Activation of phosphatidylinositol turnover by neurotensin receptors in the human colonic adenocarcinoma cell line HT29

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Association of neurotensin to its receptor in HT29 cells increases the intracellular concentration of inositol phosphates. A rapid (20–30 s), transient stimulation of inositol trisphosphate (275% of the basal level) and inositol bisphosphate (420%) is first observed, followed by a slower, stable increase in inositol monophosphate (170%). Half-maximal stimulation of the three inositol phosphates was obtained with 50–100 nM neurotensin. These results indicate that neurotensin is able to regulate intracellular Ca^{2+} levels in HT29 cells by using inositol trisphosphate as a second messenger.

Neurotensin receptor (Human colonic HT29 cell) Phosphatidylinositol turnover Inositol trisphosphate intracellular Ca^{2+}

1. INTRODUCTION

The pharmacological and biochemical properties of neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) strongly suggest that the peptide acts as a neurotransmitter or neuromodulator in the central nervous system [1] and as a hormone in the periphery [2]. Specific neurotensin receptors have been characterized not only in brain and gastrointestinal membrane preparations but also in intact cells of neural or nonneural origin [3]. We have recently taken advantage of the presence of high-affinity neurotensin binding sites in the electrically excitable neuroblastoma N1E115 clone [4] to study the intracellular consequences of neurotensin receptor occupancy in a model nerve cell. We found that association of neurotensin to its neuroblastoma receptor increased the basal cGMP level by a factor of 10 [5] and decreased the prostaglandin E_1-stimulated cAMP concentration by 55% [6]. The neurotensin-induced cGMP stimulation has also been reported by others [7].

The purpose of this work was to identify the intracellular events that are regulated by neurotensin receptors in a nonneural cell line. Since neurotensin had previously been shown to interact with specific receptors in the human colonic adenocarcinoma HT29 [8], this clone was used as a model of nonneural target cell. We show that neurotensin has little effect on cyclic nucleotide levels in HT29 cells but strongly stimulates phosphatidylinositol turnover according to a receptor-mediated mechanism.

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Abbreviations: TPA, 4β-phorbol 12-myristate 13-acetate; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate

2. MATERIALS AND METHODS

2.1. *Drugs and peptides*

Z-pro-prolinal (*N*-benzyloxycarbonyl-prolyl-prolinal) was kindly provided by Dr S. Wilk

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(Department of Pharmacology, Mount Sinai School of Medicine, NY). 1,10-Phenanthroline was from Merck; 4β-phorbol 12-myristate 13-acetate (PMA) from Sigma. *myo*-[2-^{3}H]Inositol (Amersham, 12.3 Ci/mmol) was purified before use by chromatography on a Dowex 1 × 8 column (0.8 ml, formate form) eluted with H_2O. Neurotensin, neurotensin (1–12), acetylneurotensin (8–13) and neurotensin (9–13) were generous gifts from Drs C. Granier and J. Van Rietschoten (Faculté de Médecine Nord, Marseille, France). [Monoiodo-Tyr^3]neurotensin (2000 Ci/mmol) was prepared and purified as described [9].

2.2. *Cell culture*

The HT29 cell line kindly provided by Dr J. Fogh (Sloan Kettering Institute, Rye, NY) was cultured in 75 cm^2 Falcon tissue culture flasks at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (50 U/ml) and streptomycin (50 μg/ml), in a humidified atmosphere of 5% CO_2/95% air. Cells used in binding and biochemical experiments (passages 13–26) were obtained as follows: cells were detached before confluence by incubation for 5 min in a 10 mM phosphate buffer containing 140 mM NaCl, 5 mM KCl, 50 μM EDTA and 0.02% trypsin. After centrifugation, the cellular pellet was washed and resuspended in 10 ml culture medium. The number of viable cells was determined by trypan blue exclusion. The cell suspension was distributed into Falcon 24-wells plates (~10^5 cells/well) and allowed to grow for 3–5 days before use.

2.3. *Binding experiments*

Monolayers of HT29 cells (5 × 10^5–8 × 10^5 cells/well) were incubated at 37°C with various concentrations of ^{125}I-labeled [monoiodo-Tyr^3]-neurotensin and unlabeled neurotensin or its analogues in a total volume of 200 μl incubation buffer (25 mM Hepes-Tris, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$, 0.02% bovine serum albumin, 4.5 g/l glucose, 1 mM 1,10-phenanthroline and 0.1 μM Z-pro-prolinal). After 30 min of incubation, the medium was removed by aspiration and the cell layer was rapidly washed twice with 0.5 ml of incubation buffer. Cells were detached with 1 ml of 0.1 M NaOH and the radioactivity bound to the cells was counted with an Intertechnique CG 4000 gamma counter at a counting efficiency of 80%. Nonspecific binding was determined in the presence of 1 μM unlabeled neurotensin and subtracted from total binding to obtain the specific binding.

2.4. *Cyclic nucleotide measurements*

HT29 cells were incubated at 37°C for different times with or without neurotensin (0.1–1 μM) in 200 μl of the buffer used in binding experiments. The cAMP and cGMP content of cell extracts was measured as described for neuroblastoma N1E115 cells [5].

2.5. *Labeling of HT29 cells with [^{3}H]inositol and measurement of phosphatidylinositol turnover*

Cell monolayers (5 × 10^5–8 × 10^5 cells/well) were incubated for 18 h at 37°C with 1 ml of serum-free culture medium containing 3 μCi [^{3}H]inositol. After extensive washing, cells were preincubated for 15 min at 37°C with 200 μl of a 25 mM Hepes-Tris buffer, pH 7.4, containing 110 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$, and 30 mM LiCl in order to inhibit inositol-1-phosphatase [10]. Stimulation of phosphatidylinositol hydrolysis was initiated by addition of a 20 μl-aliquot of peptide solution to the incubation medium. The reaction was terminated by aspiration of the medium and the cell layer in each well was extracted with 300 μl of 10% perchloric acid for 15 min at 4°C then washed once with 300 μl H_2O. Combined extracts were buffered with 75 mM Hepes and neutralized with 2 M KOH. The $KClO_4$ precipitate was removed by centrifugation and supernatants were analyzed for their inositol phosphate content according to the technique described by Bone et al. [11]. Briefly, soluble extracts were applied to columns (0.8 ml) of Dowex-1 × 8 (formate form; BioRad) that were eluted stepwise by solutions containing increasing concentrations of formate. Fractions were collected in the following order: free inositol (6 ml of water); glycerophosphoinositol (6 ml of 5 mM sodium tetraborate/60 mM ammonium formate); IP_1 (8 ml of 5 mM sodium tetraborate/200 mM ammonium formate); IP_2 (6 ml of 0.1 M formic acid/0.5 M ammonium formate); and IP_3 (6 ml of 0.1 M formic acid/1.5 M ammonium formate).

The radioactivity of each fraction was counted after addition of 15 ml Aquassure (New England Nuclear).

3. RESULTS

3.1. *Binding properties of neurotensin receptors in monolayers of HT29 cells*

Neurotensin receptors in HT29 cell suspension have already been characterized at 24°C with [^{3}H]neurotensin as labeled ligand [8]. Since intracellular responses to neurotensin were studied at 37°C and with plated HT29 cells, the binding properties were reinvestigated under these conditions using [monoiodo-Tyr^3]neurotensin as labeled ligand. In order to protect neurotensin and its analogues from extensive degradation by cell proteases, binding experiments were carried out in the presence of 1,10-phenanthroline, a metallopeptidase inhibitor [12], and of 0.1 μM Z-pro-prolinal, a specific inhibitor of proline endopeptidase [13]. These two inhibitors reduced the amount of neurotensin degraded by HT29 cells in the course of binding experiments (30 min at 37°C) from about 80% to less than 5%.

The binding and biological properties of [monoiodo-Tyr^3]neurotensin are identical to those of the parent peptide [9]. It was therefore possible to use mixtures of ^{125}I-labeled [monoiodo-Tyr^3]neurotensin and native neurotensin to study the binding of neurotensin to its cell receptor. These radiolabeled peptide preparations associated to specific neurotensin binding sites in monolayers of HT29 cells according to pseudo first-order kinetics (not shown). Binding equilibrium was always reached after 30 min of association. Fig.1A shows the results of equilibrium binding experiments in which radiolabeled neurotensin solu-

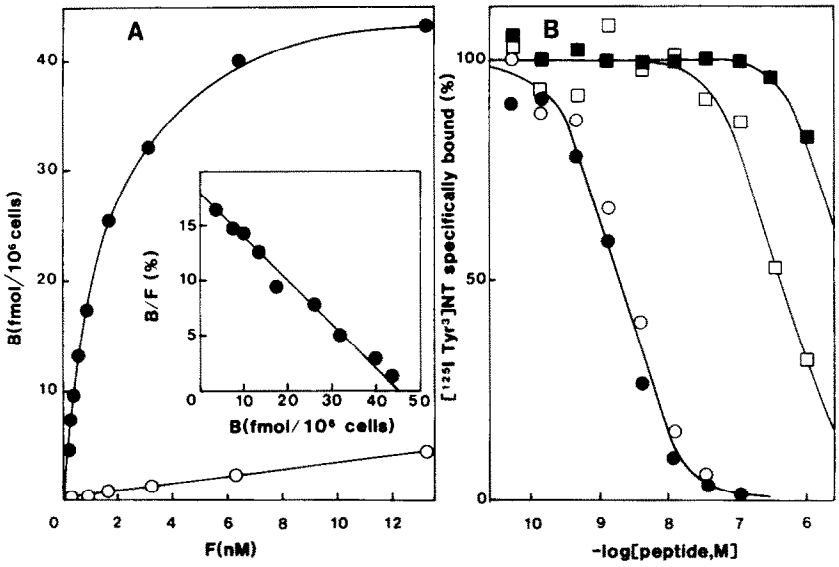

Fig.1. Binding of neurotensin and its analogues to monolayers of HT29 cells. (A) Saturation experiments. ^{125}I-labeled neurotensin solutions of various specific radioactivities were prepared by mixing ^{125}I-labeled [monoiodo-Tyr^3]neurotensin with unlabeled neurotensin. HT29 cells were incubated with increasing concentrations of ^{125}I-labeled neurotensin in the presence (nonspecific binding, ○) or absence (total binding, not shown) of 1 μM unlabeled neurotensin. Specific binding (●) is the difference between total and nonspecific binding. A Scatchard plot of the data is presented in the inset. B and F are the bound and free concentrations of labeled neurotensin. (B) Competition experiments. ^{125}I-labeled [monoiodo-Tyr^3]neurotensin ([^{125}I Tyr^3]NT, 0.15 nM) was incubated with HT29 cells and the indicated concentrations of unlabeled neurotensin (●), acetylneurotensin (8–13) (○), neurotensin (9–13) (□) or neurotensin (1–12) (■). In both saturation and competition experiments the radioactivity bound to cells was measured after incubation for 30 min at 37°C. Each point represents the mean of sextuplicate determinations from 3 different experiments.

tions of increasing concentration were incubated with a constant number of HT29 cells. The specific binding is saturable whereas the nonspecific binding increases linearly with the peptide concentration. Linearity of the Scatchard plot (inset of fig.1A) demonstrates that neurotensin associates with a single class of noninteracting binding sites. The dissociation constant of the neurotensin-receptor complex is 1.2 nM and the maximal binding capacity of HT29 cells is 45 fmol of peptide bound per 10^6 cells.

The affinity of neurotensin for its cell receptor was compared to those of three neurotensin analogues in competition experiments involving various concentrations of unlabeled peptides and constant amounts of cells and ^{125}I-labeled [monoiodo-Tyr^3]neurotensin (fig.1B). The dissociation constant of the complex formed between native neurotensin and its receptor calculated from these data [5] was 1.5 nM, a value not significantly different from that found in saturation experiments (fig.1A). This result confirms that [monoiodo-Tyr^3]neurotensin and native neurotensin bind to the same receptor with identical properties. The affinity of acetyl neurotensin (8–13) was also very similar to that of neurotensin whereas that of neurotensin (9–13) was about 200-times lower. The dissociation constant of neurotensin (1–12) was not determined since this analogue displaced the bound radiolabeled ligand by less than 20% at 1 μM.

3.2. *Effect of neurotensin on intracellular cyclic nucleotide levels in HT29 cells*

Neurotensin was found to have no effect on the basal concentration of both cAMP and cGMP in HT29 cells, even at concentrations as high as 1 μM. The inability of neurotensin to modulate cAMP levels in this cell line has already been reported [14].

3.3. *Stimulation of inositol phosphate levels by neurotensin in HT29 cells*

Water-soluble extracts of HT29 cells prelabeled with [^{3}H]inositol contained five ^{3}H-labeled inositol derivatives that could be separated by anion exchange chromatography [11]. The first two eluted fractions were free inositol, which represented more than 90% of the water-soluble radioactivity, and glycerophosphoinositol which was weakly radiolabeled. The intracellular concentrations of these two compounds were insensitive to neurotensin. By contrast, incubation of HT29 cells with 0.1 μM neurotensin in the presence of Li^+ produced a rapid and transient stimulation of IP_3 and IP_2 levels, and a slower, more persistent increase of the IP_1 concentration (fig.2). Maximal responses were obtained after 20 s, 30 s and 2 min, respectively. Concentration-response curves for the effect of neurotensin on the intracellular levels of each one of the three inositol phosphates are presented in the main part of fig.3. Maximal stimulations over basal levels of 170 (IP_1), 420 (IP_2) and 275% (IP_3) were observed at 1 μM neurotensin. The peptide concentrations that induced half-maximal effect (EC_{50}) were similar: 60, 84 and 87 nM for IP_1, IP_2 and IP_3, respectively.

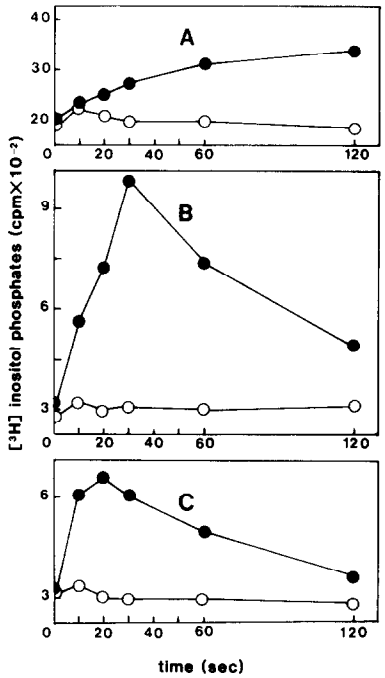

Fig.2. Time course of neurotensin-induced stimulation of inositol phosphate levels in HT29 cells. Monolayers of HT29 cells were prelabeled for 24 h with [^{3}H]inositol, then incubated at 37°C in a Hepes buffer, pH 7.4, in the presence (●) or absence (○) of 100 nM neurotensin. After the indicated incubation times, the IP_1 (A), IP_2 (B) and IP_3 (C) cell contents were measured by ion-exchange chromatography [11]. Each point is the mean of sextuplicate determinations from 3 different experiments.

Three neurotensin partial sequences were compared to the parent peptide for their ability to stimulate IP_3 formation (inset of fig.3). Neurotensin (1–12) was totally inactive, even at a concentration of 1 μM. Acetylneurotensin (8–13) was very similar to native neurotensin in terms of both efficiency (260% stimulation of the basal level) and potency (EC_{50} = 63 nM). Neurotensin (9–13) did not produce maximal stimulation at the highest concentration assayed (1 μM); its EC_{50} value was 500 nM. Similar relative potencies of neurotensin and its analogues, i.e. acetylneurotensin (8–13) $\geqq$ neurotensin > neurotensin (9–13) $\gg$ neurotensin (1–12) = 0, were obtained for stimulation of IP_1 and IP_2 (not shown).

Preincubation of cells with PMA, a phorbol ester which activates protein kinase C, resulted in a drastic attenuation of the neurotensin-promoted stimulation of inositol phosphate levels. In the course of three different experiments, the mean values of the inhibition percentages induced by 0.1 μg/ml PMA against the effect of 0.1 μM neurotensin were 72 ± 3 (IP_1), 82 ± 4 (IP_2) and 74 ± 3% (IP_3).

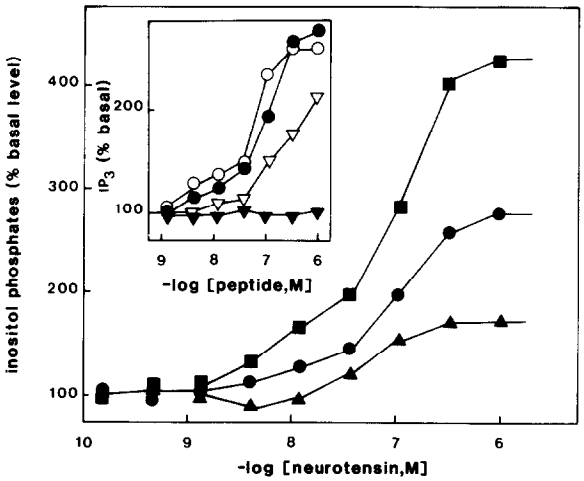

Fig.3. Concentration-response curves for the stimulation of inositol phosphate levels induced by neurotensin and its analogues in HT29 cells. Cells were prelabeled for 24 h with [^{3}H]inositol, then incubated at 37°C with the indicated concentrations of neurotensin. Intracellular inositol phosphate levels were determined after an incubation time of 20 s for IP_3 (●), 30 s for IP_2 (■) and 2 min for IP_1 (▲). (Inset) [^{3}H]Inositol-prelabeled cells were incubated at 37°C with the indicated concentrations of neurotensin (●), acetylneurotensin (8–13) (○), neurotensin (9–13) (▽) or neurotensin (1–12) (▼). IP_3 levels were determined after an incubation time of 20 s. Each point is the mean of sextuplicate determinations from 3 different experiments.

4. DISCUSSION

The main result of this work is that neurotensin stimulates the formation of inositol phosphates in HT29 cells, while not affecting cAMP and cGMP levels. The rapid and transient increase of IP_3 is observed just before that of IP_2. Both products are finally hydrolyzed into IP_1 which accumulates inside the cell in the presence of Li^+ [10]. This kinetic pattern suggests that phosphodiesterase-catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate into IP_3 and diacylglycerol is the major event initiated by neurotensin [15–18]. Binding affinities of neurotensin and three of its analogues (fig.1B) are correlated to their potencies for increasing IP_3 levels (fig.3, inset). Therefore, the intracellular effects of neurotensin on phosphatidylinositol breakdown in HT29 cells appear to be direct consequences of neurotensin receptor occupancy. As already observed with a variety of other neurotensin receptors [3], the integrity of the C-terminal 8–13 peptide sequence is essential for an optimal expression of neurotensin activities.

As far as we are aware, the only other evidence of a neurotensin-induced stimulation of phosphatidylinositol turnover has been obtained in rat brain slices [19]. In these studies, cAMP levels were found to be insensitive to neurotensin; no attempt was made to investigate the influence of neurotensin on cGMP formation. We have demonstrated that in neuroblastoma N1E115 cells, the association of neurotensin to its receptor is able not only to modulate cyclic nucleotide levels (see section 1) but also to stimulate IP_3 formation (in preparation). Taken together, these results indicate that stimulation of phosphatidylinositol breakdown is a more general response to neurotensin receptor occupancy than modulation of cyclic nucleotide levels.

It has been demonstrated that phorbol esters, which mimic diacylglycerol in activating protein kinase C, are able to inhibit agonist-induced formation of inositol phosphates in human platelets [20], rat hippocampal slices [21] and DDT_1 MF-2

smooth muscle cells [22]. In this work, we found that pretreatment of HT29 cells with PMA decreased by more than 70% the ability of neurotensin to stimulate levels of the three inositol phosphates. This observation is in agreement with the suggestion that protein kinase C activation by diacylglycerol may be involved in a general feedback inhibition mechanism leading to phosphorylation and inactivation of receptors that are coupled to the inositol phospholipid cycle [20–22].

The pharmacological properties of neurotensin on neutrophils [23], smooth muscle [24] or pituitary cells [25] have been correlated with the ability of the peptide to modulate intracellular Ca^{2+} levels. Similarly, we show here that neurotensin is also able to mobilize Ca^{2+} from intracellular stores in HT29 cells by using IP_3 as a second messenger. Since this mechanism also occurs in rat brain slices [19] and in N1E115 cells (in preparation), it may well represent a general mode of translation of the neurotensin-receptor interaction inside neurotensin target cells of different origin.

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