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FURTHER CHARACTERIZATION OF NEUROTENSIN RECEPTOR DESENSITIZATION AND DOWN-REGULATION IN CLONE N1E-115 NEUROBLASTOMA CELLS

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Abstract—Murine neuroblastoma clone N1E-115 cells possess neurotensin receptors that are coupled to polyphosphoinositide hydrolysis and cyclic guanosine 3′,5′-monophosphate (cGMP) formation. These responses rapidly desensitize and these receptors rapidly down-regulate nearly completely in about 15 min. Although neurotensin is rapidly degraded by peptidases, in this study we show that at 37° neurotensin (100 nM) in the absence of peptidase inhibitors caused this rapid desensitization and down-regulation (32 ± 5 and 24 ± 2% of control, respectively) of neurotensin receptors in N1E-115 cells. In addition, we demonstrated that this desensitization, resensitization, down-regulation and recovery of binding sites were temperature dependent. These data suggest that a certain degree of phospholipid fluidity or activity of some enzymes is required for these processes to occur. After addition of sodium nitroprusside or ionomycin to cells, cGMP increased in desensitized cells to the same degree as in control cells. Additionally, desensitization and down-regulation occurred in the absence of a change in the affinity of neurotensin for the remaining sites. These data suggest that desensitization is not caused by changes in nitric oxide synthesis, guanylyl cyclase activity or receptor affinity, but predominantly by a decrease in receptor number.

The tridecapeptide neurotensin is a putative neurotransmitter in the central nervous system. Murine neuroblastoma clone N1E-115 cells possess neurotensin receptors that are known to be coupled to phosphoinositide hydrolysis and cyclic guanosine 3′,5′-monophosphate (cGMP)† formation [1, 2]. Neurotensin is degraded rapidly not only by peptidases of brain tissue but also by those of N1E-115 cells [3]. Recently, we showed that agonist-induced rapid desensitization and down-regulation of neurotensin receptors occur in N1E-115 cells after exposure to [D-Lys[8]]neurotensin (8–13), which is degraded less rapidly by peptidase [4]. However, we also found that neurotensin (100 nM) without peptidase inhibitors causes a rapid desensitization of intracellular Ca^{2+} mobilization [5]. This concentration of neurotensin is known to cause a maximum effect on down-regulation (Yamada *et al.*, unpublished observations), phosphoinositide hydrolysis and cGMP formation [6] in N1E-115 cells.

In this study, we examined the effects of exposure of N1E-115 cells to 100 nM neurotensin without peptidase inhibitors on neurotensin receptor binding and cGMP formation in these cells. We also examined the effects of temperature on neurotensin receptor desensitization, resensitization, down-regulation and its recovery. Although, the effects of temperature on desensitization of catecholamine [7–9], nicotinic acetylcholine [10] and muscarinic acetylcholine [11] receptors are known, this information has not been determined for neurotensin receptors.

Previously, we showed that neurotensin receptor-mediated cGMP formation involves nitric oxide (NO) synthesis in N1E-115 cells [12, 13]. To gain further insight into desensitization of the neurotensin receptor, we also examined cGMP formation stimulated independently of the receptor by a calcium ionophore, ionomycin or sodium nitroprusside, in desensitized cells. The calcium ionophore ionomycin increased intracellular Ca^{2+} concentrations in a receptor-independent manner. Increased intracellular Ca^{2+} concentration stimulates NO synthesis, which is an intracellular messenger for receptor-mediated cGMP formation [14]. Sodium nitroprusside is known to produce NO by the simple breakdown of this compound [15].

MATERIALS AND METHODS

Materials. Polypropylene or polyethylene plasticware was used for all experiments. [^{3}H]Neurotensin was obtained from New England Nuclear (Boston, MA); [^{3}H]guanosine was from ICN Radiochemicals (Irvine, CA); cyclic [^{14}C]GMP was purchased from Amersham (Arlington Heights, IL); polyethylenimine and sodium nitroprusside were supplied

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† Abbreviations: cGMP, cyclic guanosine 3′,5′-monophosphate; NO, nitric oxide; PBS-GS, phosphate-buffered saline solution consisting of 110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 2.0 mM Na_2PO_4, 25 mM glucose and 70 mM sucrose, pH 7.35; 340 mOsmol; K_D, equilibrium dissociation constant; and B_{max}, maximum number of binding sites.

by the Sigma Chemical Co. (St. Louis, MO); and neurotensin and ionomycin were from Boehringer-Mannheim (Indianapolis, IN). All other reagents were analytical grade.

Cell culture. Murine neuroblastoma cells (clone N1E-115) were cultured in Dulbecco-Vogt's modified 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 (passages 12–18) were cultured in 40 mL of medium in 150 cm^2 Corning flasks (Corning Glass Works, Corning, NY) in a humidified atmosphere of 10% CO_2:90% air at 37°. The culture medium was changed daily by adding 20 mL of fresh medium and removal of 20 mL of medium. Cells were harvested during the stationary phase of growth, 14–22 days after subculture.

Measurement of relative changes in cGMP production. The assay used for measurement of intracellular cGMP formation was essentially that of Pfenning and Richelson [2], in which cyclic [^{3}H]-GMP produced in clone N1E-115 cells was isolated chromatographically from cells labeled with a radioactive precursor prior to stimulation. N1E-115 cells were harvested for use by aspiration of culture medium, incubation of the cellular monolayer for 10 min at 37° in 20 mL of modified Puck's D1 solution, and collection of the cells by centrifugation at 900 *g* for 1 min at 4°. The cellular pellet was resuspended in 10 mL of phosphate-buffered saline solution (PBS-GS), consisting of 110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 2.0 mM Na_2PO_4, 25 mM glucose and 70 mM sucrose (pH 7.35; 340 mOsmol). The washed cells were collected by centrifugation. The cellular pellet was resuspended in PBS-GS and metabolically labeled with 40 μCi of [^{3}H]guanosine at 37° for 45 min. The cells were collected by centrifugation and resuspended in a volume of PBS-GS which provided 1×10^5 cells per 270 μL. This quantity was distributed to each well of a Linbro 24-well plate (Flow Laboratories, McLean, VA), and the tray was placed on the platform of a 37° water bath and allowed to equilibrate for 3 min. Individual wells of cells at 80 oscillations/min were then tested by the addition of 30 μL of either PBS-GS (to obtain basal cyclic [^{3}H]GMP values) or stimulants. 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), and the radioactive cGMP was purified as previously described.

Neurotensin receptor binding assay. N1E-115 cells were harvested for use as described for the cGMP assay. The cellular pellet was resuspended in 10 mL of PBS-GS, and the washed cells were collected by centrifugation. This cellular pellet was then resuspended in at least 2 mL of PBS-GS, and 20 μL of the suspension was removed for enumeration of cells. The remaining volume was diluted in PBS-GS, made 0.1% (w/v) in bovine serum albumin to ensure nonspecific protection from proteases, to provide routinely 3×10^5 cells per assay tube. This suspension was equilibrated at 0° before distribution. [^{3}H]-Neurotensin binding to N1E-115 cells was performed at 0° for 30 min in polypropylene tubes (Walter Sarstedt Inc.) with 2 nM [^{3}H]neurotensin. A total assay volume of 1 mL was used, and the reaction was initiated by the addition of 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 competition binding assay was carried out using different concentrations of unlabeled neurotensin with 2 nM [^{3}H]neurotensin, thus allowing determination of the maximum number of binding sites (B_{max}) and the equilibrium dissociation constant (K_D) for neurotensin receptor. The binding reaction was routinely terminated after 30 min by rapid filtration under vacuum on glass fiber filters (GF/B, 2.4 cm, Whatman, Clifton, NJ) that had been pretreated with 0.1% polyethylenimine for 60 min just before use. Each tube and corresponding filter were rinsed immediately with three 4-mL rinses of cold 0.9% NaCl solution. Filters were then placed in plastic scintillation vials to which 7 mL of Ready-Safe counting solution was added, and the filters were allowed to incubate for at least 5 hr prior to measurement of radioactivity.

Exposure of N1E-115 cells to neurotensin. For our desensitization studies, the radioactivity labeled cells were exposed to 100 nM neurotensin in PBS-GS at 37° for 15 min without peptidase inhibitors. Intact N1E-115 cells were used for the down-regulation studies. To study the effects of temperature on desensitization and down-regulation, the cells were incubated at different temperatures (0°, 20°, 24°, 28°, 32°, or 37°). Following five washes with ice-cold PBS-GS, changes in cGMP formation and neurotensin receptor binding were examined. The cells were then incubated at 37° for 120 min, and the time course of resensitization and recovery of binding sites was studied. To examine the effects of temperature on resensitization and recovery of binding sites, the cells were incubated at different temperature (0°, 20°, 24°, 28°, 32°, or 37°).

Statistics. Values are expressed as means ± SEM. The statistical significance was evaluated using Student's *t*-test. P values less than 0.05 were considered significant.

RESULTS

Time course of desensitization, resensitization, down-regulation and recovery of binding sites. We found that exposure of cells to 100 nM neurotensin a 37° in the absence of peptidase inhibitors caused rapid desensitization (32 ± 5% of control, after 15 min) and down-regulation (24 ± 2% of control, after 15 min) of neurotensin receptors in N1E-115 cells (Fig. 1). By using a competition radioligand binding assay we confirmed that the affinity of neurotensin for the remaining neurotensin receptors was not changed (data not shown). The K_D and B_{max}

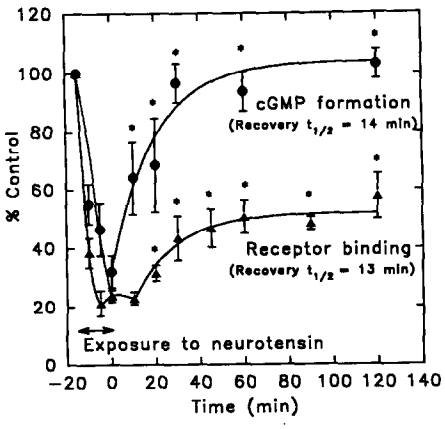

Fig. 1. Time course of neurotensin receptor desensitization and down-regulation and their recoveries. Clone N1E-115 cells were exposed to 100 nM neurotensin at 37° for 15 min without peptidase inhibitors. For desensitization and its recovery (resensitization), cells were radioactively labeled with [^{3}H]guanosine. Relative changes in cGMP stimulated by 100 nM neurotensin and neurotensin receptor binding were studied. Control basal and stimulated levels of cyclic [^{3}H]GMP were about 400 and 1400 dpm/10^6 cells, respectively. The control specific [^{3}H]neurotensin binding was about 1800 dpm/3 × 10^5 cells. After five washes with ice-cold buffer, cells were incubated at 37° at the indicated times and resensitization and recovery of neurotensin binding sites were examined. Data are means ± SEM of 4 independent experiments. Key: (*) $P < 0.05$ vs data at time = 0 min (Student's t-test). Recovery data were fit to the equation:

$$R_t = R_\infty - (R_\infty - R_0)\, e^{-k(t-t_0)}$$

where R_t = response or binding at time t; R_∞ = response or binding at infinite time; R_0 = response or binding at time 0 (t_0). The nonlinear regression (Marquardt–Levenberg algorithm) was done with the use of the curve-fitting function of *SigmaPlot* (version 4.1, Jandel Scientific, Corte Madera, CA).

of control cells was 10 ± 1 nM and 290 ± 50 fmol/10^6 cells, respectively.

Incubation of desensitized and down-regulated cells at 37° after neurotensin was removed by five washes with ice-cold buffer resulted in essentially total recovery of sensitivity (97 ± 7% of control) after 30 min. However, there was recovery of only 58 ± 8% of control binding sites even after 120 min of incubation. The recovery of binding sites, but not the recovery of sensitivity, had about a 10-min lag-time after washing away excess neurotensin (Fig. 1). The rate constants for recovery gave basically identical half-times for recovery for cGMP stimulation and receptor binding of 14 and 13 min, respectively, when the lag-time for recovery of binding (0–10 min) was excluded (Fig. 1). These results are similar to our previous data [4].

Effects of temperature on desensitization and resensitization. Neurotensin receptor desensitization was not observed after a 15-min exposure to 100 nM neurotensin at 0° (sensitivity at 98 ± 1% of control, Fig. 2A). Above 20°, the apparent rates of neurotensin receptor desensitization increased as the incubation temperature increased (90 ± 20, 70 ± 10, 53 ± 8, 40 ± 10, and 32 ± 5% of control at 20°, 24°, 28°, 32°, and 37°, respectively). After cells were desensitized to neurotensin, significant recovery of sensitivity was not observed after a 60-min incubation of cells at 0° (40 ± 3% of control, Fig. 2B). Above 20°, the apparent rates of neurotensin receptor resensitization increased as the incubation temperature increased (50 ± 5, 52 ± 7, 56 ± 3, 70 ± 10, and 94 ± 7% at 20°, 24°, 28°, 32°, and 37°, respectively). Arrhenius plots of the data for 20–37° (data not shown) yielded activation energies of 4.0 ± 0.3 and 1.4 ± 0.3 kcal/mol for desensitization and resensitization, respectively. These results suggest that thermodynamically, resensitization of neurotensin receptors in these cells was easier to achieve than desensitization. In addition, these data suggest that both of these processes were easier to achieve for the neurotensin receptor than for the muscarinic receptor of these cells [11].

Effects of temperature on neurotensin receptor down-regulation and its recovery. At 0° neurotensin (100 nM) caused about a 22% loss of binding sites after a 15-min exposure (78 ± 4% of control, Fig. 3A). Neurotensin binding sites decreased in the presence of neurotensin as the incubation temperature increased (48 ± 3, 36 ± 3, 31 ± 2, 20 ± 2, and 23.3 ± 0.4% of control at 20°, 24°, 28°, 32°, and 37°, respectively). After neurotensin receptors were down-regulated with 100 nM neurotensin, significant recovery of binding sites was not observed after an additional 60-min incubation at or below 20° (26 ± 7 and 25 ± 5% of control at 0° and 20°, respectively; Fig. 3B). Above 20°, the apparent rates of recovery increased as the incubation temperature increased (33 ± 5, 40 ± 3, 48 ± 1, and 51 ± 6% of control at 24°, 28°, 32°, and 37°, respectively). Arrhenius plots of the data for 20–37° (data not shown) yielded activation energies of 1.3 ± 0.1 and 1.7 ± 0.2 kcal/mol for down-regulation and its recovery, respectively. These results suggest that thermodynamically, down-regulation of neurotensin receptors in these cells was easier to achieve than their recovery.

Cyclic GMP formation by ionomycin and sodium nitroprusside in desensitized cells. Ionomycin (10 μM) or sodium nitroprusside (10 mM) stimulated cGMP formation in desensitized cells to the same degree as in the control cells, whereas 100 nM neurotensin stimulated cGMP to only 30 ± 2% of control in desensitized cells (Table 1). The EC_{50} values (200 nM and 100 μM for ionomycin and sodium nitroprusside, respectively) and maximal response for stimulation by ionomycin or sodium nitroprusside in desensitized cells were also identical to those obtained with control cells.

DISCUSSION

In this study, we showed that for clone N1E-115 neuroblastoma cells rapid desensitization and rapid down-regulation of neurotensin receptors occur after exposure to 100 nM neurotensin at 37° in the absence of peptidase inhibitors. Recently, we found that the down-regulation of neurotensin receptors occurred concentration dependently with an EC_{50} = 1.7 nM

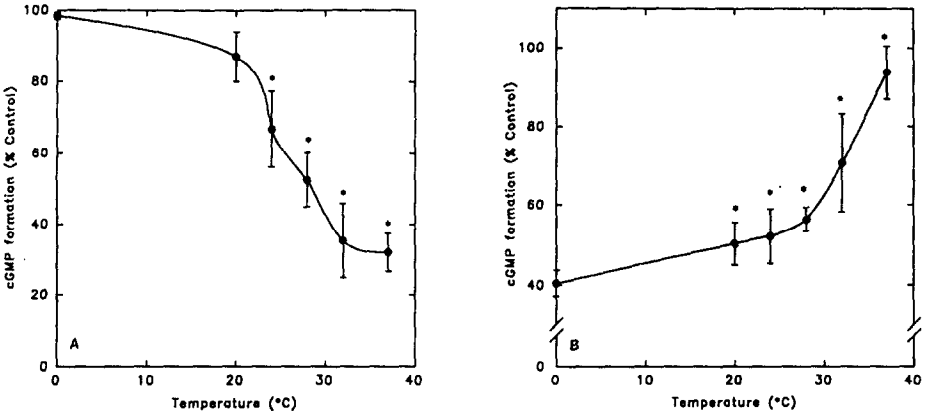

Fig. 2. Effects of temperature on desensitization and resensitization of neurotensin receptors on clone N1E-115 cells. (A) Cells radioactively prelabeled with [^{3}H]guanosine were exposed to 100 nM neurotensin for 15 min at the indicated temperatures (0°, 20°, 24°, 28°, 32° and 37°). After five washes with ice-cold buffer, cGMP formation stimulated by 100 nM neurotensin at 37° was measured. (B) Cells exposed to 100 nM neurotensin for 15 min at 37° were washed and incubated at the indicated temperatures (0°, 20°, 24°, 28°, 32° and 37°) for another 60 min. After that time cGMP formation stimulated by 100 nM neurotensin at 37° was measured. Data are shown as percent of control, mean ± SEM of 4 independent experiments. Basal and stimulated levels of cyclic [^{3}H]GMP were about 450 and 1300 dpm/10^6 cells. Key: (*) $P < 0.05$ vs control data (A) and vs data after exposure at 37° (B) (Student's *t*-test).

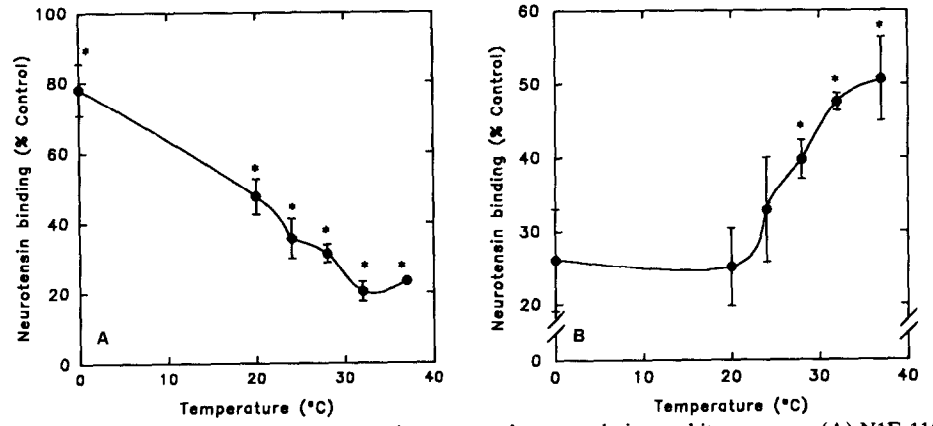

Fig. 3. Effects of temperature on neurotensin receptor down-regulation and its recovery. (A) N1E-115 cells were exposed to 100 nM neurotensin at the indicated temperatures (0°, 20°, 24°, 28°, 32° and 37°) for 15 min without peptidase inhibitors. After five washes with ice-cold buffer, the amount of neurotensin receptor binding was determined using 2 nM [^{3}H]neurotensin, and cells were incubated at 0° for 30 min. (B) Cells exposed to 100 nM neurotensin at 37° for 15 min were washed and incubated at the indicated temperatures (0°, 20°, 24°, 28°, 32° and 37°) for another 60 min. Binding of [^{3}H]neurotensin was then determined. Data are shown as percent of control, mean ± SEM of 4 independent experiments. The control specific [^{3}H]neurotensin binding was about 1800 dpm/3 × 10^5 cells. Key: (*) $P < 0.05$ vs control data (A) or vs data after exposure to neurotensin (B) (Student's *t*-test).

(Yamada *et al.*, unpublished data). This value compares favorably with our previously reported EC_{50} values for polyphosphoinositide hydrolysis (0.9 nM) and cGMP formation (1.2 nM) [6].

Agonist-induced loss of cell surface neurotensin receptors also occurs in primary cultures of rat [16] and mouse [17] neurons. If these conditions are close to physiological, these data suggest that

Table 1. Cyclic GMP formation in desensitized cells

Stimulant	cGMP formation (% of control)
Neurotensin	30 ± 2* (6)
Ionomycin	99 ± 1 (4)
Sodium nitroprusside	98 ± 1 (6)

Cyclic GMP formation stimulated by neurotensin (100 nM), ionomycin (10 μM) or sodium nitroprusside (10 mM) in desensitized N1E-115 cells was studied. Cells radioactively labeled with [^{3}H]guanosine were exposed to 100 nM neurotensin for 15 min at 37°. After five washes with ice-cold buffer, cGMP formation stimulated by neurotensin, ionomycin or sodium nitroprusside was studied. For the control, the basal level of cyclic [^{3}H]GMP was about 200 dpm/1 × 10^5 cells; the levels of cyclic [^{3}H]-GMP stimulated by neurotensin, ionomycin, and sodium nitroprusside were 800, 1100 and 1300 dpm/1 × 10^5 cells, respectively. Data are means ± SEM; the number of independent experiments is given in parentheses.

* Significantly different from control data, $P < 0.05$ (Student's *t*-test).

desensitization and down-regulation of neurotensin receptors occur in the central nervous system *in vivo*. In fact, the internalization of neostriatal neurotensin receptors followed by retrograde axonal transport to the substantia nigra pars compacta occurs in rat brain [18–20]. The mechanisms involved with neurotensin receptor down-regulation *in vitro* may be similar to those involved with receptor internalization *in vivo*.

In our previous study utilizing a more stable analog of neurotensin (8–13) [4], we obtained data suggesting that the process of rapid down-regulation of neurotensin receptors in clone N1E-115 cells involves intracellular sequestration of recyclable receptors. This was because nearly complete recovery of binding sites was achieved with a $T_{\frac{1}{2}}$ of about 15 min. In the present study, it is likely that about half of the internalized receptors were degraded, probably by lysosomal enzymes, since after cells were free of neurotensin for 120 min after exposure to neurotensin, they recovered only about 50% of control binding sites. In this case, newly synthesized neurotensin receptors would be required to obtain complete recovery. In addition, recovery of binding sites after washing showed about a 10-min lag time. This result, consistent with our previous study [4], suggests that time is required for migration of internalized receptors to the cellular surface.

Approximately 60 min after washing with ice-cold buffer, cells had essentially complete recovery of sensitivity to neurotensin. However, at this time point only about 50% of control binding sites were present. These data suggest that maximal cGMP formation requires stimulation of only a fraction of the total receptors on the membrane of the cellular surface. These results are consistent with the relationship between the EC_{50} for cGMP formation of about 1 nM and the K_D for neurotensin binding of about 10 nM obtained in intact N1E-115 cells [6]. Using these numbers and the receptor occupancy theory, we calculated that maximal response would occur around 50% receptor occupancy. Thus, 50% of the receptors are "spare."

We have demonstrated that the desensitization, resensitization, down-regulation and recovery of neurotensin receptors were temperature dependent. Above 20°, the apparent rates of desensitization, resensitization and recovery of binding sites were increased as the incubation temperature increased. These data suggest that a certain degree of phospholipid fluidity or activity of some enzyme is required for these processes to occur.

Below 20°, desensitization, resensitization and recovery of binding sites after down-regulation at 37° did not occur. However, even at 0° there appeared to be a loss of about 20% of binding sites in cells exposed to neurotensin. One difficulty in these types of experiments is knowing whether one is studying true down-regulation or simply occlusion of binding sites by bound ligand. If the latter were correct, we would expect a change in the K_D of neurotensin for these binding sites, a result we did not find. However, since we would not expect down-regulation to occur at 0°, we could estimate that at most about 20% of what we were measuring might be occlusion of binding sites by tightly bound, and undegraded neurotensin, that could not be washed away even after five washes with ice-cold buffer. Also, it is not likely that we are seeing the effect of a breakdown product of neurotensin, since we have demonstrated before that no active metabolite of neurotensin is found after incubation with N1E-115 cells at 37° [3].

Ionomycin or sodium nitroprusside stimulated cGMP formation to the same degree in the desensitized cells as in control cells. The calcium ionophore, ionomycin, is thought to increase intracellular Ca^{2+} concentrations in a receptor-independent manner. Increased intracellular Ca^{2+} concentration stimulates NO synthesis, which is an intracellular messenger for receptor-mediated guanylate activation [14]. Sodium nitroprusside is known to produce NO by a simple breakdown of this compound [15]. Our data may suggest that desensitization is not caused by changes in NO synthesis or guanylyl cyclase activity. Additionally, desensitization and down-regulation were observed without modification of the affinity of neurotensin for the remaining sites. We proposed that desensitization of neurotensin receptors in clone N1E-115 cells occurs mainly from a decrease in receptor number. This process of down-regulation may involve the coupling of this receptor to G-proteins [21].

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