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Neurotensin-Mediated Inhibition of Cyclic AMP Formation in Neuroblastoma N1E115 Cells: Involvement of the Inhibitory GTP-Binding Component of Adenylate Cyclase

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SUMMARY

The tridecapeptide, neurotensin, inhibited prostaglandin E_1-stimulated cyclic AMP production in intact plated neuroblastoma N1E115 cells. The peptide effect was concentration dependent (EC_{50} = 2 nM) and maximal inhibition reached 55% with 100 nM neurotensin. Acetyl neurotensin (8-13) was as active as neurotensin whereas neurotensins (1-8), (1-12), and (10-13) were barely active in inhibiting cyclic AMP production, thus showing the requirement of the carboxy terminal hexapeptide sequence of neurotensin for biological activity. The inhibitory effect of neurotensin on cyclic AMP production was largely prevented by pretreatment of N1E115 cells with islet-activating protein (pertussis toxin). In contrast, pertussis toxin did not inhibit neurotensin-stimulated cyclic GMP production in neuroblastoma cells. In cell membranes, the toxin promoted the selective ADP-ribosylation of a single protein having the same molecular weight (41,000) as the α-subunit of N_i, the inhibitory regulatory protein of adenylate cyclase. In membranes prepared from N1E115 cells, monoiodo[^{125}I-Tyr^3]neurotensin bound to a single population of receptors characterized, at 25° and in the absence of monovalent cations and guanyl nucleotides, by a dissociation constant (K_d) of 56 pM and a maximal binding capacity (B_m) of 30 fmol/mg of protein. Na^+ (10–100 mM) and GTP (0.1–100 μM) inhibited neurotensin binding in a concentration-dependent manner. At 100 mM Na^+ and 100 μM GTP, receptor affinity was decreased by 5- and 2-fold, respectively. Li^+ and K^+ were less effective than Na^+, and the effect of GTP was shared by GDP and guanyl-5′-yl-imidodiphosphate, but not by GMP, ATP, ADP, or adenyl-5′-yl-imidodiphosphate. It is concluded that in N1E115 cells, neurotensin attenuates cyclic AMP production by exerting an inhibitory effect on adenylate cyclase through an interaction of the peptide receptors with the regulatory GTP-binding protein N_i.

There is now a considerable body of morphological, pharmacological, neurochemical, and behavioral evidence that the tridecapeptide neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) acts as a neurotransmitter or neuromodulator in the central nervous system (see Refs. 1–3 for review). Among this evidence is the well established existence of specific high affinity neurotensin receptors on central neuronal cell bodies and fibers. Although these receptors have been well characterized with regard to their central nervous system distribution and to their pharmacological and biochemical properties (4), little is known about the molecular events subsequent to their occupancy by neurotensin.

Neuroblastoma cell lines which retain many of the properties of normal neurones have provided useful models to study receptor-mediated cellular effects of a variety of neuropeptides and classical neurotransmitters. Recently, we demonstrated that specific high affinity neurotensin receptors were expressed in the murine neuroblastoma N1E115 cell line during the course of cell differentiation *in vitro* (5, 6). In addition, we and others reported that neurotensin stimulated cGMP production in N1E115 cells (6, 7), and this was shown to be a consequence of the peptide interaction with its receptors (6). N1E115 cells therefore represent a valuable system for investigating neurotensin receptor-mediated responses in a nerve cell population.

In the present work, we have investigated the possibility that neurotensin receptors interact with the adenylate cyclase-GTP-binding regulatory protein (N) system in N1E115 cells. This research was initiated by our previous observation that neurotensin inhibited, by 20–30%, basal cAMP levels in N1E115 cells (6). The data reported here show that neurotensin receptor occupancy leads to an inhibition of stimulated cAMP production in N1E115 cells, an effect that is reversed by IAP, the toxin from *Bordetella pertussis*. Furthermore, they show that monoiodo[^{125}I-Tyr^3]neurotensin binding to N1E115 cell membranes is regulated by Na^+ and GTP. It is concluded that, in the neuroblastoma N1E115 cell line, neurotensin receptor com-

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ABBREVIATIONS: IAP, islet-activating protein; PGE_1, prostaglandin E_1; IBMX, isobutylmethylxanthine; N_i, inhibitory GTP binding protein of the adenylate cyclase system; EDTA, ethylenediaminetetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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plexes interact with the inhibitory GTP-binding protein, N_i, thereby attenuating adenylate cyclase activity. It is also concluded that neurotensin-stimulated cGMP production in N1E115 cells occurs through a mechanism that does not involve N_i since this effect is not affected by IAP. A preliminary and partial account of this work has been presented (8).

Materials and Methods

Peptides and drugs. Neurotensin and its analogues acetyl-neurotensin (8-13), neurotensin (10-13), neurotensin (1-12), and neurotensin (1-8) were generous gifts from C. Granier and J. Van Rietschoten (Faculté de Médecine Nord, Marseille, France). 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 (9). [^{3}H]Adenine (15–30 Ci/mmol) was from Amersham (France) and [α-^{32}P]NAD (50 Ci/mmol) was from New England Nuclear (France). IAP was purchased from List Biological Laboratories, Inc., Campbell, CA). PGE_1 and IBMX were from Sigma Chemical Co. (St. Louis, MO). 1,10-Phenanthroline was from Merck (Rahway, NJ). Unlabeled nucleotides were from Boehringer Mannheim (Indianapolis, IN).

Cell culture. Neuroblastoma N1E115 cells (passage number > 24 and < 31) were cultured and induced to differentiate as previously reported (6). Briefly, cells were propagated in 75-cm^2 Falcon tissue culture flasks and cultured at 37° in a humidified atmosphere of 5% CO_2/95% air, in Dulbecco's modified Eagle's medium that contained 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells cultured in the nondifferentiated state were detached before confluence by incubation for 3 min in 10 mM phosphate buffer containing 140 mM NaCl, 5 mM KCl, and 0.02% trypsin. After centrifugation, the cellular pellet was washed and resuspended in 10 ml/75-cm^2 flask of culture medium. An aliquot of this suspension was used for cell enumeration with a Coulter counter. The cell suspension was distributed into Falcon 24-well plates (10^5 cells/well) or in 100-mm Petri dishes (10^6 cells/dish), and cells were allowed to grow for 48 hr under the conditions described above. Cells were then induced to differentiate for 2–3 days in culture medium containing 0.5% fetal calf serum and 1.5% dimethyl sulfoxide. Differentiated cells in multiwell plates were reincubated for 4 hr in normal culture medium before use for cyclic nucleotide measurements (see below). For each experiment, cell number per well was evaluated by resuspending the cells from two wells per plate in trypsin solution as described above and counting each suspension with a Coulter counter. Cell number per well usually ranged from 3.10^5 to 4.10^5. Differentiated cells in Petri dishes (about $3–4.10^6$ cells/dish) were scraped off the culture dishes and harvested in 25 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose and 1 mM EDTA. The resulting cell suspensions were aliquoted and stored in liquid nitrogen.

Measurement of cellular cAMP levels. N1E115 cells in multiwell plates (3.10^5–4.10^5 cells/well) were preincubated for 30 min at 37° in incubation buffer (140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$, 0.2% bovine serum albumin, 4.5 g/liter of glucose, and 25 mM HEPES-Tris, pH 7.5). The phosphodiesterase inhibitor IBMX was then added at 1 mM and, 30 sec later, 0.1 μM PGE_1 was added to the incubation medium without or with varying concentrations of neurotensin or its analogues (final incubation volume, 200 μl). After another 30-sec period, incubations were stopped and cAMP measurements were carried out according to either of the following two procedures.

The first procedure involved treatment of each well with 0.8 ml of a 2:1 (v/v) mixture of ethanol/5 mM EDTA. Cell extracts were centrifuged. The supernatants were evaporated, reconstituted in 50 mM Tris-HCl, pH 7.5, containing 4 mM EDTA, and assayed for their cAMP content using cAMP assay kits from Amersham (France) and exactly following the procedure recommended by the manufacturer. Results were expressed as pmol of cAMP/10^6 cells.

In the second procedure, N1E115 cells in their culture medium were incubated for 3 hr at 37° with 2 μCi/ml of [^{3}H]adenine in each well separately. The culture medium was then removed and the cells were preincubated and subsequently incubated in incubation buffer as described above. Each well was then treated with 1 ml of 5% trichloroacetic acid. The resulting cell extracts were centrifuged for 10 min at 10,000 × g. The supernatant contents of [^{3}H]cAMP formed from prelabeled intracellular [^{3}H]ATP were determined by purifying [^{3}H]cAMP from other tritiated compounds according to the following procedure. The supernatants, to which were added 1 mM unlabeled ATP and cAMP, were loaded on 1.2-ml Dowex AG 50 WX 100 columns (Bio-Rad) that had been washed once with 5 ml of 2 N HCl and twice with 10 ml of H_2O. The columns were rinsed with 2 ml of H_2O which eluted most of the [^{3}H]ATP and were mixed with 7 ml of Aquassure (New England Nuclear) for counting in a liquid scintillation counter with a 40% ^{3}H counting efficiency. The Dowex columns were rinsed a second time with 10 ml of H_2O and were directly loaded on 0.8-ml neutral alumina AG7 (Bio-Rad) columns equilibrated in 100 mM imidazole-HCl buffer, pH 7.5. Each alumina column was eluted with 5 ml of imidazole buffer. Eluates which contained [^{3}H]cAMP were mixed with 7 ml of Aquassure and counted for radioactivity. The amount of radioactivity present in the first washout of the Dowex column and corresponding to the amount of [^{3}H]adenine that was incorporated in the form of [^{3}H]ATP in N1E115 cells provided an index of cell viability and cell number reproducibility for each well. The values ranged from 1.2 to $1.8.10^6$ dpm/10^6 cells/well between experiments and did not vary more than 15% within an experiment. Using pure labeled and unlabeled adenine derivatives (adenine, AMP, ADP, ATP, and cAMP) as standards, it was checked that the cAMP purification procedure described above led to the recovery of cAMP (>99% pure) with a 55–65% yield. The results, expressed as dpm/10^6 cells, were not corrected for this recovery factor.

Attempts were also made to test the effect of neurotensin on adenylate cyclase activity in N1E115 cell membrane preparations. In this system, PGE_1 readily stimulated adenylate cyclase activity, but neurotensin was without effect on either basal or PGE_1-stimulated adenylate cyclase activity. In addition, carbachol and Met-enkephalin, two agents which have been reported to inhibit stimulated cAMP production in intact N1E115 cells (10, 11), did not affect PGE_1-stimulated adenylate cyclase activity in N1E115 cell membranes. This suggests that, for as yet unknown reasons, broken N1E115 cell preparations are not suitable for studying receptor-mediated inhibition of adenylate cyclase activity.

Measurement of cellular cGMP levels. Neuroblastoma cells in multiwell plates ($3–4.10^5$ cells/well) were preincubated for 5 min in the absence of peptide and then incubated for 20 sec with 100 nM neurotensin. Incubations were carried out at 37° in 200 μl of incubation buffer (see above). Incubations were stopped and wells were extracted as described above for the first procedure used to measure cGMP levels. cGMP levels were measured using cGMP radioimmunoassay kits from Amersham (France) and exactly following the procedure recommended by the manufacturer.

IAP treatment of intact cells and IAP-mediated ADP-ribosylation of cell membranes. Differentiated N1E115 cells in their culture medium were incubated with 1 μg/ml of IAP for 3 hr. Cell incubation and cyclic nucleotide measurements were then performed as described above.

ADP-ribosylation of N1E115 cell membranes involved activation of IAP by incubating 50 μg/ml of the toxin for 30 min at 30° with 100 μM dithiothreitol in 10 mM Tris-HCl, pH 7.5, and was carried out in 100 μl of this buffer containing 200 μg/ml of cell membranes, 8 μg/ml of activated IAP, 5 mM $MgCl_2$, 100 μM GTP, 1 mM ATP, 10 mM thymidine, and 20 μCi/ml of [α-^{32}P]NAD. After 45 min at 30°, incubation was stopped by addition of 0.5 ml of ice-cold buffer and centrifugation (15 min, 10,000 × g). The pellet was resuspended in 50 μl of 10 mM sodium phosphate buffer, pH 7.5, containing 2% sodium dodecyl sulfate and 5% β-mercaptoethanol and heated at 95° for 5 min. Solubilized membranes were analyzed according to the method of Laemmli (12) by electrophoresis on 4% stacking and 10% separating polyacrylamide

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gels. Electrophoresis was carried out for 3 hr with a current of 40 mamp/gel. After staining with Coomassie blue and destaining, gels were dried and exposed for 2–3 days to Kodak X-OMAT-S films.

[^{125}I]Neurotensin binding to cell membranes. N1E115 cell membranes were prepared as follows. Frozen cell suspensions obtained as described above were thawed and centrifuged for 10 min at 9000 × *g*, and the pellet was suspended in 5 mM Tris-HCl, pH 7.5. Cells were broken by repeated passages through a syringe needle. The cell homogenate was centrifuged (10 min, 9000 × *g*) and the pellet was 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. Protein concentrations were determined using the Bio-Rad protein assay reagent following the procedure recommended by the manufacturer.

Binding assays were carried out at 25° in 250 μl of binding assay buffer (50 mM Tris-HCl, pH 7.5, 0.2% bovine serum albumin) that contained [^{125}I]neurotensin, 0.05–0.1 mg of membrane protein, and, when needed, monovalent cations, nucleotides, and unlabeled neurotensin. The binding assay buffer also contained 1 mM 1,10-phenanthroline which efficiently prevented [^{125}I]neurotensin from degradation (see below). Total, specific, and nonspecific binding were determined as previously described (5, 9). Dissociation constant, K_d, and maximal binding capacity, B_m, 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 unlabeled neurotensin were added to a fixed concentration (0.05–0.1 nM) of [^{125}I]neurotensin. It was checked that the binding of 0.1 nM [^{125}I]neurotensin reached equilibrium by 15–20 min (not shown). Therefore, in all experiments, binding was measured after 20 min of incubation. At 0.1 nM [^{125}I]neurotensin, nonspecific binding did not exceed 10% of total binding.

Integrity of [^{125}I]neurotensin exposed to cell membranes. [^{125}I]Neurotensin at 0.1 nM was incubated without or with N1E115 cell membranes in the conditions of the binding assay. At varying times of incubation, the membranes were centrifuged at 4 ° for 10 min at 10,000 × *g*. The supernatants were applied to a reverse phase RP 18 Lichrosorb (Merck) column connected to a Waters Associates HPLC apparatus. Elution was carried out as described in previous studies (9, 13). Fractions of 1 ml were collected and counted for radioactivity in a gamma spectrophotometer. In all cases, 90–100% of the radioactivity applied was recovered from the column. Intact [^{125}I]neurotensin eluted from the column with a retention time of 39 min. Integrity of [^{125}I]neurotensin was assessed by evaluating the percentage of radioactivity recovered in the peak corresponding to intact [^{125}I]neurotensin. In the case of [^{125}I]neurotensin that had not been exposed to cell homogenates, this percentage was > 95%. Correction was made to account for this fact. Exposure to cell membranes for 10, 30, and 60 min resulted in a 36, 68, and 81% loss of intact free labeled peptide, respectively. When the metalloprotease inhibitor 1,10-phenanthroline was added at 1 mM to the binding assay buffer, degradation was markedly inhibited since only 1, 14, and 24% of ligand were lost after 10, 30, and 60 min, respectively.

Results

Effects of neurotensin on cAMP levels in N1E115 cells. Previous experiments showed that neurotensin (30 nM) exerted a slight (20–30%) inhibition on basal cAMP levels in N1E115 cells (6). This led us to study the effect of neurotensin on stimulated cAMP levels. It has been reported that, in the presence of the phosphodiesterase inhibitor IBMX, PGE_1 is a potent and efficient activator of cAMP synthesis in N1E115 cells (10). In the present study, it was found that stimulation of cAMP production in N1E115 cells by PGE_1 (0.01–1 μM) in the presence of IBMX (1 mM) increased linearly during at least 2 min and reached a plateau by 5 min (data not shown). Stimulation was maximal at 1 μM PGE_1 and half-maximal at 0.1 μM, the PGE_1 concentration used in the present experiments. Fig. 1 shows basal and stimulated cAMP levels after 30 sec in N1E115 cells as measured by the two procedures described in Materials and Methods. IBMX at 1 mM increased cAMP levels 2–3-fold over basal. Addition of 0.1 μM PGE_1 to IBMX resulted in a further 3.5-fold increase in cAMP levels. Note that both procedures detected similar relative cAMP variations in the different conditions tested. This makes it possible to establish a correspondence between dpm of [^{3}H]cAMP (second procedure, Fig. 1, *right*) and pmol of cAMP (first procedure, Fig. 1, *left*). In all subsequent experiments, the second procedure was used to measure relative changes in cAMP content of N1E115 cells.

Neurotensin added concomitantly to PGE_1 for 30 sec exerted a concentration-dependent inhibition of PGE_1-stimulated cAMP production in neuroblastoma cells (Fig. 2). It was checked that, during the 30-sec exposure of neurotensin to N1E115 cells at 37°, no significant degradation of the peptide occurred. An incubation time of 30 sec was found to be sufficient for neurotensin to reach the maximal extent of inhibition of PGE_1-stimulated cAMP production (data not shown). The EC_{50} for neurotensin was 2 nM and a maximal inhibition of 55% was reached at 100 nM neurotensin. Of the neurotensin analogues tested, acetyl-neurotensin (8-13) was as potent (EC_{50}, 2 nM) and efficient as neurotensin in inhibiting cAMP production,

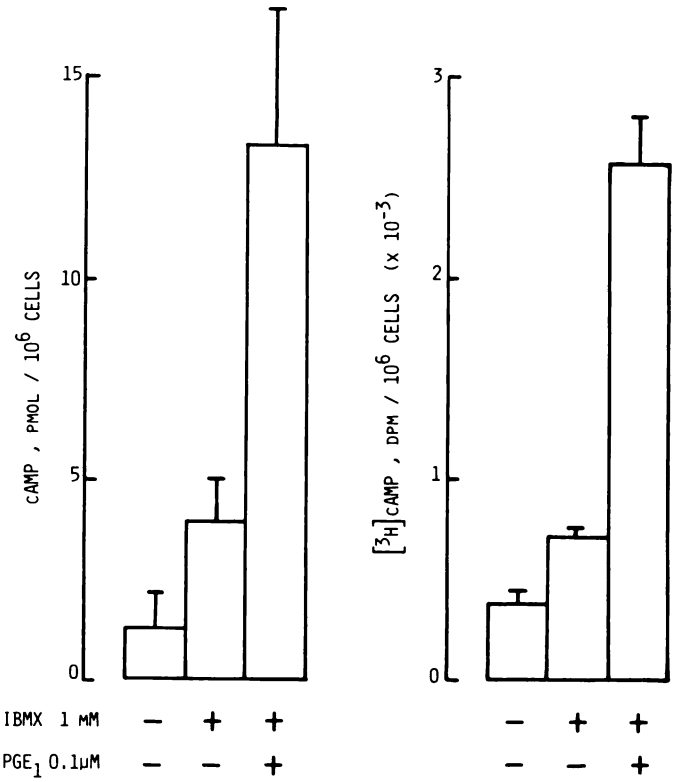

Fig. 1. Basal and stimulated cAMP levels in N1E115 cells. Cells in multiwell plates (3–4.10^5 cells/well, passage number > 24 and < 31) were incubated at 37° in the absence of agent, or in the presence of 1 mM IBMX alone for 1 min, or in the presence of 1 mM IBMX alone for 30 sec followed by another 30-sec incubation with 1 mM IBMX + 0.1 μM PGE_1. cAMP levels were measured according to either of the two procedures described in Material and Methods. They are expressed in pmol/10^6 cells and dpm/10^6 cells for the first (*left panel*) and second (*right panel*) procedures, respectively. Data are the means ± SE from three independent experiments with 5–10 determinations within each experiment.

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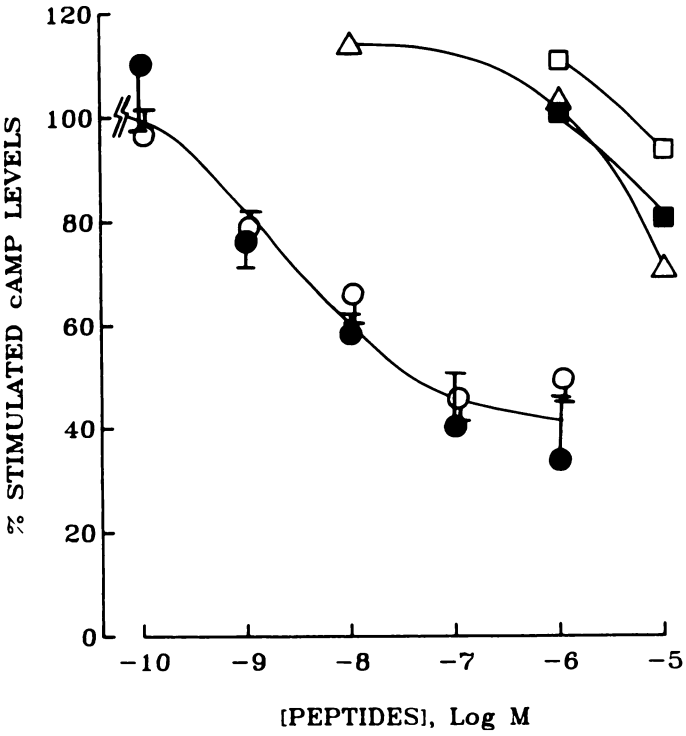

Fig. 2. Concentration response curves for the inhibition by neurotensin and its analogues of PGE_1-stimulated cAMP production in N1E115 cells. Cells in multiwell plates (3–4.10^5 cells/well, passage number > 24 and < 31) were incubated at 37° in the presence of 1 mM IBMX alone for 30 sec and then in the presence of 1 mM IBMX + 0.1 μM PGE_1 without or with neurotensin or its analogues at the indicated concentrations. cAMP measurements were carried out as described in Materials and Methods. Mean cellular cAMP levels in the presence of IBMX alone and in the presence of IBMX + PGE_1 were 931 ± 23 dpm/10^6 cells and 2656 ± 66 dpm/10^6 cells, respectively (mean ± SE from eight experiments with 5–10 determinations within each experiment). For each experiment, percentage of stimulated cAMP levels were calculated as (cAMP levels in the presence of IBMX, PGE_1, and peptide − cAMP levels in the presence of IBMX alone)/(cAMP levels in the presence of IBMX and PGE_1 − cAMP levels in the presence of IBMX alone). Data are the means ± SE from eight experiments with neurotensin (○), four with acetyl-neurotensin (8-13) (●), and one with neurotensins (1-12) (△), (1-8) (□), and (10-13) (■), with duplicate determinations within each experiment.

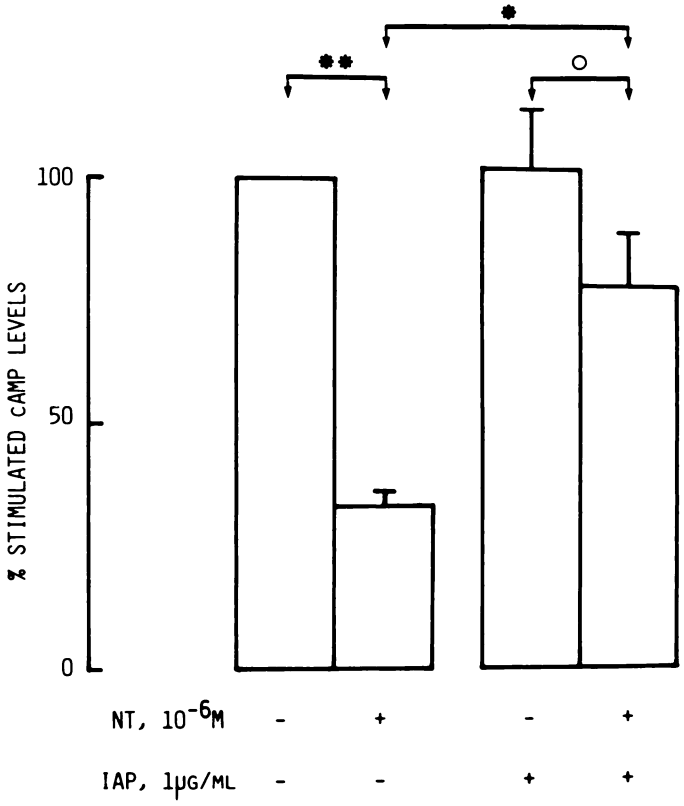

Fig. 3. Effect of IAP on neurotensin (*NT*)-induced inhibition of PGE_1-stimulated cAMP production in N1E115 cells. Cell treatment with IAP was carried out at 37° for 3 hr in culture medium containing 1 μg/ml of IAP. Other experimental conditions were as described in the legend to Fig. 2. cAMP measurements were carried out as described in Materials and Methods. Mean cellular cAMP levels in the presence of IBMX alone were 1330 ± 77 and 1390 ± 116 dpm/10^6 cells in IAP untreated and treated cells, respectively. cAMP levels in PGE_1-stimulated, untreated cells were 2816 ± 250 dpm/10^6 cells. For each experiment, percentage of stimulated cAMP levels were calculated as explained in the legend to Fig. 2. Data are the means ± SE from three experiments with duplicate determinations within each experiment. Means are compared using the Student's *t* test. ○, not significant; *, $p < 0.02$; **, $p < 0.005$.

whereas neurotensins (10-13), (1-12), and (1-8) were ineffective at 1 μM and showed little to moderate activity at 10 μM (Fig. 2).

Effect of pertussis toxin (IAP) on cAMP and cGMP responses to neurotensin. Pretreatment of N1E115 cells with IAP did not significantly affect PGE_1-stimulated cAMP production (Fig. 3). In contrast, the toxin markedly reduced the inhibition by 1 μM neurotensin of stimulated cAMP levels (Fig. 3). IAP has been shown to ADP-ribosylate the α-subunit (M_r = 41,000) of the membrane GTP-binding protein N_i (the inhibitory regulatory component of the adenylate cyclase system) in a number of cell types (14). In order to test for the presence of N_i in N1E115 cell membranes, crude membrane preparations were treated with IAP in the presence of [α-^{32}P] NAD. This resulted in the selective labeling of only one membrane protein component of molecular weight 41,000 as estimated by autoradiography of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4). Another protein band of M_r = 112,000 was also labeled. However, the labeling was not IAP mediated since it persisted in the absence of the toxin.

It has been reported that neurotensin stimulated cGMP production in N1E115 cells (6, 7) and that this appeared to be a neurotensin receptor-mediated response (6). It was therefore of interest to test the effect of IAP on neurotensin-stimulated cGMP production in N1E115 cells. It has been shown in this laboratory that cGMP levels were maximally elevated after 20–30 sec in the presence of 100 nM neurotensin (6). In the present series of experiments, basal and neurotensin (100 nM)-stimulated cGMP levels were 14.8 ± 3.9 and 90.5 ± 8.5 pmol/10^6 cells (n = 10), respectively, as measured after 20 sec in control cells, whereas the corresponding values in cells treated with IAP (see Materials and Methods) were 11.6 ± 3.8 and 125.1 ± 7.7 pmol/10^6 cells (n = 10). Thus, in conditions similar to those in which IAP markedly reduced the cAMP response to neurotensin in N1E115 cells, the toxin did not prevent the peptide from stimulating cGMP production. If anything, IAP treatment slightly potentiated neurotensin-induced cGMP formation.

Effects of monovalent cations and guanyl nucleotides on [^{125}I]neurotensin binding to cell homogenates. The results of the preceding section are consistent with an interaction of neurotensin receptors with the GTP-binding protein N_i, as will be discussed later. A characteristic of ligand binding to

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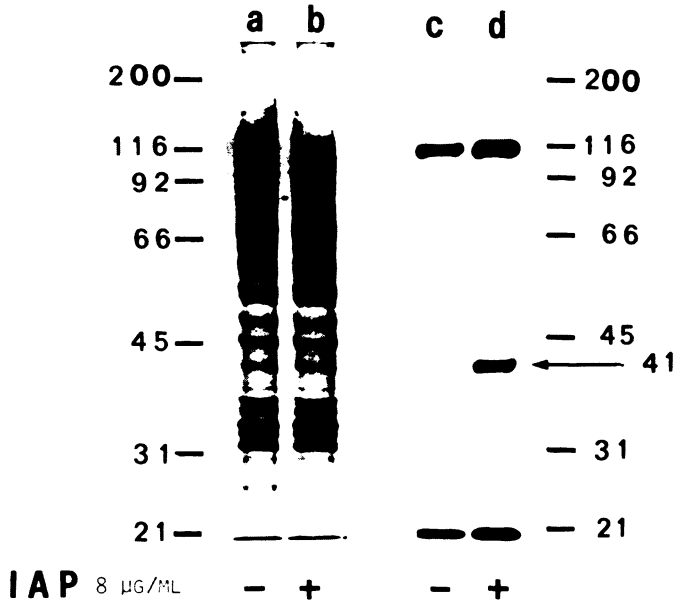

Fig. 4. IAP-mediated ADP-ribosylation of a 41,000-Da protein in N1E115 cell membranes. Cell membranes were incubated with [α-^{32}P]NAD, and without (*lanes a* and *c*) or with (*lanes b* and *d*) IAP, and then submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and to autoradiography as described in Materials and Methods. *Lanes a* and *b*, Coomassie blue staining; *lanes c* and d, corresponding autoradiograms. Marker proteins were, from *top* to *bottom;* myosine (200,000), β-galactosidase (116,250), phosphorylase *b* (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500). The band observed just below the 21-kDa marker corresponds to the gel front.

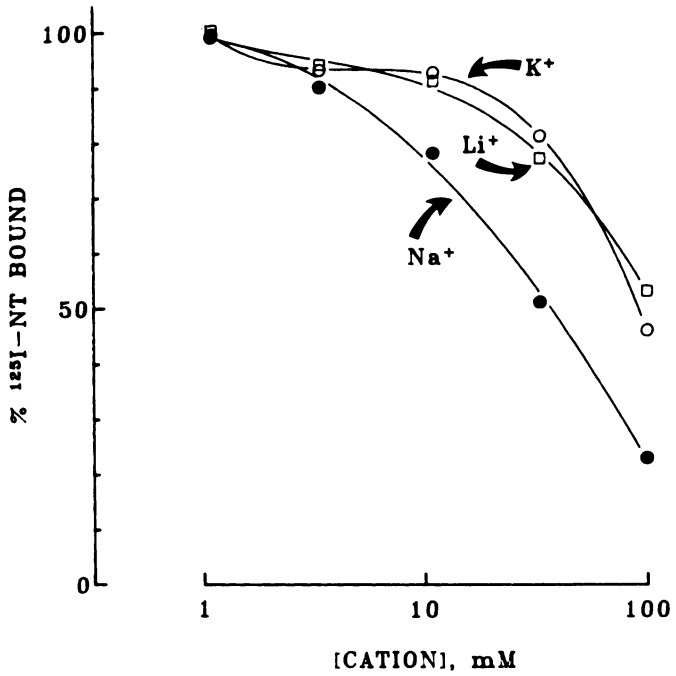

Fig. 5. Effect of increasing concentrations of monovalent cations on the binding of 0.1 nM [^{125}I]neurotensin (*NT*) to N1E115 cell membranes. Binding assays were carried out for 20 min at 25° as described in Materials and Methods. The results are expressed as the percentage of [^{125}I]neurotensin binding in the absence of cation. Data are the means from three experiments with duplicate determination within each experiment.

receptors that interact with N_i is the regulation of binding affinity by sodium and GTP (15). We therefore investigated the effects of monovalent cations and guanyl nucleotides on the binding of [^{125}I]neurotensin to a crude particulate fraction obtained from N1E115 cells.

Na^+ and, to a lesser extent, K^+ and Li^+ inhibited in a concentration-dependent manner the binding of 0.1 nM [^{125}I] neurotensin to N1E115 cell homogenates (Fig. 5). The EC_{50} for Na^+ was 35 mM. At this concentration, K^+ and Li^+ inhibited [^{125}I]neurotensin binding by 19 and 23%, respectively. A typical Scatchard analysis of [^{125}I]neurotensin binding in the absence and presence of 33 and 100 mM Na^+ is shown in Fig. 6. Scatchard plots were linear, indicating the existence of one population of independent neurotensin receptors, as previously reported in N1E115 cell homogenates at 0° (5) and in intact N1E115 cells at 37° (6). Mean K_d and B_m values derived from several experiments like that in Fig. 6 are shown in Table 1. At 33 mM, Na^+ induced a 4-fold increase in K_d and a 20% decrease B_m. These effects were amplified at 100 mM Na^+.

The guanyl nucleotides GTP and GDP, and the nonhydrolyzable GTP analogue, guanyl-5′-yl imidophosphate, inhibited in a concentration-dependent manner the binding of 0.1 nM [^{125}I]neurotensin to N1E115 cell homogenates (Fig. 7). EC_{50} values were 0.3 μM for GTP and GDP and 1.6 μM for guanyl-5′-yl imidophosphate. For all three nucleotides, maximum inhibition reached about 40% at 10 μM. At this concentration, other nucleotides such as ATP (Fig. 7), ADP, adenyl-5′-yl imidophosphate, and GMP (not shown) exerted no inhibitory effect on [^{125}I]neurotensin binding. Scatchard plots from several binding experiments in the presence of 0.1 mM GTP were linear (not shown) and indicated that the nucleotide increased K_d (about 2-fold) and decreased B_m (about 30%) (Table 1).

Discussion

The present data clearly show that neurotensin inhibits cAMP production in the neuroblastoma N1E115 cell line. This is to our knowledge the first demonstration that neurotensin can regulate intracellular cAMP levels. This effect of neurotensin appears to be triggered by an interaction of the peptide with the receptors that have been previously characterized in binding studies using intact N1E115 cells at 37° (6). Thus, from the limited number of neurotensin analogues tested in the present and previous studies (6), it appears that the carboxy-terminal hexapeptide portion of the neurotensin molecule contains all of the structural requirements for both receptor binding and inhibition of cAMP production. In this respect, the functional neurotensin receptors characterized in N1E115 cells are similar to neurotensin receptors previously characterized in other tissues of neural and extraneural origins (4).

It is now well established that coupling between occupancy of its receptor by an inhibitory effector of adenylate cyclase and resulting inhibition of the enzyme occurs through a third membrane component designated N_i or G_i which belongs to a family of guanine nucleotide-binding regulatory proteins (15, 16). Several lines of evidence strongly support the hypothesis that neurotensin-induced inhibition of cAMP production in the neuroblastoma N1E115 cell line results from an interaction of neurotensin receptors with N_i.

The strongest evidence comes from experiments with IAP (pertussis toxin). Like other N proteins, N_i consists of three protein subunits α, β, and γ. IAP has been shown to inactivate

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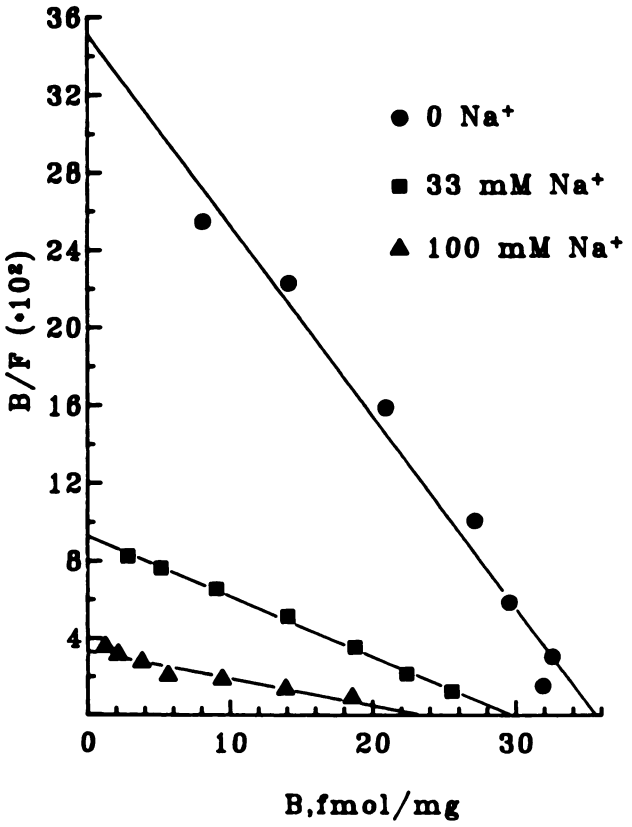

Fig. 6. Scatchard analysis of [^{125}I]neurotensin binding to N1E115 cell membranes in the absence (●) or presence of 33 mM (■) and 100 mM (▲) Na^+. The binding assay was carried out for 20 min at 25° as described in Materials and Methods. Data are duplicate determinations in a typical experiment.

N_i by catalyzing the ADP-ribosylation of its α-subunit (14). This in turn attenuates receptor-mediated inhibition of adenylate cyclase (14). The present data show that IAP markedly decreases the ability of neurotensin to inhibit cAMP production in N1E115 cells. This is not a nonspecific effect of the toxin since IAP-treated N1E115 cells retain full ability to respond to neurotensin by an increase in cGMP. Furthermore, the only protein which is selectively ADP-ribosylated by IAP in N1E115 cell membranes has the same molecular weight (41,000) as that reported for the α-subunit of N_i (14, 16).

Other evidence that neurotensin receptors interact with N_i in N1E115 cells comes from binding experiments. An interesting feature of receptors which mediate adenylate cyclase inhibition is the decrease in receptor affinity that occurs in the presence of guanine nucleotides (15, 17–19). This decrease presumably results from a conformational change of the effector-receptor-N_i complex that is brought about by the binding of GTP to the α-subunit of N_i (14, 15). In addition, the binding affinity of inhibitory effectors of adenylate cyclase for their receptors is strongly negatively regulated by Na^+ (15, 17–19). It is shown here that in N1E115 cell membranes, both guanyl nucleotides and monovalent cations decrease the affinity of neurotensin for its receptors without significantly changing receptor number. The specificity and EC_{50} values reported here for these agents are comparable to what has been reported for other receptor systems that mediate adenylate cyclase inhibition (18, 19). Examples of receptors that mediate adenylate cyclase inhibition and for which GTP and Na^+ modulate agonist binding include α_2-adrenergic receptors in platelets (20), cardiac muscarinic cholinergic receptors (11), pituitary D-2 dopamine receptors (21), and brain opiate receptors (22). In most of these and other systems, GTP and Na^+ convert a high affinity, agonist-sensitive state of the receptor to a low affinity state without changing maximal binding capacity. In some cases, receptor number appears to decrease (22), but this may reflect the inability to measure ligand binding to those receptors in the low affinity state (21).

Altogether, the above observations are compatible with the existence in N1E115 cell membranes of neurotensin receptor-N_i complexes that would exhibit a high affinity for neurotensin in the absence of GTP and Na^+. GTP, through binding to N_i (16), and Na^+, through a less clear mechanism, change the conformation of the receptor-N_i complex and decrease its affinity for neurotensin. This decrease probably explains the difference in neurotensin receptor-binding affinity derived from binding studies using intact N1E115 cells in physiological medium (K_d = 0.75 nM, Ref. 6) as compared to the value obtained with N1E115 cell membranes in the absence of GTP and cation (K_d = 0.06 nM, present study).

It is quite interesting that, in N1E115 cells, neurotensin inhibits cAMP production and at the same time stimulates cGMP production through apparently different transduction mechanisms. Indeed, IAP treatment of N1E115 cells under nearly identical conditions markedly attenuates neurotensin-induced decrease in stimulated cAMP levels, whereas it does not prevent neurotensin-stimulated cGMP production. At this point, the question arises as to whether these two responses occur through the same or through distinct sets of neurotensin receptors. Before attempting to answer this question, the following experimental evidence should be taken in consideration: 1) Scatchard analysis of neurotensin binding to N1E115 cells in a variety of conditions has always revealed the existence of a single class of noninteracting binding sites; 2) EC_{50} values for the ability of neurotensin to stimulate cGMP production, to inhibit PGE_1-stimulated cAMP production, and to inhibit [^{125}I]

TABLE 1

Effect of Na^+ and GTP on neurotensin binding parameters in N1E115 cell membranes

Binding experiments were performed as described in Materials and Methods. Binding parameters were derived from linear regression analysis of Scatchard plots such as those shown in Fig. 6. Data are the means ± SE from six control experiments and three experiments in the presence of Na^+ and GTP with duplicate determination within each experiment. Means are compared using the Student's *t* test for paired data.

Binding parameter	Control	Na^+		GTP (0.1 mM)
		33 mM	100 mM	
K_d, nM	0.056 ± 0.010	0.21 ± 0.04[a]	0.28 ± 0.04[a]	0.090 ± 0.014[a]
B_m, fmol/mg	30 ± 4	24 ± 6[b]	18 ± 4[b]	20 ± 7[b]

[a] Significantly different from control, $p < 0.05$.
[b] Not significantly different from control.

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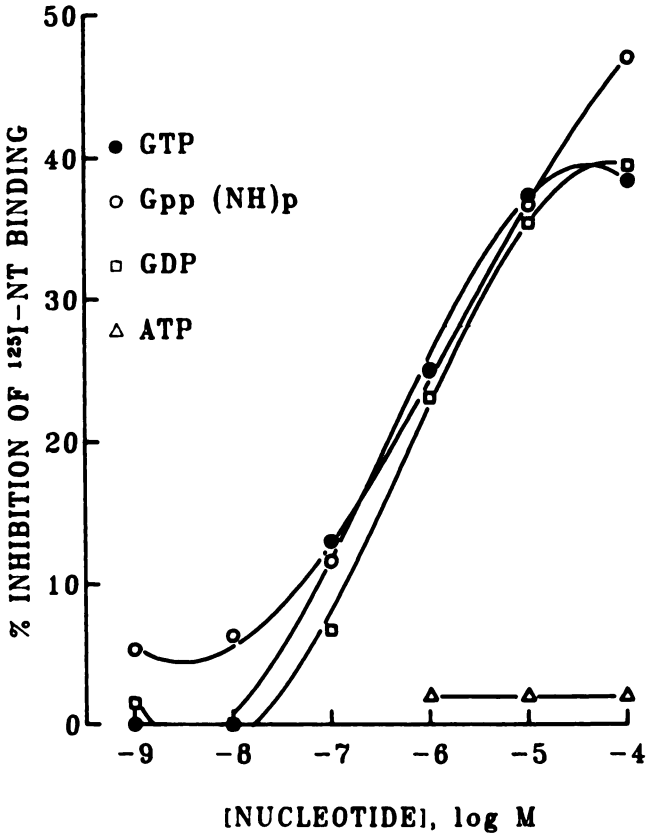

Fig. 7. Effects of nucleotides on the binding of 0.1 nM [^{125}I]neurotensin (*NT*) to N1E115 cell membranes. Binding assays were carried out for 20 min at 25° as described in Materials and Methods. The results are expressed as the percentage of inhibition of [^{125}I]neurotensin binding in the absence of nucleotide. Data are the means from three experiments with duplicate determinations within each experiment. *Gpp(NH)p*, guanyl-5′-yl imidodiphosphate.

neurotensin binding in intact N1E115 cells under identical conditions of incubation are very close (1–2 nM); 3) structure-activity data indicate that it is the same region of the neurotensin molecule, i.e., the carboxy-terminal hexapeptide sequence, which is responsible for binding, stimulation of cGMP, and inhibition of cAMP production in N1E115 cells (present study, Refs. 5 and 6).

Given this evidence, two hypotheses may be proposed to answer the above question. In the first hypothesis, a unique population of neurotensin receptors would mediate both the cAMP and cGMP responses. This would imply that the same neurotensin receptor molecule bears two distinct intracellular transduction domains, one interacting with N_i and thereby promoting adenylate cyclase inhibition, and the other interacting with a cGMP-generating system. Alternatively, since there is no direct evidence for a unique population of neurotensin receptors in N1E115 cells, it might be proposed as a second hypothesis that two separate sets of neurotensin receptors with similar recognition sites and affinities for the peptide but with different transduction domains would each mediate one of the responses to neurotensin in N1E115 cells. In this context, it is interesting that muscarinic agonists have been shown to increase cGMP production and to inhibit PGE_1-stimulated cAMP production in N1E115 cells (10, 23), and that recent evidence has been presented that the two effects occur via occupation of distinct populations of muscarinic receptors (24).

Whether neurotensin effects in N1E115 cells are mediated by one or two populations of receptors, it is clear that the cAMP and cGMP responses involve different transduction mechanisms. Neurotensin inhibition of cAMP production requires the GTP-binding protein N_i as a coupling component, as documented here. The mechanism that leads from neurotensin receptor occupancy to stimulation of cGMP production is less clear. It has been recently proposed that effector-mediated increase in cGMP levels in N1E115 cells involves the formation of arachidonic acid possibly as the consequence of a stimulation of phosphatidylinositol turnover (25). Preliminary experiments suggest that, indeed, neurotensin increases phosphatidylinositol turnover in N1E115 cells.[1] If the hypothesis that neurotensin stimulates cGMP production in N1E115 cells through an increase in phosphatidylinositol turnover is confirmed, our data would suggest that this response does not involve N_i as a coupling component.

It will be interesting to see whether the effects generated by the interaction of neurotensin with its receptor in N1E115 cells also occur in other cell types and tissues. Neurotensin has been reported to increase cGMP levels in slices of guinea pig cerebellum (26) and to stimulate phosphatidylinositol turnover in rat brain slices (27). Previous attempts to demonstrate a stimulatory or inhibitory effect of neurotensin on brain adenylate cyclase have failed[2] (27). However, brain tissue is highly heterogeneous and neurotensin receptors are likely to represent only a minor proportion of all brain adenylate cyclase-coupled receptors. It may therefore be inherently difficult to detect an effect of neurotensin on brain adenylate cyclase, particularly if this effect is inhibitory as in N1E115 cells.

It may also be that other mechanisms, besides those discussed here, are involved in coupling cellular response to neurotensin-receptor interaction. Thus, it has been recently reported that neuroblastoma × glioma NG108-15 hybrid cells possess neurotensin receptors that mediate facilitation of synaptic transmission, and that this response is not coupled to adenylate cyclase activity or phosphatidylinositol turnover (28). Further work will be needed in order to elucidate the molecular mechanisms responsible for the wide range of neurotensin actions that have been reported in a variety of neuronal and non-neuronal tissues.

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