]. The lack of effect of pertussis toxin on the inositol phosphate and Ca^{2+} responses to neurotensin suggests that this G-protein corresponds to G_p , the putative pertussis-toxin-insensitive G-protein involved in phospholipase C activation [26]. This situation is different to what has been reported in N1E115 cells, where the neurotensin effect on inositol phosphate production was partly inhibited by pertussis

toxin [5]. This suggests that the G-proteins involved in phospholipase C activation by neurotensin are different in HT29 and N1E115 cells.

In conclusion, the present data show that neurotensin receptors in HT29 cells are coupled to phospholipase C through a pertussis-toxin-insensitive G-protein. Activation of phospholipase C by the peptide leads to an increase in inositol phosphate levels, and this in turn results in Ca^{2+} mobilization. The other branch of the bifurcating phospholipase C pathway, i.e. the activation of PKC through *sn*-1,2-diacylglycerol production, does not seem to be significantly operated by neurotensin. Thus Ca^{2+} appears to be the major second messenger involved in neurotensin effects in HT29 cells. This is in agreement with the reported actions of the peptide in other systems, such as, for instance, its effects on prolactin secretion in pituitary cells [28] and on intestinal smooth muscle contractility [29], which have been shown to involve a rise in $[\text{Ca}^{2+}]_i$. In this context, it is interesting that in a subclone of HT29 cells that differentiate into mucus-secreting cells, neurotensin has recently been shown to stimulate mucus secretion (M. Laburthe & C. Labois, personal communication). If this proves to be the consequence of Ca^{2+} mobilization, as is likely, then this cell line would represent a useful model in which neurotensin effects on second messenger production could be linked to a physiological response at the cellular level.

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