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REGULATION OF CYCLIC GMP LEVELS BY NEUROTENSIN IN NEUROBLASTOMA CLONE N1E115

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The binding of ^{125}I-labeled (monoiodo-Tyr^{3})neurotensin to intact neuroblastoma N1E115 cells and the effect of neurotensin on the intracellular concentration of cyclic nucleotides were studied at 37°C and under physiological conditions of pH and ionic strength. The radiolabeled neurotensin analogue bound specifically to differentiated cells with a dissociation constant of 0.75 nM and a maximal binding capacity of 45 fmol/10^6 cells. Incubation of neuroblastoma cells with neurotensin in the presence of calcium ions resulted in a transient increase of 10 fold over basal level of the intracellular cyclic GMP concentration. Half-maximal stimulation was obtained with 2 nM neurotensin. Under identical conditions the cyclic AMP concentration only decreased by 20-30 %. These results suggest that cyclic GMP is a second messenger of neurotensin in neuroblastoma clone N1E115.

Neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) satisfies a variety of criteria as a neurotransmitter or neuromodulator in the central nervous system (1). The peptide is present in the brain of mammals where it is associated with the synaptosomal fraction (2). Depolarisation of hypothalamic slices triggers a release of neurotensin-like immunoreactivity according to a Ca^{2+}-dependent process (3). Neurotensin affects the electrical activity of central neurons (4,5). Rat brain synaptic membranes contain high affinity neurotensin receptors (6,7) and also peptidases that are able to efficiently inactivate neurotensin (8,9). In spite of all these data, a number of questions should be answered before the physiological role of neurotensin in

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ABBREVIATIONS:
HEPES, 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid ; EDTA, ethylenediaminetetraacetate ; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

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the central nervous system could be fully understood. For example, the biochemical effects of the peptide on its target nerve cell remain essentially unknown.

We recently demonstrated that specific neurotensin binding sites are present in homogenates of differentiated neuroblastoma N1E115, an electrically excitable cell line (10). This clone provides a useful model to investigate the cellular events that are modulated by the association of neurotensin to its neuronal receptors. We show in the present paper that neurotensin increases the intracellular cyclic GMP concentration in neuroblastoma N1E115 cells and that the cyclic GMP response is mediated by the association of neurotensin to its specific receptors.

MATERIALS AND METHODS

Drugs and peptides

Z-Pro-Prolinal(N-benzyloxycarbonyl-prolyl-prolinal) was a generous gift from Dr. Wilk (Department of Pharmacology, Mount Sinaï School of Medicine, N.Y.). 1,10-Phenanthroline was purchased from Merck. Neurotensin, acetyl neurotensin(8-13), neurotensin(9-13) and neurotensin(1-12) were synthesized (11) and kindly provided by C. Granier and J. Van Rietschoten (Faculté de Médecine Nord, Marseille, France). [monoiodo-Tyr^3]neurotensin (100 or 2000 Ci/mmol) was prepared by purification of the iodination products of neurotensin on Sulfopropyl-Sephadex C-25 (12).

Cell culture

Neuroblastoma N1E115 cells were propagated in 75 cm^2 Falcon tissue culture flasks and cultured at 37°C in Dulbecco's modified Eagle's medium (H21) supplemented with 10 % fetal calf serum, in a humidified atmosphere of 5 % CO -95 % air. The culture medium also contained penicillin (50 units/ml) and streptomycin (50 µg/ml). Differentiated cells used in binding and cyclic GMP assays were obtained as follows. Cells cultured in the nondifferentiated state were detached before confluence by incubation for 3 min in a 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 of culture medium. An aliquot (100 µl) of this suspension was used for enumeration of cells with a Coulter counter. The cell suspension was distributed into Falcon 24-wells plates (10^5 cells/well) and allowed to grow for 48 h under the standard conditions described above. Cells were then induced to differentiate for 48 to 72 h (10) in a H21 medium containing 0.5 % fetal calf serum and 1.5 % dimethyl sulfoxide (13). Differentiated cells were reincubated for 4 h in normal culture medium before use in biochemical assays.

Binding experiments

Monolayers of neuroblastoma N1E115 cells (about 3.10^5 cells/well) were incubated at 37°C with various concentrations of [monoiodo-Tyr^3] neurotensin (100 or 2000 Ci/mmol) in a total volume of 200 µl of incubation buffer (25 mM HEPES-Tris, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 3.6 mM $MgCl_2$, 0.2 % bovine serum albumin, 4.5 g/l glucose, 1 mM 1,10-phenanthroline and 0.1 µM Z-Pro-Prolinal). In competition experiments, the incubation medium also contained different concentrations of unlabeled neurotensin or its

synthetic analogues. After 30 min of incubation, the medium was removed by suction and the cell layer was rapidly washed twice with 1 ml of incubation buffer. Cells were suspended in 1 ml of 0.1 M NaOH and the radioactivity bound to the cells was counted with an Intertechnique C G 4000 gamma counter at a counting efficiency of 80 %. Nonspecific binding was determined in parallel experiments in the presence of an excess (1 μM) of unlabeled neurotensin and subtracted from total binding to obtain the specific binding.

Measurement of neurotensin-induced changes in cyclic nucleotide levels

Neuroblastoma cells in multiwell plates (about 3.10^5 cells/well) were preincubated for 5 min in the absence of peptide then incubated for different times with various concentrations of neurotensin or its synthetic analogues. These incubations were carried out at 37°C in 200 μl of the buffer used in binding experiments. The reaction was terminated by aspiration of the medium and the cell layer in each well was extracted with 0.8 ml of a 2/3-1/3 (v/v) mixture of ethanol-5 mM EDTA, pH 7.4. Extracts were centrifuged and supernatants were evaporated in a speed vacuum concentrator. Extracts were reconstituted with a 50 mM Tris-HCl buffer, pH 7.5, containing 4 mM EDTA and their cyclic nucleotide content was measured with the cyclic AMP assay kit and the cyclic GMP RIA kit from Amersham.

RESULTS AND DISCUSSION

The purpose of this work was to show that intracellular levels of cyclic nucleotides are modulated by the binding of neurotensin to its specific receptors in neuroblastoma N1E115 cells. In order to compare results of binding experiments and cyclic nucleotide measurements, both types of assay should be carried out under identical experimental conditions. Since cyclic nucleotide measurements involve the use of intact cells, it was necessary to work at 37°C and under physiological conditions of pH and ionic strength. We demonstrated recently that neurotensin is extensively degraded by N1E115 homogenates at 20 and 37°C (10). In the present study, we found that incubation of (monoiodo-Tyr^3)neurotensin with intact N1E115 cells for 30 min at 37°C resulted in a 75 % degradation of the iodopeptide as measured by high performance liquid chromatography. Addition to the incubation medium of 1 mM 1,10-phenanthroline, a metallopeptidase inhibitor, and of 0.1 μM Z-Pro-Prolinal, a specific inhibitor of proline endopeptidase (14), produced an almost complete (> 95 %) protection of the peptide towards degradation. We have checked that these inhibitor concentrations did not modify the binding and biochemical properties of neurotensin and its analogues. Therefore, all the experiments described below were carried out in the presence of 1 mM 1,10-phenanthroline and 0.1 μM Z-Pro-Prolinal.

Binding parameters of neurotensin receptors in intact neuroblastoma N1E115 cells

The radiolabeled analogue [monoiodo-Tyr^3]neurotensin associated to intact N1E115 cells that had been previously differentiated according to pseudo first-order kinetics (results not shown). Independently of the radioligand concentration used, binding equilibrium was attained after an incubation time of 30 min at 37°C. Fig. 1 shows the results of equilibrium binding experiments in which increasing concentrations of radiolabeled iodoneurotensin were incubated with a fixed number of differentiated cells, either in the presence (nonspecific binding) or in the absence (total binding) of a large excess of unlabeled neurotensin (1 μM). The specific binding which is defined as the difference between total and nonspecific binding is saturable (Fig. 1A). Linearity of the Scatchard plot (Fig. 1B) demonstrates that [monoiodo-Tyr^3]neurotensin associates with a single class of noninteracting binding sites. The dissociation constant of the complex formed between the radiolabeled neurotensin analogue and its receptor is 0.75 nM and the maximal

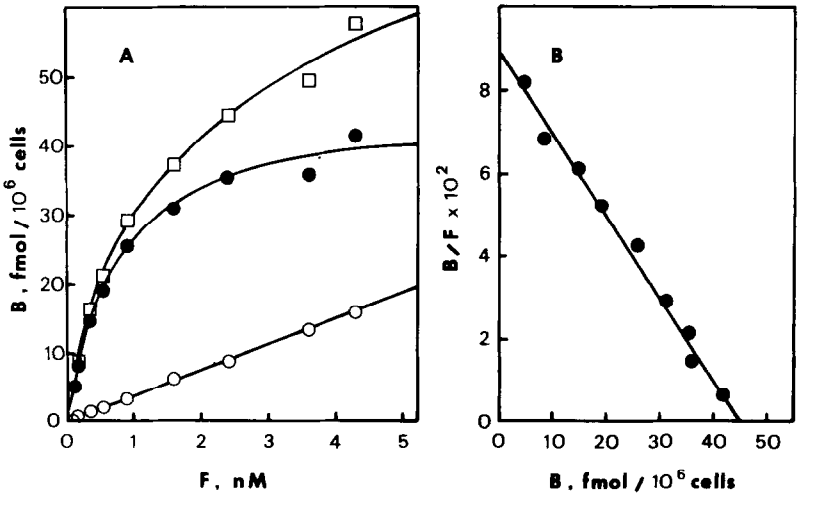

Figure 1. Binding of ^{125}I-labeled[monoiodo-Tyr^3]neurotensin to N1E115 cells. Differentiated cells (3 x 10^5 cells/well) were incubated with increasing concentrations of radiolabeled neurotensin. After incubation for 30 min at 37°C the radioactivity bound to cells was determined as described in "Materials and Methods". Nonspecific binding (○) was measured in the presence of 1 μM unlabeled neurotensin. Specific binding (●) is the difference between total binding (□) and nonspecific binding. Each point represents the mean of quadruplicate determinations from two different experiments.

binding capacity of N1E115 cells is 45 fmol of ligand bound per 10^6 cells, which corresponds to 27,000 neurotensin binding sites per cell. As already observed with N1E115 homogenates (10) neurotensin receptors could not be detected in undifferentiated cells.

The affinity of native neurotensin for its N1E115 receptor was compared to those of three neurotensin analogues in competition experiments involving various concentrations of unlabeled peptides and constant amounts of cells and (monoiodo-Tyr^3)neurotensin (Fig. 2). The concentration of unlabeled peptide that induces half-displacement of bound radiolabeled ligand (IC_{50}) is related to the dissociation constant of the unlabeled peptide-receptor complex (K_d) by the expression

$$IC_{50} = K_d\left(1 + \frac{(L^*)_{50}}{K^*_d}\right)$$

where $(L^*)_{50}$ is the concentration of free labeled ligand at half-displacement and K^*_d is the dissociation constant of the labeled ligand-receptor complex. The value of K_d was 0.75 nM (see above) and the value of $(L^*)_{50}$ was 0.1 nM under the experimental conditions of Fig. 2. The calculated dissociation constant of the native neurotensin-receptor complex was 0.88 nM, a value not significantly different from that of (monoiodo-Tyr^3)neurotensin. The affinity of acetyl neurotensin(8-13) was identical to that of native neurotensin whereas that of neurotensin(9-13) was about 100 times lower. Neurotensin(1-12) was unable to compete with (monoiodo-Tyr^3)neurotensin for binding to N1E115 cells even at concentrations as high as 1 μM (Fig. 2). These data underline the importance of the integrity of the C-terminal 8-13 sequence for the interaction between neurotensin and its receptor in N1E115 cells. Similar results have been observed with other neurotensin receptors of neural (6,7,10) or nonneural (15,16) origin.

Effect of neurotensin and its analogues on the intracellular level of cyclic nucleotides in neuroblastoma N1E115 cells

Incubation of N1E115 cells with 30 nM neurotensin produced a rapid and transient increase of 10 fold over basal level of the intracellular cGMP

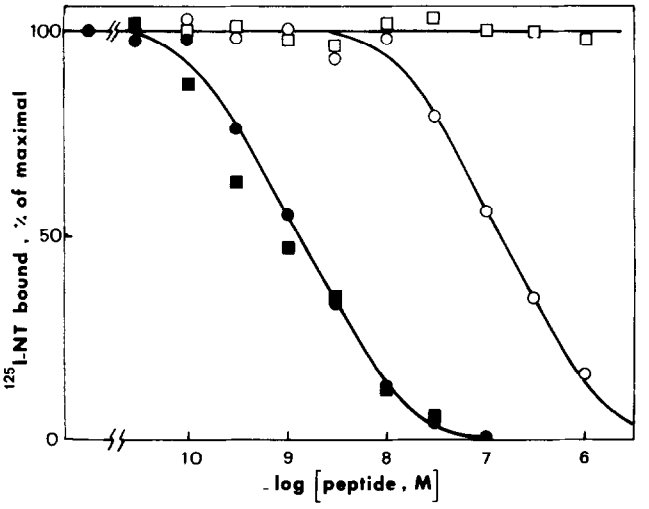

Figure 2. Competition between ^{125}I-labeled[monoiodo-Tyr^3]neurotensin and unlabeled neurotensin analogues for binding to N1E115 cells. [monoiodo-Tyr^3]neurotensin (0.1 nM) was incubated with N1E115 cells (3 x 10^5 cells/well) and the indicated concentrations of neurotensin (●), acetyl neurotensin(8-13) (■), neurotensin(9-13) (○) and neurotensin(1-12) (□). The radioactivity bound to cells was determined after incubation for 30 min at 37°C. Results are expressed in percentage of the maximal specific binding. Each point represents the mean of quadruplicate determinations from two different experiments.

concentration (Fig. 3). The response was maximal after 20-30 s then decreased progressively. cGMP stimulation was no longer detectable after 2 min of incubation. Although calcium ions were unable by themselves to modify cGMP levels, no neurotensin-induced stimulation was observed when $CaCl_2$ was omitted and replaced by EGTA in the incubation medium. It should also be noted that the intracellular cGMP concentration of nondifferentiated N1E115 cells was insensitive to neurotensin.

Concentration-response curves for the effect of neurotensin and its analogues on cGMP levels in N1E115 cells are presented in Fig. 4. Neurotensin(1-12) was unable to induce any cGMP increase. The two other neurotensin analogues produced the same 10-fold increase of the basal cGMP concentration as native neurotensin. However, the potencies of these two analogues measured as the peptide concentration that induced half-maximal stimulation of the cGMP level (EC_{50}) were different. Thus, acetyl neurotensin(8-13) (EC_{50} = 0.5 nM) and neurotensin(9-13) (EC_{50} = 80 nM) were 4 times more potent and 40 times less potent respectively than neurotensin

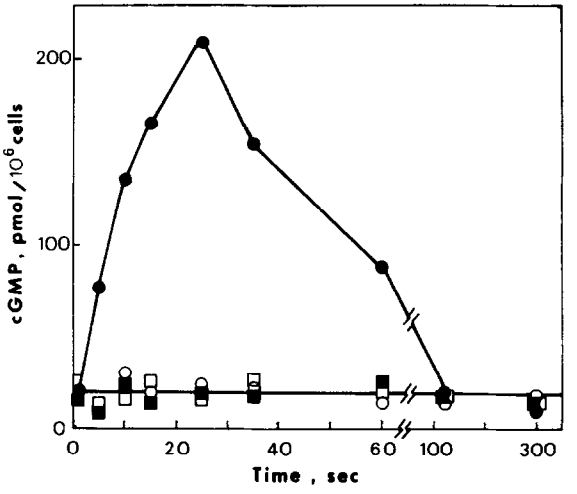

Figure 3. Time-course of neurotensin-induced cGMP stimulation in neuroblastoma clone N1E115. Differentiated cells (3 x 10^5 cells/well) were incubated in a Tris-Hepes buffer, pH 7.4, containing 1.8 mM $CaCl_2$ in the presence (●) or in the absence (■) of 30 nM neurotensin. After the indicated incubation time, the cGMP cell content was measured with the Amersham kit. In control experiments, no neurotensin-induced cGMP stimulation was observed with undifferentiated cells (□) or after replacement of $CaCl_2$ by EGTA in the incubation buffer (○). Each point is the mean of sextuplicate determinations from three different experiments.

itself (EC_{50} = 2 nM). Results in Fig. 4 also show that the efficiency of neurotensin was maximal at 30 nM and decreased at higher concentration suggesting that neurotensin receptors are regulated by a desensitization mechanism.

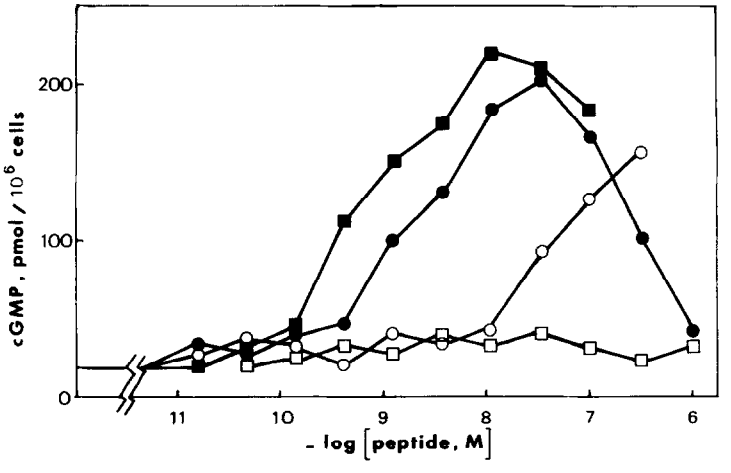

Figure 4. Concentration-response curves for the cGMP stimulation induced by neurotensin and its analogues in neuroblastoma clone N1E115. Cells (3 x 10^5cells/well) were incubated with the indicated concentrations of neurotensin (●), acetyl neurotensin(8-13) (■), neurotensin(9-13) (○), or neurotensin(1-12) (□). The cGMP cell content was measured after incubation for 25 s at 37°C. Each point is the mean of quadruplicate determinations from two different experiments.

Neurotensin produced small variations of cAMP levels in neuroblastoma N1E115 cells. A maximal 20-30 % decrease of the basal intracellular cAMP concentration was observed after a 20-30 s incubation of N1E115 cells with 30 nM neurotensin. No further characterization of this effect was attempted in this work.

Results presented above show that the neurotensin-induced increase of the cGMP concentration in neuroblastoma clone N1E115 is a direct consequence of neurotensin receptor occupancy. The cGMP level of undifferentiated cells that are devoid of neurotensin receptors is insensitive to neurotensin. Moreover, binding affinities of neurotensin and three of its analogues are strongly correlated to their potencies for increasing the intracellular cGMP concentrations.

The ability of neurotensin to stimulate cGMP formation in N1E115 cells was recently reported by Gilbert and Richelson in a short communication (17). Most of the data presented in this preliminary communication were in agreement with our own results, apart from the EC_{50} values for neurotensin which differed in the two studies : 13 nM in (17) as compared to 2 nM in the present work. The same authors also reported in an abstract on the ability of (^{3}H)-neurotensin to bind to N1E115 cells (18). However, binding experiments were carried out at 0°C, which makes it difficult to compare their binding data with ours. Furthermore, the different temperature conditions used for studying neurotensin binding and neurotensin-stimulated cGMP production may hamper attempts to correlate these two processes. In the present study, comparison of neurotensin binding and effect in N1E115 cells under identical physiological conditions was made possible by blocking neurotensin degradation which readily occurs during exposure of the peptide to N1E115 cells at 37°C.

In conclusion, results presented here suggest that cGMP is a second messenger of neurotensin in neuroblastoma clone N1E115. We are now trying to determine whether this translation mechanism occurs in other cells that possess neurotensin receptors.

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