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NEUROTENSIN AND ITS ANALOGS—CORRELATION OF SPECIFIC BINDING WITH STIMULATION OF CYCLIC GMP FORMATION IN NEUROBLASTOMA CLONE N1E-115

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Abstract—The receptors which mediate neurotensin-stimulated intracellular cyclic GMP formation in murine neuroblastoma clone N1E-115 [J. A. Gilbert and E. Richelson, *Eur. J. Pharmac.* **99**, 245 (1984)] were further characterized. The binding of [^{3}H]neurotensin to intact N1E-115 cells at 0° displayed specificity, saturability, reversibility, and tissue linearity. A single class of neurotensin receptors was demonstrated with an apparent K_D of 9–11 nM and a B_{max} of 180–250 fmoles/10^6 cells, determined by the type of serum employed in the cellular culture medium. A number of neurotensin analogs and fragments were compared for their ability to inhibit [^{3}H]neurotensin binding and stimulate intracellular cyclic GMP formation with intact N1E-115 cells. A direct correlation was found to exist between the K_D and EC_{50} for each peptide. The carboxyl-terminal portion of neurotensin proved to be responsible for the binding and biochemical activities of this peptide with clone N1E-115. Neurotensin(8–13) was, in fact, fifty times more potent than native neurotensin in stimulating intracellular cyclic GMP formation and had an 18-fold higher affinity for the neurotensin receptor on this neuronal cell type.

Neurotensin is an endogenous tridecapeptide that is found primarily in the gastrointestinal system and brain and which effects a variety of biological activities [see reviews in Refs. 1–4]. We provided support for the hypothesis that neurotensin is a neurotransmitter or neuromodulator in the central nervous system by demonstrating in a neuronal cell type (neuroblastoma clone N1E-115) [5] and in rat cerebellar slices [6] the first known biochemical event associated with activation of neurotensin receptors, i.e. the formation of intracellular cyclic GMP, a compound generally considered the second messenger of some neurotransmitters in the mammalian nervous system [7].

Murine neuroblastoma clone N1E-115 is a widely studied clone which has a number of properties of adrenergic neurons [8]. These include enzymes for catecholamine synthesis, electrically excitable membranes, physical characteristics of neurons, and several types of receptors. Neurotensin-mediated cyclic GMP synthesis in N1E-115 cells has a time course and calcium dependency essentially identical to those for other receptors on this clone which have the same biochemical activity, e.g. muscarinic acetylcholine [9], histamine H_1 [10], bradykinin [11], thrombin [12], and angiotensin II [13].

Here we report the further characterization of receptors on intact N1E-115 cells responsible for mediating the formation of neurotensin-stimulated intracellular cyclic GMP formation. Furthermore, a comparison is made of the abilities of several neurotensin analogs and fragments to stimulate intracellular cyclic GMP formation and inhibit [^{3}H]-neurotensin binding to intact N1E-115 cells. These results have been presented in preliminary form [6, 14].

MATERIALS AND METHODS

Cell culture. Murine neuroblastoma cells (clone N1E-115) were cultured in Dulbecco–Vogt's modification of Eagle's medium (DMEM†, Grand Island Biological Co., Grand Island, NY) without antibiotics. Due to a change in product usage during the course of these experiments, cells used for establishing the characteristics of neurotensin receptors were maintained in medium supplemented with 10% (v/v) newborn calf serum (Grand Island Biological Co.), while those employed for studies of the binding and biological activities of neurotensin analogs and fragments were cultured in DMEM containing 10% fetal bovine serum (Grand Island Biological Co.).

Clone N1E-115 (passage number <22) was grown in 20 ml of medium in 75 cm^2 Corning flasks (Corning Glass Works, Corning, NY) in a humidified atmosphere of 10% CO_2:90% air at 37°. Flasks were routinely inoculated with 0.5 to 1.0 × 10^6 cells which were fed daily beginning day 5 or 6 after subculture by removal of 10 ml of growth medium and replacement with 10 ml of fresh medium. Cells were harvested during the stationary phase of growth, 10–22 days after subculture for cyclic GMP assays and within 8–26 days for neurotensin binding experiments.

Measurement of relative changes in cyclic GMP production. The assay used for measurement of intracellular cyclic GMP formation was essentially that of Richelson *et al.* [9], in which cyclic [^{3}H]GMP

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† Abbreviations: DMEM, Dulbecco–Vogt's modification of Eagle's medium; NT, neurotensin; K_D, equilibrium dissociation constant; and B_{max}, maximum number of receptor sites.

produced in clone N1E-115 was isolated chromatographically from cells labeled with radioactive precursor prior to receptor stimulation. N1E-115 cells were harvested for use by aspiration of culture medium, incubation of the cellular monolayer for 10 min at 37° in 10 ml of modified Puck's D_1 solution without antibiotics and phenol red [15], disruption of the layer by agitation of the flask, and collection of the cells by centrifugation at 300 *g* for 1 min at 4° in a CRU-5000 centrifuge (Damon/IEC Division, Needham Heights, MA). The cellular pellet was resuspended in 10 ml of phosphate-buffered saline solution (solution I) consisting of 110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 2.0 mM Na_2HPO_4, 25 mM glucose, and 70 mM sucrose (pH 7.35; 340 mOsm), and the washed cells were collected by centrifugation.

The cellular pellet was resuspended in 2–4 ml of solution I and transferred to a 25-ml Erlenmeyer flask. Twenty microliters of the suspension was removed for enumeration of cells (Coulter Electronics, Hialeah, FL). To the remaining volume was added 20–40 μCi of [^{3}H]guanosine, after which the flask was covered and placed on the shaking platform of a 37° water bath at 60 oscillations/min (GCA/Precision Scientific, Chicago, IL). After an incubation time of 45 min, the cells were collected by centrifugation for 20–30 sec at 300 *g* and were resuspended in a volume of solution I which provided 100,000 cells/270 μl. This quantity was distributed to each well of a Linbro multiwell plate (Cat. No. 76-033-05, Flow Laboratories, McLean, VA), and the tray was placed on the platform of a 37° water bath and allowed to equilibrate for 15–30 min. Individual wells of cells were then stimulated at 80 oscillations/min by the addition of 30 μl of solution I (to obtain basal cyclic [^{3}H]GMP values) or the appropriate concentration of neurotensin or one of its analogs. As the neurotensin-induced cyclic [^{3}H]GMP formation in clone N1E-115 reaches a maximum in this assay 30 sec after stimulation [5], each reaction was routinely terminated after 30 sec by the addition of 30 μl of 50% (w/v) trichloroacetic acid solution.

The intracellular cyclic [^{3}H]GMP produced was isolated from each well following the addition of 1400 dpm of cyclic [^{14}C]GMP as a recovery marker. The contents of each individual well were applied to separate columns (Kontes disposable chromaflex columns; No. K-420160, Vineland, NJ) of Dowex AG 50W-X2 resin (0.8 cm × 8.0 cm; 200–400 mesh; Bio-Rad Laboratories, Richmond, CA) which had been prewashed with 5 ml of 0.1 N HCl. The resin columns were then rinsed with 4.9 ml of 0.1 N HCl and 1.0 ml of water after which the cyclic [^{3}H]GMP was eluted with 1.5 ml of water collected directly into 2.0 ml micro tubes (Walter Sarstedt Inc., Princeton, NJ). Residual multivalent guanine nucleotides were removed from the eluate of each column upon addition of 30 μl of 2.67 M $ZnSO_4$ and 30 μl of 2.67 M Na_2CO_3 followed by mixing and centrifugation for 2 min at ~11,000 *g* in a Microfuge 12 centrifuge (Beckman Instruments, Palo Alto, CA). The supernatant fraction from each tube was then decanted into a plastic scintillation vial (Research Products International, Mount Prospect, IL). Seven milliliters of Safety-Solve counting solution (Research Products International) was added, and the radioactivity was measured in an Isocap/300 liquid scintillation counter (Searle, Des Plaines, IL).

Neurotensin receptor binding assay. N1E-115 cells were harvested for use as described for the cyclic GMP assay. The cellular pellet was resuspended in 10 ml of solution I made 1% (w/v) in bovine serum albumin to ensure nonspecific protection from proteases (solution II), and the washed cells were collected by centrifugation. This cellular pellet was then resuspended in at least 2 ml of solution II, and 20 μl of the suspension was removed for enumeration of cells. The remaining volume was diluted in solution II to routinely provide 300,000–400,000 cells per assay tube and was equilibrated at 0° before distribution.

[^{3}H]Neurotensin binding to N1E-115 cells was performed with solution II in 5-ml polypropylene tubes (Walter Sarstedt Inc.) at 0°, to inhibit uptake of radiolabel by the intact cells. A total assay volume of 1 ml was used, and the reaction was initiated by the addition of cells. In the experiments characterizing the specificity, saturability, reversibility, and tissue linearity of [^{3}H]neurotensin binding to N1E-115 cells, nonspecific binding was determined for each point by inclusion of a 1 μM concentration of unlabeled neurotensin in parallel reaction tubes. During the incubation at 0°, all assay tubes were mixed every 10 min to prevent settling of the cells. The binding reaction was routinely terminated after 20 min by rapid filtration under vacuum on glass fiber filters (GF/B, 2.4 cm, Whatman, Clifton, NJ) which had been pretreated with 0.1% polyethylenimine for 30–60 min just before use. Each tube and corresponding filter were immediately rinsed with three 4-ml rinses of cold solution II. The filters were then placed in plastic scintillation vials to which 7 ml of Safety-Solve counting solution was added, and the filters were allowed to incubate for at least 4 hr prior to radioactivity measurement.

The neurotensin binding assay was modified as follows for use in competition studies employing neurotensin fragments and analogs: The content of bovine serum albumin in solution II was lowered to 0.1% to enable rapid filtration of numerous samples concurrently; the inhibition experiments were designed with one set each of total and non-specific tubes and with a radiolabel concentration of 2–3 nM; the assay was routinely terminated by simultaneous filtration of twenty-four samples at a time on a cell harvester filtering unit (Brandel, Gaithersburg, MD) equipped with a GF/B filter strip; and the data were analyzed with the LIGAND program [16] on a 9845B desktop computer (Hewlett-Packard, Fort Collins, CO) connected by the VA3451 telephone modem (Racal-Vadic, Sunnyvale, CA) to a Cyber mainframe computer (Control Data Corporation, Minneapolis, MN).

Materials. Polypropylene plasticware or silanized glassware were used for all experiments. [^{3}H]Guanosine was from ICN Radiochemicals (Irvine, CA), and [^{3}H]neurotensin was obtained from New England Nuclear (Boston, MA). Sigmacote, bovine serum albumin (A-7906), polyethylenimine, neurotensin (1–11), [D-Phe^{11}]neurotensin, [Gln^4]neurotensin, neurotensin (1–8), and [D-Trp^{11}]neurotensin were

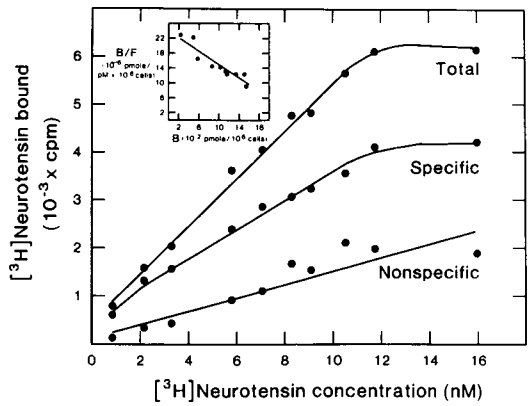

Fig. 1. [^{3}H]Neurotensin binding to clone N1E-115 as a function of radioligand concentration. The binding of increasing concentrations of [^{3}H]neurotensin to intact N1E-115 cells was performed at 0° as described under Materials and Methods with 0.4×10^6 cells/assay tube (cells in stationary phase of growth; passage number 9; 12 days after subculture in DMEM with 10% newborn calf serum). Specific binding was obtained by subtracting the average of duplicates for nonspecific binding at a given radiolabel concentration (measured in the presence of 1 μM unlabeled neurotensin) from the average of duplicates for total binding. Each point represents the average of duplicates, and the data presented are from one of four similar neurotensin binding assays. Inset represents a Scatchard plot of the given specific binding data fitted with the least squares method of linear regression analysis.

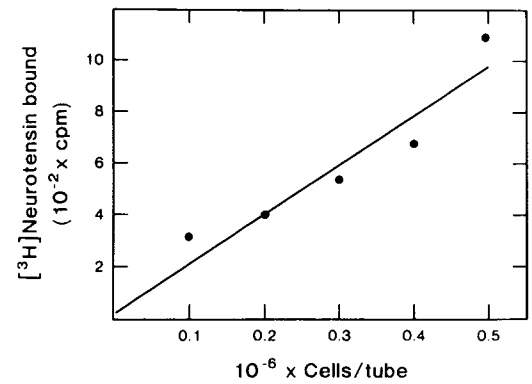

Fig. 2. [^{3}H]Neurotensin binding to clone N1E-115 as a function of cell number. The binding of 6 nM [^{3}H]neurotensin to intact N1E-115 cells was performed at 0° as described under Materials and Methods with an increasing number of cells/assay tube (cells in stationary phase of growth; passage number 8; 11 days after subculture in DMEM with 10% newborn calf serum). Each point represents the specific binding of [^{3}H]neurotensin at a given cell number and was obtained by subtracting the average of duplicates for nonspecific binding (measured in the presence of 1 μM unlabeled neurotensin) from the average of duplicates for total binding. The data presented are from one of two similar experiments. The data were fitted by the least squares method of linear regression analysis with a correlation coefficient of 0.97.

supplied by the Sigma Chemical Co. (St. Louis, MO). Neurotensin (8–13) was purchased from Bachem (Torrance, CA), and neurotensin was from Boehringer-Mannheim (Indianapolis, IN). The following neurotensin (NT) analogs were provided by Dr. Charles Nemeroff, Department of Psychiatry, Duke University Medical Center, Durham, NC: [D-Pro^7]NT, NT(1–6), NT-NHMe, NT(1–10), [Phe^{11}]-NT, NT(9–13), [D-Arg^8]NT, [D-Tyr^{11}]NT, and [D-Pro^{10}]NT. All other reagents were analytical grade.

RESULTS

Radioligand binding assays. A binding assay employing intact cells cultured in DMEM with 10% newborn calf serum and incubated with [^{3}H]neurotensin at 0° was used to characterize the neurotensin receptors present on neuroblastoma clone N1E-115. As indicated in Fig. 1, binding of [^{3}H]neurotensin in the concentration range of 1–16 nM to N1E-115 cells was both specific and saturable. At higher concentrations of [^{3}H]neurotensin, sharply increasing specific binding was seen with saturation not being approached. A straight line was consistently obtained in Scatchard analyses of saturable binding data, which indicated a homogeneous class of neurotensin receptors. This finding was confirmed by Hill plots which gave coefficients essentially equal to unity (see Table 2). Scatchard analyses demonstrated an apparent equilibrium dissociation constant (K_D) of 9 ± 2 nM (mean ± S.E.) and a maximum number of receptor sites (B_{max}) of 180 ± 40 fmoles/10^6 cells or about 110,000 sites/cell (N = 4). In comparison, when intact cells cultured in DMEM with 10% fetal bovine serum were employed in the modified neurotensin binding assay, Scatchard analyses indicated an apparent K_D for neurotensin of 11 ± 1 nM (see Table 2) and a B_{max} of 250 fmoles/10^6 cells (N = 10).

Binding of [^{3}H]neurotensin to intact N1E-115 cells displayed tissue linearity (Fig. 2). The number of specific counts bound increased in a linear fashion when the cell number per assay tube was increased from 0.1 to 0.5 million. Association and dissociation studies (Fig. 3 a and b) indicated that specific binding of radioligand to intact cells at 0° reached a maximum by 20 min. Furthermore, the binding of [^{3}H]neurotensin to clone N1E-115 was rapidly reversible, with displacement of 72% of the radiolabel by an excess of unlabeled neurotensin within 10 min at 0°. The association and dissociation rate constants were calculated with the aid of plots of $\ln(B_{eq}/(B_{eq} - B))$ versus association time, which produced straight lines, and graphs of $\ln(B/B_o) = -k_{-1}t$, which were linear during the first 5 min of the dissociation reaction. The dissociation rate constant, 0.22 min^{-1}, was then divided by the association rate constant, 0.08 min^{-1} nM^{-1}, to provide the K_D value, 2.8 nM. This kinetically determined K_D agreed well with the K_D of 9 nM obtained from Scatchard analyses. Under the assay conditions described, i.e. incubation with intact cells for 20 min at 0°, [^{3}H]neurotensin was demonstrated to undergo little, if any, degradation by endogenous proteases, while incubation of this radiolabel with clone N1E-115 at 37° resulted in its significant proteolysis after 1 min and its almost complete degradation by 20 min.*

* P. Hanson and E. Richelson, manuscript in preparation.

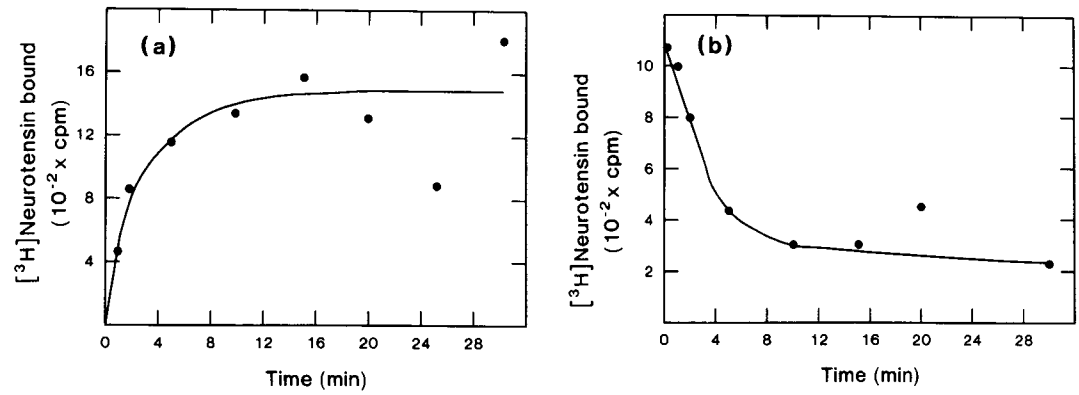

Fig. 3. Time course for association (a) and dissociation (b) of [^{3}H]neurotensin binding to clone N1E-115. (a) The binding of 5 nM [^{3}H]neurotensin to intact N1E-115 cells was performed at 0° as described under Materials and Methods for increasing lengths of incubation with 0.4×10^6 cells/assay tube (cells in stationary phase of growth, passage number 11, 21 days after subculture in DMEM with 10% newborn calf serum). Each point represents the specific binding of [^{3}H]neurotensin at a different time and was obtained by subtracting the average of triplicates for nonspecific binding (measured in the presence of 1 μM unlabeled neurotensin) from the average of triplicates for total binding. The data presented are from one of three similar experiments. (b) [^{3}H]Neurotensin (18 nM) was incubated for 10 min at 0° as described under Materials and Methods with 0.4×10^6 intact N1E-115 cells/assay tube (cells in stationary phase of growth, passage number 12, 15 days after subculture in DMEM with 10% newborn calf serum). The tubes were then all made 1 μM in unlabeled neurotensin by the rapid addition of 10 μl of a 0.1 mM solution, and the dissociation of labeled neurotensin from the cells was measured at increasing lengths of incubation at 0°. Each point represents the specific binding of [^{3}H]neurotensin at a different time and was obtained by subtracting the average of triplicates for non-specific binding (measured in the presence of 1 μM unlabeled neurotensin) from the average of triplicates for total binding. The data presented are from one of two similar experiments.

Stimulation of cyclic GMP production and inhibition of radioligand binding by neurotensin fragments and analogs. Results of the investigation of a number of neurotensin fragments and analogs (see Table 1) for their ability to stimulate the intracellular formation of cyclic GMP in clone N1E-115 cells cultured in DMEM with 10% fetal bovine serum are presented in Table 2. Typical dose–response curves from a given cyclic GMP experiment are illustrated in Fig. 4. Table 2 also provides data obtained when the same peptides were assessed for their potency in inhibiting [^{3}H]neurotensin binding to intact N1E-115 cells cultured in DMEM with 10% fetal bovine serum. Representative competition curves obtained in the neurotensin binding assay are shown in Fig. 5. As indicated by the Hill coefficients listed in Table 2, neurotensin and its analogs and fragments were competing for a single class of non-cooperative binding sites on clone N1E-115.

In support of the majority of structure–activity studies, which typically employ biological assays with whole animals, intact organs, or tissue dissected from rodent heart, stomach, or intestine, our results indicated that the carboxyl-terminal of neurotensin, spe-

Table 1. Differences in structure between neurotensin and its analogs

Analog name	1 pGlu	2 Leu	3 Tyr	4 Glu	5 Asn	6 Lys	7 Pro	8 Arg	9 Arg	10 Pro	11 Tyr	12 Ile	13 Leu
[Gln^4]NT				Gln									
[D-Pro^7]NT							D-Pro						
[D-Arg^8]NT								D-Arg					
[D-Pro^{10}]NT										D-Pro			
[Phe^{11}]NT											Phe		
[D-Trp^{11}]NT											D-Trp		
[D-Tyr^{11}]NT											D-Tyr		
[D-Phe^{11}]NT											D-Phe		
NT-NHMe													NHMe
NT(1–6)							—	—	—	—	—	—	—
NT(1–8)									—	—	—	—	—
NT(1–10)											—	—	—
NT(1–11)												—	—
NT(8–13)	—	—	—	—	—	—	—						
NT(9–13)	—	—	—	—	—	—	—	—					

Table 2. Potencies of neurotensin, its analogs, and fragments in biological and binding activities with clone N1E-115

Peptide	Stimulation of cyclic [^{3}H]GMP formation in intact cells	Competition with [^{3}H]neurotensin binding to intact cells	
	EC_{50} (nM)	K_D (nM)	Hill coefficient
Neurotensin	1.5 ± 0.6(11)*	11 ± 1(10)	0.97 ± 0.02(10)
NT(8–13)	0.03 ± 0.01(3)	0.61 ± 0.02(3)	1.03 ± 0.02(3)
[Gln^4]NT	0.6 ± 0.5(3)	3.9 ± 0.6(4)	1.06 ± 0.04(4)
[D-Pro^7]NT	0.7 ± 0.3(3)		
[D-Arg^8]NT	1.1 ± 0.4(3)	24 ± 2(3)	0.90 ± 0.08(3)
[Phe^{11}]NT	3 ± 1(3)	72 ± 4(3)	0.94 ± 0.06(3)
NT(9–13)	7 ± 3(3)	210 ± 20(3)	0.83 ± 0.06(3)
[D-Trp^{11}]NT	210 ± 60(3)	~10,000 (1)	
[D-Tyr^{11}]NT	420 ± 130(3)	~13,000 (1)	
[D-Pro^{10}]NT	No response at 1 μM or less (3)		
[D-Phe^{11}]NT	No response at 10 μM or less (2)	~36,000 (1)	
NT-NHMe	No response at 10 μM or less (2)	No displacement at 10 μM or less (3)	
NT(1–6)	No response at 10 μM or less (2)	No displacement at 10 μM or less (2)	
NT(1–8)	No response at 10 μM or less (3)	No displacement at 10 μM or less (2)	
NT(1–10)	No response at 10 μM or less (2)	No displacement at 10 μM or less (3)	
NT(1–11)	No response at 10 μM or less (3)	No displacement at 10 μM or less (2)	

Values are means ± S.E.
* Number of independent experiments performed.

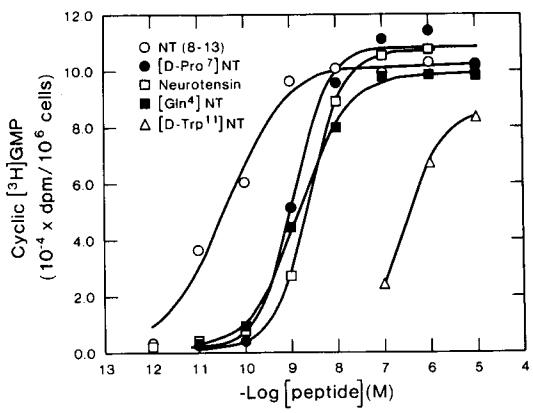

Fig. 4. Effect of concentration on stimulation of intracellular cyclic GMP synthesis in clone N1E-115 by neurotensin and some of its analogs and fragments. The data presented are from one experiment and are representative of the dose–response curves obtained when increasing concentrations of neurotensin and its analogs and fragments were assayed for their ability to induce cyclic GMP formation in intact cells with the procedure described under Materials and Methods. Cells employed for these assays were cultured in DMEM with 10% fetal bovine serum. Each point is the average of triplicates from which has been subtracted the average of triplicate basal values. Complete data on all the neurotensin peptides and their cyclic GMP responses are presented in Table 2.

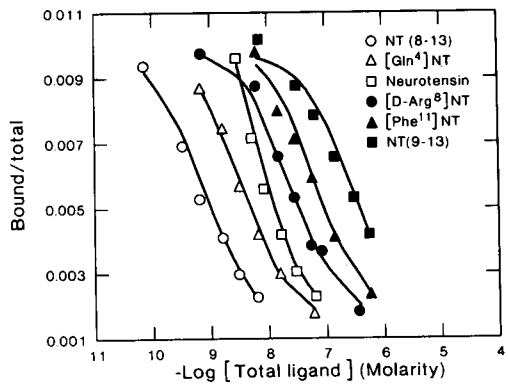

Fig. 5. Effect of concentration on inhibition of [^{3}H]neurotensin binding to clone N1E-115 by neurotensin and some of its analogs and fragments. The data presented are representative of the competition curves obtained when increasing concentrations of neurotensin and its analogs and fragments were assessed for their ability to compete with [^{3}H]neurotensin for binding to intact cells employing the modified assay procedure described under Materials and Methods. Cells used for these assays were cultured in DMEM with 10% fetal bovine serum. Each point is the average of duplicates, and the inhibition curves were generated with the LIGAND computer program [16]. Complete data on all the neurotensin peptides and their apparent equilibrium dissociation constants and Hill coefficients are presented in Table 2.

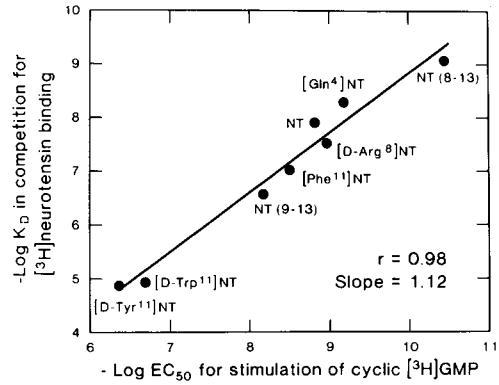

Fig. 6. Correlation between K_D for binding to neurotensin receptors and EC_{50} in stimulating intracellular cyclic GMP formation for neurotensin and its analogs and fragments with neuroblastoma clone N1E-115.

cifically residues 8–13, was the biochemically active portion of this peptide in a neuronal cell type. Interestingly, in this system, NT(8–13) proved to be fifty times as potent as intact neurotensin itself in stimulating intracellular cyclic GMP formation and 18-fold more active in competing with [^{3}H]neurotensin binding. By contrast, NT(1–6) and NT(1–8) displayed no binding or biochemical activities while modifications of amino acids in the amino-terminal half of neurotensin did not change significantly the biochemical activity of those analogs (e.g. [Gln^4]NT and [D-Pro^7]NT).

Modifications of the carboxyl half of neurotensin, i.e. the sequence of Arg-Arg-Pro-Tyr-Ile-Leu, resulted in drastic changes of activity. Removal of neurotensin's carboxyl-terminal di- and tripeptides in NT(1–11) and NT(1–10), respectively, produced inactive molecules as did blocking the free carboxyl group, e.g. NT-NHMe. Substitution of a D-arginine into the 8 position of neurotensin did not change significantly the biochemical potency although it decreased by half the binding affinity, and the accompanying removal of the 8th amino acid with the N-terminal of neurotensin reduced the biochemical activity for NT(9–13) almost 5-fold while increasing the equilibrium dissociation constant to twenty times that of native neurotensin. The importance of the ring structures in positions 10 and 11 to the ability of neurotensin to function was demonstrated. A D-phenylalanine substituted into position 11 almost completely destroyed the biochemical and binding activities of neurotensin as did a D-proline inserted into position 10. However, [D-Trp^{11}]NT and [D-Tyr^{11}]NT retained some functional capabilities at very high concentrations. Replacement of the usual tyrosine in position 11 of neurotensin with a phenylalanine had no significant effect on the EC_{50} for stimulating cyclic GMP formation but it increased the K_D almost 7-fold. All of the neurotensin peptides which were active in stimulating intracellular cyclic GMP production induced the same maximal response as neurotensin itself except for those analogs with D-amino acids in positions 10 or 11, which produced a maximal cyclic GMP level that was routinely lower than that for neurotensin.

When the potency for each of the neurotensin peptides in stimulating intracellular cyclic GMP formation was compared diagrammatically to its ability to compete for neurotensin receptors on intact N1E-115 cells, a direct correlation between each EC_{50} and corresponding K_D was found to exist (Fig. 6). Interestingly, with these N1E-115 cells cultured in DMEM supplemented with 10% fetal bovine serum, the EC_{50} values for neurotensin and its analogs and fragments were an average of 18-fold lower than the corresponding K_D values, suggesting that fewer than 50% of the neurotensin receptors needed to be occupied for production of 50% of the maximal biochemical activity. When clone N1E-115 was maintained in DMEM containing 10% newborn calf serum, a condition which yielded a lower number of receptors per cell (*vide supra*), the EC_{50} for native neurotensin in the cyclic GMP assay, 13 ± 5 nM [5], more closely agreed with its K_D in the receptor binding assay, 9 ± 2 nM. Regardless of the type of serum employed in the cellular culture medium, neurotensin demonstrated no ability to inhibit prostaglandin E_1-induced cyclic AMP formation in clone N1E-115 at concentrations of 1 μM or less.

DISCUSSION

With an assay measuring [^{3}H]neurotensin binding at 0° to intact cells cultured in DMEM with newborn calf serum, neuroblastoma clone N1E-115 was demonstrated to possess a single class of neurotensin receptors having an apparent K_D of 9 nM and a B_{max} of 180 fmoles/10^6 cells. [^{3}H]Neurotensin binding to clone N1E-115 displayed specificity, saturability, reversibility, and tissue linearity. Therefore, the neurotensin receptors characterized on these intact neuroblastoma cells, with a K_D of 9–11 nM as determined by the type of serum employed in the cellular culture medium, were comparable to the single class of neurotensin receptors found in rat brain membranes, slices, and synaptic membranes, having a K_D value in the range of 2–8 nM [17–21]. They were also similar to the low-affinity class of neurotensin receptors demonstrated in rat brain synaptic membranes [22] and human substantia nigra [23] where two classes of receptors were found with K_D values of 0.1 and 4.7 nM, and 0.26 and 4.3 nM, respectively.

The presence of neurotensin receptors on clone N1E-115 was also reported recently by Poustis *et al.* [24]. Measuring the binding of monoiodo[Trp^{11}] neurotensin to homogenates of differentiated N1E-115 cells at 0°, these authors report a single class of neurotensin receptors having a K_D of 0.15 nM and a B_{max} of 9 fmoles/mg protein or 12,500 binding sites per cell. The discrepancy between these values for neurotensin receptors on clone N1E-115 and the data reported here may be explained by differences between the assay employed by Poustis *et al.*, i.e. binding of an iodinated neurotensin analog having a very high specific radioactivity to cell homogenates, and the conditions presented in this paper, i.e. binding of tritiated neurotensin to intact cells. Furthermore, cell culture techniques differed between the two laboratories. Poustis *et al.* [24] cultured cells in the presence of penicillin and streptomycin and differentiated clone N1E-115 for use in binding

assays by employing 0.5% fetal calf serum and 1.5% dimethyl sulfoxide. Cells were cultured in our laboratory without antibiotics and confluent cells 8–26 days past subculture were used in all binding assays.

A number of neurotensin fragments and analogs were employed with neuroblastoma N1E-115 cells cultured in DMEM with fetal bovine serum to investigate the potency of these peptides for stimulating intracellular cyclic GMP formation and inhibiting [^{3}H]neurotensin binding. Comparison of these activities for each peptide indicated a direct correlation with EC_{50} values in inducing cyclic GMP synthesis being an average of 18-fold lower than the corresponding K_D values for binding to the neurotensin receptor. These data demonstrated that, in N1E-115 cells cultured in the presence of fetal bovine serum, an average of only 7% of the receptors required occupancy for the production of the half-maximal biochemical response. In comparison, cells grown in newborn calf serum had a 28% lower maximum number of neurotensin receptors per cell, and this reduction in receptor number was associated with a requirement for approximately 60% of the receptors to be occupied by neurotensin for the induction of half-maximal response. This result suggested that the type of serum used in culturing N1E-115 cells influenced not only the maximum number of neurotensin receptors per cell but also the coupling of the agonist–receptor complex to its effector(s) which occurs prior to the production of the biological response, i.e. the stimulation of intracellular cyclic GMP production.

The structure–activity results reported here supported those obtained from biological assays typically employing whole animals, intact organs, or tissue dissected from rodent heart, stomach, or intestine, in that the carboxyl-terminal of neurotensin, specifically residues 8–13, contained the binding and biochemical activities of this peptide while the amino-terminal fragment was inactive. Certain modifications in the carboxyl portion of neurotensin greatly reduced or destroyed the binding and biological activities, e.g. removing the carboxyl-terminal di- or tripeptide, blocking the free carboxyl group, or substituting D-amino acids into positions 10 and 11. Interestingly, neurotensin(9–13) was relatively active, possessing one-fifth and one-twentieth the biological and binding activities, respectively, of native neurotensin.

A novel finding resulting from this study of neurotensin's binding and biochemical activities in an adrenergic neuron-like cell, however, was that neurotensin(8–13) was fifty times more potent than neurotensin itself in stimulating intracellular cyclic GMP formation in clone N1E-115 and had an 18-fold higher affinity for neurotensin receptors on the intact cells. These data suggested that neurotensin might function as a precursor form of neurotensin(8–13), for which the neurotensin receptors on this neuronal cell type had a higher affinity and a greater receptiveness.

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