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Synergistic Effects of Neurotensin and β-Adrenergic Agonist on 3′,5′-Cyclic Adenosine Monophosphate Accumulation and DNA Synthesis in Prostate Cancer PC3 Cells

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ABSTRACT. Since neurotensin is often co-stored with catecholamines and since it can excite the release of dopamine and norepinephrine, responses to this peptide might depend upon the activity of catecholaminergic systems. In this study, we used prostate cancer PC3 cells, which express neurotensin receptors and β_2-adrenergic receptors, to demonstrate that neurotensin can potentiate the effects of isoproterenol on 3′,5′-cyclic adenosine monophosphate (cAMP) formation and on inhibition of DNA synthesis. While neurotensin had only a slight effect on basal cAMP levels, it nearly doubled the response to isoproterenol even at maximal levels without altering potency. Neurotensin increased the rate of cAMP accumulation and the steady-state level achieved. Consistent with the known antimitogenic action of dibutyryl-cAMP in PC3 cells, isoproterenol was found to inhibit DNA synthesis concentration-dependently, measured using [^{3}H]thymidine. Neurotensin enhanced DNA synthesis when given alone. However, it inhibited DNA synthesis when given with a threshold level of isoproterenol, which by itself had no significant effect. These results, demonstrating cross-talk in the neurotensin and β-adrenergic signaling pathways, suggest that there may be other physiologic instances of similar interactions between neurotensin and catecholamines. BIOCHEM PHARMACOL **57**;12:1391–1397, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. neurotensin; β-adrenergic; cAMP; PC3 cells; DNA synthesis

NT† is a regulatory peptide found primarily in endocrine cells of the intestinal mucosa, but it is also present in various glands, in the central nervous system, and in peripheral nerves innervating vascular smooth muscle throughout the body [1–3]. Among the diverse biologic actions of NT are trophic effects *in vivo* on rat gastroenteropancreatic tissues [4–6], as well as mitogenic effects *in vitro* on multiple types of cancer cells [7–9]. NT also has been reported to promote the carcinogenic process in rats under certain conditions [10].

The findings that NT is co-stored with dopamine and norepinephrine in some tissues [11] and that NT can excite the release of norepinephrine, acetylcholine, dopamine, and histamine [12–14] suggest that NT may act in concert with these and perhaps other transmitters. Not only might some of the actions of NT be mediated by such monoamines, but there is also the possibility of modulatory and synergistic interactions.

Recently [9], we demonstrated that NT binds with high affinity to human prostate cancer PC3 cells and stimulates cell growth at concentrations near the K_d for binding. Our work also shows that this cell line expressed the NT1 receptor mRNA and that elevations in inositol 1,4,5-triphosphate, Ca^{2+}, cAMP, and cGMP were seen in response to NT.‡ The fact that PC3 cells also express abundant β_2-adrenergic receptors, which can mediate an elevation in cAMP [15], and that dibutyryl-cAMP can inhibit the growth of these cells [16] led us to use these cells to investigate possible interactions between NT and the β-adrenergic system.

In the present study, we examined the influence of NT on the responsiveness of PC3 cells to ISO, and we report for the first time that NT synergistically enhanced both cAMP generation and growth inhibition by this β-adrenergic agonist. Furthermore, we report opposite effects of NT on growth in the presence and absence of threshold levels of ISO.

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† *Abbreviations*: NT, neurotensin; ISO, isoproterenol; cAMP, 3′,5′-cyclic adenosine monophosphate; cGMP, 3′,5′-cyclic guanosine monophosphate; IBMX, isobutylmethylxanthine; THEO, theophylline; TCA, trichloroacetic acid; PMSF, phenylmethylsulfonyl fluoride; and AC, adenylyl cyclase.

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MATERIALS AND METHODS

Cell Culture

PC3 cells were grown in F12K medium supplemented with 10% fetal bovine serum and containing 1 mM glutamine, streptomycin sulfate (0.1 mg/mL), and penicillin G (100 U/mL) as described by us [9].

Studies on cAMP

For the cAMP work, PC3 cells were plated in 12-well dishes and used just before they reached confluency, which gave $\sim 4 \times 10^5$ cells/well. Thirty minutes before the experiment, the medium was replaced with HEPES-buffered Locke-BSA (buffer): 148 mM NaCl, 5.6 mM KCl, 6.3 mM HEPES, 2.4 mM $NaHCO_3$, 1.7 mM $CaCl_2$, 0.7 mM $MgCl_2$, 5.6 mM glucose, and 0.1% BSA. Aliquoted stock solutions of NT (1 mM in 0.1% acetic acid) and ISO (10 mM in 0.05 M HCl, 0.1 M ascorbic acid) were stored at −20° for less than 3 weeks. On the day of the experiment, buffer and NT diluted into buffer were brought to 37°. Just before use, ISO was diluted to 10× the desired concentrations using buffer containing 1 mM ascorbic acid (ascorbate buffer). Experiments were started by aspirating the buffer from the adherent cells and adding 0.9 mL of buffer or NT, followed in 15 sec by 0.1 mL ascorbate buffer or ISO. After the incubation at 37°, solutions were aspirated, reactions were stopped with 0.5 mL of ice-cold 6% TCA, and plates were kept at −20°. To assay for cAMP, 4.5 mL of 0.05 M sodium acetate (pH 6) was added to each well, and the pH was adjusted to 6 using 2 M NaOH. Aliquots were acetylated and assayed according to directions supplied with the Amersham radioimmunoassay kit. Sigmoid curve fitting of concentration–response data was performed using the Sigma-Plot program.

Binding Studies

The binding of ^{125}I-labeled NT to PC3 cell membranes was performed as described by us [9]. Briefly, ^{125}I-labeled NT (10^5 cpm) was incubated while shaking for 1 hr at 22° with an aliquot of the membrane preparation (5–10 μg protein) and various amounts of unlabeled NT in 1 mL of 10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 20 μM bacitracin, 1 mM 1,10-phenanthroline, 1 mM benzamidine HCl, and 1 mM PMSF. Non-specific binding was measured in the presence of 1 μM NT. The mixtures were filtered using GF/C filter strips and a Brandell Cell Harvester and washed three times with 5 mL of ice-cold 10 mM Tris-acetate (pH 7.4). Filter-bound radioactivity was determined by γ-radiation spectrometry, and the binding data were analyzed by non-linear regression using the IN-PLOT program for curve fitting.

Growth Experiments

For the growth studies, 10^4 cells were plated per well in 24-well dishes. After the cells were allowed to attach for 24 hr, they were washed once to remove serum and then cultured in the incubator for 18 hr in medium without serum. Mixtures of NT and ISO were added to achieve the concentrations indicated. After 24 hr, solutions were aspirated and fresh medium with test mixtures was added, followed by [^{3}H]thymidine (1 μCi/well). After 18 hr, DNA was isolated, and thymidine incorporation was measured by scintillation counting [9].

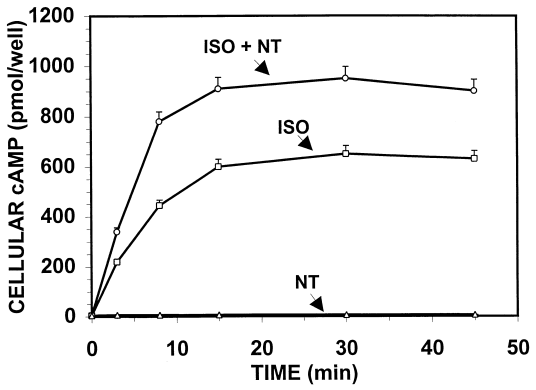

FIG. 1. Time course for effect of ISO on cAMP levels in NT-treated and control PC3 cells. Cells in 12-well dishes were pretreated at 37° with 0.9 mL of 100 nM NT or buffer for 15 sec, and then ISO was added (10 μM final concentration). Reactions were stopped at the times indicated by aspiration and TCA addition, and dishes were kept at −20° until cAMP was assayed (see Materials and Methods). Data are means ± SEM for N = 4 from a representative experiment, which was duplicated with similar results.

Statistical Analyses

The experimental values obtained were expressed as means ± SEM with N indicating the number of independent observations. Statistical analyses were performed using Student's *t*-test for single comparisons and an ANOVA with Fisher's PSLD post hoc for multiple comparisons.

RESULTS

Effect of ISO and NT on cAMP Levels

When PC3 cells were stimulated with the β-adrenergic agonist ISO (10 μM), cellular cAMP levels increased in a time-dependent manner ($T_{1/2}$ = 4.5 min), reaching a plateau after ~15 min (Fig. 1). Although 1 μM NT by itself had only a modest effect on the basal levels of cAMP after 15 min (Fig. 1: basal, 5.1 ± 0.3 pmol; NT, 6.9 ± 0.3 pmol; N = 4; $P \leq 0.05$), NT almost doubled the response to ISO throughout the time course (Fig. 1). The response to NT and ISO in combination was more than additive (i.e. synergistic; Fig. 1). NT increased the rate of cAMP accumulation (~1.7 fold) as well as the steady-state level achieved (~1.5 fold).

The results of a concentration–response study indicated that NT almost doubled the response to each concentration of ISO tested (Fig. 2). Since the EC_{50} for ISO in the presence of NT (~11 nM; Fig. 2) was similar to that in its

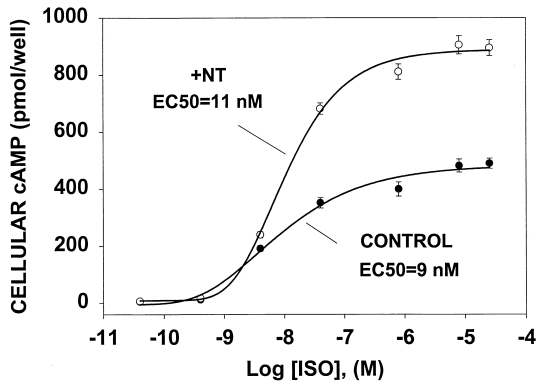

FIG. 2. Log concentration–response relationship for effect of ISO on cAMP in NT-treated and control PC3 cells. PC3 cells as in Fig. 1 were preincubated with 0.9 mL of 500 nM NT or buffer for 15 sec and then stimulated with ISO at the indicated concentrations. Reactions were stopped after 12 min, and cAMP was measured. Data are means ± SEM for N = 4 from a representative experiment, which was duplicated with similar results.

absence (~9 nM; Fig. 2), NT increased the efficacy of ISO without altering its potency.

Effects of IBMX and THEO

When cells were pretreated with the phosphodiesterase inhibitors THEO and IBMX, more cAMP was recovered; however, the response to NT expressed as percent increase was not altered (Fig. 3). In the presence and absence of these drugs, NT enhanced basal cAMP accumulation by 30–35% (Fig. 3A) and increased the response to ISO by 60–80% (Fig. 3B). Thus, the effect of NT was on cAMP formation, not on cAMP degradation.

Concentration–Response Relationship for the Effect of NT on Cells

NT caused a small (~35%) but significant elevation in the basal levels of cAMP, and the concentration–response curve gave an EC_{50} of ~1.5 nM (Fig. 4A). The effect of NT on ISO-stimulated cAMP generation was more pronounced (~80% enhancement), and the concentration–response curve gave an EC_{50} of ~0.3 nM (Fig. 4B). The slightly higher potency of NT in the presence of ISO was not due to an interaction at the NT receptor since ISO did not alter the binding of ^{125}I-labeled NT to PC3 membranes. Binding parameters for control membranes, 1.0 fmol/μg (B_{max}) and 53 pM (K_d), did not differ from those determined in the presence of 10 μM ISO, 1.0 fmol/μg (B_{max}) and 48 pM (K_d).

Effect of NT and ISO on DNA Synthesis

Consistent with its ability to generate cAMP, which is antimitogenic in PC3 cells, the exposure of PC3 cells to ISO for 2 days decreased [^{3}H]thymidine incorporation into

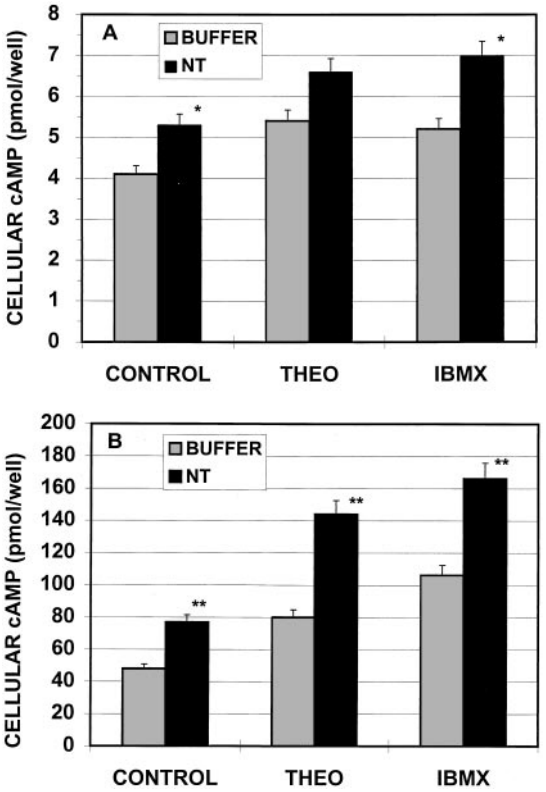

FIG. 3. Effect of NT on basal cAMP levels (A) and on the cAMP response to ISO (B) in PC3 cells pretreated with buffer or 1 mM THEO or 0.5 mM IBMX. PC3 cells were preincubated with 0.9 mL buffer or buffer containing IBMX or THEO for 15 min. NT (500 nM final concentration) was added, followed in 15 sec by 10 nM ISO. Reactions were stopped after 10 min, and cellular cAMP was measured. Data are means ± SEM for N = 4 from a representative experiment, which gave similar results when repeated. ANOVA indicated that there were significant differences. Key: (*) $P \leq 0.05$ and (**) $P \leq 0.01$ as compared with the respective control.

DNA by as much as 30 ± 4% (mean ± SEM, 6 experiments) (Fig. 5A). The response to ISO was enhanced in the presence of 100 nM NT, shifting the concentration–response curve to the left and increasing the maximal inhibition to 43 ± 5% (Fig. 5A).

When given by itself, NT (0.1 to 100 nM), which is a known mitogen, stimulated DNA synthesis (Fig. 5B). However, when NT was given in the presence of a threshold level of ISO (10 or 100 nM), which by itself had no significant effect, NT inhibited DNA synthesis (Fig. 5B). Thus, NT produced opposite effects in the absence and presence of ISO.

Since the IC_{50} for the antimitogenic effect of NT in the presence of ISO (~1 nM; Fig. 5B) was similar to its EC_{50} for enhancing cAMP generation (Fig. 4B), we hypothesized that the enhanced inhibition of DNA synthesis was mediated by the increased level of cAMP.

Effect of NT and Dibutyryl-cAMP on DNA Synthesis

To further investigate this point, we examined the effect of NT on the antimitogenic response to dibutyryl-cAMP. The

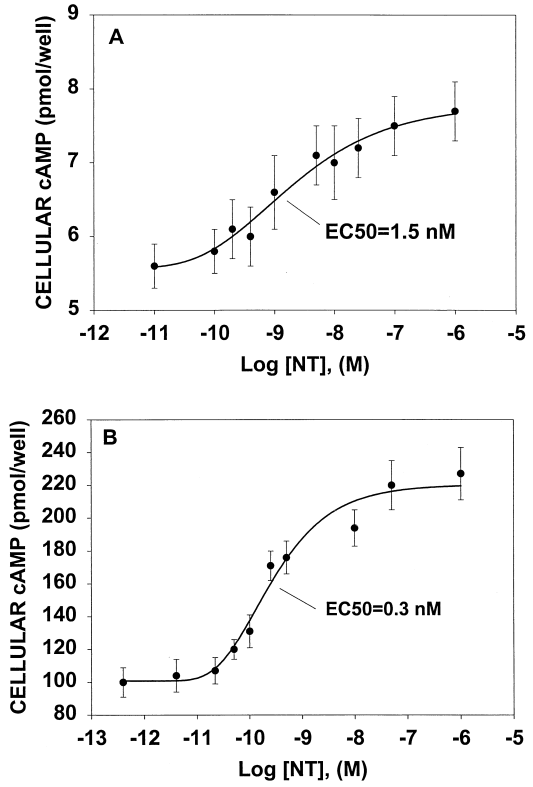

FIG. 4. Log concentration–response plots for the effect of NT on (A) basal levels of cAMP and (B) cAMP levels in response to ISO. Confluent PC3 cells were preincubated with various concentrations of NT as indicated for 15 sec followed by stimulation with 50 nM ISO. Reactions were stopped after 10 min, and cAMP was measured. Data are means ± SEM for N = 4 from a representative experiment, which was duplicated with similar results.

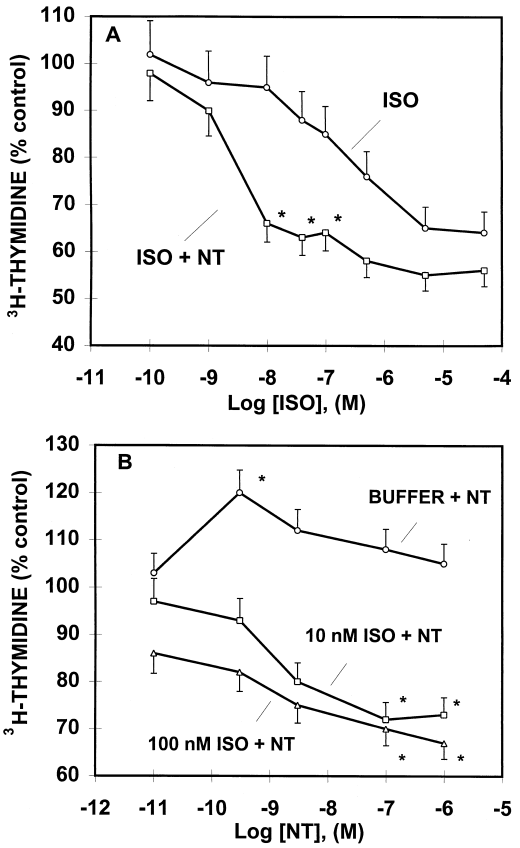

FIG. 5. Log concentration–response plots for effects of NT, ISO, and NT plus ISO on DNA synthesis in PC3 cells. (A) Inhibition of DNA synthesis by ISO in the absence and presence of 100 nM NT. (B) Stimulation of DNA synthesis by NT alone and inhibition of DNA synthesis by NT given with ISO (10 and 100 nM). Serum was withdrawn for 18 hr from cells in 24-well plates, and combinations of NT and ISO were added. After 24 hr, fresh medium with reagents was added, and cells were pulse-labeled overnight with [^{3}H]thymidine. The data, expressed as percent of control with means ± SEM for N = 4, were pooled from three to six experiments showing similar tendencies. A typical experiment for (A) gave 10,840 cpm (control) and 13,400 cpm (NT) as 100%. A typical experiment for (B) gave 9720 cpm (control), 9140 cpm (10 nM ISO), and 8450 cpm (100 nM ISO) as 100%. ANOVA showed significant differences. Key: (*) $P \leq 0.05$ as compared with the respective control.

results in Fig. 6 show that the IC_{50} for the inhibitory effect of dibutyryl-cAMP on DNA synthesis was similar in the absence (IC_{50} = 0.9 mM) and the presence of NT (IC_{50} = 0.9 mM). Thus, the ability of NT to enhance the antimitogenic response to ISO (Fig. 5, A and B) was due solely to the increased cAMP level and not to an altered responsiveness to cAMP.

DISCUSSION

This study has used the human prostate (PC3) cell line, which is known to express both NT receptor [9] and β-adrenergic receptor [15], to demonstrate that NT can potentiate cAMP generation in response to ISO. While NT itself barely increased basal cAMP levels, NT in combination with ISO had a synergistic effect, and as demonstrated here, this interaction greatly influenced the growth response to NT. Thus, NT alone was found to stimulate DNA synthesis, whereas NT in the presence of a threshold level of ISO inhibited DNA synthesis. This implies that physiologic responses to NT may depend upon the activity of catecholaminergic systems. Since NT is found co-stored with catecholamines in the CNS [11] and in adrenal medullary cells where it is released by nerve stimulation [17–19], there may be instances of coincident signaling by NT and catecholamines, the resultant of which may differ significantly from their individual effects. This phenomenon appears not be confined to the adrenergic system since NT can also potentiate cAMP generation in response to PGE_2, cholera toxin, and forskolin [20]. Thus, it is possible that NT could modulate responses to a number of monoamines and peptides whose actions involve ACs (e.g. dopamine, VIP, CCK, LH, glucagon).

The fact that NT increased the efficacy of ISO without altering its potency (Fig. 2) suggests that NT did not act on the β-adrenergic receptor itself but rather at some post-receptor step to enhance the cAMP response. This is supported by our finding that NT also potentiated cAMP

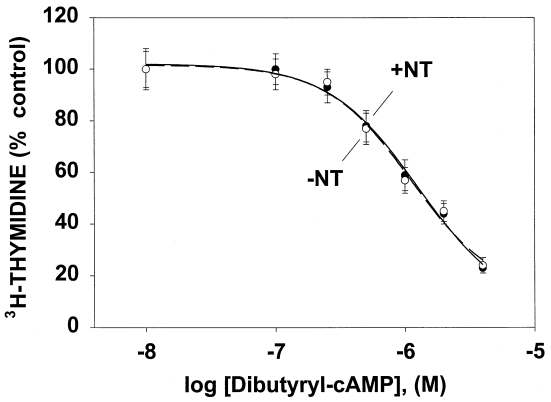

FIG. 6. Log concentration–response plots for effects of dibutyryl-cAMP on DNA synthesis in the absence and presence of NT. Cells in 24-well plates were withdrawn from serum and incubated for 24 hr with dibutyryl-cAMP alone or dibutyryl-cAMP plus 100 nM NT. Fresh medium and reagents were added, and cells were pulse-labeled overnight with [^{3}H]thymidine. The data, expressed as percent of control (means ± SEM, N = 6) were pooled from three experiments giving similar results. A typical experiment gave 23,100 cpm (control) and 25,240 cpm (NT) as 100%. ANOVA showed no significant differences in the presence and absence of NT for maximal inhibition or IC_{50}.

formation in response to a number of other agents, including both direct (forskolin) and indirect (PGE_2, cholera toxin) activators of AC [20]. That NT acted primarily on the rate of cAMP formation and not on cAMP degradation was evidenced by the inability of phosphodiesterase inhibitors to diminish its effect (Fig. 3). Further support for this contention is provided by measurements of cAMP decay rates after blocking the action of ISO with propranolol [20]. Although NT increased the responsiveness of AC(s) to a variety of activators including ISO, NT alone had little ability to activate the enzyme (Fig. 1). Interestingly, the activity of two of the nine known isoforms of AC (AC-II and AC-IV) is subject to modulation in a similar fashion by G-protein βγ subunits [21] and activators of protein kinase C [22]. Thus, determining which AC isoforms are expressed in PC3 cells may help us to delineate the mechanism(s) involved in the action of NT.

Other studies by us suggest that NT may work by facilitating the activation of Ca^{2+}-dependent ACs. First, we found that NT transiently elevates cytosolic $[Ca^{2+}]_i$ in PC3 cells,* as has been shown in some other cell lines [8, 23]. Second, the cAMP-enhancing effect of NT was blocked by inhibiting phospholipase C and was inhibited concentration-dependently by buffering the rise in $[Ca^{2+}]_i$ using a chelator. Furthermore, elevators of $[Ca^{2+}]_i$ such as ionomycin and thapsigargin mimicked the action of NT [20]. Whether the NT-stimulated rise in $[Ca^{2+}]_i$ is associated with an activation of protein kinase C is not known. However, activation of protein kinase C using phorbol myristic acid was found to enhance cAMP responses to ISO and forskolin in PC3 cells.†

In regard to PC3 cell growth, the stimulating effect seen here for NT is in keeping with our earlier work using these cells [9] and studies by others using small-cell lung cancer cells [7], prostate cancer LnCaP cells [24], and pancreatic cancer MIA PaCa-2 cells [8]. In several of these systems including PC3 cells, a bell-shaped concentration–response relationship was observed for the growth-promoting effect of NT. It has been proposed that the inhibition of the positive growth effect of NT in MIA PaCa-2 cells at the high end of the concentration–response curve may be due to the increasing levels of cAMP [8]. This seems unlikely in PC3 cells, where NT alone showed little ability to generate cAMP (Fig. 1), and a more likely explanation is that desensitization and/or down-regulation of NT receptors inhibited the growth response.

Work by others using PC3 or PC3M cells has shown that dibutyryl-cAMP can inhibit DNA synthesis, cell growth [16], and clonogenicity [25]. Consistent with this, the present results show concentration-dependent inhibition of DNA synthesis by ISO (Fig. 5A) at concentrations shown to generate increased amounts of cAMP (Fig. 2). Although higher concentrations of ISO were needed to inhibit DNA synthesis (IC_{50}, ~150 nM) than to elevate cellular cAMP (EC_{50}, ~10 nM), this discrepancy may be attributable to the known lability of catechols and the 200-fold difference in time course for these responses. That cAMP mediated the antimitogenic effect of ISO was supported by the fact that NT enhanced both cAMP generation and growth inhibition with similar concentration dependences (compare Figs. 4B and 5B). The fact that NT did not alter the response to dibutyryl-cAMP (Fig. 6) indicated that NT modulated DNA synthesis by elevating cAMP levels and not by enhancing the responsiveness to cAMP. Having shown that prostate cancer cells treated with dibutyryl-cAMP undergo terminal differentiation, Bang *et al.* [25] suggested that hydrolysis-resistant cAMP analogues may be useful in the treatment of metastatic prostate cancer. The results presented here indicate that investigating the use of Ca^{2+}-elevating agents in combination with stimulators of cAMP formation might also be worthwhile.

In the present study, NT not only potentiated the action of a β-agonist on cAMP generation but it also caused a 30–40% elevation in the basal levels of cAMP. The fact that an identical effect can be induced by ionomycin and thapsigargin suggests that this action is also mediated by the rise in $[Ca^{2+}]_i$, although work by others suggests that NT receptors in some systems can couple to G_s [26]. Presumably, cAMP formation under basal conditions in PC3 cells is driven at least partly by endogenous AC activator(s) whose action(s) is Ca^{2+}-dependent.

This is the first report describing a synergistic effect of NT on cAMP generation. Previous work has shown that NT can stimulate or inhibit cAMP accumulation depend-

*Mitra SP, Yamaguchi H and Carraway RE, Manuscript in preparation.

†Carraway RE, unpublished results.

ing on the cell system used. Whereas NT stimulated cAMP formation in pancreatic MIA-PaCa-2 cells [8] and in CHO cells transfected with the NT1 receptor [26], NT inhibited the rise in cAMP levels in response to forskolin [27] and PGE_2 [28] in neuroblastoma N1E115 cells. On the other hand, NT had little effect on cAMP levels in colon cancer HT29 cells [29]. Whether these differences reflect the expression of different NT receptors or arise from variations in the associated G-proteins or second messenger pathways is not known. At this point, two NT receptor isoforms have been identified and cloned [30, 31]. Although PC3 cells were shown by mRNA analysis to express the NT1 receptor [9], it is still possible that other NT receptors are present. The binding properties of NT receptors in PC3 cells [9] resemble those reported for the NT1 receptor [30] and differ from those of the NT2 receptor, which exhibits a lower affinity and recognizes levocabastine [32]. Thus, the current evidence would suggest that the synergistic response observed here is likely to be a property of the NT1 receptor.

In regard to other pharmacologic and physiologic responses to NT, the present finding that NT synergizes with a cAMP-generating agent suggests that it may also be capable of interacting with endogenous regulators that utilize the cAMP signaling pathway. Examples that may represent instances of cAMP synergy include the following: (a) NT potentiates the effects of cholinergic stimulation on pancreatic exocrine secretion [33]; (b) NT modulates dopamine-feedback inhibition of neuronal firing [34]; and (c) NT modulates glucagon and insulin secretion in a glucose-dependent manner [35]. Based on the current study, it might be worthwhile to examine further the mechanisms of these effects.

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