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Short communication

LANT-6, xenopsin and neuromedin N stimulate cyclic GMP at neurotensin receptors

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The naturally occurring analogs of neurotensin-(8-13), xenopsin, [Lys^8,Asn^9]neurotensin-(8-13) (LANT-6) and neuromedin N stimulated the production of intracellular cyclic GMP in murine neuroblastoma clone N1E-115, an adrenergic neuronal cell type. The order of potency was neurotensin-(8-13) > neurotensin > xenopsin > neuromedin N > LANT-6. Furthermore, xenopsin, LANT-6 and neuromedin N each inhibited the specific binding of [^{3}H]neurotensin to intact N1E-115 cells in a dose-related fashion. The order of affinity of the peptides for the neurotensin receptor was neurotensin-(8-13) > xenopsin > neurotensin > neuromedin N > LANT-6.

Neurotensin-(8-13); Xenopsin; [Lys^8,Asn^9]neurotensin-(8-13); Neuromedin N; Clone N1E-115; cyclic GMP

1. Introduction

Neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu), a putative neurotransmitter in the central nervous system, was first demonstrated to stimulate the formation of intracellular cyclic GMP, considered a second messenger for some neurotransmitters, in murine neuroblastoma clone N1E-115, an adrenergic neuronal cell type of mammalian origin (Gilbert and Richelson, 1984). Binding studies with intact N1E-115 cells demonstrated the existence of receptors which bound [^{3}H]neurotensin with a high affinity and specificity (Gilbert and Richelson, 1984; Gilbert et al., 1986). A comparison was 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 which had been cultured in growth medium supplemented with fetal bovine serum (Gilbert et al., 1986). It was found that the carboxyl-terminal region of neurotensin, neurotensin-(8-13) (Arg-Arg-Pro-Tyr-Ile-Leu; NT-(8-13)), was 50 times more potent than native neurotensin in stimulating intracellular cyclic GMP formation and had an 18-fold higher affinity for the neurotensin receptor.

Three naturally occurring analogs of neurotensin-(8-13) have been isolated and identified. Xenopsin (see table 1 for structure) was found in extracts from the skin of frog, *Xenopus laevis* (Araki et al., 1973), and was later detected in neural and gastrointestinal tissues from numerous amphibia (Carraway et al., 1982). More recently, peptides related to xenopsin in their carboxyl-terminal regions have been demonstrated with immunohistochemistry and radioimmunoassay in canine and human stomach (Feurle et al., 1985). [Lys^8,Asn^9]neurotensin-(8-13) (see table 1) was first isolated from extracts of chicken intestine (Carraway and Ferris, 1983), and was subsequently detected by radioimmunoassay in the brain and gastrointestinal tract of chicken (Carraway et al., 1983). It has also been demonstrated im-

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TABLE 1

Structures of neurotensin and peptides of the neurotensin-(8-13)-like family.

Peptide	Structure
Neurotensin	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
Neurotensin-(8-13)	Arg-Arg-Pro-Tyr-Ile-Leu
Xenopsin	pGlu-Gly-Lys-Arg-Pro-Trp-Ile-Leu
Neuromedin N	Lys-Ile- Pro-Tyr-Ile-Leu
LANT-6	Lys-Asn-Pro-Tyr-Ile-Leu

munohistochemically in neural tissue of animals from several vertebrate classes, including mammals (Reiner and Carraway, 1985). Neuromedin N (see table 1) was discovered in extracts of porcine spinal cord (Minamino et al., 1984), and radiolabeled neuromedin N has since been found to bind specifically to rat brain membranes (Emson and Richards, 1985). Here we report that xenopsin, LANT-6 and neuromedin N stimulated intracellular production of cyclic GMP and inhibited the binding of radiolabeled neurotensin to intact N1E-115 cells, results suggesting that these peptides are agonists at the neurotensin receptor.

2. Materials and methods

2.1. Cell culture

Murine neuroblastoma cells (clone N1E-115) were cultured in Dulbecco-Vogt's modification of Eagle's medium (Grand Island Biological Co., Grand Island, NY) without antibiotics and supplemented with 10% (v/v) fetal bovine serum (Grand Island Biological Co.). Cells (passage number < 21) were cultured 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°C. Flasks were routinely inoculated with $0.5\text{-}1.0 \times 10^6$ cells which were fed daily beginning day 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.

2.2. Measurement of relative changes in cyclic GMP production

The measurement of intracellular cyclic GMP formation was by a method in which cyclic [^{3}H]GMP 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°C in 10 ml of modified Puck's D_1 solution without antibiotics and phenol red, disruption of the layer by agitation of the flask, and collection of the cells by centrifugation at $300 \times g$ for 1 min at 4°C 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 (mM) 110 NaCl, 5.3 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 2.0 Na_2HPO_4, 25 glucose and 70 sucrose (pH 7.35; 340 mOsm), and the washed cells were collected by centrifugation.

The cellular pellet was resuspended in 2 ml of solution I and transferred to a 25 ml Erlenmeyer flask. Twenty microliters of the suspension were removed for enumeration of cells (Coulter Electronics, Hialeah, FL). To the remaining volume was added 20 μCi of [^{3}H]guanosine, after which the flask was covered and placed on the shaking platform of a 37°C 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 s at $300 \times g$ and were resuspended in a volume of solution I which provided 100 000 cells/270 μl. This quan-

tity 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°C water bath. 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 peptide. Each reaction was routinely terminated after 30 s 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 d.p.m. 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 × 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 approximately 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) were added, and the radioactivity was measured in an Isocap/300 liquid scintillation counter (Searle, Des Plaines, IL).

2.3. Neurotensin receptor binding assay

Competition studies measuring the inhibition in binding of [^{3}H]neurotensin to intact N1E-115 cells were performed as follows. 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, and the washed cells were collected by centrifugation. This cellular pellet was then resuspended in at least 2 ml of solution I, and 20 μl of the suspension were removed for enumeration of cells. The remaining volume was diluted in solution I made 0.1% (w/v) in bovine serum albumin (solution II), to routinely provide 350 000-400 000 cells per assay tube, and was equilibrated at 0°C before distribution.

[^{3}H]Neurotensin binding to N1E-115 cells was performed in 5 ml polypropylene tubes (Walter Sarstedt Inc.) at 0°C. A total assay volume of 1 ml was used, and the reaction was initiated by the addition of cells. During the incubation at 0°C, all assay tubes were mixed every 10 min to prevent settling of the cells. Non-specific binding was determined with a 1 μM concentration of unlabeled neurotensin in reaction tubes, and routinely was 30% of the total binding. 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 after 20 min by simultaneous filtration of 24 samples at a time on a cell harvester filtering unit (Brandel, Gaithersburg, MD) equipped with a GF/B filter strip 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 I. 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 h prior to radioactivity measurement.

The data were analyzed with the LIGAND program of P.J. Munson and D. Rodbard 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).

2.4. Materials

Polypropylene plasticware or silanized glassware were used for all experiments. [^{3}H]Guanosine was from ICN Radiochemicals (Irvine, CA), [^{3}H]neurotensin was obtained from New England Nuclear (Boston, MA), and cyclic [^{14}C]GMP was

purchased from Amersham (Arlington Heights, IL). Sigmacote, bovine serum albumin (A-7906) and polyethylenimine were supplied by Sigma Chemical Co. (St. Louis, MO). Neurotensin-(8-13) and xenopsin were purchased from Bachem (Torrance, CA), and LANT-6 and neuromedin N were from Peninsula (Belmont, CA). Neurotensin was supplied by Boehringer-Mannheim (Indianapolis, IN). All other reagents were analytical grade.

3. Results

Xenopsin, LANT-6 and neuromedin N all stimulated the production of intracellular cyclic GMP in N1E-115 cells in dose-related fashions, with an average of 8 ± 1, 12 ± 5 and 10 ± 1 fold, respectively (means $\pm$ S.E., number of experiments = 3) over basal levels (5088 ± 1135 dpm/10^6 cells, means $\pm$ S.E., number of experiments = 9). The EC_{50} for each of these analogs, i.e. the concentration of peptide inducing 50% of the maximal response, is indicated in table 2. The order of potency in stimulating intracellular cyclic GMP production was neurotensin-(8-13) > neurotensin > xenopsin > neuromedin N > LANT-6. The three naturally occurring analogs of neurotensin-(8-13) all induced the same maximal cyclic GMP production as both neurotensin and neurotensin-(8-13).

Furthermore, xenopsin, LANT-6 and neuromedin N all demonstrated a concentration-dependent ability to inhibit the specific binding of [^{3}H]neurotensin to intact N1E-115 cells. The K_d for each of these analogs, i.e. the apparent equilibrium dissociation constant, is presented in table 2. The order of affinity of the peptides for the neurotensin receptor was neurotensin-(8-13) > xenopsin > neurotensin > neuromedin N > LANT-6. Hill plots of the specific binding data gave coefficients essentially equal to unity for the neurotensin-(8-13)-like peptides, confirming that the three naturally occurring analogs were competing for binding at the homogeneous class of neurotensin receptors previously described on intact N1E-115 cells (Gilbert and Richelson, 1984; Gilbert et al., 1986).

When the potency for each of the neurotensin-(8-13)-like peptides in stimulating intracellular cyclic GMP formation was compared graphically to its ability to compete for neurotensin receptors on intact N1E-115 cells by plotting ($-\log EC_{50}$) versus ($-\log K_d$), a linear regression analysis by the least squares method gave a direct correlation between each EC_{50} and corresponding K_d ($r = 0.98$; data not shown).

4. Discussion

In our studies of neurotensin's binding and biochemical activities with clone N1E-115, an

TABLE 2

Potencies of neurotensin and peptides of the neurotensin-(8-13)-like family in biological and binding activities with clone N1E-115. Values are means $\pm$ S.E.

Peptide	Stimulation of cyclic [^{3}H]GMP formation in intact cells EC_{50} (nM)	Competition with [^{3}H]neurotensin binding to intact cells	
		K_d (nM)	Hill coefficient
Neurotensin-(8-13)	0.32 [a] $\pm$0.04 (8) [b]	0.61 $\pm$ 0.02 (3) [c]	1.03 $\pm$ 0.02
Neurotensin [c]	1.5 $\pm$0.6 (11)	11 $\pm$ 1 (10)	0.97 $\pm$ 0.02
Xenopsin	1.7 $\pm$0.4 (3)	6 $\pm$ 1 (5)	1.00 $\pm$ 0.07
Neuromedin N	15 $\pm$1 (3)	46 $\pm$ 7 (5)	0.93 $\pm$ 0.07
LANT-6	77 $\pm$8 (3)	90 $\pm$29 (6)	0.90 $\pm$ 0.06

[a] EC_{50} values for neurotensin-(8-13) and its natural analogs varied as much as 10-fold with passages originating from different, low passage stocks of clone N1E-115, although the rank order of potency remained identical. [b] Number of independent experiments performed. [c] Values are from Gilbert et al. (1986).

adrenergic neuronal cell type of mammalian origin, we found that the carboxyl-terminal portion of native neurotensin, neurotensin-8-(13), was more potent than the parent peptide itself in stimulating intracellular cyclic GMP formation and had a higher affinity for neurotensin receptors on intact cells. As this result suggested that the neurotensin-(8-13) fragment might be of physiological importance, it was of interest to examine the naturally occurring analogs of neurotensin-(8-13) for their binding and biochemical activities.

We report here that xenopsin, LANT-6 and neuromedin N, the naturally occurring analogs of neurotensin-(8-13) found originally in frog skin, chicken intestine and porcine spinal cord, respectively, induced biochemical activity in these neuronal-like cells. The potency of the three NT-(8-13)-like peptides in stimulating the formation of intracellular cyclic GMP in clone N1E-115 was less than that of neurotensin-(8-13) and native neurotensin, which, in this study, was 5-fold less potent than NT-(8-13). Furthermore, the potency for each of the analogs in inducing intracellular cyclic GMP production was directly correlated with its ability to compete for neurotensin receptors on intact N1E-115 cells. These data suggested that xenopsin, LANT-6 and neuromedin N were inducing biological activity in clone N1E-115 upon binding to the neurotensin receptor.

That these three naturally occurring analogs of neurotensin-(8-13) inhibited the binding of [^{3}H] neurotensin to intact neuroblastoma cells less well than did either unlabeled neurotensin-(8-13) or (except for xenopsin) neurotensin itself, gives support to the idea that these peptides could interact with neurotensin receptors and thereby induced biochemical activity, but does not rule out the possibility that these analogs might have their own specific receptors in mammalian neural tissue. These results also raise the possibility that these neurotensin-(8-13)-like peptides might induce biochemical activity in mammalian neural tissue following binding to any specific receptors that might exist. At any rate, the natural occurrence of xenopsin, LANT-6 and neuromedin N indicates the physiological importance of the neurotensin-(8-13)-like family of peptides.

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