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13. ABSTRACT (Maximum 200 Words)
The aims are (a) to determine the mechanism by which neurotensin (NT) enhances prostate cancer growth and (b) to test if NT released by eating contributes to cancer-promoting effects of high fat diets. Experiments show that NT transactivated the EGF receptor by liberating HB-EGF form the cell-surface by a metallo-protease-dependent and PKC-dependent mechanism. Activation of EGFR was followed by activation of MAP-kinase, PI3-kinase, AKT and p70-S6-kinase, and in 24 hrs by a stimulation of DNA synthesis. We found that NT and EGF stimulated 3H-AA release form PC3 cells and that lipoxygenase (LOX) inhibitors blocked basal and NT-induced DNA synthesis. This suggests that EGF and NT act by way of PLA2 f-LOX and 12-LOX. Cell growth responses depend on the hormonal milieu. In the presence of a Gs stimulus, NT enhances cAMP production and his results in an inhibition of DNA synthesis. Thus, NT effects involve actions on protein kinase, LOX, EGFR, and cross-talk with adenylyl cyclases. Drug effects on NT-receptor function include calcium channel blockers, such as dihydropyridines, which indirectly block the first step in the signaling pathway, the formation of inositol phosphates. The mechanism is under study.

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Table of Contents

Introduction-

The overall goal of this work is to examine the involvement of the intestinal hormone neurotensin (NT) in the regulation of prostate cancer cell growth *in vivo*. The initial hypothesis under study is that NT, which is released from the intestine by ingestion of fatty meals, contributes to the enhanced growth of prostate cancer cells in animals consuming high fat food, and that the effects of NT may explain the worldwide association between high fat intake and growth / incidence of prostate cancers in humans. Since NT has been shown to exert important effects on growth of both androgen-independent (PC3) and androgen-dependent (LNCaP) human prostate cancer cells *in vitro*, a key goal is to understand the mechanism(s) involved in its growth promoting effect(s) in these two cell lines (1).

To examine the mechanism(s) involved in NT-induced growth promotion, we have performed experiments using PC3 and LNCaP cells in tissue culture, and we studied the interaction of NT with major growth factor pathways. We have characterized NT binding to the cells and examined its regulation. Of particular interest are the effects of NT on the EGF pathway but TGFβ and PDGF may also be examined. Attention has been given to growth regulation by the MAPK-, Akt- and CDK-pathways. We have identified NT inputs into the phosphorylation of the key enzymes in these pathways and we have investigated relationships to inositol phosphate (IP) formation, Ca^{2+}-release, Ca^{2+}-influx, PKC-activation and cAMP formation (2, 3).

To examine the contribution of NT to prostate cancer cell growth *in vivo*, we have utilized an animal model involving PC3 cells xenografted into nude mice. We also developed an NT knockout-nude mouse to address this issue. Some animals are maintained on high and low fat diets and the effects of NT antagonist SR48692 on tumor growth are also being measured.

Body

Task #1—To compare the effects of NT antagonist on the growth of PC3 and LNCaP cells in nude mice (months 1-30).

Initial studies were performed using PC3 cells and the preliminary data showed that daily injections of NT antagonist SR48692 slowed the growth rate of the PC3 tumors in nude mice. However, we were not satisfied with the test system and we thought it best to improve on the method. Previously, we described our quandary as to whether xenografting was best achieved by the injection of cancer cells or the implantation of cubes of tumor tissue using a trocar syringe. In our last report, we indicated that we would receive instruction from another cancer laboratory (Dr. Timothy Wang, Dept of Gastroenterology, UMMS) where the cell injection method was optimized to avoid the difficulty of multiple tumor development. We did this and decided that we would apply the techniques that we learned to perform an experiment measuring tumor growth

rates in NT knockout-nude mice as compared to nude mice controls fed high fat food.

Fourteen knockout mice and thirteen controls were inoculated with tumor cells that were settled as a mass into the end of a 26-gauge needle. By injecting them as a clump, we intended to favor the formation of a single tumor. Although single tumors were obtained, it took 2 months before they were evident and the entire experiment has lasted for 6 months. On the positive side, the results were encouraging. Tumor growth rates, measured weekly, were less at all times for the NT knockout mice than for the controls. For example, on 6-20-03 tumor volumes for NT knockout and controls were 7.2±1.9 and 11.9±2.5 mm^3; on 8-01-03 they were 61±26 and 129±58; on 8-29-03 they were 64±19 and 102±33 mm^3. Although statistical significance was not reached, there was a clear trend to the measurements obtained in this experiment. We now plan to perform additional experiments to confirm this difference. As animals became moribund, tumors were collected and blood was sampled for analyses by histological, radioreceptor and radioimmunoassay methods and these results are not yet available.

Since the slow tumor development greatly increased the cost of animal, we used tumor cubes in the next experiment. We learned the trocar syringe method and found that it could be done on nude mice with minimal stress and injury. Donor mice (wild type nude) were chosen from the earlier described experiment and we implanted 2 tumor cubes (3 mm^3) at separate sites in 12 nude mice and 12 NT knockout nude mice. We hope that tumor development will proceed quicker with this method and that the variability in tumor size will also be less.

Task #2—Perform dietary studies to correlate NT levels with fat intake and cancer cell growth (months 18-36).

These studies will be done in the final year of this project.

Task #3—To develop NT knock-out / nude mouse strain from our NT knock-out and nu/nu purchased from Jackson Labs (months 1-24).

As reported earlier, we have succeeded in accomplishing this task.

Task #4—Test effect of fat on cancer growth in NT-knockout/nude mice (months 16-36).

These studies will be performed in the final year of the project.

Task #5—Examine effects of NT on arachidonic acid metabolism and on lipoxygenase expression in prostate cancer cells (months 1-12).

We have extended the observations reported earlier regarding the effects of NT on arachidonic acid (AA) metabolism. NT and EGF were previously shown to liberate [3H]-AA from labeled PC3 cells and the effect of NT was inhibited by PKC inhibitor (staurosporine), PI3-kinase inhibitor (LY294002 and wortmannin), MEK inhibitor (U0126), calcium channel blocker (nifedipine) and EGFR-tyrosine kinase inhibitor (AG1478). These results supported the idea that NT activated MAP-kinase and that this enzyme, which is known to activate PLA2, liberates AA

from the phospholipid pool. Now, we have shown by western blotting that PC3 cells express cPLA2 but not sPLA2 and iPLA2. In addition, the cells expressed 5-lipoxygense and 12-lipoxygenase but not 15-lipoxygenase. These results are consistent with the pattern of ^{3}H-AA metabolites detected after NT treatments.

Task #6- Test NT for ability to alter growth responses to EGF in PC3 cells (months 13-24).

As reported earlier, we have completed most of this task. In additional studies, we have now shown that NT stimulates PKC-dependent PKD activation in PC3 cells. EGF also activated PKD and our results thus far are consistent with the idea that NT acts by transactivating the EGF-receptor. It may be that an AA-metabolite mediates the activation of PKC.

Other work shows that NT-induced EGFR/AKT/MAPK activation and DNA synthesis was attenuated by metallo-endopeptidase inhibitor phosphoramidon. This is in agreement with the involvement of HB-EGF in the NT pathway. Using western blotting, we also showed that NT activates p70 S6-kinase which is downstream of AKT and MAPK and a reliable indicator of mitogenesis.

In the course of investigating the Ca^{2+} dependence of NT signaling, we also discovered that L-type Ca^{2+}-channel blockers enhanced NT binding and inhibited NT-induced IP formation. This is discussed at length under "task #7".

Task #7—Test NT for ability to activate MAP-kinases and to induce indicators of mitogenic stimulation (months 25-36).

In the last report, we indicated that NT dose-responsively (0.1 to 30 nM) enhanced the activation (phosphorylation) of EGFR, ERK1/2 and Akt, but it did not affect JNK/SAPK and p38 MAPK. The NT-induced transactivation of EGFR and the enhancement in DNA synthesis that followed were blocked by heparin (10μg/ml) but were unaffected by neutralizing anti-EGF (10μg/ml), indicating the involvement of HB-EGF instead of EGF itself. Since HB-EGF can be liberated from the cell surface by PKC-dependent steps, we examined the involvement of PKC. The EGFR transactivation, the ERK activation and the stimulation of DNA synthesis in response to NT were all blocked by PKC inhibitor (staurosporine) and by downregulation of PKC, indicating a mediator role for PKC. Our model at this time was that NT interacated with its Gq-coupled receptor to stimulate PLC-mediated formation of inositol phosphates (which elevated Ca^{2+}) and DAG (which activated PKC). PKC then releases HB-EGF, activating the EGFR-MAP-kinase cascade and stimulating DNA synthesis.

Although NT-induced EGFR transactivation and ERK phosphorylation were not blocked by intracellular Ca^{2+}-chelator (BAPTA-AM), nor by extracellular chelator (EGTA), these effects were inhibited by Ca^{2+}-channel blockers (e.g., nifedipine). To better understand the mechanism(s) involved, we screened

numerous Ca^{2+}-channel blockers for effects on NT-receptor binding and on NT-induced inositol phosphate (IP) formation. We hypothesized that Ca^{2+}-channels might somehow interact directly with NT-receptors. Ca^{2+}-channel blockers from 7 chemical categories altered NT binding and NT-induced IP formation. 1,4-dihydropyridines, which are specific for L-type Ca^{2+}-channels, were the most effective agents; however, their effects correlated best to inhibition of store-operated Ca^{2+}-channels (SOCC). Since NT is known to empty internal Ca^{2+}-stores, we hypothesized that it stimulated Ca^{2+}-influx through SOCC. We then showed that NT caused an influx of $^{45}Ca^{2+}$ that was blocked by nifedipine. The results of further studies were in keeping with the idea that IP formation in response to NT involved both PLCβ and PLCδ. NT initially stimulated PLCβ which (via IP3) emptied internal Ca^{2+} stores and stimulated SOCC-mediated Ca^{2+}-influx. The Ca^{2+}-influx then activated PLCδ, generating further IP. This is the subject of a manuscript accepted for publication in JPET.

1,4-dihydropyridines also exerted major effects on NT-receptor binding (3-fold enhancement) which we are still sorting out. Our current findings are in keeping with the idea that this effect results from an indirect antioxidative action. These drugs do not alter NT-receptor binding to cellular membranes, indicating a requirement for cellular metabolism or architecture. Enhanced binding was associated with decreased function. Interestingly, the EGF-receptor displays a similar behavior in that Tyr-kinase inhibitors block function while greatly enhancing binding. For NT-receptor, there is a 3-fold shift in receptor affinity without a change in receptor number or receptor internalization. The effects of a series of 1,4-dihydropyridines correlates to their ability to react with superoxide anion. Furthermore, some polyphenolic antioxidants could mimic these effects. This is the subject of a manuscript submitted to JPET.

To follow this up, we are currently investigating the effects of Ca^{2+}-channel blockers on cell growth induced by NT, EGF and serum. We are pursuing this line of research since it is likely to reveal key steps in the NT-signaling cascade. One hypothesis, for example, is that NT-receptor activates NADH-oxidase in the plasma membrane, generating reactive oxygen species (ROS). Since ROS (e.g., superoxide) can transactivate EGF-receptor, it is possible that 1,4-dihydropyridines inhibit this step by scavenging ROS. ROS can also activate certain isoforms of PKC, and PKC can activate the MAP-kinase pathway. Since NT-receptor binding is desensitized by PKC, this provides a feedback loop that could explain how NT binding could be increased in the face of inhibited effects. There is precedence for the involvement of NADH-oxidase in growth promotion and other actions of angiotensin-II on vascular cells in culture.

KEY RESEARCH ACCOMPLISHMENTS-

- **Developed NT-knockout/nude mouse strain.**

- **Demonstrated that NT stimulated DNA synthesis in PC3 and LNCaP cells at near physiologic concentrations (0.1 to 1 nM range).**
- **Demonstrated that NT transactivated EGFR in PC3 and in LNCaP cells.**
- **Demonstrated that formation of HB-EGF in response to NT led to the activation of EGFR.**
- **Demonstrated that NT activated ERK in PC3 and in LNCaP cells.**
- **Demonstrated that NT activated AKT (PI3-kinase) in PC3 cells.**
- **Demonstrated that NT-induced activation of EGFR, ERK and AKT in PC3 cells was Ca^{2+}-independent, PKC-dependent and contact inhibited.**
- **Demonstrated that cAMP inhibited NT-induced activation of ERK, and that NT could enhance cAMP formation in response to Gs-stimuli.**
- **Demonstrated ^{125}I-NT binding to PC3 cells and the presence of NTR1 by western blotting. Related the binding Kd to the EC50 for IP-formation and to activation of EGFR and ERK.**
- **Demonstrated the absence of NTR1 in LNCaP cells by western blotting and the inability of LNCaP to bind ^{125}I-NT and to respond to NT by increasing IP-formation.**

NEW ACCOMPLISHMENTS THIS PERIOD

- **Measured slower tumor growth rates in NT knockout-nude mice as compared to controls.**
- **Demonstrated NT effects on AA-metabolism involving LOX-pathway are important for growth promotion.**
- **Demonstrated Effects of Ca^{2+}-channel blockers on NT-receptor binding and IP formation.**
- **Demonstrated Antioxidative mechanism for regulation of NT-binding and IP formation.**
- **Showed PC3 cell expression of cPLA2, 5-LOX and 12-LOX.**
- **Showed PKC-dependent PKD activation by NT and by EGF.**
- **Showed p70 S6-kinase activation by NT.**
- **Showed metallo-protease involvement in NT-induced EGFR activation.**

REPORTABLE OUTCOMES:

Abstracts have been written and presented at meetings:

1.- S. Hassan and R.E. Carraway. Involvement of MAP-kinase, PI3-kinase and EGF receptor in the Stimulatory Effect of Neurotensin on DNA-replication in PC3 Cells. Reg Pep 2002 Meeting, Boston MA, Aug 31 - Sept 3,2002.

2.- R.E. Carraway, S. Hassan, P.R. Dobner. Positive and Negative Effects of Neurotensin on DNA synthesis in PC3 cells Mediated by EGF receptor and Adenylyl Cyclase, respectively. 9th Prouts Prostate Cancer Meeting, Nov 7-10, 2002.

3. S. Hassan and RE Carraway. Signaling mechanisms involved in the acativation of arachidonic acid metabolism in PC3 cell growth by neurotensin and its interaction with epidermal growth factor receptor. Summer Neuropeptide Conference, June 8-12, 2003. (Neuropeptides 37(2003) 159-199.

Manuscripts are in preparation:

1.- S. Hassan and R.E. Carraway. Involvement of EGF-receptor, MAP-kinase and PI3-kinase in the Stimulatory Effect of Neurotensin on DNA-replication in PC3 Cells. Submitted to *Molecular Cancer Research,* September, 2003.

2.- R.E. Carraway, X. Gui and D.E. Cochrane. Ca^{2+}-channel Blockers Enhance Neurotensin (NT) Binding and Inhibit NT-induced Inositol Phosphate Formation in Prostate Cancer PC3 Cells. *J Pharmacol Exp Therap* , accepted for publication 2003.

3.- RE Carraway, S Hassan and DE Cochrane. 1,4 Dihydropyridines Alter Neurotensin Receptor Function in PC3 Cells Primarily By An Antioxidative Mechanism. Submitted to *J Pharmacol Exp Therap,* September, 2003.

4.- RE Carraway, M Sanderson and DE Cochrane. Role of Ca^{2+} and PKC in the cAMP-enhancing Effect of Neurotensin in PC3 cells. *in preparation.*

5.- R.E. Carraway and S. Hassan. Role of Lipoxygenase-mediated Arachidonic Acid Metabolism in PC3 Cell Growth Responses to EGF and Neurotensin. *in preparation*

6.- R.E. Carraway and S. Hassan. Role of PKC in ^{125}I-neurotensin (NT) Binding and Biologic Effects leading to Growth of Prostate Cancer PC3 cells. *in preparation*

CONCLUSIONS:

The importance of the completed work is in regards to the mechanism by which NT enhances the growth of prostate PC3 and LNCaP cells in culture. Our work indicates that in PC3 cells, NT binds to NTR1, transactivating EGFR by liberating HB-EGF from the cell-surface by a metallo-protease-dependent and PKC-dependent mechanism. In LNCaP, the mechanism is PKC-independent and a different receptor may be involved. Temporally associated with activation of EGFR is an activation of MAP-kinase, PI3-kinase and AKT, which is followed a day later by a stimulation of DNA synthesis. These responses depend on the hormonal milieu. In the presence of a Gs-stimulus, NT can enhance cAMP formation, resulting in an inhibition of DNA synthesis. Since we found that LOX inhibitors can reduce basal and NT-stimulated DNA replication, it seems likely that NT & EGF both act by way of PLA2, 5-LOX and 12-LOX. Thus, growth

enhancement by NT & EGF involves a coordinate enhancement of LOX(s) and protein kinase(s), and the growth response can be influenced by the activity of adenylyl cyclase(s).

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Abstracts

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potency of galanin was 100-fold higher in SY5Y/GalR2 (EC_{50} 1.1 nM) than in SY5Y/GalR1 cells (EC_{50} 190 nM). In SY5Y/GalR2 cells activation of receptor expression and exposure to galanin resulted in apparent morphological changes which were comparable to apoptosis. Induction of cell death in these cells by the apoptotic process was supported by Poly-(ADP-ribose)-polymerase cleavage, caspase-3 activation, and by the typical laddering of DNA. The controlled expression of GalR2 receptors in neuroblastoma xenografts will elucidate whether GAL has an antiproliferative role on neuroblastoma development in vivo and if GAL receptors could serve as therapeutical targets.

These studies were supported by a grant of the Austrian Science Foundation (P15415) the Medizinische Forschungsgesellschaft Salzburg and the Children's Cancer Foundation Salzburg.

Neurotensin (NT) potentiates the mitogenic effects of insulin and epidermal growth factor (EGF) in human embryonic lung fibroblasts

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Neurotensin (NT) is a neuroendocrine peptide identified in brain, gut, peripheral nerves and in several endocrine organs. NT receptors (NTR) are found in a variety of tissues and cell types, including some cancer cell lines, where NT induces proliferation. Whether it has similar proliferative effects in non-cancerous cells is unclear. Here we report that NT significantly potentiates the mitogenic effects of insulin (I) and EGF in human lung fibroblasts (IMR90), without itself causing mitogenesis. IMR90 cells were cultured under standard conditions and the incorporation of [^{3}H]thymidine used to measure proliferation. NT (100 nM) induced an ~25% increase in proliferation versus I alone. SR48692, a NTR type 1 (NTR1) antagonist blocked this increase. The PLC inhibitor, U73122, also blocked this potentiation, without inhibiting the mitogenic effects of I. Maximum potentiation was seen at 100 nM NT. Addition of the NTR type 2 (NTR2) antagonist, levocabastine, restored the potentiating effect of NT at concentrations over 100 nM. The EGF receptor tyrosine kinase inhibitor, AG1478, had no effect on the mitogenic effect of I or the potentiating effect of NT. Addition of the p70s6k inhibitor, rapamycin, partially inhibited I-dependent proliferation, but did not effect NT's ability to potentiate this diminished proliferation. NT also potentiated the mitogenic effects of EGF in a dose-dependent manner, with a maximal increase of ~80% for 10 μM NT. U73122 did not inhibit EGF or the potentiating effects of NT. Addition of rapamycin partially inhibited EGF-dependent proliferation, but did not effect NT's ability to potentiate. The effect of NT on EGF and I is rapid (a 1-min exposure to NT is sufficient) and lasts for 2 h. These results suggest an interplay between NTR's and the signaling cascades induced by EGF and I.

Signaling mechanisms involved in the activation of arachidonic acid metabolism in PC3 cell growth by neurotensin and its interaction with epidermal growth factor receptor

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The consumption of excess dietary fat is associated with an increased risk of prostate cancer (PC). This may be due to elevation of arachidonic acid (AA) levels since metabolism of AA by cyclooxygenase (COX) and lipoxygenase (LOX) generates eicosanoids implicated in the pathogenesis of PC. Neurotensin (NT), a growth promoting gut hormone released by fat, stimulates leukotriene formation in animals. This led us to investigate the involvement of AA metabolism in the growth-promoting effect of NT in PC3 cells and the relationship to EGFR and the MAPK pathways. Both NT and EGF dose-dependently release [^{3}H]AA from labeled PC3 cells and this was prevented by specific EGFR tyrosine-kinase inhibitors AG1478 and PD153035. Cell proliferation was measured by [^{3}H]thymidine incorporation, MAPK activation was measured by Western blot analysis, and release of EGF-like substance was measured by EGFR binding assay. NT (0.1–30 nM) stimulated time-dependent (1–12 min) phosphorylation of EGFR, MAPK and Akt/PKB, followed by release of EGF-like substance and an increase in cell DNA synthesis. PLA2 inhibitor arachidonyltrifluoromethyl ketone (AACOF3) diminished the NT effects on MAPK activation and DNA synthesis. Non-selective inhibitors of LOX nordihydroguaiaretic acid (NDGA) and 5,8,11,14-eicosatetranoic acid (ETYA), 5-LOX activating protein (FLAP) inhibitor MK886, selective 5-LOX inhibitors Rev 5901 and AA861, and 12-LOX inhibitor baicalein significantly inhibited NT- and EGF-induced MAPK activation and DNA synthesis. COX inhibitor indomethacin had no effect. Radiolabeled products from [^{3}H]AA incubated with PC3 cells in the presence and absence of NT were extracted and separated by C18 reverse phase HPLC using 15–100% methanol gradient. NT caused a 3-fold enhancement of radioactivity eluting at the positions of LOX products and AA, identified by coelution with the standards. Based on these data, we propose a model whereby NT-induced activation of MAPK and DNA synthesis in PC3 cells is mediated thorough transactivation of EGFR followed by stimulation of PLA_2, AA release and generation of AA derivatives in PC3 cells.

The substance P receptor, neurokinin 1, is transiently expressed by cholinergic amacrine cells in the early postnatal rabbit retina

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In the mature rabbit retina, neurokinin 1 (NK1) receptors are expressed by a population of ON-type cone bipolar cells and by the population of tyrosine hydroxylase (TH) containing, dopaminergic amacrine cells. We studied the developmental expression of NK1 receptors in postnatal rabbit retinas. Double label immunofluorescence experiments showed

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Ca^{2+} Channel Blockers Enhance Neurotensin (NT) Binding and Inhibit NT-Induced Inositol Phosphate Formation in Prostate Cancer PC3 Cells

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ABSTRACT

Neurotensin (NT) stimulates Ca^{2+} release and Ca^{2+} influx in many cells. Its contractile effects in smooth muscle are inhibited by removal of Ca^{2+} and by Ca^{2+} channel blockers (CCBs). To better understand NT signaling in prostate cancer PC3 cells, blockers of voltage-gated and store-operated Ca^{2+} channels (VGCC and SOCC) were tested for effects on NT-binding and signaling. Eight chemical types of agents, including VGCC-blocker nifedipine and SOCC-blocker SKF-96365 (1-[β-[3-(4-methoxyphenyl)-propoxy]-4-methoxyphenyl]-1*H*-imidazole), enhanced cellular NT binding up to 3-fold, while inhibiting (by ≅70%) NT-induced inositol phosphate (IP) formation. The ability to enhance NT binding correlated with the ability to inhibit NT-induced IP formation, and both effects were relatively specific for NT. Although cellular binding for β_2-adrenergic, V_{1a}-vasopressin, and epidermal growth factor receptors was not enhanced by these drugs, bombesin receptor binding was increased ≅19% and bombesin-induced IP formation was inhibited ≅15%. One difference was that the effect on NT binding was Ca^{2+}-independent, whereas the effect on IP formation was Ca^{2+}-dependent (in part). The Ca^{2+}-dependent part of the IP response seemed to involve SOCC-mediated Ca^{2+} influx to activate phospholipase C (PLC)δ, while the Ca^{2+}-independent part probably involved PLCβ. Photoaffinity labeling of the NT receptor NTR1 was enhanced in CCB-treated cells. NTR1 affinity was increased but NTR1 number and internalization were unchanged. Since CCBs did not alter NT binding to isolated cell membranes, the effects in live cells were indirect. These results suggest that CCBs exert two effects: 1) they inhibit NT-induced IP formation, perhaps by preventing Ca^{2+} influx-dependent activation of PLCδ; and 2) they enhance NTR1 affinity by an unexplained Ca^{2+}-independent mechanism.

Neurotensin (NT), a peptide found primarily in brain and intestine, exerts many effects (Ferris, 1989; Rostene and Alexander, 1997) by activating type 1, G-protein-linked NT receptor NTR1 (Vincent et al., 1999). NTR1 is present on excitable cells (neuroendocrine, smooth muscle) and nonexcitable cells (epithelial, fibroblast) where it activates multiple signaling pathways (Hermans and Maloteaux, 1998). NTR1 is coupled to $G_{q/11}$ since NT stimulates phosphatidylinositol-specific phospholipase C (PLC)-mediated formation of inositol phosphate (IP) and release of intracellular Ca^{2+}. NT also induces Ca^{2+} influx into excitable (Trudeau, 2000) and nonexcitable (Gailly, 1998) cells.

Some actions of NT depend on extracellular [Ca^{2+}] and are inhibited by Ca^{2+} channel blockers (CCBs). Based on the effects of 1,4-dihydropyridines (DHPs) such as nifedipine (NIF), Donoso et al. (1986) and Mule and Serio (1997) suggested that NT-induced intestinal contraction involved Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCC). However, in some systems, VGCC current is inhibited by NT (Belmeguenai et al., 2002), and DHPs inhibit NT effects independently of Ca^{2+} (Golba et al., 1995). These contradictory findings led us to investigate the effects of CCBs on NT

This work was supported by Department of Defense (DOD) Grant DAMD17-00-1-0528 and by National Institutes of Health (NIH) Center Grant 5P30-DK32520, although the opinions expressed in the manuscript are not necessarily those of the DOD or the NIH. Part of this material was presented as Abstract P3-576 at the 81st Annual Meeting of the Endocrine Society in June, 1999.

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ABBREVIATIONS: NT, neurotensin; NTR1, type 1 NT receptor; PLC, phospholipase C; IP, inositol phosphate; CCBs, Ca^{2+} channel blockers; DHP, dihydropyridine; NIF, nifedipine; VGCC, voltage-gated Ca^{2+} channel; SOCC; store-operated calcium channel; HOLVA, des-Gly-[$Phaa^1$, D-$Tyr(Et)^2$,Lys^6,Arg^8]-vasopressin; SKF-96365, 1-[β-[3-(4-methoxyphenyl)-propoxy]-4-methoxyphenyl]-1*H*-imidazole; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PMSF, phenylmethyl sulfonylfluoride; EGFR, EGF receptor; GPCR, G-protein-coupled receptor; SR48692, {2-[(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3-yl)carbonylamino]tricyclo(3.3.1.1.^{3,7})decan-2-carboxylic acid}; AG-1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline; PD-153035, 4-[(3-bromophenyl)amino]6,7-dimethoxyquinazoline.

binding and signaling in prostate cancer PC3 cells, which express functional NTR1 (Seethalakshmi et al., 1997). We hypothesized that CCBs could exert effects at multiple levels of the signaling pathway.

Our studies in PC3 cells indicate that NTR1 is linked to $G_{q/11}$ and that stimulation by NT activates PLC, enhances IP formation, and elevates cellular [Ca^{2+}]. This signaling pathway contributes to the regulation of cellular growth by NT (Seethalakshmi et al., 1997) and is linked to a conditional activation of adenylyl cyclase (Carraway and Mitra, 1998). Ca^{2+} is required for NT to stimulate DNA synthesis and to enhance cAMP formation, and these effects are inhibited by the VGCC blocker NIF (R. E. Carraway, unpublished results). Although this suggests that Ca^{2+} influx participates in NT signaling, the roles of Ca^{2+} channels, Na^+/Ca^{2+} exchange, and Ca^{2+} pumps are not defined. The inhibitory effects of NIF implicate VGCC in NT signaling, but this must be questioned since PC3 cells are epithelial and nonexcitable (Putney and Bird, 1993). Another process, which occurs in excitable and nonexcitable cells (Parekh, 2003) subsequent to Ca^{2+} store emptying (Putney, 1999), is capacitative Ca^{2+} entry through store-operated Ca^{2+} channels (SOCC). Given that NT stimulates capacitative Ca^{2+} entry in Chinese hamster ovary cells (Gailly, 1998) and that NT elevates cellular [Ca^{2+}] in PC3 cells (R. E.Carraway, unpublished results), it seems likely that NT stimulates SOCC-mediated Ca^{2+} influx in PC3 cells. Thus, additional studies are required to determine whether NT induces Ca^{2+} influx and to define the channels and mechanisms involved.

Our ability to distinguish mechanisms of Ca^{2+} entry depends largely on the selectivity of CCBs (Harper and Daly, 1999; Triggle, 1999). Based on its possible relevance to NT signaling as discussed above, we focus the following discussion on blockers of L-type VGCC and SOCC. Blockers of VGCC include DHPs (e.g., NIF), phenylalkylamines (e.g., verapamil), and benzothiazepines (e.g., diltiazem). Inhibitors of SOCC include imidazoles (e.g., SKF-96365) and tricyclics (e.g., trifluoperazine). Unfortunately, these agents exhibit some nonspecificity, and their actions can be complex (Harper et al., 2003; Triggle, 2003). Although selective for VGCC at nanomolar levels, DHPs inhibit capacitive Ca^{2+} entry in the micromolar range (Harper et al., 2003). Ligand-gated ion channels are also targets of DHPs. Ca^{2+} influx involving the *N*-methyl-D-aspartate receptor was inhibited by 1 to 10 μM nitrendipine (Skeen et al., 1993), nicotinic acetylcholine receptor-induced currents were abolished by 10 μM NIF (Lopez et al., 1993), and the 5-hydroxytryptamine receptor was inhibited by 10 μM nimodipine (Hargreaves et al., 1996). These findings attest to the need to examine multiple agents and to assess the effects on each step leading to downstream events. Yet, in performing experiments to order signaling steps, it is often assumed that the agents tested (e.g., CCBs) do not alter the agonist-receptor interaction. Even when this is examined, cellular membranes are commonly used, which provides a limited assessment. These considerations have led us to focus attention on the early steps in the signaling pathway, NT binding, and NT-induced IP formation in live cells.

The current study investigates the effects of CCBs on NT binding and signal transduction in PC3 cells. Screening ion channel agents for effects on NT signaling, we unexpectedly find that VGCC and SOCC blockers dramatically enhance NT binding and cause a parallel inhibition of NT-induced IP formation. We document the specificity of these effects in regard to agent and receptor, studying eight classes of CCBs and five receptors. We find that the efficacy order to enhance NT binding is similar to that for inhibition of IP production, and these effects display similar receptor selectivity. Detailed studies examine the effects on NTR1 and investigate the involvement of Ca^{2+}-dependent PLC(s).

Materials and Methods

Materials. The radiochemicals, [^{125}I]sodium iodide (2000 Ci/mmol), [1,2-^{3}H(N)]myoinositol (60 mCi/mmol), and [^{45}Ca]calcium chloride (>10 Ci/g) were obtained from PerkinElmer Life Sciences (Boston, MA). Phloretin, 2-aminoethoxydiphenylborate (2-APB), tetrandrine, human EGF, ω-conotoxin, ionomycin, thapsigargin, and veratridine were purchased from Calbiochem (San Diego, CA). Glibenclamide, diazoxide, ryanodine, dantrolene, and 1-[β-[3-(4-methoxyphenyl)-propoxy]-4-methoxyphenyl]-1*H*-imidazole (SKF-96365) were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Des-Gly-[Phaa^1,D-Tyr(Et)^2,Lys^6,Arg^8]vasopressin (HOLVA) was purchased from Peninsula Laboratories (Belmont, CA). Nimodipine, verapamil, diltiazem, NT, NIF, miconazole, tetraethylammonium, flunarizine, phenylarsine oxide, amiloride, pindolol, and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). [4-azido-Phe^6]NT was synthesized using reagents obtained from Novabiochem (San Diego, CA). SR48692 was generously provided by Danielle Gully at Sanofi-Synthelabo (Toulouse, France). Stocks of test agents were prepared daily (10 mM in DMSO) and diluted into Locke's solution just before use, except for SKF-96365, miconazole, and trifluoperazine (dissolved in Locke's solution).

Iodinations. Iodinations of ligands (EGF, 3 nmol; all others, 15 nmol) were performed using chloramine T (10 μg) as described (Carraway et al., 1993). All reactions were stopped using sodium metabisulfite (30 μg) except for EGF (stopped by dilution). The monoiodinated products were purified by reverse-phase HPLC using μBondapak-C18 (3.9 × 300 mm column) eluted at 1.5 ml/min with a linear gradient (60 min) from 0.1% trifluoroacetic acid to 60% CH_3CN. The specific activity of the purified ^{125}I-NT was 1000 to 2000 cpm/fmol as determined by radioimmunoassay (Carraway et al., 1993).

Binding to PC3 Cells. PC3 cells, obtained from the American Type Culture Collection (Manassas, VA) were maintained (passage 4) by our tissue culture facility at the University of Massachusetts Medical School (Seethalakshmi et al., 1997). Cells, passaged no more than 30 times, were grown to 95% confluency in 24-well culture plates. For binding studies, cells were washed with 2 ml/well Hepes-buffered Locke-BSA (Locke) (148 mM NaCl, 5.6 mM KCl, 6.3 mM Hepes, 2.4 mM $NaHCO_3$, 1.0 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.6 mM glucose, 0.1% BSA; pH 7.4). Equilibrium binding at 37°C was performed for 25 min using 10^5 cpm/ml of each ^{125}I-labeled ligand in 1.0 ml Locke with varying amounts of unlabeled ligand (0–1 μM). The reaction was stopped on ice for 15 min, the medium was aspirated, and the cells were washed twice with 2 ml and once with 4 ml of ice-cold saline. During this 5-min washing procedure dissociation of ^{125}I-NT from cell receptors was <1%. Total cellular binding was assessed by measuring radioactivity and protein (Bio-Rad assay; BSA standard) in cells extracted in 0.6 ml of 0.2 M NaOH. A Packard 10-well gamma counter was used to measure radioactivity. Specific binding was defined as that displaceable by 1 μM ligand.

Cell surface binding and internalization of ^{125}I-NT were assessed by washing cells at 22°C for 2 min with 0.6 ml of 0.2 M acetic acid, 0.5 M NaCl, pH 3.0 (Beaudet et al., 1994). Binding at 4°C achieved equilibrium within 3 h, at which time >90% of the radioactivity was on the cell surface. Binding at 37°C reached equilibrium in 25 min, at which time ≅70% of total binding was internalized. To measure rates of internalization for ^{125}I-NT prebound to cells, the following procedure was used. ^{125}I-NT (10^5 cpm) was prebound to PC3 cells in 24-well plates at 4°C for 3 h. After washing the cells three times in

ice-cold phosphate-buffered saline (PBS), >90% of ^{125}I-NT was located on the cell surface as determined by acid washing. Agents (10 mM in DMSO) were diluted to 50 μM in Locke and incubated with the cells at 37°C for 5 min. The control was 0.5% DMSO. Cell-surface and internalized ^{125}I-NT were measured, and the percentage of internalization per minute was calculated.

Binding displacement curves were constructed for each set of treatments and binding parameters were determined by Scatchard analysis. The K_i value was determined by using the equation $K_i = IC_{50}/1 + [L]/K_d$, where K_d and $[L]$ are the dissociation constant and the concentration of the ligand, respectively (Cheng and Prusoff, 1973).

Assessment of Binding Assay Artifacts. CCBs did not alter the ability of cells to adhere to plates, as evidenced by protein assay. Typically, each well contained 188 ± 11 μg (control, $n = 6$), 183 ± 10 μg (50 μM NIF, $n = 6$), 190 ± 12 μg (50 μM phloretin, $n = 6$), and 181 ± 11 μg of protein (100 μM verapamil, $n = 6$) after binding and washing.

CCBs did not alter the stability of ^{125}I-NT during binding at 37°C. After binding at 37°C, >90% of the radioactivity in the medium eluted during HPLC at the position of ^{125}I-NT for cells incubated in buffer, 50 μM NIF, or 100 μM verapamil. HPLC was performed at 1.5 ml/min on μBondapak-C18 (8 × 100 mm) with linear gradient (20 min) from 0.1% trifluoroacetic acid to 30% CH_3CN. ^{125}I-NT eluted at 25.0 min. During binding at 37°C, ≅4% of the added ^{125}I-NT was bound to the cells. Therefore, the medium was sampled in time and tested for the ability to bind to fresh cells. The loss of binding ability was ≅5% after 25 min. The protease inhibitors *o*-phenanthroline and PMSF (0.5 mM), had no effects on HPLC profiles and on loss of binding ability over time.

CCBs did not alter the dissociation rate of ^{125}I-NT from cellular receptors during washing with ice-cold saline. When cells were labeled with ^{125}I-NT in buffer, 50 μM NIF, or 100 μM verapamil, dissociation of cell-associated radioactivity was negligible (<6%) during incubation in ice-cold saline for 15 min.

Binding to PC3 Cell Membranes. PC3 cell membranes were prepared and collected by centrifugation at 30,000*g* as described by us (Seethalakshmi et al., 1997). Binding of ^{125}I-NT (10^5 cpm) to membranes (10–50 μg) was performed at 20°C for 60 min in 10 mM Tris-HCl (pH 7.5), containing 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% BSA, and protease inhibitors as described. Membranes were collected and washed onto glass fiber (GF-B) filters using a Brandel cell harvester, and the filters were counted (Seethalakshmi et al., 1997).

Cross-Linking of ^{125}I-[4-azido-Phe^6]-NT to NTR1. [4-azido-Phe^6]-NT was iodinated and purified by HPLC to ≅1500 Ci/mmol. PC3 cells in 10-cm dishes were incubated with 0.3 × 10^6 cpm/ml ^{125}I-[4-azido-Phe^6]-NT in 8 ml of Locke for 25 min at 37°C in the presence and absence of Ca^{2+} channel agents; 1 μM NT was added to controls. Cells were placed on ice for 30 min, irradiated at 254 nm with a hand-held UV light for 5 min at 3 cm, washed in ice-cold PBS, and lysed in 10 mM Hepes, 1 mM EDTA, 0.5 mM *o*-phenanthroline, PMSF, and *N*-tosyl-L-phenylalanine chloromethyl ketone (pH 7.4). Membranes, collected by centrifugation (30,000*g*, 20 min) were solubilized in 250 μl of 50 mM Tris buffer (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 0.5% NP-40, and 5% glycerol at 4°C for 2 h. Solubilized NTR1, diluted 2-fold in buffer without detergent, was immunoprecipitated by addition of our rabbit antiserum (Ab-NTR1) toward the C-terminal 15 residues of human NTR1 (final 1:100). During Western blotting, Ab-NTR1 detected two major bands in extracts of PC3 cells, the parent protein of 50 kDa and a breakdown product of 33 kDa, in keeping with results in other cells (Boudin et al., 1995). After 18 h at 4°C, protein A-agarose (10 mg; Sigma-Aldrich) was added for 6 h. After the agarose beads were washed three times with PBS at 4°C, associated radioactivity was measured using a gamma counter. Usually the immunoprecipitate contained ≅5% of the solubilized cpm for samples prepared in the absence of NT. SDS-PAGE was used in some cases to validate that the radiolabeled material represented NTR1. For this, the beads were boiled for 5 min in an equal volume of 2× SDS sample buffer, and extracts were subjected to SDS-PAGE using 10% polyacrylamide gels, followed by autoradiography.

Western Blotting. PC3 cells in 60-mm dishes were washed in Locke containing inhibitors 0.5 mM EDTA, 0.5 μM PMSF, 0.5 μM *N*-tosyl-L-phenylalanine chloromethyl ketone, and 0.5 μM *o*-phenanthroline. Cells were lysed in 100 μl of 2× SDS loading buffer with inhibitors, scraped into microfuge tubes, and sonicated (20 s) on ice. Membranes were isolated from regions of adult rat brains (Carraway et al., 1993), and P2 pellets were extracted in 2× SDS loading buffer and sonicated. Cell and tissue extracts were boiled 5 min and separated by SDS-PAGE on 10% polyacrylamide minigels. Proteins were electroeluted onto polyvinylidene difluoride (Immobilon P; Millipore Corporation, Bedford, MA). After blocking in 5% nonfat milk in TTBS (0.05% Tween 20, 20 mM Tris, 0.5 M NaCl) for 1 h and washing 3 times with TTBS, blots were incubated with the primary antiserum (1:1000) in blocking buffer for 18 h at 4°C. Our rabbit antiserum (Ab-NTR1) was raised using a synthetic peptide corresponding to residues 398–418 of human NTR1 conjugated to keyhole limpet hemocyanin. The antibodies were affinity-purified before use. Blots were washed in TTBS, then incubated with horseradish peroxidase-linked goat anti-rabbit antibody (1:1000) for 1 h at 20°C, and washed again in TTBS. Enhanced chemiluminescence was performed according to the manufacturer's instructions (Santa Cruz Biochemicals, Santa Cruz, CA). After stripping (1 h at 70°C in 62.5 mM Tris-HCl, 2% SDS, 0.1 M β-mercaptoethanol, pH 6.8) and washing in TBS, blots were reprobed with antigen-adsorbed antisera to validate the results.

Influx of $^{45}Ca^{2+}$ into PC3 Cells. The method of Katsura et al. (2000) was used to measure $^{45}Ca^{2+}$ influx in response to NT. Briefly, confluent PC3 cells in 24-well dishes were washed with Ca^{2+}-free Locke and pretreated for 10 min with 0 to 36 μM NIF (600 μl/well). The reaction was initiated by the addition of 200 μl of NT followed in 2 min by 2.5 mM $CaCl_2$ (5 μCi $^{45}Ca^{2+}$/well). After 8 min, the cells were washed three times with ice-cold Locke and solubilized in 1.0 ml of 0.25 M NaOH. The cell extract was neutralized with acetic acid, and an aliquot was subjected to liquid scintillation spectrometry to measure $^{45}Ca^{2+}$ radioactivity.

Measurement of IP Formation. IP formation was measured by the method of Chen and Chen (1999) wherein [^{3}H]inositol was used to label the phosphoinositide pool. PC3 cells in 24-well plates were incubated 48 h with myo-[^{3}H]inositol (2.5 μCi/ml) in medium 199 and 5% fetal calf serum. Medium 199 (Difco, Detroit, MI) was chosen because of its low inositol content. After washing with 2 ml of Locke, cells were preincubated 10 min with test agents in Locke and 15 mM LiCl, and reactions were started by adding a maximal dose of NT (30 nM) or vehicle. After 30 min at 37°C, the medium was aspirated, ice-cold 0.1 M formic acid in methanol (1 ml) was added, and plates were placed at −20°C overnight. Samples were transferred to tubes using 2× 2-ml water washes, and [^{3}H]IP was adsorbed to 0.25 ml AG-1 × 8 slurry (formate form; Bio-Rad, Hercules, CA), which was washed five times in 5 mM myoinositol (5 ml) and eluted in 0.75 ml of 1.5 M ammonium formate and 0.1 M formic acid. Scintillation counting was performed on 0.5 ml of eluate in 5 ml of Ecoscint (National Diagnostics, Atlanta, GA). For experiments involving removal of Ca^{2+} from the buffer, cells were washed with Ca^{2+}-free buffer and used immediately to minimize any disturbance to internal Ca^{2+} stores.

Statistics. Statistical comparisons were made using the Student's *t* test. Results were calculated as mean ± S.E.M. and $p < 0.05$ was considered significant.

Results

CCBs Enhanced Cellular Binding of NT. Specific binding of ^{125}I-NT (10^5 cpm/ml) to PC3 cells at 37°C was >95% of total binding and was 16.8 ± 0.81 cpm ^{125}I-NT bound/μg

protein (n = 12), which corresponded to ≅3000 cpm ^{125}I-NT bound per well. Table 1 gives the binding parameters determined for NT binding to PC3 cells. Data given under *Materials and Methods* attests to the validity of the assay, showing that the ^{125}I-NT remained intact during incubation and that dissociation did not occur during washing. CCBs did not alter these conditions.

CCBs (e.g., NIF, phloretin, and verapamil) increased the apparent rate[1] of and the steady-state level of NT binding to PC3 cells (Fig. 1A). NIF enhanced specific binding without altering nonspecific binding, and it was effective across a 10-fold range in cell density (Fig. 1B). Similar effects were displayed by five L-type VGCC blockers, two L-type/T-type VGCC blockers, and two blockers of SOCC, representing seven different classes of chemicals. The order of efficacy (NIF > phloretin > verapamil > diltiazem) for VGCC blockers was similar to that for peripheral vasodilation (Triggle, 1999). NT binding was increased up to 3.1-fold by NIF, 2.9-fold by phloretin, 2.0-fold by verapamil, and 1.4-fold by diltiazem (Fig. 1C). Nimodipine and NIF were the most potent agents, elevating NT binding at submicromolar concentrations [control, 100 ± 4%; 0.3 μM nimodipine, 116 ± 5% (p < 0.05); 0.9 μM NIF, 115 ± 5% (p < 0.05)]. Although less specific CCBs (flunarizine, tetrandrine, trifluoperazine, and chlorpromazine) had only modest effects (Table 2), well defined blockers of SOCC (SKF-96365, miconazole) enhanced NT-binding up to 2.9-fold (Fig. 1D; Table 2).

CCBs Inhibited NT-Induced IP Formation. NT increased IP formation ≅5-fold in PC3 cells with an EC_{50} value of ≅1 nM (Fig. 2A). L-type VGCC blockers inhibited the response to a maximal dose of NT (Fig. 2B), with an efficacy order (NIF > phloretin > verapamil) similar to that for enhancement of NT binding (Table 2). SOCC blockers also inhibited the response to NT (Fig. 2B), giving an efficacy order (SKF-96365 > miconazole > trifluoperazine) similar to that for enhancing NT binding (Table 2). For each of these agents the EC_{50} value for enhancing NT binding was similar to the IC_{50} value for inhibiting NT-induced IP formation (Table 2), and there was a strong statistical correlation (r^2 = 0.842). These results indicated that the drug effects on NT binding and NT-induced IP formation had a similar chemical sensitivity and/or that the two effects were linked, e.g., that one led to the other.

TABLE 1
Parameters determined for binding of ^{125}I-labeled ligands to PC3 cells

Ligand[a]	Specific Binding[b]	B_{max}[c]	K_i[c]
	% total	*fmol/mg*	*nM*
^{125}I-NT	95	158 ± 9	1.0 ± 0.07
^{125}I-[Nle^{14}]bombesin	95	1016 ± 64	0.6 ± 0.09
^{125}I-EGF	95	151 ± 11	0.6 ± 0.07
^{125}I-pindolol	66	86 ± 6	0.3 ± 0.05
^{125}I-HOLVA	77	156 ± 12	0.5 ± 0.07

[a] NT, [Nle^{14}]bombesin and EGF are agonists for NTR1, bombesin receptor, and EGF receptor, respectively. Pindolol and HOLVA are antagonists for β_2-adrenergic receptor and vasopressin (V1a) receptor, respectively.
[b] All ligands were HPLC-purified (specific activity >1000 Ci/mmol). Specific binding was measured to near-confluent cells (≅185 μg protein/well) using 10^5 cpm ^{125}I-ligand in 1.0 ml Locke's buffer (see under *Materials and Methods*).
[c] Scatchard analysis was performed using 12 ligand concentrations and results were from three to nine experiments.

Tyrosine Kinase Inhibitors Increased ^{125}I-EGF Binding to PC3 Cells. Tyrosine kinase inhibitors have been identified that specifically bind to the ATP binding site of EGFR and block kinase function (Arteaga et al., 1997). Since these drugs were known to greatly elevate EGF binding in some cancer cells (Lichtner et al., 2001), we tested their effects in PC3 cells. Initially, we showed that the PC3 cell surface displayed EGFR with high affinity for ^{125}I-EGF (Table 1). Testing the effects of tyrosine kinase inhibitors, we found that AG1478 and PD153035 increased EGF binding to PC3 cells by as much as 4.3-fold (Fig. 3A), whereas they had little effect on NT binding (Fig. 3B). In contrast, CCBs NIF and SKF-96365 had little effect on EGF binding (Fig. 3A,

[1] This does not necessarily indicate that the association rate constant is increased, since the apparent rate is a function of association, dissociation, internalization and other processes.

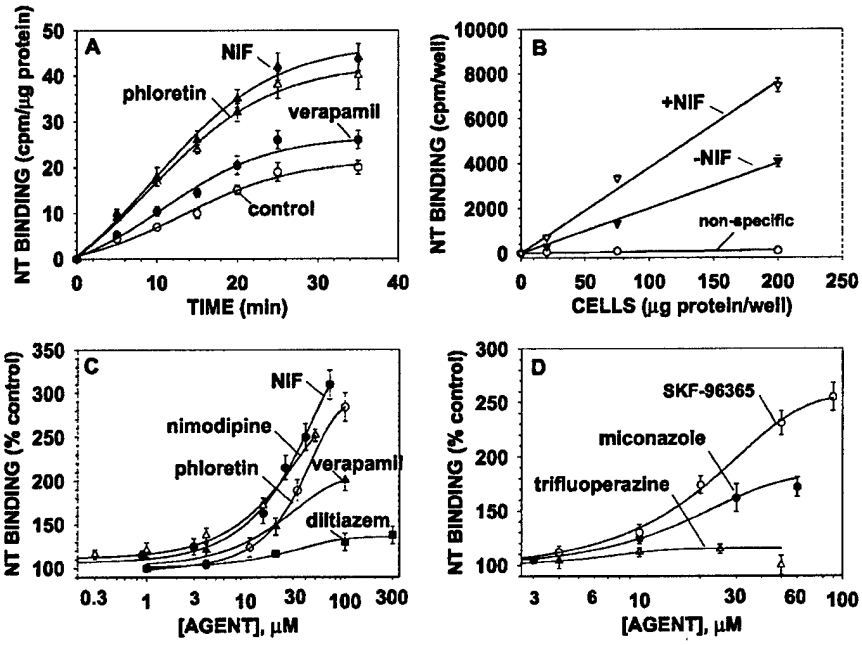

Fig. 1. CCBs increased the rate and steady-state level of NT binding to intact PC3 cells. A, PC3 cells were preincubated with 30 μM NIF, phloretin, or verapamil or with 0.3% DMSO at 37°C. After 10 min, ^{125}I-NT (10^5 cpm; 50 pM final) was added, along with NT (1 μM final) or control. Incubation continued for times indicated, reactions were stopped, and binding was expressed as cpm/μg protein. Results are for n = 3 in a typical experiment that was repeated. B, cells, diluted 1:1, 1:3, and 1:9, were plated, yielding ≅200 μg, 75 μg, and 20 μg of protein/well, respectively. Cells were preincubated 10 min with 30 μM NIF or vehicle, and NT binding (37°C, 25 min) was measured. Results are n = 6 in a typical experiment performed three times. C and D, cells were pretreated 10 min with indicated agents and NT binding was measured. The minimum dose that significantly (p < 0.05) elevated NT binding above control was 0.3 μM (nimodipine), 0.9 μM (NIF), 20 μM (verapamil), 10 μM (phloretin), 4 μM (SKF-96365), 10 μM (miconazole), and 100 μM (diltiazem). Results are from four experiments.

TABLE 2
Activity of CCBs on NT binding and NT-induced IP formation

Channel	Agent	NT Binding[a]		IP Formation[b]	
		Efficacy	EC_{50}	Efficacy	IC_{50}
		% increase	*μM*	*% decrease*	*μM*
VGCC	NIF[c]	210	15	74	15
	Phloretin[c]	186	27	70	23
	Verapamil[c]	85	43	58	53
	Diltiazem[c]	38	>300	ND	ND
	Flunarizine[d]	45	>100	ND	ND
	Tetrandrine[d]	35	>100	ND	ND
SOCC	SKF-96365	155	23	69	26
	Miconazole	75	60	54	51
	Trifluoperazine	16	>100	14	>100
	Chlorpromazine	36	>100	ND	ND

ND, no data.
[a] Efficacy was defined as the maximal percentage of increase in NT binding observed for each agent. ED_{50} was defined as the [agent] at which NT binding was increased by 80%. The data are means determined in three to eight experiments for each agent.
[b] Efficacy was defined as the maximal percentage of decrease in NT-induced IP formation observed for each agent. IC_{50} was defined as the [agent] at which IP formation was decreased by 50%. The data are means determined in three to eight experiments for each agent. nd, not determined.
[c] L-type CCBs.
[d] L-type/T-type blockers.

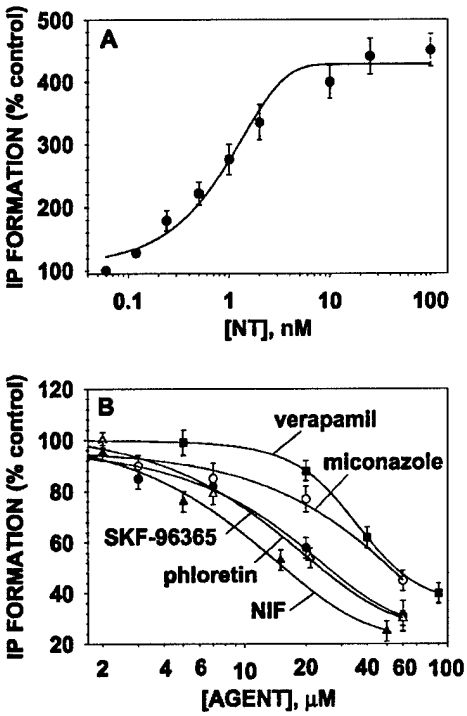

Fig. 2. NT-induced IP formation (A) was inhibited by CCBs (B). A, log dose-response plot showing that NT enhanced IP formation 4.5-fold with $EC_{50} \cong 1.0$ nM. The minimum dose of NT that significantly ($p < 0.05$) elevated IP formation above control was 0.2 nM. B, log dose-response plots showing that IP formation in response to a maximal dose of NT (30 nM) was inhibited by CCBs. The minimum dose that significantly ($p < 0.05$) decreased IP formation below control was 5 μM (NIF), 7 μM (phloretin), 7 μM (SKF-96365), 20 μM (miconazole), and 40 μM (verapamil). Results are from 10 experiments (A) and 3 to 4 experiments (B).

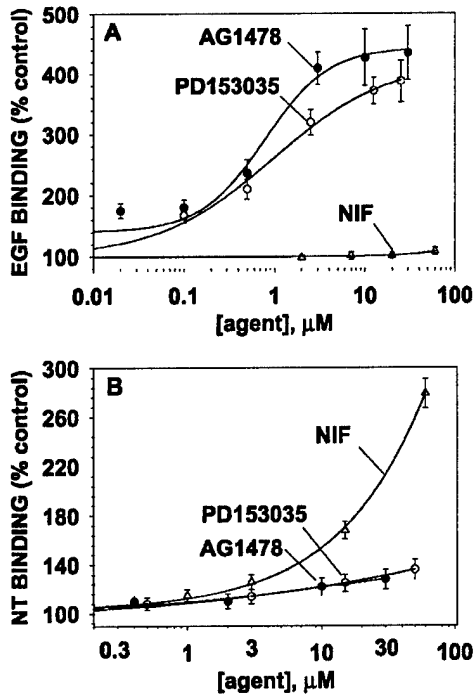

Fig. 3. Tyrosine kinase inhibitors increased ^{125}I-EGF binding to PC3 cells (A) but had little effect on NT binding (B). PC3 cells were preincubated 10 min with indicated agents, and binding was performed at 37°C. Results from five experiments (A) and three experiments (B) were expressed as percentage of control and plotted as mean ± S.E.M. A, whereas EGF binding was not altered by CCBs ($p > 0.1$), it was increased up to 4-fold by AG1478 and PD153035. B, in contrast, NT binding was increased only slightly (<35%) by tyrosine kinase inhibitors, although it was increased up to 3-fold by NIF.

Table 5), although they enhanced NT binding ≅3-fold (Figs. 1, C and D, and 3B). These results not only demonstrated specificity, but also a degree of similarity to these systems, since the elevated binding in both cases was associated with an inhibition of the response to receptor activation.

CCBs Did Not Act Directly on NTR1. Since tyrosine kinase inhibitors were thought to act directly on the EGFR to elevate binding (Lichtner et al., 2001), we wondered whether this was also the case for the effects of CCBs on NTR1. To address this, we tested the effects of CCBs on ^{125}I-NT binding to isolated PC3 cell membranes in vitro. NT binding to cell membranes was not increased by NIF, phloretin, and verapamil (Table 3), indicating that these agents were unable to act directly on NTR1. Although a key participant in the reaction might have been lost during membrane isolation, it seems more likely that there was a requirement for cellular metabolism and/or architecture. Thus, the increase in NT

TABLE 3
Effects of CCBs on NT binding to PC3 cell membranes

Agent[a]	Specific NT Binding (% Control) at Dose of Agent[b]			
	10 μM	25 μM	75 μM	100 μM
NIF	97 ± 5		102 ± 5	108 ± 8
Nimodipine	99 ± 2	101 ± 2	94 ± 4	
Phloretin	110 ± 7	105 ± 8	97 ± 8	
Verapamil	100 ± 2		101 ± 5	103 ± 4

[a] Agents were freshly dissolved in DMSO at 10 mM and diluted into binding buffer just before use.
[b] PC3 cell membranes were preincubated 10 min with agents or control, and NT binding was performed at 22°C for 60 min (see under *Materials and Methods*). Specific binding was measured in four to six experiments and expressed as percentage of control (mean ± S.E.M.). Results for the various agents were not significantly different from control ($p > 0.1$).

binding observed in live cells most likely reflected an indirect effect of CCBs, possibly by way of a change in ion movement or by some other means.

Other Channel Agents Did Not Increase NT Binding. To assess drug specificity, we tested agents toward other ion channels for effects on NT binding. We focused on agents that might alter the movement of Ca^{2+}, Na^+, and K^+ since NT binding to isolated membranes was known to be inhibited by these metal ions (Carraway et al., 1993). A variety of agents toward other types of channels did not enhance NT binding to PC3 cells (Table 4). These included an N-type Ca^{2+} channel blocker (ω-conotoxin), Ca^{2+} release inhibitors (ryanodine, dantrolene), K^+ channel blockers (glibenclamide, diazoxide, tetraethylammonium), an Na^+ channel blocker (amiloride), and an Na^+ channel opener (veratridine). These results indicated that the NT binding response displayed a degree of drug specificity.

Enhancement of Cell Binding by CCBs Was Relatively Specific to NT. To assess receptor specificity, we tested CCBs for effects on PC3 cell binding of ligands specific for other GPCRs and for EGFR. Radioreceptor assays were developed for β_2-adrenergic, bombesin, and V_{1a}-vasopressin receptors as well as for EGFR. Table 1 shows the ligands used and the binding parameters determined. For NT, bombesin, and EGF receptors agonist ligands were used; the others were antagonists. Assessing the effects of CCBs, we found that NIF, phloretin, verapamil, and SKF-96365 did not enhance β_2-adrenergic, V_{1a}-vasopressin, and EGF receptor binding to PC3 cells (Table 5). However, bombesin receptor binding was elevated slightly (≅19%) by NIF (Table 5). β_2-adrenergic receptor binding was, in fact, decreased by these

TABLE 4
Effects of various channel-directed agents on NT binding to PC3 cells

Agent[a]	Specific NT Binding (% Control) at Dose of Agent[b]			
	2 μM	10 μM	30 μM	100 μM
ω-Conotoxin	95 ± 8	104 ± 7		
Ryanodine		108 ± 7	101 ± 6	114 ± 10
Dantrolene		106 ± 6	93 ± 10	112 ± 10
Glibenclamide	102 ± 6		112 ± 9	117 ± 10
Diazoxide	106 ± 5	101 ± 5	96 ± 6	
Tetraethylammonium	103 ± 5	95 ± 6	83 ± 5	
Amiloride		108 ± 5	102 ± 6	101 ± 6
Veratridine	99 ± 2	101 ± 3	105 ± 6	

[a] Agents were freshly dissolved in DMSO at 10 mM and diluted into Locke's buffer just before use.
[b] PC3 cells were preincubated with each agent and vehicle control for 10 min; NT binding was performed at 37°C for 25 min. Specific NT binding is given as percentage of control (mean ± S.E.M.) for at least three independent experiments. Results for the various agents were not significantly different from control ($p > 0.1$).

TABLE 5
Effects of CCBs on PC3 cell binding of ligands specific for bombesin, vasopressin, β_2-adrenergic, and EGF receptors

Ligand	Agent	Specific Binding (% Control) at Dose of Agent[a]	
		12 μM	60 μM
^{125}I-[Nle^{14}]bombesin	NIF	108 ± 4	119 ± 4**
	Phloretin	104 ± 3	111 ± 4
	Verapamil	104 ± 4	104 ± 4
	SKF-96365	99 ± 2	106 ± 4
^{125}I-Pindolol[b]	NIF	105 ± 5	82 ± 3**
	Phloretin	102 ± 4	93 ± 3
	Verapamil	68 ± 5**	35 ± 6**
	SKF-96365	86 ± 2**	51 ± 2**
^{125}I-HOLVA[c]	NIF	95 ± 4	59 ± 5**
	Phloretin	92 ± 4	73 ± 4**
	Verapamil	85 ± 4*	58 ± 4**
	SKF-96365	80 ± 2**	50 ± 2**
^{125}I-EGF	NIF	100 ± 4	108 ± 4
	Phloretin	98 ± 2	95 ± 4
	Verapamil	103 ± 4	96 ± 4
	SKF-96365	103 ± 3	94 ± 3

[a] Specific binding of each ^{125}I-ligand was measured to PC3 cells (see under *Materials and Methods* and Table 1). Binding was expressed as percentage of control (mean ± S.E.M.) for three to six independent experiments.
[b] Verapamil and SKF-96365 resemble pindolol structurally. Thus, the decrease in binding was due to direct competition with the ligand (percentage of cross-reaction ≅ 0.0005). This conclusion was supported by the fact that these agents also inhibited the binding of ^{125}I-pindolol to PC3 cell membranes (see under *Materials and Methods* and Table 3). Binding (percentage of control ± S.E.M.) for three experiments in duplicate was 60 μM verapamil (9 ± 2); 60 μM SKF-96365 (18 ± 5).
[c] These agents did not resemble HOLVA structurally and they did not inhibit the binding of ^{125}I-HOLVA to PC3 cell membranes. Binding (percentage of control ± S.E.M.) for three experiments in duplicate was 60 μM verapamil (91 ± 5); 60 μM SKF-96365 (110 ± 3); 60 μM NIF (90 ± 3).
* Result was significantly different from control ($p < 0.05$).
** Result was significantly different from control ($p < 0.01$).

agents (Table 5), but this was due to a direct competition with ^{125}I-pindolol. This conclusion was based on the structural resemblance of these agents to pindolol and the fact that ^{125}I-pindolol binding to PC3 cell membranes was inhibited in a similar manner (for results, see the footnote to Table 5). Cell binding for the vasopressin receptor was also diminished by these drugs (Table 5); however, this could not be attributed to a direct competition with ^{125}I-HOLVA (Table 5 footnote). These data indicated that the robust elevation in cell binding was specific to NTR1, although the bombesin receptor responded to a lesser degree.

Inhibition of IP Formation by CCBs Was Relatively Specific to NT. To examine receptor specificity, we tested the ability of NIF to inhibit IP formation in response to GPCR agonists known to stimulate PLC. Preliminary dose-response experiments showed that a maximal dose of NT (30 nM), bombesin (20 nM), and ATP (10 μM) stimulated IP formation by ≅5-fold, ≅15-fold, and ≅17-fold, respectively. When PC3 cells were pretreated with varying amounts of NIF, we found that the response to this dose of NT was inhibited as much as ≅69%, whereas that for bombesin was inhibited ≅19%, and that for ATP was not inhibited (Fig. 4A). When the dose of each agonist was varied, we found that the percentage of inhibition by 15 μM NIF was independent of the level of stimulation. Thus, at each dose the response to NT was inhibited ≅64%, whereas that for bombesin was inhibited ≅15% and that for ATP was not inhibited (Fig. 4B). These results indicated that the robust inhibition of IP formation by

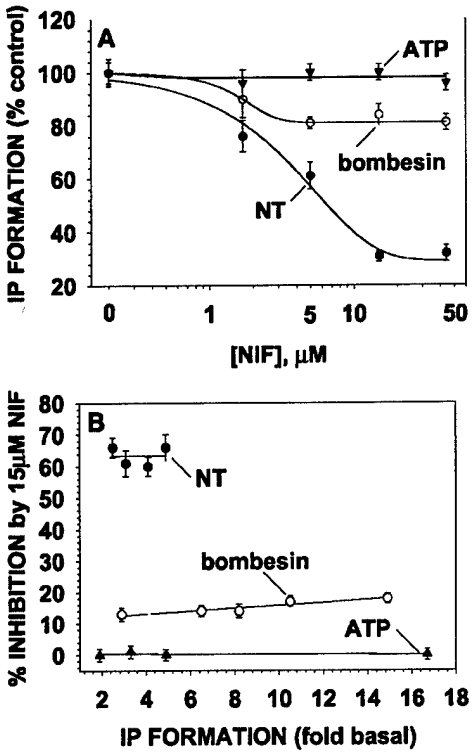

Fig. 4. NIF inhibited NT- and bombesin-, but not ATP-induced, IP formation. Agonist-induced IP formation was measured in PC3 cells. A, plots show inhibitory effects of various doses of NIF on the response to a maximal dose of NT (20 nM), bombesin (10 nM), and ATP (10 μM). Results are from three experiments. B, plots show inhibitory effects of 15 μM NIF on responses to various doses of NT (0.2–20 nM), bombesin (0.1–10 nM), and ATP (0.2–10 μM), plotted as a function of fold enhancement of basal IP formation. Results are from three experiments.

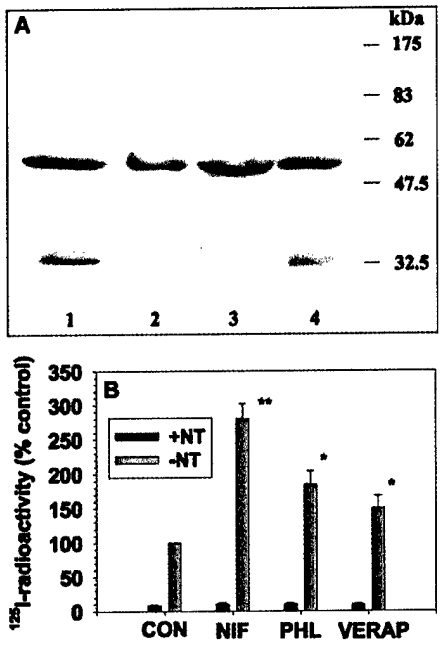

Fig. 5. CCBs enhanced photoaffinity labeling of NTR1. A, Western blot, representative of three experiments, verifying the specificity of Ab-NTR1 toward human NTR1. Lane numbering: PC3 cells (lanes 1, 4), rat cerebral cortex (lane 2), rat hypothalamus (lane 3). B, plot showing the effect of CCBs on cross-linking of ^{125}I-(4-azido-Phe^6)-NT to immunoprecipitated NTR1. PC3 cells were incubated 10 min in 50 μM NIF, 50 μM phloretin, 100 μM verapamil, or 0.5% DMSO at 37°C. ^{125}I-(4-azido-Phe^6)-NT (3×10^5 cpm/ml; 0.15 nM) was added to all dishes and 1 μM NT to some dishes. After 25 min, cells were washed, UV-irradiated, and lysed. Cell membranes were isolated and solubilized NTR1 was immunoprecipitated. After washing, precipitates were counted using a gamma counter and data were expressed relative to control (100%), which typically gave ≅5000 cpm. The drugs enhanced cross-linking 1.5- to 2.8-fold, and 1 μM NT reduced it by >90%. Results are from four experiments, except NIF (eight experiments).

NIF was specific to NT, although the response to bombesin was also inhibited to a lesser degree.

CCBs Enhanced Photoaffinity Labeling of NTR1. NTR1 is a 46-kDa protein that has been immunologically characterized (Boudin et al., 1995) and labeled using UV-activatable cross-linkers (Mazella et al., 1988). Initially, we used Western blotting to verify the specificity of our antiserum (Ab-NTR1) raised toward the C terminus of human NTR1. Whereas extracts of rat brain gave a single band at ≅50 kDa, PC3 cells gave this parent protein, along with a 33-kDa fragment (Fig. 5A), in keeping with published results (Boudin et al., 1995). Next, we used UV light to incorporate ^{125}I-(4-azido-Phe^6)-NT into PC3 cells treated with CCBs or control, and we assessed the incorporation of radioactivity into immunoprecipitated NTR1. The results (Fig. 5B) showed that the radioactivity associated with NTR1 was enhanced by NIF (2.8-fold; $p < 0.001$), phloretin (1.8-fold; $p < 0.05$), and verapamil (1.5-fold; $p < 0.05$) as compared to the control. For each agent the increase in immunoprecipitated radioactivity (Fig. 5B) was similar to the increase in NT binding to PC3 cells seen at the appropriate dose (Fig. 1C). SDS-PAGE and autoradiography on selected samples verified the presence of 50- and 33-kDa radiolabeled proteins (data not shown). These results indicated that CCBs enhanced NT binding by increasing the association of ^{125}I-NT with NTR1; however, they did not rule out possible interactions with other NT receptors.

Cell-Surface Binding versus Internalization. Cell-surface binding of ^{125}I-NT was enhanced by NIF to a similar extent when assessed by three different methods (Fig. 6). NIF increased surface binding 2.4-, 2.2-, and 2.7-fold, respectively, as measured at 4°C (Fig. 6A), 37°C in the presence of phenylarsine oxide (Fig. 6A), and 37°C by acid washing (Fig. 6B). Internalization of ^{125}I-NT was 68 to 72% of total binding in the presence or absence of NIF (Fig. 6B). In addition, the internalization rate at 37°C for cell-surface ^{125}I-NT, previously bound to cells at 4°C in the absence of drugs, was unaffected by 50 μM NIF, 50 μM phloretin, and 50 μM verapamil. Internalization rates (%/min; $n = 12$ from two experiments) were control, 8.6 ± 0.6; NIF, 8.0 ± 0.6; phloretin, 8.1 ± 0.7; verapamil, 9.2 ± 0.7, which did not differ significantly ($p > 0.1$). These results indicated that these agents increased cellular NT binding by enhancing the interaction of NT with NTR1 rather than by enhancing the internalization rate for the NT-NTR1 complex.

NTR1 Affinity versus NTR1 Number. CCBs enhanced binding and increased the steepness of the NT displacement curve. When the NT displacement data were expressed as percentage of maximal binding, CCBs shifted the displacement curves to the left by a factor of 2 to 3 (Fig. 7A). In three experiments the K_i value for NT was decreased from 0.95 ± 0.1 nM (control) to 0.36 ± 0.04 nM (50 μM NIF; $p < 0.01$), 0.40 ± 0.05 nM (50 μM phloretin; $p < 0.01$), and 0.61 ± 0.06

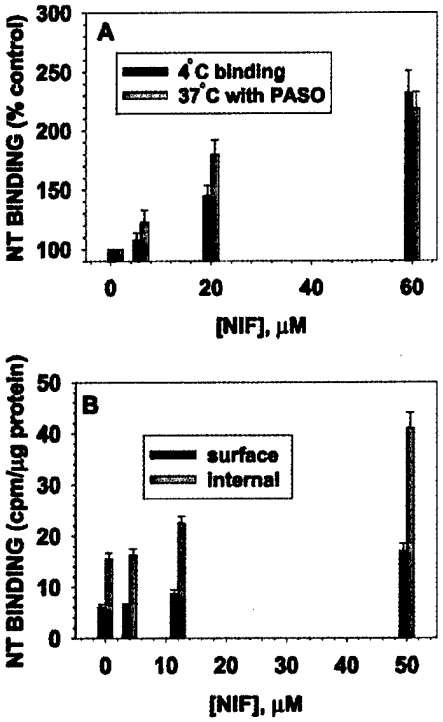

Fig. 6. NIF enhanced cell-surface binding of ^{125}I-NT (A) without altering the percentage of ^{125}I-NT internalized by PC3 cells (B). A, to study the cell-surface component of ^{125}I-NT binding we used method 1, incubation for 2 h at 4°C, and method 2, incubation at 37°C in the presence of 10 μM phenylarsine oxide. NIF enhanced cell-surface binding by 2.3-fold (method 1) and 2.2-fold (method 2). B, to study cell-surface binding and internalization we used method 3, incubation at 37°C followed by acid washing. Internalization of ^{125}I-NT was 71 ± 2% of total binding (control) and 68–72% (NIF). NIF (50 μM) enhanced cell-surface binding (2.8-fold) and internalization (2.6-fold) similarly. Results are from three experiments (A) and four experiments (B).

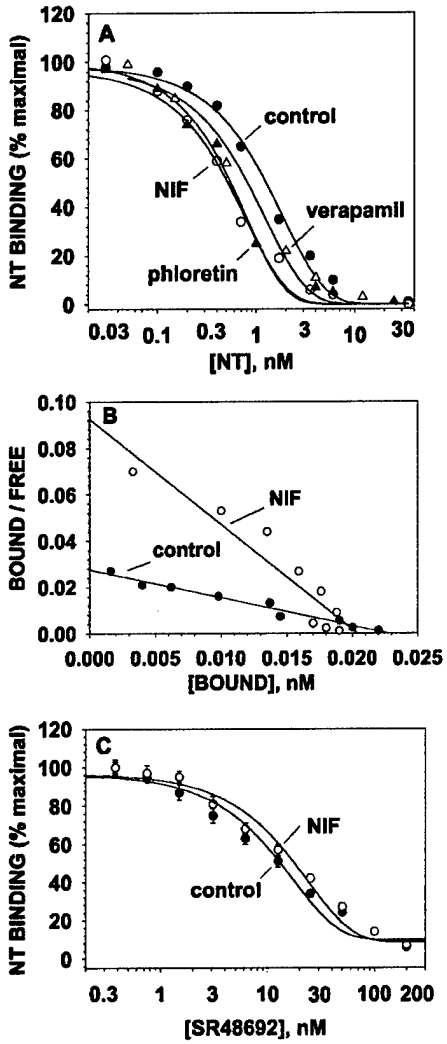

Fig. 7. Binding displacement curves (A, C) and Scatchard plots (B) for ^{125}I-NT binding to PC3 cells in the presence and absence of CCBs. Binding of ^{125}I-NT to PC3 cells (15.8 cpm/μg of protein) was increased 2.7-fold by 50 μM NIF, 2.6-fold by 50 μM phloretin, and 2.0-fold by 100 μM verapamil. A, plots show displacement of ^{125}I-NT binding by NT, in which binding was expressed as percentage of control. The agents shifted the curves to the left. The IC_{50} value for NT was 1.2 nM (control), 0.8 nM (verapamil), 0.5 nM (phloretin), and 0.5 nM (NIF). Results are from a typical experiment repeated twice. B, Scatchard plots for typical experiment showing that NIF increased NTR affinity (apparent K_i: control, 0.93 nM; NIF, 0.33 nM) without increasing receptor number (B_{max}: control, 23 fmol/well; NIF, 21 fmol/well). C, plots show displacement of ^{125}I-NT binding by SR48692. In the presence of 50 μM NIF, the curve was shifted slightly (but not significantly) to the right. Results are from typical experiment performed four times.

nM (100 μM verapamil; $p < 0.05$). Scatchard analyses indicated that NIF increased the affinity of NTR1 for NT without changing the NTR1 number (Fig. 7B). The calculated NTR1 number (158 ± 9 fmol NTR1/mg protein; $n = 9$) corresponded to ≅50,000 receptors/cell (Table 1).

In contrast, the binding displacement curve for the antagonist SR48692 was shifted slightly to the right in the presence of 50 μM NIF (Fig. 7C), although the K_i was not changed significantly (K_i: control, 12 ± 1.0 nM; NIF, 14 ± 0.8; $n = 4$; $p > 0.1$). Taken together, these results indicated that CCBs shifted NTR1 toward a state that displayed an increased affinity for the agonist NT and an unchanged affinity for the antagonist SR48692.

NIF Inhibited NT-Induced Ca^{2+} Influx. Since NT stimulated Ca^{2+} influx in Chinese hamster ovary cells transfected with NTR1 (Gailly, 1998), we tested NT for this ability in PC3 cells. NT enhanced the influx of $^{45}Ca^{2+}$ into PC3 cells, giving an EC_{50} value (≅1 nM) similar to that for NT-induced IP formation (results not shown). At doses shown to enhance NT binding (Fig. 1C) and to inhibit NT-induced IP formation (Fig. 2B), NIF inhibited the influx of $^{45}Ca^{2+}$ in response to NT (Fig. 8A).

Ca^{2+}-Dependence of NT-Induced IP Formation. Since some PLC isozymes are Ca^{2+}-dependent (Rhee and Bae, 1997), the inhibition of NT-induced IP formation by doses of NIF that diminished NT-induced Ca^{2+} influx suggested that Ca^{2+} influx might participate in the stimulation of PLC. Consistent with this, NT-induced IP formation was inhibited by omitting Ca^{2+} from Locke buffer, by adding Ca^{2+} chelator EGTA to Locke, or by adding NIF to Locke (Fig. 8B). Paradoxically, the removal of Ca^{2+} elevated basal IP production ≅2-fold (see Fig. 8 legend), perhaps by mobilizing internal Ca^{2+} stores. However, inhibition of the NT response was not due to a ceiling effect, since IP production could be elevated 15–20-fold by bombesin and ATP (see Fig. 8 legend).

Ionomycin stimulated IP formation, reproducing as much as 63% of the response to NT. IP formation (percentage of control) was 2 μM ionomycin, 139 ± 6% ($p < 0.01$); 20 μM

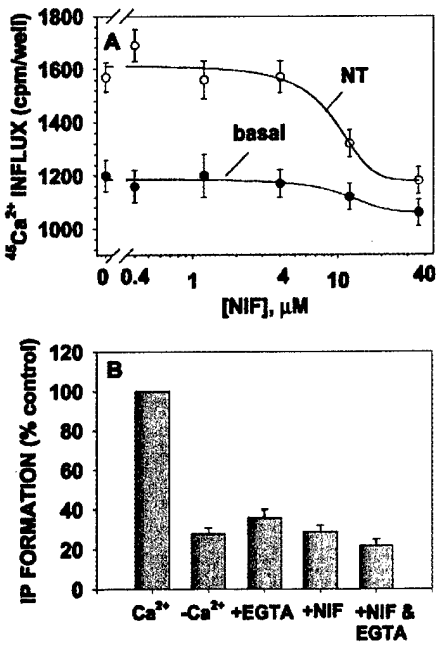

Fig. 8. NT-induced ^{45}Ca influx was inhibited by NIF (A), and NT-induced IP formation was Ca^{2+}-dependent (B). A, in experiments not shown, $^{45}Ca^{2+}$ influx into PC3 cells was enhanced ≅30% by NT (EC_{50} ≅1.2 nM). The log dose-response plot shows that a 10-min pretreatment of cells with varying doses of NIF inhibited the response to 20 nM NT (IC_{50} ≅12 μM), without much effect on basal $^{45}Ca^{2+}$ influx. Results are from three experiments. B, IP formation in PC3 cells in Locke (1 mM Ca^{2+}) was enhanced ≅4.5-fold by 20 nM NT (shown as 100% response). The response to NT was inhibited by omitting Ca^{2+} from Locke, by adding to Locke either 1.1 mM EGTA or 50 μM NIF, or 1.1 mM EGTA plus 50 μM NIF. Although basal IP formation was unaffected by NIF it was increased ≅2-fold by omitting Ca^{2+} from Locke or by adding EGTA to the Ca^{2+}-containing Locke. Inhibition of the response to NT was not due to a ceiling effect, since IP formation was stimulated ≅15-fold by 10 nM bombesin and ≅17-fold by 10 μM ATP in similar experiments. Results are from three experiments.

ionomycin, 324 ± 14% ($p < 0.01$); 30 nM NT, 457 ± 12% ($p < 0.01$); $n = 4$ experiments. When added 2 min after a maximal dose of NT (30 nM), low doses of ionomycin (2–10 μM) enhanced the response to NT. IP formation (percentage of control) was 10 μM ionomycin, 157 ± 5 ($p < 0.01$); NT, 366 ± 20 ($p < 0.01$); NT plus ionomycin, 465 ± 9 ($p < 0.001$); $n = 4$ experiments. In contrast, a maximal dose of ionomycin gave less than additive enhancement of the response to NT. IP formation (percentage of control) was 25 μM ionomycin, 322 ± 11 ($p < 0.001$); NT, 384 ± 14 ($p < 0.001$); NT plus ionomycin, 476 ± 15 ($p < 0.001$); $n = 4$ experiments. These data suggested that the inhibition of NT-induced IP formation by CCBs may have been partly attributable to a change in Ca^{2+} influx.

Ca^{2+}-Dependence of NT Binding. In six experiments, removal of Ca^{2+} from the buffer elevated NT binding significantly [NT binding (percentage of control) 2 mM EGTA, 125 ± 5; $p < 0.01$]. However, relative to the effects of CCBs (Fig. 1), this effect was very small. These data suggested that only a small part (≅10%) of the effect of Ca^{2+} channel agents on NT binding might be attributed to a change in Ca^{2+} influx.

Discussion

This is the first report that CCBs exert major effects on NTR1 binding or even, for that matter, on GPCR binding. Although NT binding to NTR1 was increased dramatically by these agents, NTR1-mediated effects on Ca^{2+} influx and IP formation were inhibited. Drugs representing three major classes of VGCC blockers enhanced NT binding, giving an efficacy order similar to that for peripheral vasodilation (Triggle, 1999). Although the most potent agents, DHPs, were regarded as specific for L-type VGCC (Triggle, 2003), their effects on NT binding and bioactivity occurred in a dose range shown to alter SOCC behavior (Harper et al., 2003). Furthermore, CCBs selective for SOCC elevated NT binding and inhibited NT-induced IP formation. Thus, the effects on NTR1 function were associated predominately with agents having the ability to block SOCC, although SOCC involvement in these actions was not proven.

Enhancement of NT binding by CCBs was drug-specific, receptor-specific, and could not be explained by enhanced tracer stability, membrane partitioning, or metabolic trapping. Under the same conditions that increased NT binding ≅3-fold and using ^{125}I-ligands with similar specific activities, binding for β_2-adrenergic, V_{1a}-vasopressin, and EGF receptors was not increased, and binding for bombesin receptor was increased <20% by CCBs. Although the results suggested that the effect was specific for NTR1, it was possible that other GPCRs could respond under proper conditions, e.g., agonist ligands might have been necessary for the enhancing effect to manifest itself. The two GPCR binding assays that gave increases in response to CCBs, NTR1 (≅200% increase) and bombesin (≅20% increase), used agonist ligands. Although difficult to understand at this time, it is interesting that the V_{1a}-vasopressin assay, which used an antagonist ligand, gave decreased binding in response to CCBs. Like the increase observed in NT binding, the decrease in vasopressin binding required intact cells (Table 5), and thus was not due to competition at the ligand binding site. Since NTR1, bombesin, and V_{1a}-vasopressin receptors signal via $G_{q/11}$, this suggests that the associated G-protein may be an important determinant of these effects. Although some CCBs decreased ^{125}I-pindolol (antagonist) binding to β_2-adrenergic receptors that signal via G_s, this was due to competition at the ligand binding site (Table 5).

That the increase in NT binding involved an enhanced interaction of NT with NTR1 was shown by photoaffinity labeling of immunoprecipitated NTR1. Augmentation of NT binding was not due to an increase in cell-surface receptors or to a change in receptor internalization. The binding of NT has been shown to initiate internalization of the NT-NTR1 complex, a process involving sortilin (Chabry et al., 1993). Stimulation of this process could conceivably lead to an apparent increase in cellular NT binding. However, we found that CCBs did not promote NT internalization and they did not change the apparent number of receptors participating in binding. Instead, the NT-displacement curve was shifted to the left, with an associated decrease in K_i and no change in NTR1 number. Classically, GPCRs display higher affinity for agonists, but not for antagonists, when they are in the coupled state as compared to the uncoupled state. CCBs increased the affinity of NTR1 for agonist NT without altering that for antagonist SR48692. Based on this, we propose that CCBs trap NTR1 in a G-protein-coupled state that exhibits increased affinity for NT. Although NT-induced IP formation was also inhibited, it is not known whether the "high-affinity" state of NTR1 exhibits a reduced ability to activate PLC.

However, Paton's rate theory of drug-receptor interaction would predict that increased affinity (associated with a decreased offset rate) would lead to decreased potency (Paton, 1961). Thus, if NTR1 is unable to release NT, it may be less efficacious.

An unexpected outcome was the finding that Ca^{2+} influx participated in the activation of PLC by NT. While other workers have shown that Ca^{2+} was required for PLC action in vitro, agonist-induced IP formation in cells was generally insensitive to removal of extracellular Ca^{2+} (Rhee and Bae, 1997). In contrast, we found 1) that NT-induced IP formation was enhanced by Ca^{2+} ionophore and inhibited by Ca^{2+} removal; 2) that Ca^{2+} ionophore stimulated IP formation, reproducing about half the NT response; and 3) that NT increased Ca^{2+} influx. Since NT can stimulate capacitative Ca^{2+} entry through SOCC (Gailly, 1998), it is likely that SOCCs contribute to the Ca^{2+} component of PLC activation by NT. Consistent with this, we found that the ability to inhibit NT-induced IP formation was associated with SOCC-directed agents. In addition, NIF inhibited NT-induced $^{45}Ca^{2+}$ uptake and NT-induced IP formation over the same dose-range. Unfortunately, we did not test other SOCC blockers for effects on $^{45}Ca^{2+}$ uptake.

Determining the PLC isotype(s) expressed by PC3 cells may be key to understanding these findings. PLCs are classified into three categories (PLCβ, PLCγ, and PLCδ) that exhibit distinct regulatory properties. While PLCβ is activated by α-subunits of $G_{q/11}$-type G-proteins and G$\beta\gamma$-subunits from other G-proteins, and PLCγ is regulated by tyrosine kinases (Rhee and Bae, 1997), PLCδ is activated by $[Ca^{2+}]$ in the physiologic range (Allen et al., 1997). Rhee and Bae (1997) proposed that PLCδ activation might occur secondary to receptor-mediated activation of PLCβ via the ensuing elevation in intracellular Ca^{2+}. This could provide an explanation for our results, given that Kim et al. (1999) have shown PLCδ1-activation mediated by the capacitative Ca^{2+} entry following bradykinin-stimulation of PC12 cells. Since PC3 cells express PLCβ and PLCδ isoforms (Carraway, unpublished results), it is possible that PLCδ might be activated by capacitative Ca^{2+} entry following NT-induced stimulation of PLCβ. Given that removing Ca^{2+} from the buffer inhibited NT-induced IP formation by ≅70%, this mechanism could account for the majority of IP formed during prolonged NT stimulation. Paradoxically, removing Ca^{2+} from the buffer was by itself a weak stimulus. Basal IP formation increased ≅2-fold when Ca^{2+} was omitted from or EGTA was added to the Ca^{2+}-containing buffer. This effect may have involved the release of Ca^{2+} from internal stores. In preliminary experiments we have shown that thapsigargin, a stimulator of internal Ca^{2+} release, elevates basal IP formation ≅2-fold.

Enhancement of NT binding by CCBs was always associated with inhibition of NT-induced IP formation. The efficacy order and potencies in these two assays were similar for the agents tested (Table 2). Furthermore, NIF altered bombesin receptor binding and bombesin-induced IP formation precisely as it did for NT, only to a lesser extent. These similar drug and receptor dependencies suggested that these effects came about coordinately or that they were separate events with similar chemical sensitivity. Supporting the latter hypothesis was the different Ca^{2+}-dependence of these effects. Whereas NT binding was largely Ca^{2+}-independent, NT-induced IP formation was partly Ca^{2+}-dependent. Although both effects were associated with SOCC-inhibiting drugs, the rank order of potency (NIF > SKF-96365 > miconazole > trifluoperazine) differed from that for inhibition of SOCC conductance (miconazole > NIF > trifluoperazine > SKF-96365) measured in HL-60 cells (Harper and Daly, 1999, 2003). One possibility is that PC3 and HL-60 cells express different Ca^{2+} channels, e.g., the six mammalian *Trp* genes can create multiple, functionally diverse Ca^{2+} channels that give complex responses to GPCR agonist activation and store depletion (Zhu et al., 1998). Another possibility is that Ca^{2+} channel occupation per se mediated CCB action, since Ca^{2+} channels interact with GPCRs (Grazzini et al., 1996) and G-proteins (De Waard et al., 1997), and since channel structure and conductance could depend on different drug properties. Another possibility is that CCBs target sites other than Ca^{2+} channels to alter NTR1 structure, and that this action alone enhances binding and obviates NT-induced IP forma-

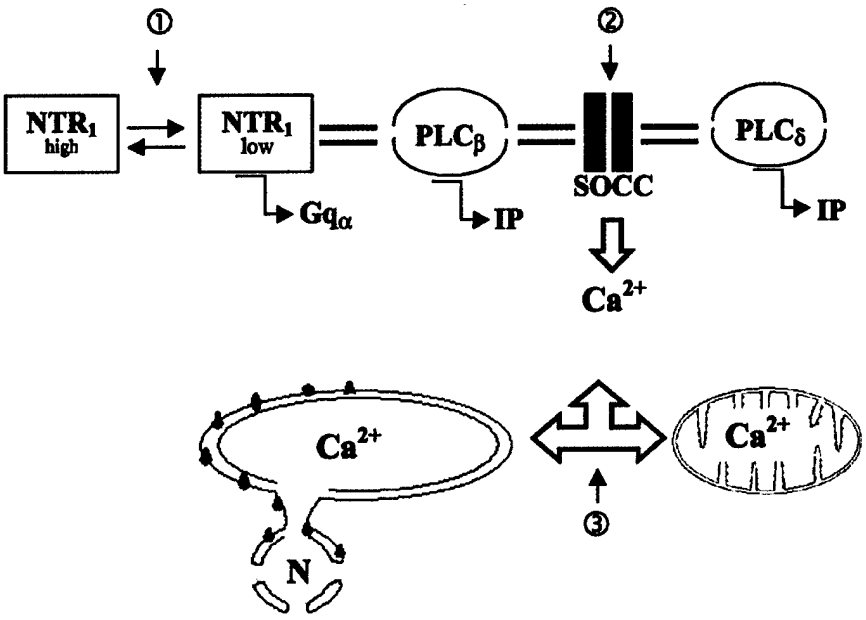

Fig. 9. Model depicting the effects of CCBs on NT binding and NT-induced IP formation. 1, by an indirect, Ca^{2+}-independent mechanism, these drugs shift NTR1 into a "high-affinity" state. If the high-affinity state of NTR1 is unable to activate PLC, this would explain the associated inhibition of NT-induced IP formation. 2, alternatively, NT-induced IP formation is inhibited by the blocking of the SOCC, which mediates the Ca^{2+} entry involved in activation of PLCδ. 3, another possibility is that these drugs alter some aspect(s) of cellular Ca^{2+} handling such that influxed Ca^{2+} is unable to activate PLCδ.

tion. At this time, the simplest explanation is that CCBs produce two effects: 1) they enhance NTR1 binding (and to a lesser extent) bombesin binding; and 2) they inhibit NT-induced (and to a lesser extent) bombesin-induced IP formation. Although changes in Ca^{2+} influx and Ca^{2+} channel interactions might contribute, especially to (2), it seems likely that other targets are also involved. These findings can be summarized as depicted in Fig. 9.

The effects of CCBs on NTR1 resemble those observed when EGFR is treated with tyrosine kinase inhibitors (Arteaga et al., 1997). Although EGF binding is increased greatly by AG1478 and PD153035 (as shown here), EGFR is unable to autophosphorylate in response to EGF and downstream responses are blocked (Lichtner et al., 2001). Tyrosine kinase inhibitors interact directly with EGFR, and the high-affinity state has been identified as an inactive dimer (Lichtner et al., 2001). CCBs do not interact directly with NTR1 since they do not increase NT binding to isolated cell membranes. However, it might be worthwhile to test the possibility that the phosphorylation state or polymerization state of NTR1 is indirectly altered by CCBs.

The DHPs, nimodipine and NIF, were the most potent (threshold dose, $\cong 1\ \mu M$) and most efficacious agents tested to elevate NT binding. NIF was also the most effective agent to inhibit NT-induced IP formation. Given that blood levels of DHPs in patients can approach the micromolar range and that DHPs concentrate in membrane fractions (Mason et al., 1992), it is possible that NT binding and bioactivity are altered in humans receiving these drugs. Whether any of the effects of these drugs on cardiovascular function involve NT is not known; however, NT is present throughout the cardiovascular system, where it can produce vasodilation and exert ionotropic and chronotropic effects (Ferris, 1989).

In conclusion, CCBs exert indirect effects in PC3 cells leading to 1) a dramatic increase in cellular NT binding and a smaller increase in bombesin binding; and 2) a dramatic inhibition of NT-induced IP formation and a smaller inhibition of the response to bombesin. Although changes in Ca^{2+} influx and Ca^{2+} channel interactions might contribute, especially to the latter response, it seems likely that other targets are involved.

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