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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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NT, neurotensin; NTR1, type 1 NT receptor; CCBs, Ca^{2+}-channel blockers; EGF, epidermal growth factor; EGFR, EGF receptor; IP, inositol phosphates; PLC, phosphatidylinositol-specific phospholipase C; HOLVA, des-Gly-[$Phaa^1$, D-$Tyr(Et)^2$, Lys^6, Arg^8]-vasopressin; VGCC, voltage-gated Ca^{2+}channel; SOCC; store operated calcium channel; G-protein, GTP-binding protein; GPCR, G-protein coupled receptor; NIF, nifedipine PMSF, phenylmethyl sulfonylfluoride; PASO, phenylarsine oxide; TPCK, tosyl-phenylalanine chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; TEA, tetraethylammonium hydroxide; cAMP, 3',5'-cyclic adenosine monophosphate; DMSO, dimethylsulfoxide.

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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 (VGCC) and store-operated (SOCC) Ca^{2+}-channels were tested for effects on NT-binding and signaling. Eight chemical types of agents, including VGCC-blocker nifedipine (NIF) and SOCC-blocker SKF-96365, enhanced cellular NT binding up to 3-fold, while inhibiting (by ≅70%) NT-induced inositol phosphate (IP)-formation. Ability to enhance NT binding correlated to ability to inhibit NT-induced IP-formation, and both effects were relatively specific for NT. Although cellular binding for β2-adrenergic, V_{1a}-vasopressin and EGF 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 PLCδ, while the Ca^{2+}-independent part probably involved PLCβ. Photoaffinity labeling of 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: (a)- they inhibit NT-induced IP-formation, perhaps by preventing Ca^{2+}-influx-dependent activation of PLCδ; (b)- they enhance NTR1 affinity by an unexplained Ca^{2+}-independent mechanism.

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Neurotensin (NT)[1], a peptide found primarily in brain and intestine, exerts many effects (Rostene and Alexander, 1997; Ferris, 1989) by activating type 1, G-protein-linked NT receptor NTR1 (Vincent et al, 1999). NTR1 is present on excitable cells (neuroendocrine, smooth muscle) and non-excitable 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 phosphates (IP), and release of intracellular Ca^{2+}. NT also induces Ca^{2+}-influx into excitable (Trudeau, 2000) and non-excitable cells (Gailly, 1998).

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 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

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NT to stimulate DNA synthesis and to enhance cAMP formation, and these effects are inhibited by VGCC-blocker NIF (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 non-excitable (Putney and Bird, 1993). Another process, which occurs in excitable and non-excitable 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 CHO cells (Gailly, 1998) and that NT elevates cellular [Ca^{2+}] in PC3 cells (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 (Triggle, 1999; Harper and Daly, 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 benzothiazipines (e.g., diltiazem). Inhibitors of SOCC include imidazoles (e.g., SKF-96365) and tricyclics (e.g., trifluoperazine). Unfortunately, these agents exhibit some non-specificity, and their actions can be complex (Triggle, 2003; Harper et al, 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 NMDA receptor was inhibited by 1-10μM nitrendipine (Skeen et al, 1993); nicotinic acetylcholine receptor-induced currents were abolished by 10μM NIF (Lopez, 1993); and 5-hydroxytrypamine receptor was inhibited by 10μM nimodipine (Hargreaves et al, 1996). These findings attest to

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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 find unexpectedly 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 regards 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 that these effects display similar receptor selectivity. Detailed studies examine the effects on NTR1 and investigate the involvement of Ca^{2+}-dependent PLC(s).

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METHODS

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

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

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Binding to PC3 Cells- PC3 cells, obtained from American Type Culture Collection (Rockville, MD), were maintained (passage 4) by our tissue culture facility at UMass 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 2ml/well of hepes-buffered Locke-BSA (Locke): 148mM NaCl; 5.6mM KCl, 6.3mM hepes; 2.4mM $NaHCO_3$; 1.0mM $CaCl_2$; 0.8mM $MgCl_2$; 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 1.0ml Locke with varying amounts of unlabeled ligand (0-1μM). The reaction was stopped on ice for 15min, the medium was aspirated and the cells were washed twice with 2ml and once with 4ml ice-cold saline. During this 5min 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 0.6ml 0.2M NaOH. A Packard 10-well γ-counter was used to measure radioactivity. Specific binding was defined as that displaceable by 1μM ligand.

Cell surface binding and internalization of ^{125}I-NT were assessed by washing cells at 22°C for 2min with 0.6ml 0.2M acetic acid, 0.5M NaCl, pH 3.0 (Beaudet et al, 1994). Binding at 4°C achieved equilibrium within 3 hrs, 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 pre-bound to PC3 cells in 24-well plates at 4°C for 3 hrs. After washing the cells three times in ice-cold PBS, >90% of ^{125}I-NT was located on cell surface as determined by acid washing. Agents (10mM in DMSO) were diluted to 50μM 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 % internalization per min was calculated.

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Binding displacement curves were constructed for each set of treatments and binding parameters were determined by Scatchard analysis. Ki was determined by using the equation {Ki = IC50 / 1+[L] / Kd} where Kd 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±11µg (control, n=6), 183±10µg (50µM NIF, n=6), 190±12µg (50µM phloretin, n=6) and 181±11µg protein (100µM 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 or 50µM NIF or 100µM verapamil. HPLC was performed at 1.5ml/min on µBondapak-C18 (8x100mm) with linear gradient (20min) from 0.1% trifluoroacetic acid to 30% CH_3CN. ^{125}I-NT eluted at 25.0min. 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 ability to bind to fresh cells. The loss of binding ability was ≅5% after 25min. Protease inhibitors, o-phenanthroline and PMSF (0.5mM), 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 or 50µM NIF or 100µM verapamil, dissociation of cell-associated radioactivity was negligible (<6%) during incubation in ice-cold saline for 15min.

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Binding to PC3 Cell Membranes- PC3 cell membranes were prepared and collected by centrifugation at 30,000g as described by us (Seethalakshmi et al, 1997). Binding of ^{125}I-NT (10^5cpm) to membranes (10-50μg) was performed at 20°C for 60min in 10mM Tris-HCl (pH 7.5), containing 1mM $MgCl_2$, 1mM 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 (Mitra and Carraway, 1994).

Crosslinking of ^{125}I-[4-azido-Phe^6]-NT to NTR1- [4-azido-Phe^6]-NT was iodinated and purified by HPLC to ≅1500Ci/mmol. PC3 cells in 10cm dishes were incubated with $0.3x10^6$ cpm/ml ^{125}I-[4-azido-Phe^6]-NT in 8ml Locke 25min at 37°C in presence and absence of Ca^{2+}-channel agents. 1μM NT was added to controls. Cells were placed on ice for 30min, irradiated at 254nm with a handheld UV light for 5 min at 3 cm, washed in ice-cold phosphate-buffered saline (PBS) and lysed in 10mM Hepes, 1mM EDTA, 0.5mM o-phenanthroline, PMSF, TPCK (pH 7.4). Membranes, collected by centrifugation (30,000g, 20min) were solubilized in 250μl 50mM Tris buffer (pH 7.4), 150mM NaCl, 0.5% Triton X-100, 0.5% NP-40, 5% glycerol at 4° C for 2 hr. Solubilized NTR1, diluted 2-fold in buffer without detergent, was immunoprecipitated by addition of our rabbit antiserum (Ab-NTR1) towards 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[2], the parent protein of 50kDa and a breakdown product of 33kDa, in keeping results in other cells (Boudin et al, 1995). After 18 hrs at 4°C, protein A-agarose (10mg, Sigma) was added for 6 hrs. After the agarose beads were washed 3 times with PBS at 4°C, associated radioactivity was measured using a γ-counter. Usually the immunoprecipitate contained ≅5% of the solubilized cpm for samples prepared in the absence of NT. SDS-PAGE was used in some

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cases to validate that the radiolabeled material represented NTR1. For this, the beads were boiled 5 min in an equal volume of 2 x SDS sample buffer and extracts were subjected to SDS-PAGE using 10% polyacylamide gels, followed by autoradiography.

Western Blotting-- PC3 cells in 60mm dishes were washed in Locke containing inhibitors: 0.5mM EDTA, 0.5μM PMSF, 0.5μM TPCK and 0.5 μM o-phenanthroline. Cells were lysed in 100μl of 2 x SDS loading buffer with inhibitors, scraped into microfuge tubes and sonicated (20 sec) on ice. Membranes were isolated from regions of adult rat brains (Carraway et al, 1993) and P2 pellets were extracted in 2 x 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 PVDF (Immobilon P, Millipore). After blocking in 5% non-fat milk in TTBS: 0.05% Tween 20, 20 mM Tris, 0.5M NaCl for 1 h and washing 3X 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. Enanced chemiluminescence was performed according to manufacturer (Santa Cruz). After stripping (1 h at 70°C in 62.5 mM Tris-HCl, 2% SDS, 0.1M 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+}-

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free Locke and pretreated for 10min with 0-36µM NIF (600µl per well). The reaction was initiated by addition of 200µl NT, followed in 2min by 2.5mM $CaCl_2$ (5µCi $^{45}Ca^{2+}$ per well). After 8min, the cells were washed three times with ice-cold Locke and solubilized in 1.0ml 0.25M 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 hrs with myo-[^{3}H]-inositol (2.5µCi/ml) in medium 199, 5% fetal calf serum. Medium 199 (Difco) was chosen because of its low inositol content. After washing with 2ml Locke, cells were preincubated 10 min with test agents in Locke, 15mM LiCl and reactions were started by adding a maximal dose of NT (30nM) or vehicle. After 30 min at 37°C, medium was aspirated, ice-cold 0.1M formic acid in methanol (1ml) was added and plates were placed at –20°C overnight. Samples were transferred to tubes using 2 x 2ml water washes and [^{3}H]-IP was adsorbed to 0.25ml AG-1X8 slurry (formate form, Bio-Rad), which was washed five times in 5mM myo-inositol (5ml) and eluted in 0.75ml 1.5M ammonium formate, 0.1M formic acid. Scintillation counting was performed on 0.5ml eluate in 5ml Ecoscint (National Diagnostics). 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 t-test. Results were calculated as mean±SEM and $p<0.05$ was considered significant.

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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/well. Table 1 gives the binding parameters determined for NT binding to PC3 cells. Data given in 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[2] of and the steady state level of NT binding to PC3 cells (Fig 1A). NIF enhanced specific binding, without altering non-specific 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 sub-micromolar concentrations {control, 100±4%; 0.3µM nimodipine, 116±5% ($p<0.05$); 0.9µM NIF, 115±5% ($p<0.05$)}. While less specific CCBs (flunarizine, tetrandrine, trifluaperazine 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).

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CCBs Inhibited NT-induced IP Formation- NT increased IP formation ≅5-fold in PC3 cells with an EC50 ≅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 EC50 for enhancing NT binding was similar to the IC50 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.

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, 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), while they had little effect on NT binding (Fig 3B). In contrast, CCBs NIF and SKF-96365 had little effect on EGF binding (Fig 3A; Table 5), while they enhanced NT binding ≅3-fold (Fig 3B; Fig 1CD). 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.

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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 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 towards 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 towards other types of channels did not enhance NT binding to PC3 cells (Table 4). These included: N-type Ca^{2+}-channel blocker (ω-conotoxin), Ca^{2+}-release inhibitors (ryanodine, dantrolene), K^{+}-channel blockers (glibenclamide, diazoxide, TEA), Na^{+}-channel blocker (amiloride) and 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

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and for EGFR. Radioreceptor assays were developed for β2-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 β2-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). β2-adrenergic receptor binding was, in fact, decreased by these 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 (results in Table 5 footnote). 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 (see Table 5 footnote). These data indicated that the robust elevation in cell binding was specific to NTR1, although 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 (30nM), bombesin (20nM) and ATP (10μM) 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 % inhibition by 15μM NIF was independent of the

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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 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 46kDa protein that has been immunologically characterized (Boudin et al, 1995) and labeled using UV-activatable crosslinkers (Mazella et al, 1988). Initially, we used western blotting to verify the specificity of our antiserum (Ab-NTR1) raised towards the C-terminus of human NTR1. While extracts of rat brain gave a single band at ≅50kDa, PC3 cells gave this parent protein, along with a 33kDa 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 50kDa and 33kDa 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.

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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), at 37°C in the presence of phenylarsine oxide (Fig 6A), and at 37°C by acid washing (Fig 6B). Internalization of ^{125}I-NT was 68-72% 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 50μM NIF, 50μM phloretin and 50μM verapamil. Internalization rates (% / min; n=12 from 2 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 was expressed as % maximal binding, CCBs shifted the displacement curves to the left by a factor of 2-3 (Fig 7A). In three experiments, the Ki for NT was decreased from 0.95±0.1nM (control) to 0.36±0.04nM (50μM NIF; $p<0.01$), 0.40±0.05nM (50μM phloretin; $p<0.01$) and 0.61±0.06nM (100μM verapamil; $p<0.05$). Scatchard analyses indicated that NIF increased the affinity of NTR1 for NT, without changing 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 50μM NIF (Fig 7C), although the Ki was not changed significantly (Ki: control, 12±1.0 nM; NIF, 14±0.8; n=4; $p>0.1$). Taken together, these results

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indicated that CCBs shifted NTR1 towards 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 CHO 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 EC50 (≅1nM) 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 the Locke buffer, by adding Ca^{2+}-chelator EGTA to the Locke, or by adding NIF to the 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 (% control) was: 2μM ionomycin, 139±6% ($p<0.01$); 20μM ionomycin, 324±14% ($p<0.01$); 30nM NT, 457±12% ($p<0.01$); n=4 experiments). When added 2 min after a maximal dose of NT (30nM), low doses of ionomycin (2-10μM) enhanced the response to NT. IP formation (% control) was: 10μM ionomycin, 157±5 ($p<0.01$); NT, 366±20 ($p<0.01$), NT plus

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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 (% control) was: (25μM 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 (% control): 2mM 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.

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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 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 β2-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), employed agonist ligands. Although difficult to understand at this time, it is interesting that the V_{1a}-vasopressin assay, which employed 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

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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 β2-adrenergic receptors which 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 Ki and no change 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.

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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 a)- that NT-induced IP formation was enhanced by Ca^{2+}-ionophore and inhibited by Ca^{2+}-removal; b)- that Ca^{2+}-ionophore stimulated IP formation, reproducing about half the NT response; and c)- that NT increased Ca^{2+}-influx. Since NT can stimulate capacitative Ca^{2+}-entry through SOCC (Gailly, 1998), it is likely that SOCC 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

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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 > trifluoperizine) differed from that for inhibition of SOCC conductance (miconazole > NIF > trifluoperizine > 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 perse 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-

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induced IP formation. At this time, the simplest explanation is that CCBs produce two effects: (a)- they enhance NTR1 binding (and to a lesser extent) bombesin binding; and (b) 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 (b), 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 (Lichner 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 (threshhold dose, ≅1μM) 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 (a)- a dramatic increase in cellular NT binding, and a smaller increase in bombesin binding; and (b) a dramatic inhibition of

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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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FOOTNOTES

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[2] 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.

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FIGURE LEGENDS

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

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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 EC50 ≅ 1.0nM. The minimum dose of NT that significantly ($p<0.05$) elevated IP formation above control was 0.2nM. B- Log dose-response plots showing that IP formation in response to a maximal dose of NT (30nM) was inhibited by CCBs. The minimum dose that significantly ($p<0.05$) decreased IP formation below control was 5μM (NIF), 7μM (phloretin), 7μM (SKF-96365), 20μM (miconazole) and 40μM (verapamil). Results are from 10 experiments (A) and 3-4 experiments (B).

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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 10min with indicated agents, and binding was performed at 37°C. Results from 5 experiments (A) and 3 experiments (B) were expressed as % control and plotted as mean±SEM. 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.

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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 (20nM), bombesin (10nM) and ATP (10μM). Results are from 3 experiments. B- Plots show inhibitory effects of 15μM NIF on responses to various doses of NT (0.2-20nM), bombesin (0.1-10nM) and ATP (0.2-10μM), plotted as a function of fold enhancement of basal IP formation. Results are from 3 experiments.

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Fig 5- CCBs enhanced photoaffinity labeling of NTR1. A- Western blot, representative of 3 experiments, verifying the specificity of Ab-NTR1 towards 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 crosslinking of ^{125}I-(4-azido-Phe^{6})-NT to immunoprecipitated NTR1. PC3 cells were incubated 10min in 50µM NIF, 50µM phloretin, 100µM verapamil or 0.5% DMSO at 37°C. ^{125}I-(4-azido-Phe^{6})-NT (3x10^{5} cpm/ml; 0.15nM) was added to all dishes and 1µM 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 γ-counter and data were expressed relative to control (100%), which typically gave ≅5000cpm. The drugs enhanced crosslinking 1.5- to 2.8-fold, and 1µM NT reduced it by >90%. Results are from 4 experiments, except NIF (8 experiments).

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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 hrs at 4°C and method #2: incubation at 37°C in presence of 10μM PASO. 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 (50μM) enhanced cell-surface binding (2.8 fold) and internalization (2.6 fold), similarly. Results are from 3 experiments (A) and 4 experiments (B).

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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 protein) was increased 2.7-fold by 50µM NIF, 2.6-fold by 50µM phloretin and 2.0-fold by 100µM verapamil. (A)- Plots show displacement of ^{125}I-NT binding by NT, in which binding was expressed as % control. The agents shifted the curves to the left. IC50 for NT was 1.2nM (control), 0.8nM (verapamil), 0.5nM (phloretin) and 0.5nM (NIF). Results are from a typical experiment repeated twice. (B)- Scatchard plots for typical experiment showing that NIF increased NTR affinity (apparent Ki: control, 0.93nM; NIF, 0.33nM) without increasing receptor number (Bmax: control, 23fmol/well; NIF, 21fmol/well). (C)- Plots show displacement of ^{125}I-NT binding by SR48692. In the presence of 50µM NIF, the curve was shifted slightly (but not significantly) to the right. Results are from typical experiment performed 4 times.

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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 (EC50, ≅1.2nM). The log dose-response plot shows that a 10min pretreatment of cells with varying doses of NIF inhibited the response to 20nM NT (IC50≅12μM), without much effect on basal $^{45}Ca^{2+}$ influx. Results are from 3 experiments. B- IP formation in PC3 cells in Locke buffer (1mM Ca^{2+}) was enhanced ≅4.5 fold by 20nM NT (shown as 100% response). The response to NT was inhibited by omitting Ca^{2+} from Locke, by adding to Locke either 1.1mM EGTA, or 50μM NIF, or 1.1mM EGTA plus 50μM 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 10nM bombesin and ≅17-fold by 10μM ATP in similar experiments. Results are from 3 experiments.

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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 SOCC, which mediate 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.

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Table 1. Parameters Determined for Binding of ^{125}I-labeled Ligands to PC3 Cells

Ligand [a]	Specific Binding [b] (% of total)	Bmax [c] (fmol/mg)	Ki [c] (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 β2-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 (≅185μg protein/well) using 10^5 cpm ^{125}I-ligand in 1.0ml Locke (see Methods).

c Scatchard analysis was performed using 12 ligand concentrations and results were from 3 to 9 experiments.

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Table 2 – Activity of CCBs on NT Binding and NT-induced IP Formation

Channel	Agent	NT Binding [a]		IP Formation [b]	
		Efficacy (% increase)	EC50 (μM)	Efficacy (% decrease)	IC50 (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

[a] Efficacy was defined as the maximal % increase in NT binding observed for each agent. ED50 was defined as the [agent] at which NT binding was increased by 80%. The data are means determined in 3-8 experiments for each agent.

[b] Efficacy was defined as the maximal % decrease in NT-induced IP formation observed for each agent. IC50 was defined as the [agent] at which IP formation was decreased by 50%. The data are means determined in 3 to 8 experiments for each agent. nd, not determined.

[c] L-type CCBs.

[d] L-type/T-type blockers.

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Table 3. Effects of Ca^{2+}-Channel Blockers on NT-binding to PC3 Cell Membranes

Agent [a]	Specific NT-Binding (% control) at Dose of Agent [b]			
	10μM	25μM	75μM	100μM
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 10mM and diluted into Locke just before use.

[b] PC3 cell membranes were preincubated 10min with agents or control, and NT binding was performed at 22°C for 60 min (see Methods). Specific binding was measured in 4-6 experiments and expressed as % control (mean±SEM). Results for the various agents were not significantly different from control ($p > 0.1$).

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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]			
	2μM	10μM	30μM	100μM
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	
tetraethyl ammonium hydroxide	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 10mM and diluted into Locke 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 % control (mean±SEM) for at least 3 independent experiments. Results for the various agents were not significantly different from control ($p>0.1$).

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Table 5. Effects of CCBs on PC3 Cell-binding of Ligands Specific for Bombesin-, Vasopressin-, β2-adrenergic- and EGF-receptors

Ligand	Agent	Specific Binding (% control) at Dose of Agent [a]	
		12μM	60μM
^{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 Methods and Table 1). Binding was expressed as % control (mean ±SEM) for 3 to 6 independent experiments.

[b] Verapamil and SKF-96365 resemble pindolol structurally. Thus, the decrease in binding was due to direct competition with the ligand (% crossreaction, ≅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 Methods and Table 3). Binding (% control±SEM) for 3 experiments in duplicate was: 60μM verapamil (9±2); 60μM 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 (% control±SEM) for 3 experiments in duplicate was: 60μM verapamil (91±5); 60μM SKF-96365 (110±3); 60μM NIF (90±3).

* Result was significantly different from control ($p<0.05$).

** Result was significantly different from control ($p<0.01$).

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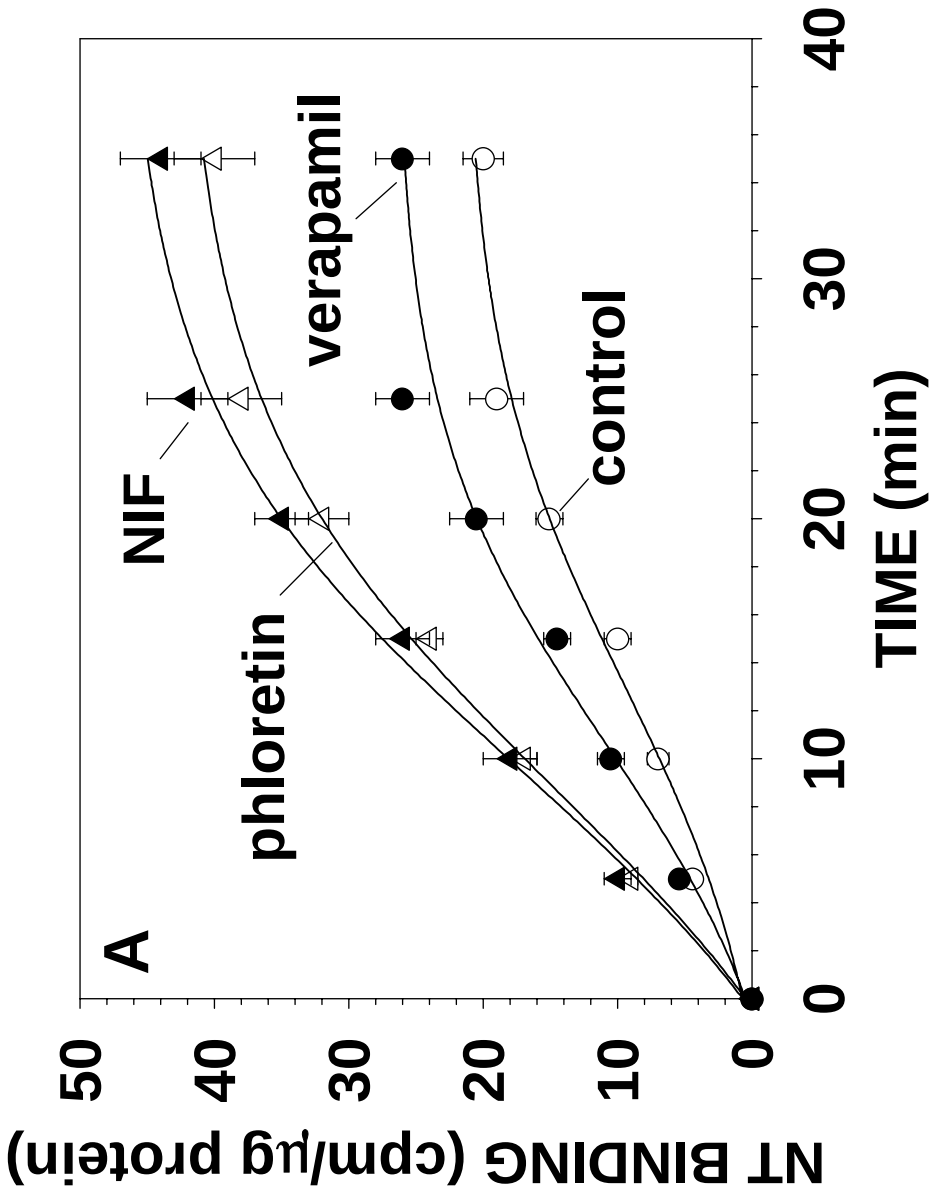

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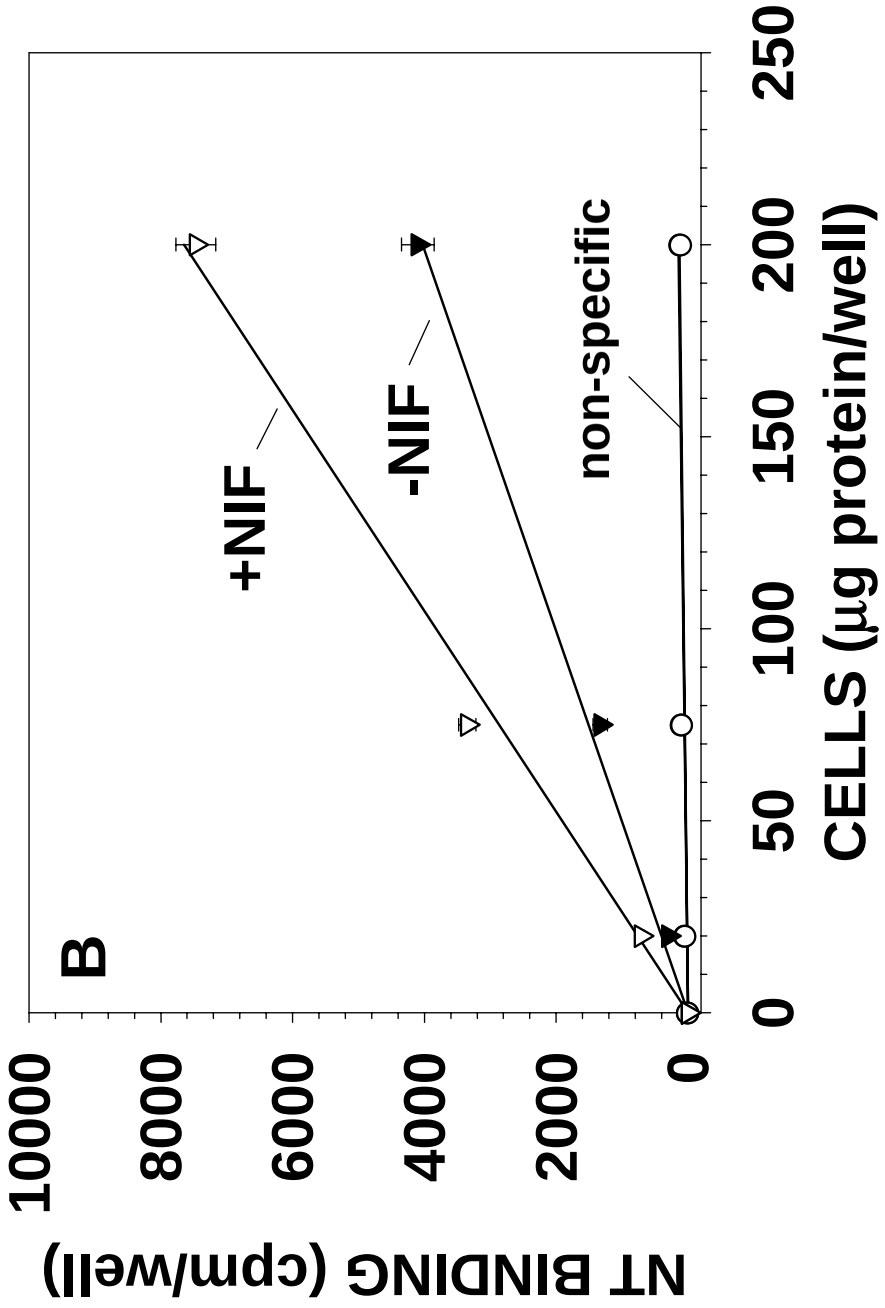

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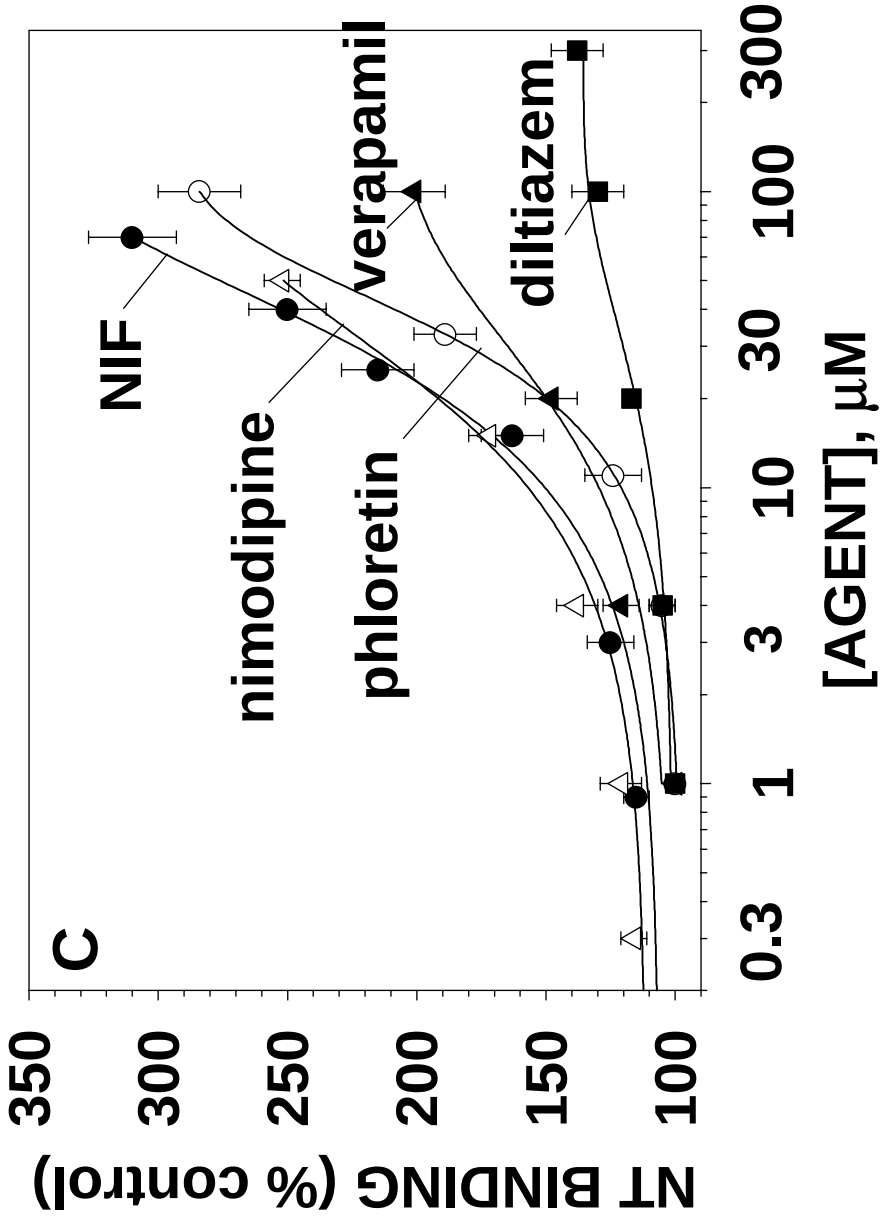

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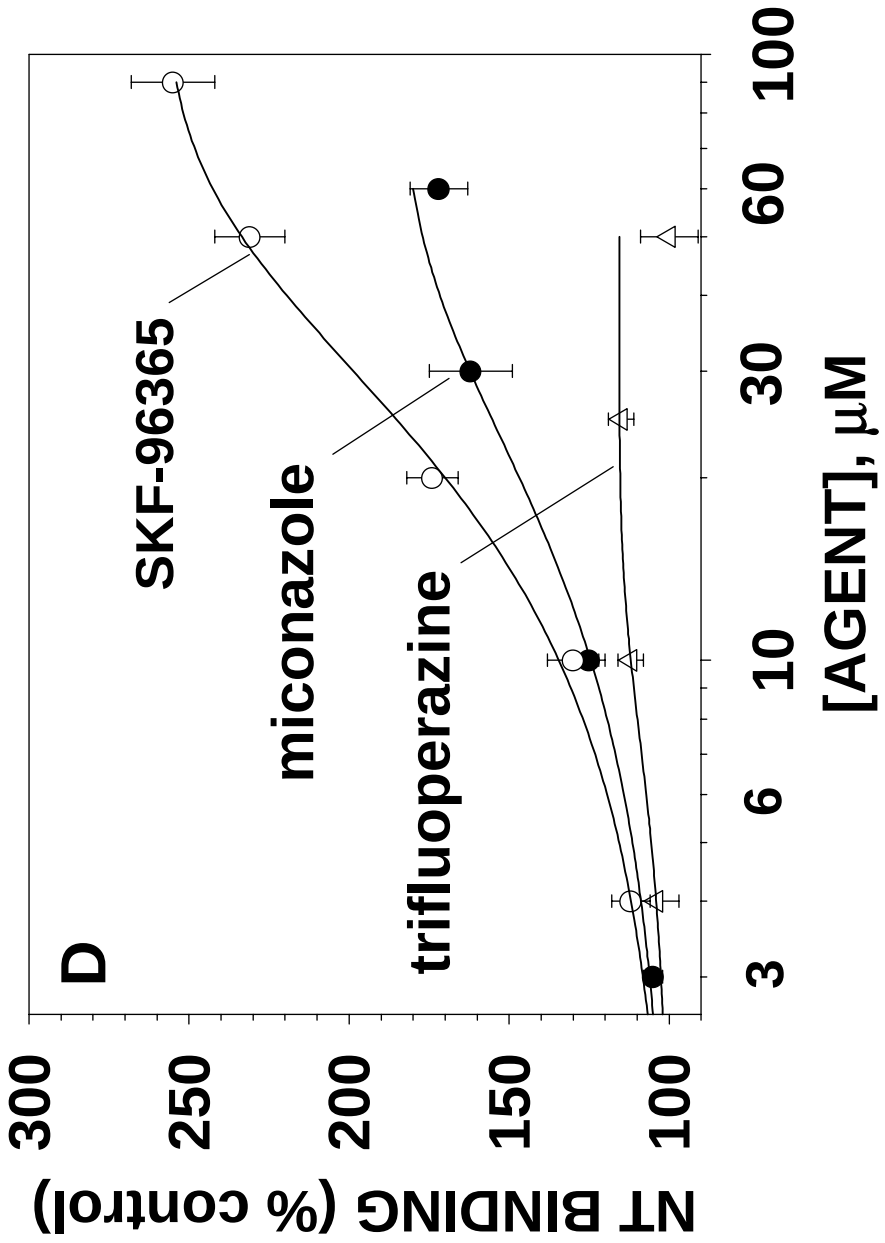

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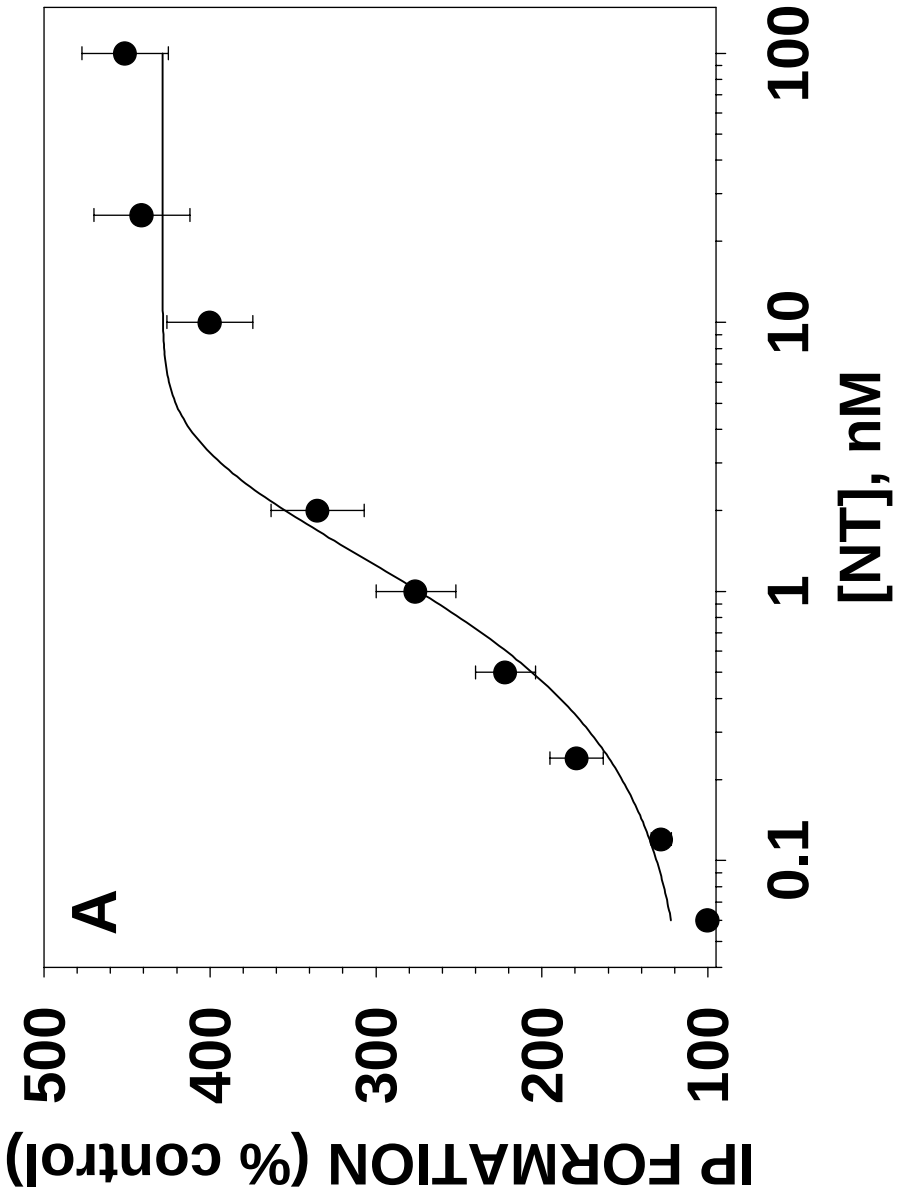

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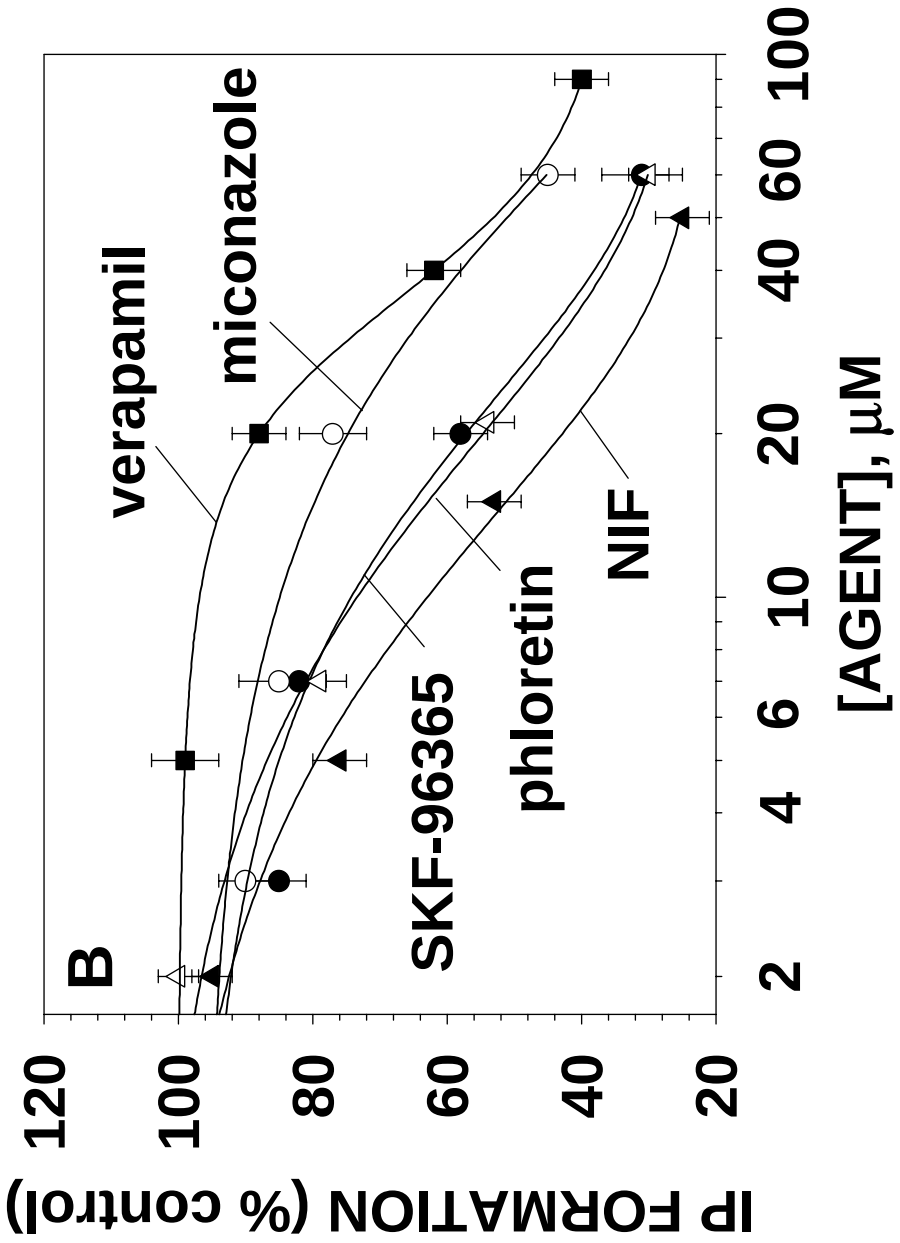

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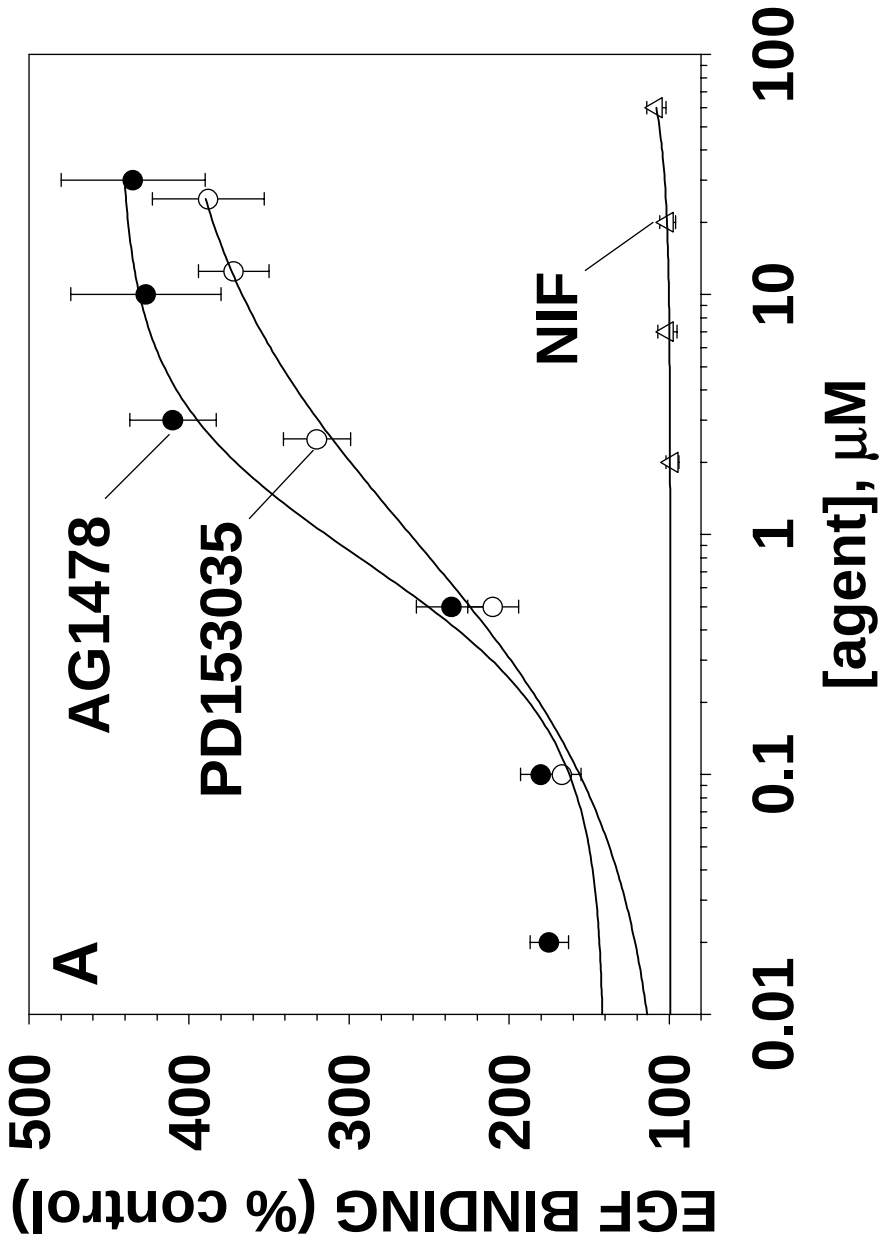

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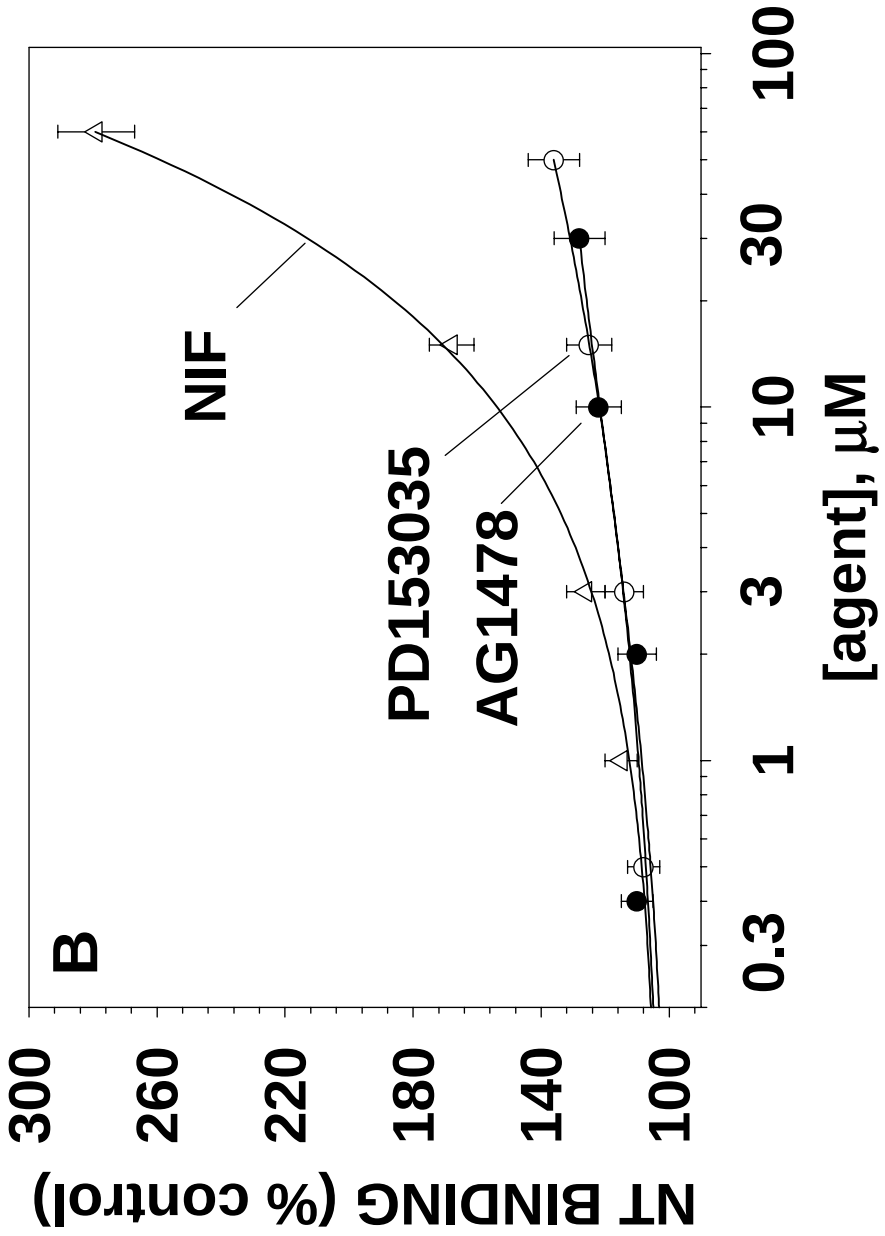

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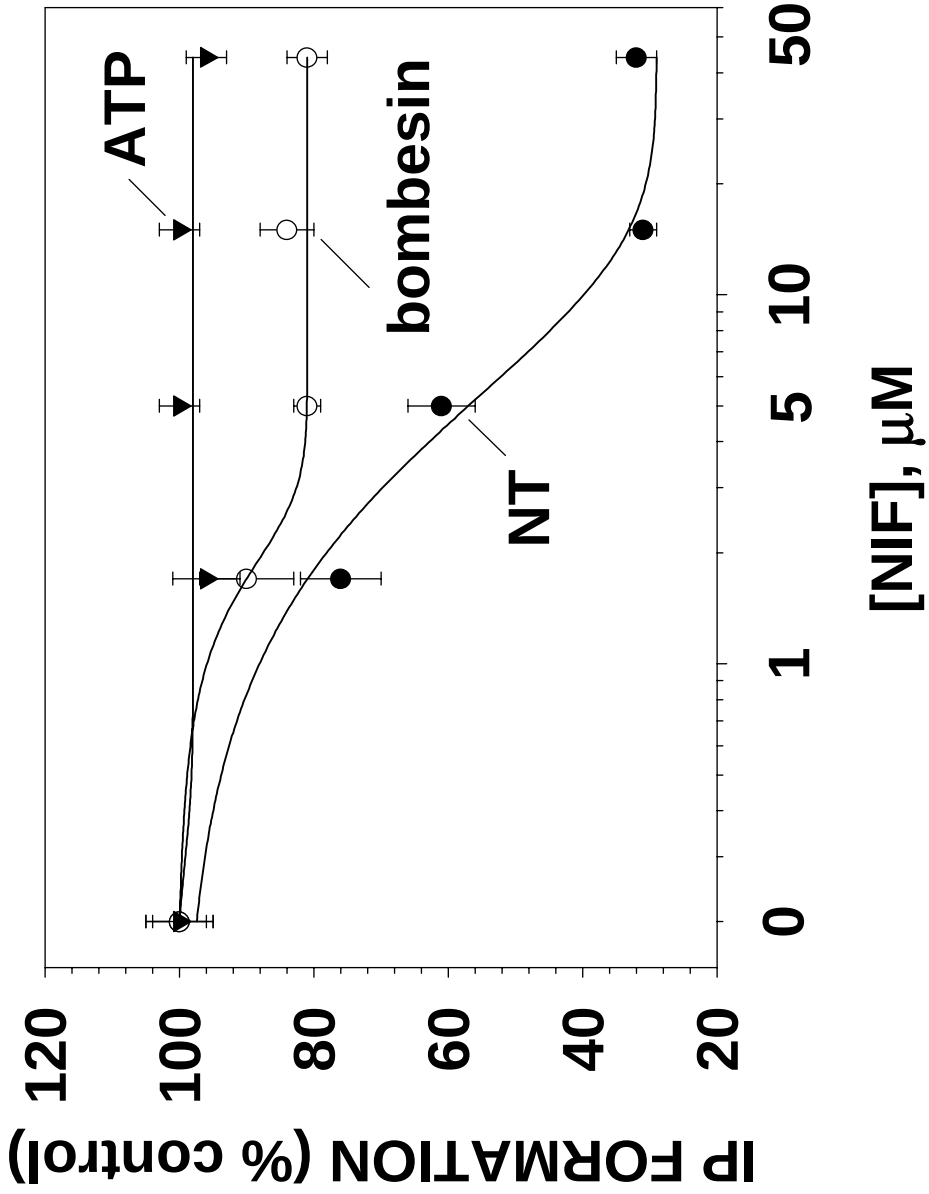

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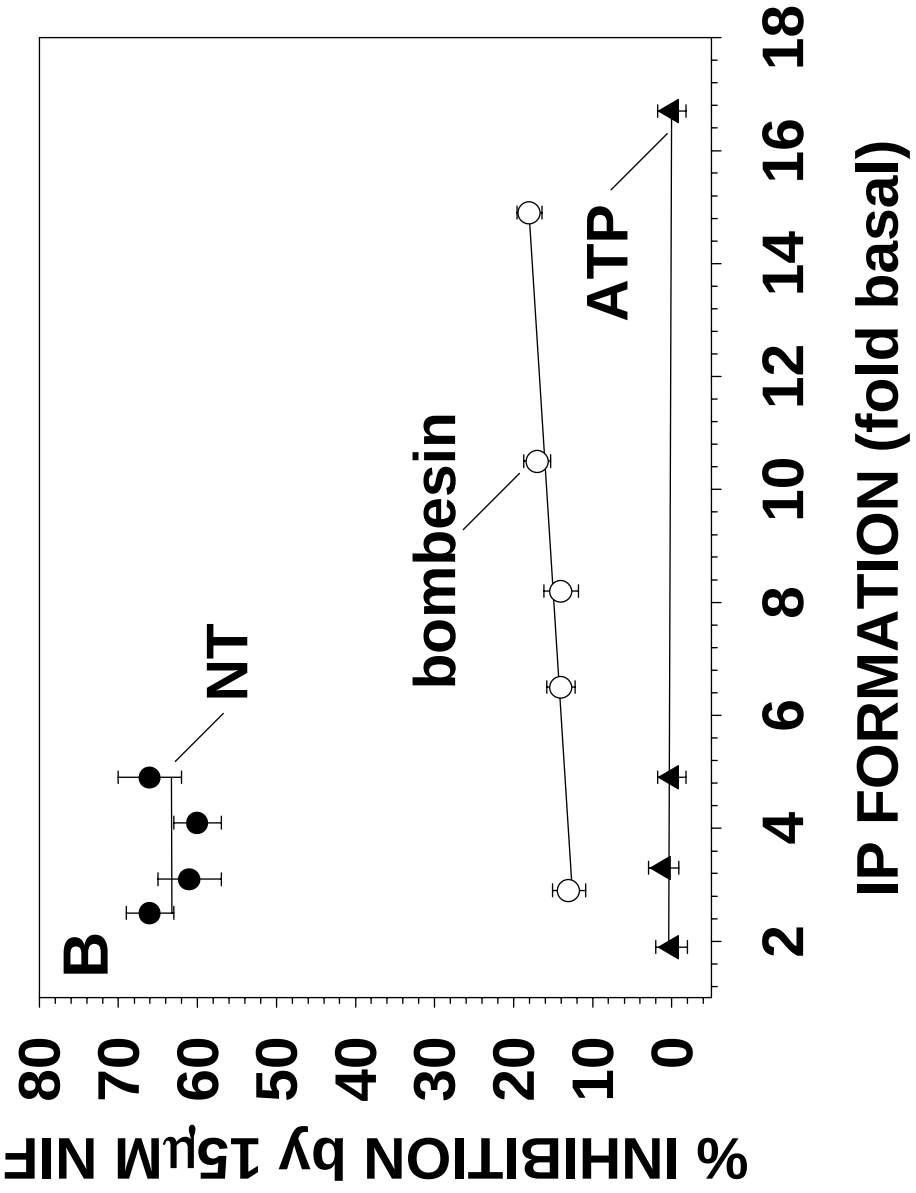

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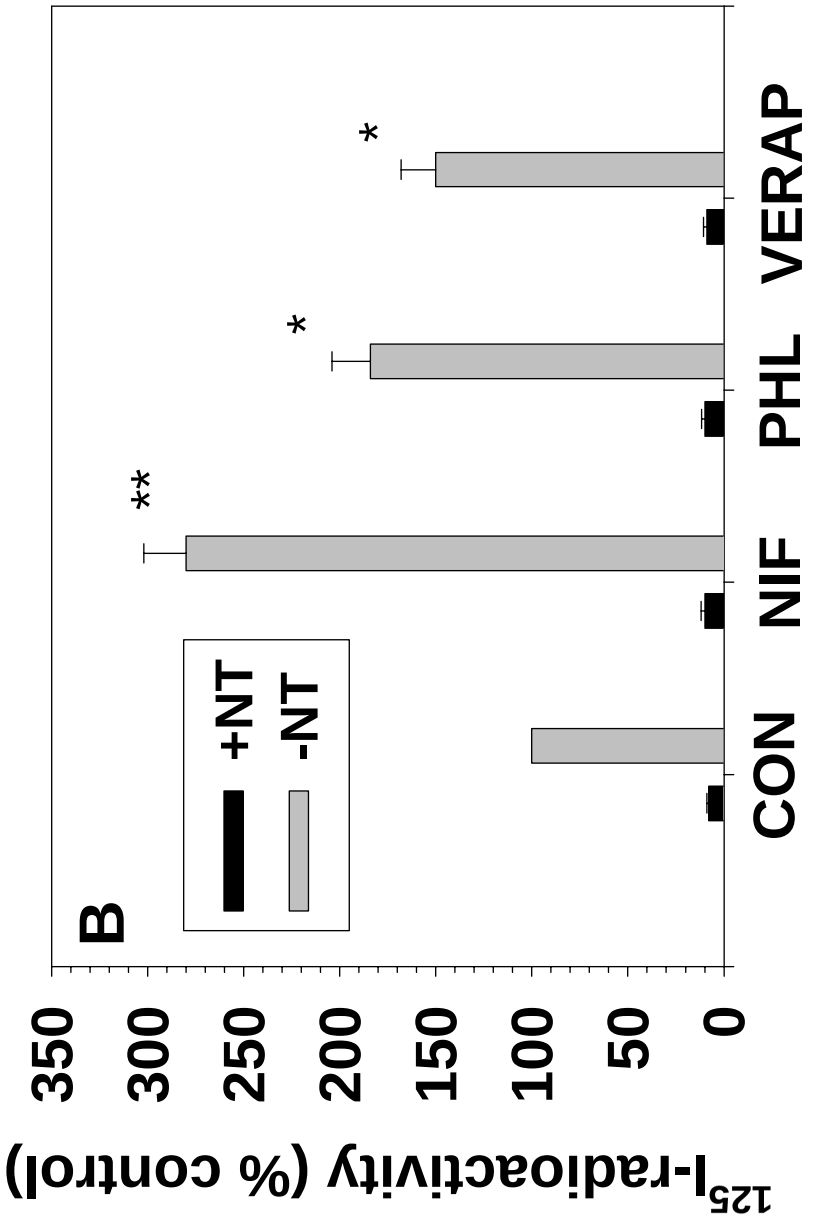

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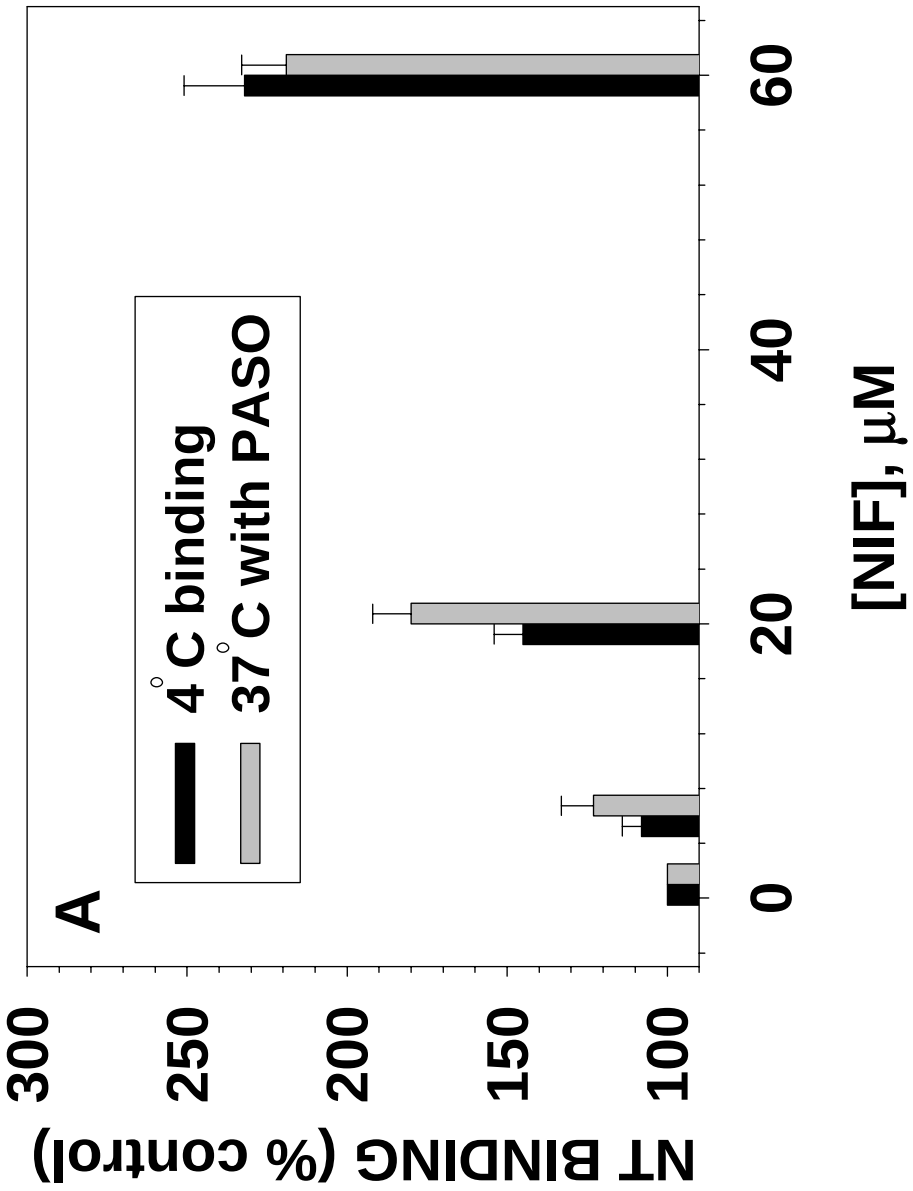

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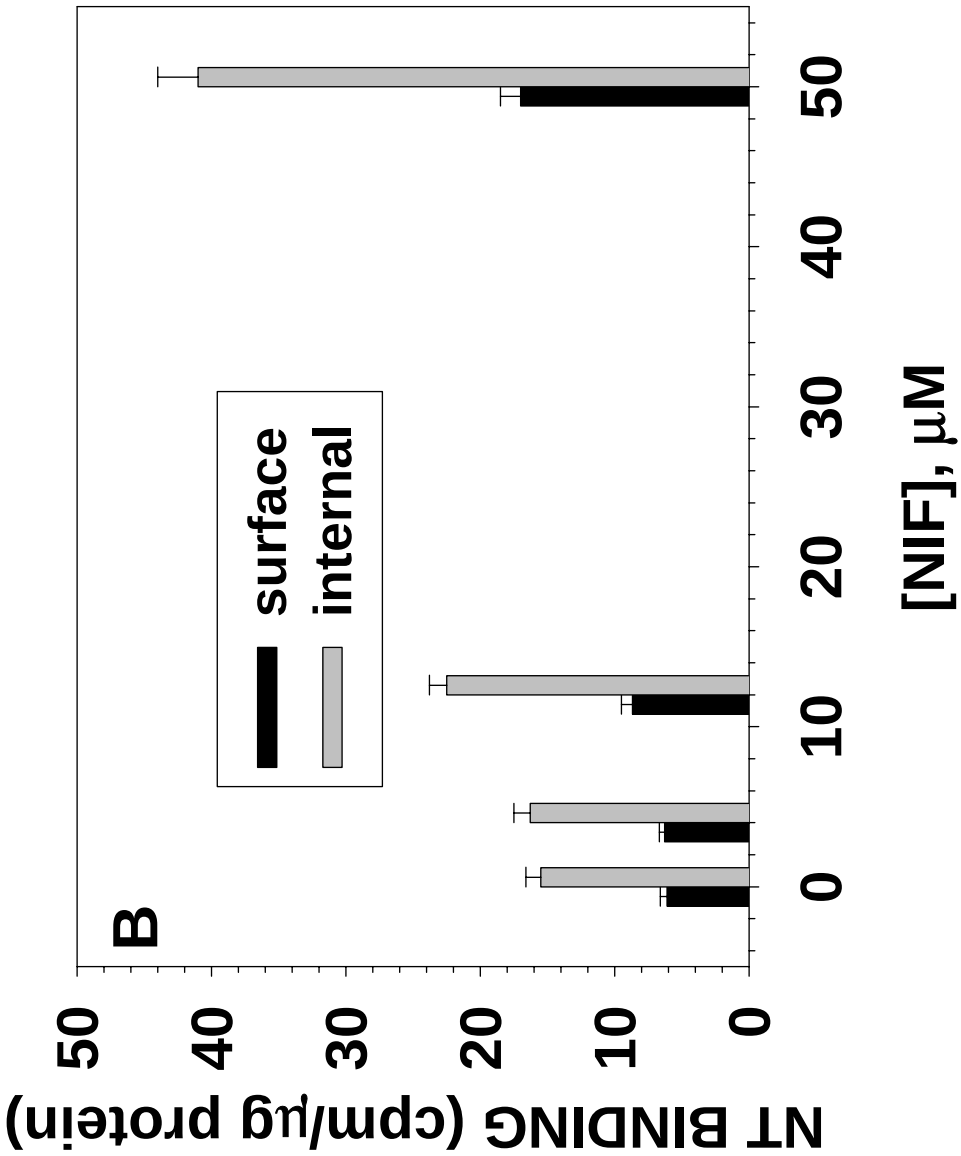

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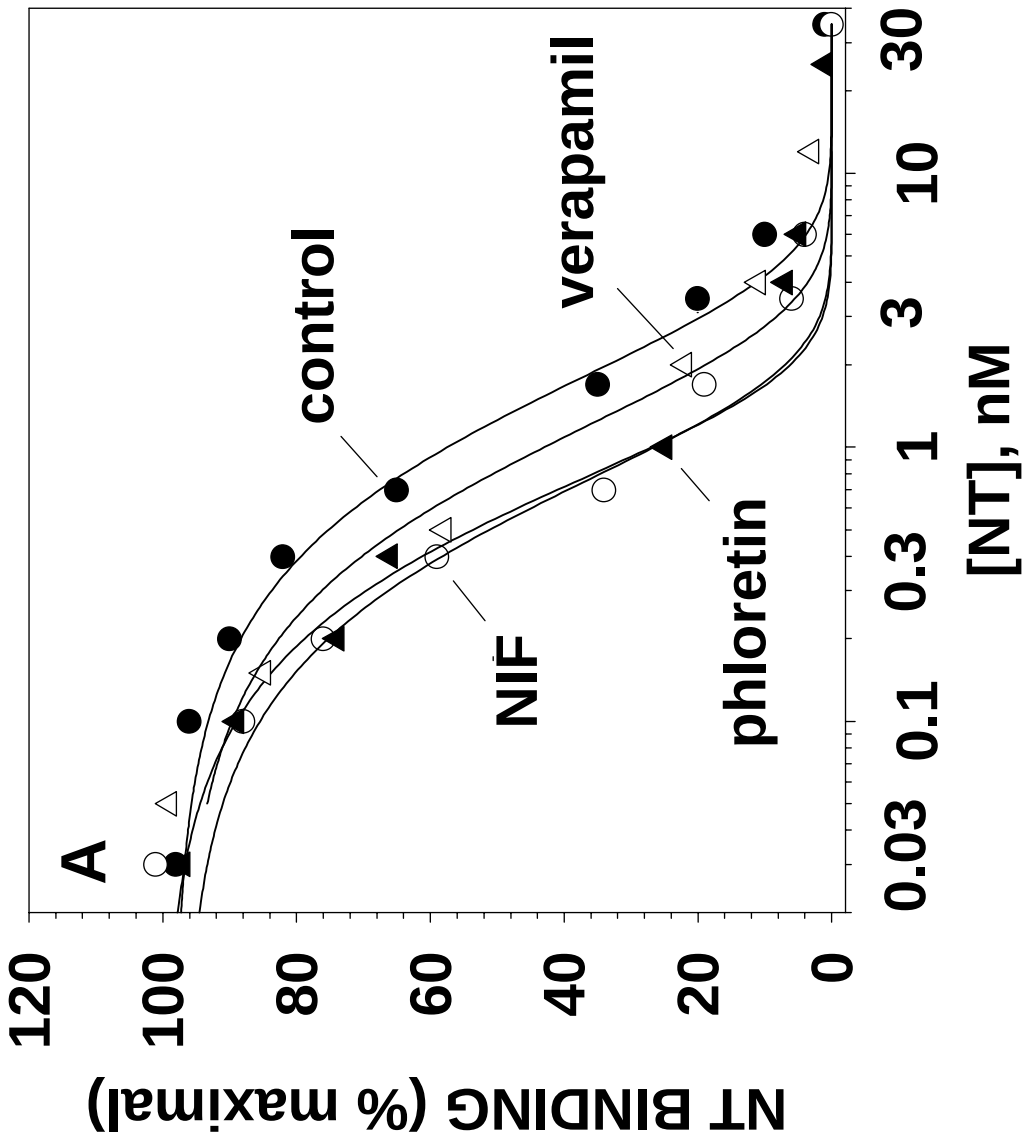

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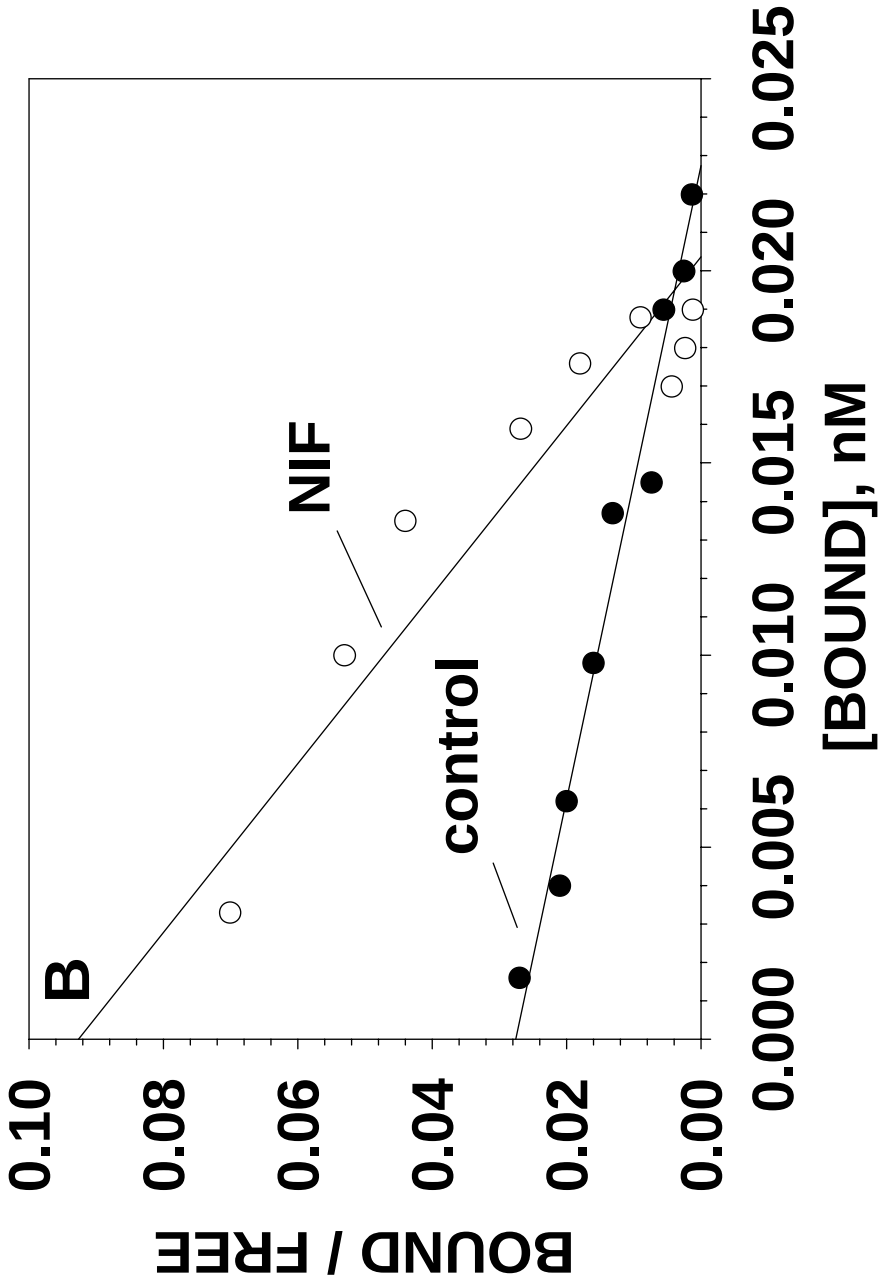

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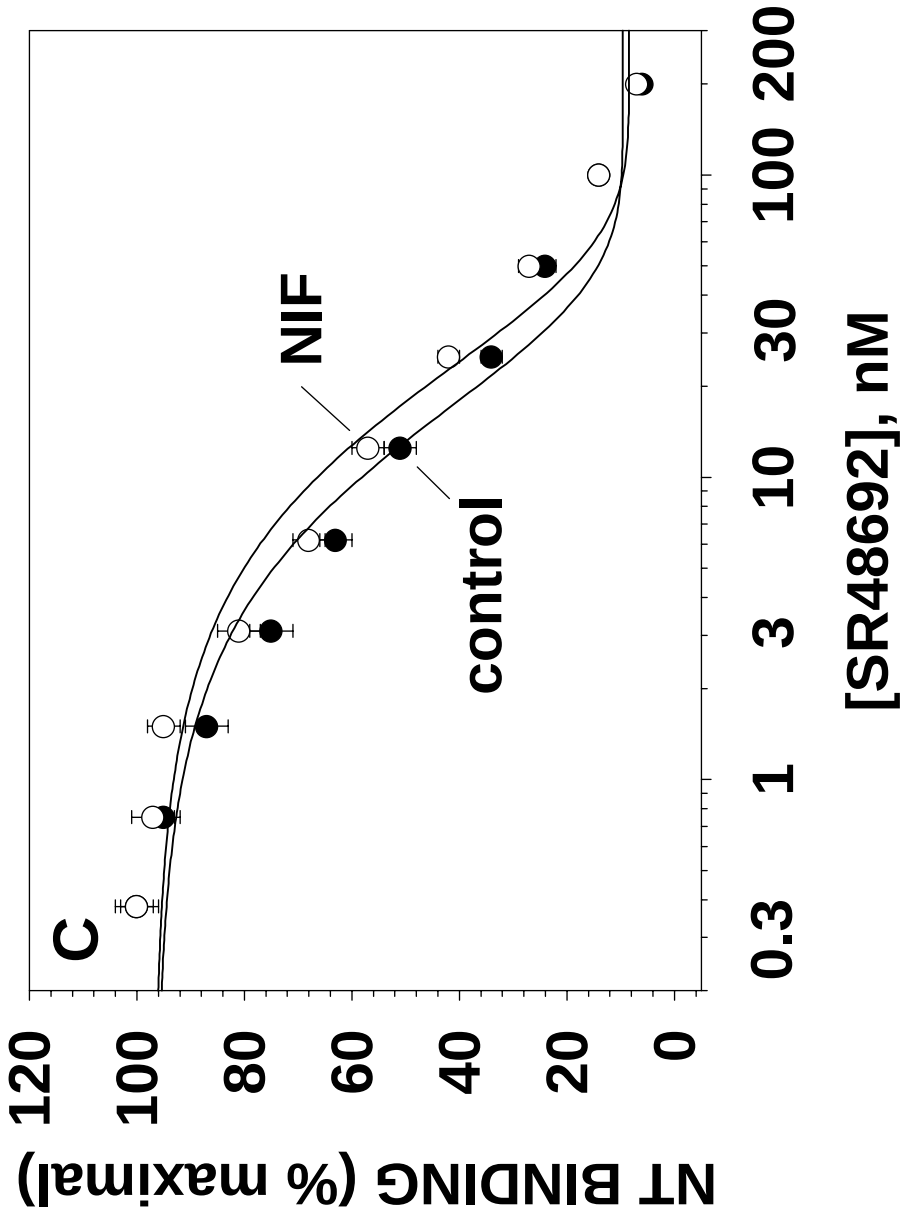

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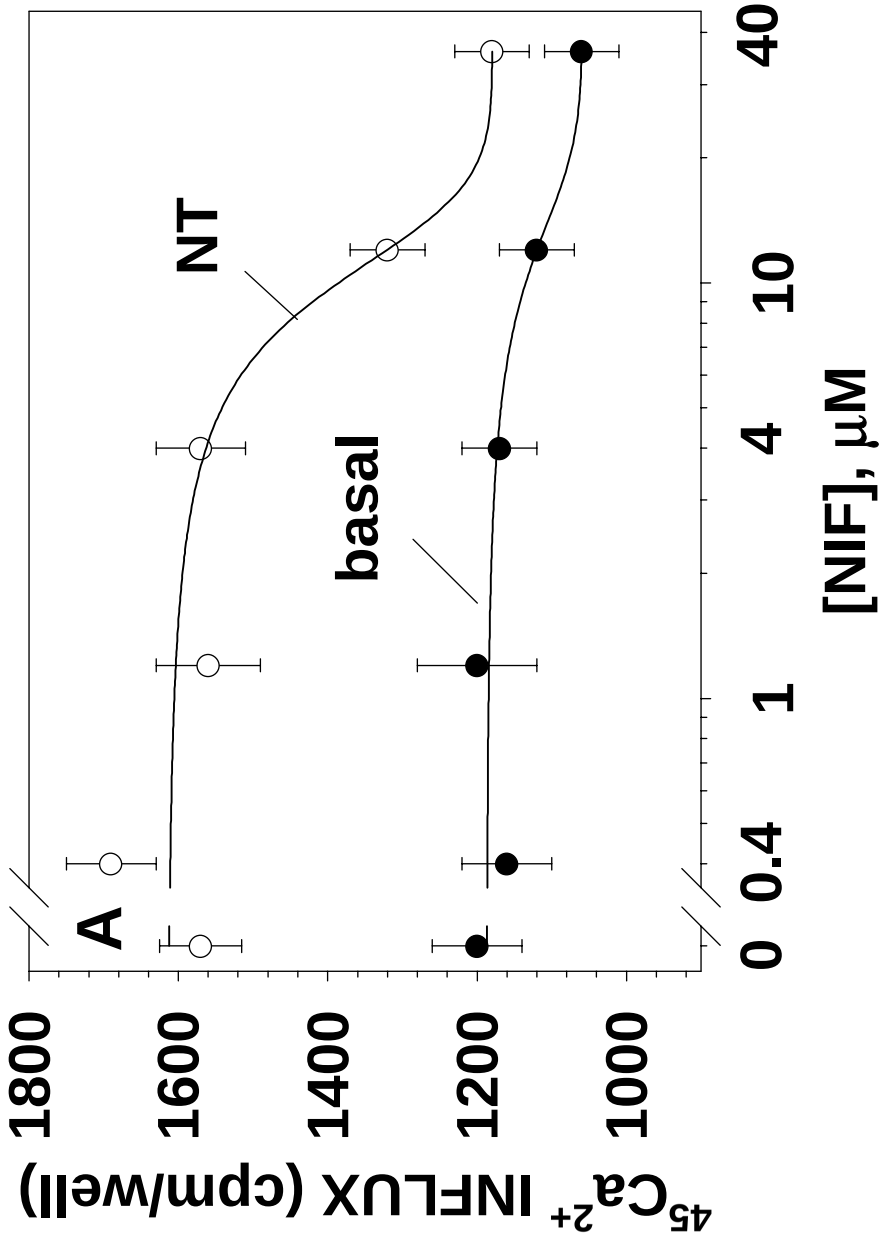

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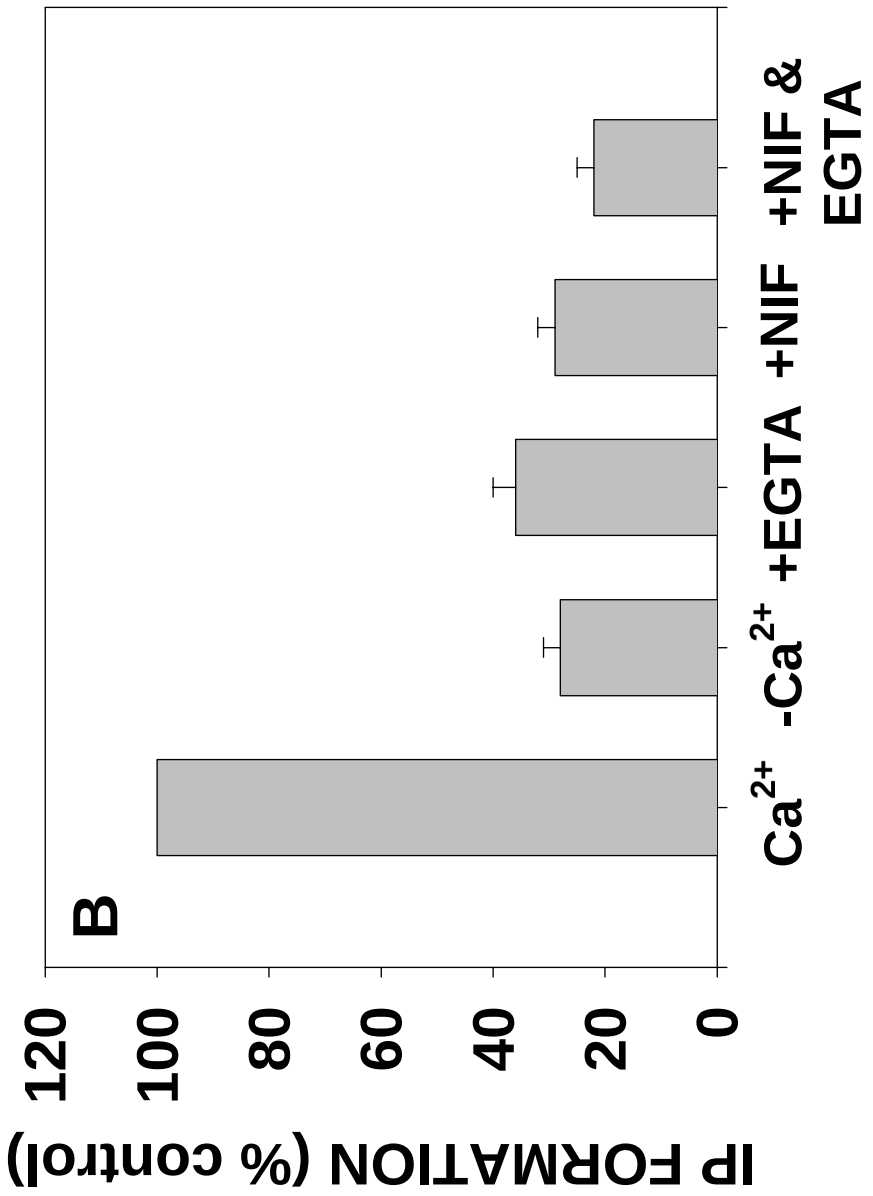

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