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14. ABSTRACT
The purpose is to define the relationship between neurotensin (NT) and protein kinases and to investigate the mechanism by which flavonoids (FLAV) inhibit NT growth signaling in PC3 cells. The long-range scope is to determine the significance of NT in the negative effects of high fat intake on PC incidence and the positive effects of diets containing FLAV. Our results show that NT-induced growth signaling involves activation of PKC, that arachidonic acid metabolism and EGF receptor activation participate in the NT signaling process, that cell metabolism and ATP levels can influence NT receptor function, and that activated PKC (most notably PKC alpha and beta) can feed back to regulate the ability of NT receptor to bind NT and to initiate signaling. FLAV was found to exert differential effects on PKC isotype activation and downregulation. In addition, FLAV was shown to inhibit protein tyrosine kinase activity in PC3 cells and evidence supports the notion that this can input to regulate NT receptor function. NT receptor was shown to localize in caveolae and to be displaced when activated by NT. FLAV appeared to influence this response to NT. These findings have implications regarding mechanisms that regulate NT receptor function and the design of agents to block NT-induced growth signaling in PC.

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neurotensin, PC3 cells, cancer growth, PKC, protein tyrosine kinase, caveolae, flavonoids

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Prostate Cancer cell growth: stimulatory role of neurotensin and the mechanism of inhibition by flavonoids as related to protein kinase C.

Principal Investigator, Robert E. Carraway

1. INTRODUCTION

Neurotensin (NT), an intestinal growth factor that is also involved in inflammation, has a potential role in prostate cancer (PC) since NT receptor (NTR) is over-expressed in PC and NT stimulates growth of cultured human PC cells. We found higher levels of NTR in aggressive androgen-independent PC3 cells than in the less aggressive androgen-dependent LNCaP cells. We demonstrated that NT stimulates DNA synthesis and growth of PC3 cells via a protein kinase C (PKC)-dependent transactivation of EGF receptor. Since NTR is Gq-coupled and NT promotes inositol phosphate (IP) formation in PC3 cells, the initial signaling event appears to involve the activation of phospholipase C, which is followed by diacylglycerol (DAG)-mediated activation of PKC. Although multiple downstream effectors could mediate NT's growth effect, a major mechanism seems to involve the liberation of heparin binding (HB-EGF), which then initiates the activation of the PI3-kinase / AKT pathway and the MAP-kinase / ERK pathway.

Fat intake, which correlates with PC incidence around the world, is the strongest stimulus for NT secretion. When injected into rats, NT promotes the conversion of arachidonic acid (AA) into growth-promoting eicosonoids via activated lipoxygenase (LOX). Our work in PC3 cells has shown that inhibitors of phospholipase A2 (PLA2), DAG lipase and LOX block NT-induced growth signaling, supporting the idea that the effects of NT involve the liberation of AA and its conversion to eicosanoids. Thus, NT and fatty acids could work in concert to mediate fat-induced PC growth. In contrast to the "high fat" diet, Asian and Mediteranean diets that are rich in antioxidant flavonoids (FLAVs) correlate with decreased PC incidence. One possibility is that FLAVs inhibit the cancer promoting effects of NT. Our work has shown that FLAVs inhibit NT growth signaling in PC3 cells, which provides a rationale for their beneficial effects. A key goal in this grant is to understand the mechanism(s) by which FLAVs inhibit NT signaling, to identify the most potent FLAVs and to evaluate the overall significance to the inhibition of PC growth *in vivo*.

Since FLAVs have been reported to inhibit PKC and LOX *in vitro*, as well as a number of receptor tyrosine kinases (RTK) and protein kinases, the mechanism for inhibition of NT could be complex. However, particularly intriguing is our finding that FLAVs can exert upstream effects on the

NT receptor (NTR), altering its binding properties *in vivo*. In PC3 cells, FLAVs caused an immediate increase in NTR binding affinity, while inhibiting the ability of NT to stimulate IP production and to induce DNA synthesis. Based on this, we have hypothesized that FLAVs block kinases (e.g., PKC or RTK) that somehow regulate NTR function. Indeed, we showed that PKC can regulate NTR function, and as detailed in last year's report, we identified the PKC isotypes that were involved. However, our findings indicated that FLAVs do not exert remarkable effects on the endogenous PKC activity in live cells, suggesting that other kinases are likely to mediate the major effects.

The current year's work has focused primarily on a second hypothesis. The idea is that FLAVs could disrupt the integrity of caveolar modules where NTR interacts with G proteins and effector molecules to promote signaling. The maintenance of these signaling modules could require energy, substrates and kinase activities that are subject to inhibition by FLAVs. Defining the mechanism(s) by which FLAVs inhibit NT-stimulated growth could shed light on general aspects of G-protein receptor action, signal transduction and pathway relationships, which could have a widespread impact due to the number of growth stimuli involved.

1. BODY

The work accomplished during this year was severely limited by the loss of the primary laboratory worker, Dr. Sazzad Hassan (originally 100% effort) who accepted a higher level position elsewhere on April 1, 2008. With only 9 months of funding remaining in the budget, it was not possible to attract a replacement for Dr. Hassan. In an attempt to alleviate this problem, Dr. Robert Carraway (originally 30% effort) increased his effort towards the project. The lack of any other technical help made the burden of learning the methods, maintaining the cells and performing all the experiments a considerable challenge. Despite these limitations, significant progress was achieved. By applying for a no-cost extension, Dr. Carraway also increased the project period by one year, which will provide more time to achieve the original goals.

Task 5. Determine effects of FLAV on the activation of PKC-isotypes (months 12-24).

As noted above and in our earlier reports, our work shows that FLAVs are not very effective PKC inhibitors *in vivo* using the PC3 cell system, although the literature indicates that they are potent inhibitors using *in vitro* assays. Since FLAVs are also known to inhibit protein tyrosine kinases (PTKs) and PI3 kinase, these were other possible targets to explore. To investigate the molecular specificity of FLAV actions, workers have compared the effects of various FLAVS on the activity of PTK, PI3 kinase and PKC in a number of systems. These studies have shown that the positioning of the hydroxyl groups

in the FLAV structure strongly influences the inhibitory effects and that the results differ quite remarkably for these different kinases (Biol Pharm Bull 31, 1403-1409, 2008; Biochem Pharmacol 53, 1649-1657, 1997; Brit J Pharmacol 128, 999-1010, 1999). These findings led us to characterize the hydroxyl group dependence for the effect of FLAVs on NT binding in PC3 cells, thinking that this information might help us to determine which of these kinases is involved.

The chemical structures of the various FLAVs investigated are depicted in Fig 1 and Table 1. The basic structure consists of two benzene rings (rings A & B) linked through a heterocyclic pyran or pyrone (ring C). Ring B is usually attached at C2 of ring C (Fig 1). The different categories are defined by the presence of various substituents, most important being the oxy group at C4 (ring C), the C2-C3 double bond (ring C) and an OH group at C3 (ring C). Catechins lack the oxy group at C4. Flavones, isoflavones, flavonols, and chalcones have the C2-C3 double bond, whereas this structure is missing in flavanones and catechins. Flavonols have an OH group at C3, whereas isoflavones have the B ring is attached at this position. The C ring is not closed in chalcones, which lack the oxy group at C1. The C ring is also missing in stilbenes but they do have the central double bond (**Fig 1**).

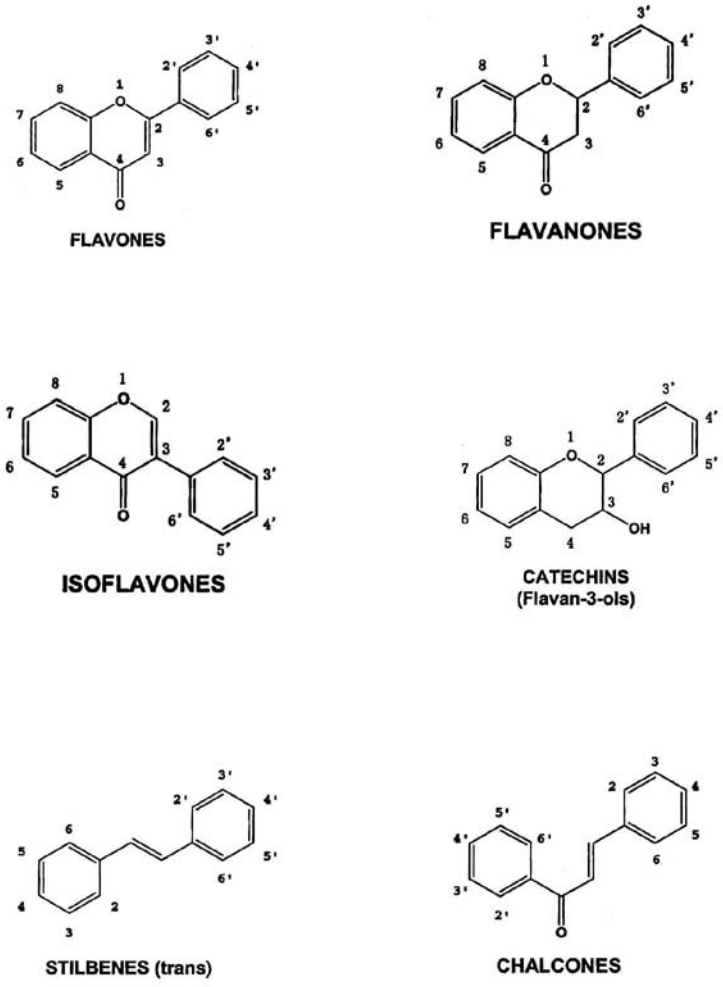

Figure 1- Chemical Structures of Flavonoids and Polyphenols

Table 1- EC50 for the Effect of Specific Categories of Polyphenols on NT Receptor Binding

	Ring Position Number							# OH * Groups	EC50 ** (μM)
	3	5	6	7	3'	4'	5'		
FLAVONES									
3-OH flavone	OH							1	58
5-OH flavone		OH						1	60
7-OH flavone				OH				1	62
chrysin		OH		OH				2	35
apigenin		OH		OH		OH		3	24
luteolin		OH		OH	OH	OH		4	23
baicalein		OH	OH	OH				3	55
ISOFLAVONES									
Genistein		OH		OH		OH		3	47
Daidzein				OH		OH		2	>100
FLAVONOLS									
galangin	OH	OH		OH				3	16
fiscetin	OH			OH	OH	OH		4	28
kaempferol	OH	OH		OH		OH		4	16
quercetin	OH	OH		OH	OH	OH		5	28
myricetin	OH	OH		OH	OH	OH	OH	6	45
FLAVANONES									
naringenin		OH		OH		OH		3	>200
taxifolin	OH	OH		OH	OH	OH		5	>200
CATECHINS									
catechin	OH	OH		OH	OH	OH		5	>200
ECGC	galate	OH		OH	OH	OH	OH	5	>100
STILBENES									
cis-stilbene								0	>200
trans-stilbene								0	>100
resveratrol	OH	OH				OH		3	65
diethylstilbestrol	4-OH				OH			2	12
CHALCONES									
Phloretin	4-OH			2'-OH	4'-OH		6'-OH	4	30

* Total number of OH groups present in the molecule.
** EC50 for the change in NT binding to intact PC3 cells at 37 C for 30 min.

Using our standard procedure, we measured the effects of multiple doses of each FLAV on NT binding to intact cells during 30 min at 37° C, and the average EC50 determined in 4 experiments is given in Table 1. Comparing the results for the stilbenes, it can be seen that stilbenes lacking OH groups on the benzene rings are unable to alter NT binding whereas resveratrol and diethylstilbestrol are quite active. Comparing the results for the flavones, it can be seen that the potency for the effect on NT binding increases with the total number of OH groups (peaking at 4). This is also true for the flavonols where potency peaks at 4 total OH groups and then decreases for 5 and 6 total OH groups. This trend is

likely due to the negative effect of increased polarity on penetration into the cells. The essential nature of the C2-C3 double bond is clear from the inactivity of all of the flavanones and catechins. This is best seen by comparing naringenin to apigenin and taxifolin to quercetin. The importance of the OH group at C3 can be seen by comparing galangin to chrysin and kampferol to apigenin, each of which shows enhanced potency with addition of the C3 OH. By comparing results for apigenin and phloretin, one concludes that the ether group that closes the middle ring is not essential for the effect on NT binding.

Comparing our results for the effects of FLAVs on NT binding to published results for effects on various isolated kinases, it is clear that our results are most similar to those for PTKs. For example, the order of potency for the NT system was similar to that for inhibition of PTK activity (luteolin > quercetin > genistein > myricetin >> taxifolin and catechin). Both responses showed an absolute requirement for the C2-C3 double bond. On the other hand, for the inhibition of PI3 kinase, myricetin was the most potent and galangin was the least potent of the flavonols, which is opposite to our results for the effect on NT binding. Similarly for PKC inhibition, myricetin, quercetin and luteolin were more effective than apigenin and kaempferol, whereas the opposite was obtained for the effect on NT binding. Also consistent with an involvement of RTKs in the regulation of the NT receptor by FLAVs was the ineffectiveness of daidzein, which is the standard inactive control for the RTK inhibitor genistein. In total, these results suggested that the effects of FLAVs on NTR function in PC3 cells could involve an inhibition of RTK activity in the cells.

To further test this idea, we next assessed the ability of a number of FLAVs to inhibit RTK initiated activation of the MAP kinase pathway in PC3 cells. Cells were pretreated 30 min with FLAVs and then stimulated for 15 min with 1% human serum plus 10 nM EGF. Cell extracts were processed for western blotting to determine the levels of activated ERK. The results in **Fig 2** show that FLAVs inhibited this RTK-mediated response. As for the effects on NT receptor, luteolin was more effective than quercetin and resveratrol.

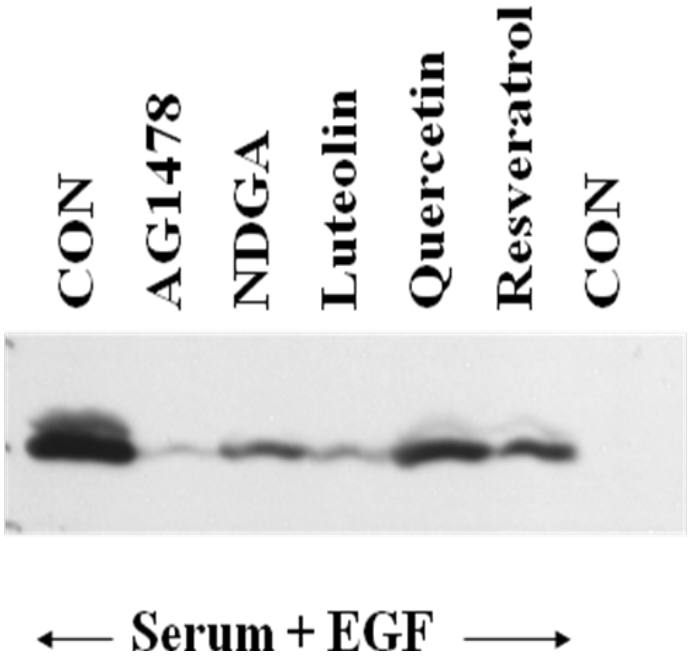

Figure 2 – FLAVs inhibit RTK – mediated MAP kinase activation by serum and EGF. Quiescent cells were pre treated with the agents (30 uM) or the DMSO control for 30 min and then stimulated for 15 min. Cell extracts were subjected to western blotting for phosphorylated ERK.

Since RTK activity is generally opposed by the action of tyrosine phosphatases (Tyr P'ase), our idea that RTKs regulated the NT receptor could also be tested by targeting Tyr P'ase. These enzymes possess viscinal sulfhydryl groups that are highly sensitive to certain heavy metals that bind to them avidly. The most active inhibitors of Tyr P'tase are peroxovanadate and zinc. To prove that these Tyr P'tase inhibitors would enhance RTK activity in PC3 cells, we assessed the effects of peroxovanadate and zinc on MAPK phosphorylation, an indicator of RTK activity in these cells. **Fig 3** and **Fig 4** show that the expected results were obtained. Peroxovanadate (Fig 3) enhanced the endogenous RTK activity, and this response was blocked by the antioxidant N-acetyl cysteine (Nac) and the viscinal sulfhydryl agent 2,3-dimercapto pyridine (MP). Zinc pyrithione (Fig 4) gave precisely the same effect. Pyrithione was used since this ionophore facilitates the entry of zinc ion into cells. Again this response was blocked by NAc and MP, which is in keeping with the mechanism involving viscinal sulfhydryl groups.

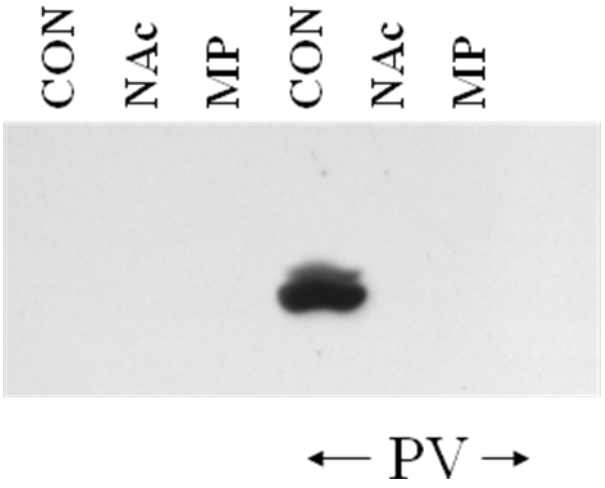

Figure 3 – Peroxovanadate (P'Tase inhibitor) stimulates RTK – mediated activation of MAP kinase, which is inhibited by antioxidant N-acetyl cysteine (NAc, 10mM) and viscinal SH-agent 2,3 –dimercapto-pyridine (MP, 0.5 mM).

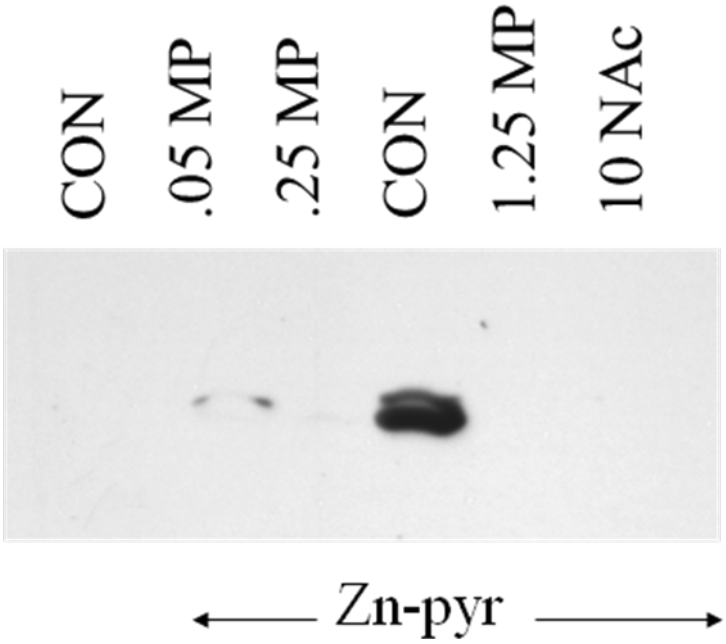

Figure 4 – Zinc - pyrithione (P'Tase inhibitor) stimulates RTK – mediated activation of MAP kinase, which is inhibited by 10 mM NAc and MP at 0.05 – 1.25 mM concentrations.

To test our hypothesis that Tyr P'tase could regulate the NT receptor, we next assessed the effect of peroxovanadate on NT binding, the expectation being that by inhibiting Tyr P'tase, peroxovanadate would enhance RTK activity, thereby inhibiting NT binding. Other workers have shown for the classical Tyr kinase EGFR that peroxovanadate is far more effective in enhancing kinase activity than vanadate or peroxide. Therefore, we assessed the effects of these 3 agents on NT binding, and the results in **Fig 5** show that peroxovanadate was far more potent than vanadate or peroxide in diminishing NT binding.

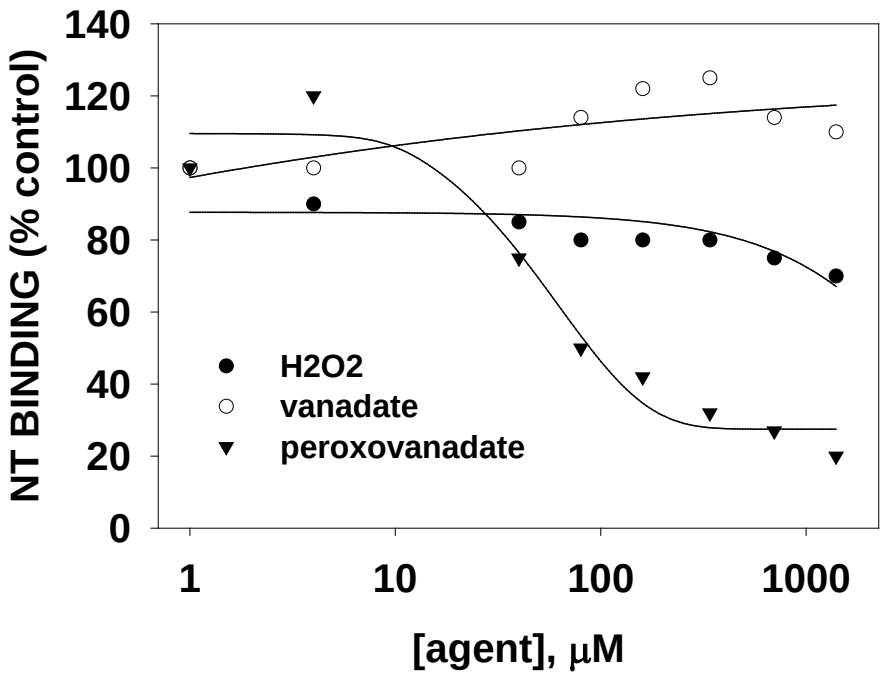

Figure 5 – Effect of peroxovanadate, vanadate and peroxide on NT binding to intact PC3 cells. Cells were pretreated with the agents for 10 min and binding was performed at 37 C for 30 min. Note that peroxovanadate was far more active than the other compounds in inhibiting NT binding. Results are representative of at least 3 experiments.

To further test our idea that Tyr P'tase could regulate NT binding, we examined the effects of zinc ion, the expectation being that zinc would enhance RTK activity, thereby inhibiting NT binding. Fig 6 shows that the expected results were obtained. NT binding to intact PC3 cells was inhibited progressively by the addition of zinc sulfate to the medium, especially at levels just above 10μM, the physiologic extracellular zinc concentration. However, addition of the ionophore pyrithione to facilitate entry of zinc ion into the cells dramatically potentiated the effect. These results indicate that intracellular levels of zinc ion can greatly inhibit NT binding to the cell surface, most likely by altering Tyr P'tase activity.

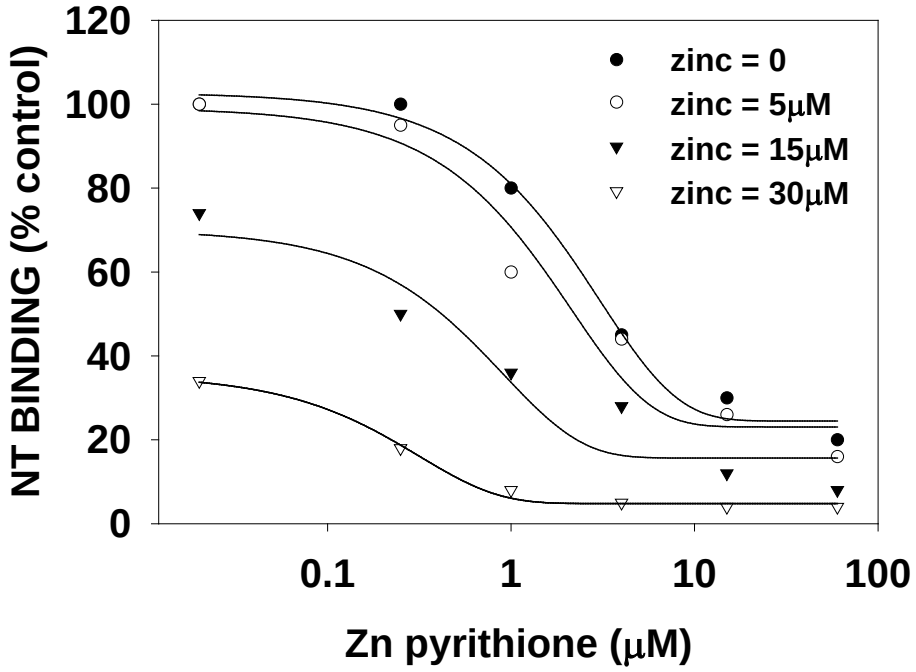

Figure 6 – Effect of zinc ion and zinc pyrithione on NT binding to intact PC3 cells. NT binding was progressively decreased as zinc levels were raised above the physiologic level of 10 μM. The effect of the zinc ionophore pyrithione was to greatly potentiate the inhibition by zinc ion. Results are representative of at least 5 experiments.

To further test the involvement of Tyr P'tase in the regulation of NT binding, we assessed the effects of zinc chelators on NT binding in intact PC3 cells. Zinc chelators would be expected to have the opposite effect as the zinc ionophore since they would reduce intracellular zinc levels, thereby incresing Tyr P'tase activity. Fig 7 shows that the expected results were obtained. Each of the zinc chelators tested increased NT binding and the order of potency was the same as that for their zinc affinity (TPEN > phenanthroline > bipyridyl).

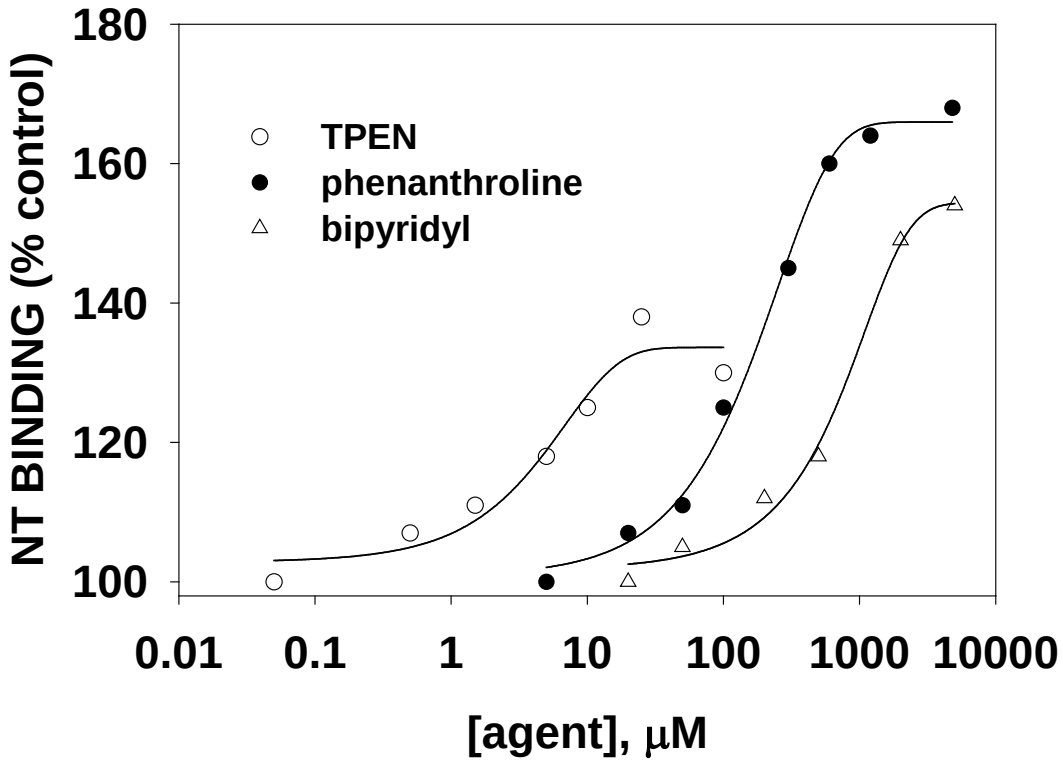

Figure 7 – Effect of zinc chelators TPEN, phenanthroline, and bipyidyl on NT binding to intact PC3 cells. Cells were pretreated for 15 minutes and binding was performed for 30 minutes. The order of potency of the agents to increase NT binding was the same as the order of affinity for zinc. Results are representative of at least 3 experiments

In total, these results support the notion that tyrosine phosphorylation events can regulate NT receptor function. However, it is not yet clear whether PTKs directly phosphorylate the NT receptor itself or exert indirect effects that involve phosphorylation of G protein(s) or other regulators. It is also unclear whether this regulation involves a change in receptor affinity (as seen with PKC) or a change in receptor number and internalization.

Task 6. Determine effects of FLAV on NT signaling modules in caveolae (months 20-30).

Since the apparent upstream effects of FLAVs on NT binding and signaling can be mimicked by β-cyclodextrin which disrupts caveolar signaling modules by adsorbing cholesterol, we hypothesized that FLAVs might disrupt caveolae. Using sucrose gradient ultracentrifugation of membrane fractions from PC3 cells **(Pike et al, Biochemistry 41, 2075-2088, 2002**), we demonstrated the localization of NTR in light density membranes that contained caveolin. Pretreatment of the cells with β-cyclodextrin

diminished the levels of caveolin, NTR, Gαq and PLCβ in the light membrane fraction **(Fig 8).** Thus, β-cyclodextrin appeared to displace NTR from its signaling module, which is consistent with the fact that it alters NT binding and signaling. Although quercetin and Bis-1 also inhibited NT signaling, these compounds had much less effect on the distribution of NTR, caveolin, Gαq and PLCβ in the sucrose gradient **(Fig 8).** These results suggest that quercetin and Bis-1 inhibit NT signaling by a different mechanism than β-cyclodextrin.

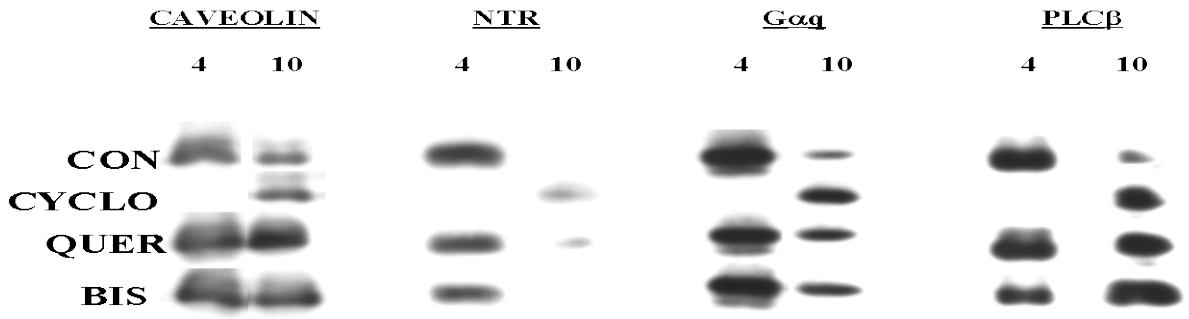

Figure 8 - Effect of β-cyclodextrin, quercetin and BIS-1 on the distribution of NT receptor (NTR), G protein (Gαq) and phospholipase C (PLCβ) in caveolae and heavy membranes. PC3 cells were treated with 5 mM β-cyclodextrin or 50 μM quercetin or 10 μM BIS-1. Sucrose centrifugation was used to separate caveolae from heavy membranes. Western blotting was performed for caveolin, NTR, Gαq and PLCβ in the 10 fractions. Shown are the results for the peak fractions (#4 = caveolae; #10 = dense membranes). For the control, NTR, caveolin, Gαq and PLCβ were primarily in fraction #4. β-cyclodextrin decreased their levels in fraction #4. Quercetin and BIS-1 had less effect.

Since it appeared that FLAVs did not displace NTR from caveolar signaling modules, we hypothesized that they may have altered NTR function in a more subtle way, perhaps by modulating the response to NT itself. Therefore, we investigated the effect of NT on the membrane distribution of NTR. PC3 cells were exposed to 100 nM NT for 30 min and membranes were separated by sucrose density centrifugation. NTR was measured by western blotting each of the 10 fractions**. Fig 9** shows the results of a typical experiment. Under control conditions, NTR was primarily present in the light density caveolae (fraction #5). Exposure to NT reduced the level of NTR in caveolae by >60%.

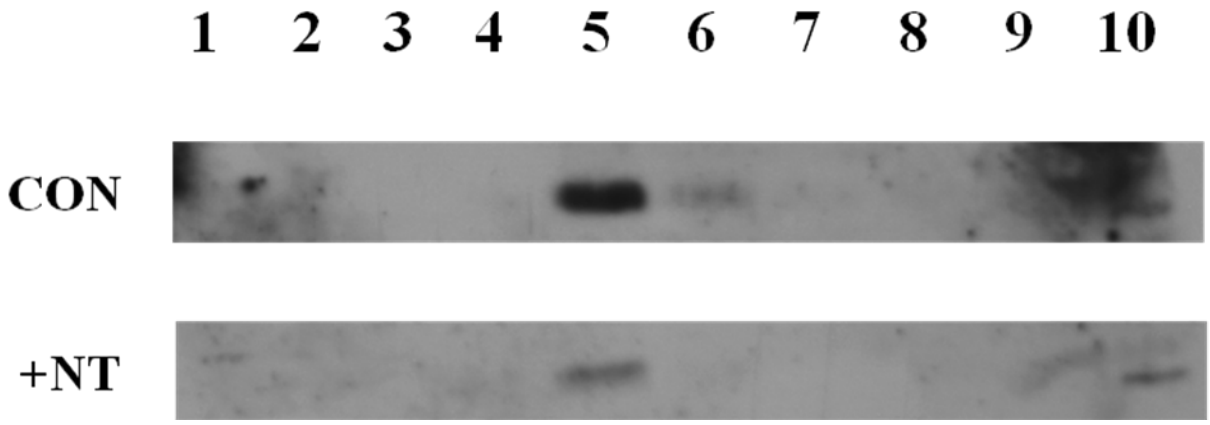

Figure 9 – Western blot showing the levels of NTR in fractions obtained during sucrose density gradient centrifugation of membranes from PC3 cells treated with 100 nM NT or control. Fraction 1 is the top and fraction 10 is the bottom of the gradient. For the control, NTR was primarily located in the caveolae (fraction 5) and it was displaced by treatment with NT. Representative of multiple experiments.

To further characterize this effect, we next examined the time course for the response to 100 nM NT. **Fig 10** shows that 100 nM NT diminished the level of NTR in caveolae within 1 min. The fact that NT can stimulate rapid elevations in cellular calcium that peak within 1 min suggests that this caveolar pool of NTR could represent cell surface receptors that mediate responses to NT. Since many of the responses to NT display a rapid tachyphylaxis, the rapid decrease in the level of NTR could represent a desensitization response.

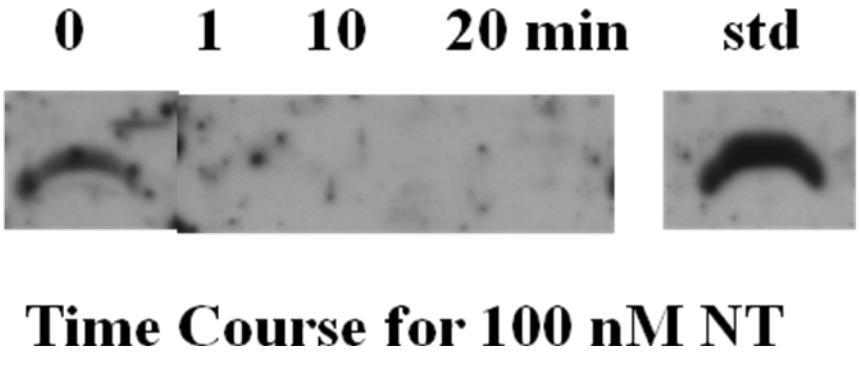

Figure 10 - The time course for the effect 100 nM NT on the level of NTR in caveolae. PC3 cells were treated with 100 nM NT for times indicated in cellular membranes were separated by sucrose density centrifugation. Western blotting was performed to measure NTR in the caveolar fraction.

Our next goal was to compare the dose dependence for this effect on the distribution of NTR to the dose dependence for binding of NT to the receptor. Using our standard procedure, NT binding to intact PC3 cells comes to equilibrium in 30 min and the displacement curve indicates that the IC50 is about 1 nM. Therefore, we used these identical conditions (30 min at 37° C) to determine the dose dependence for the ability of NT to displace NTR from caveolae. Fig 11 shows that increasing doses of NT progressively diminished the level of NTR in the caveolar fraction and that the IC50 was about 1 nM. These results are in keeping with the binding data and suggest that the binding of NT to the receptor initiates the displacement of NTR from the caveolar fraction.

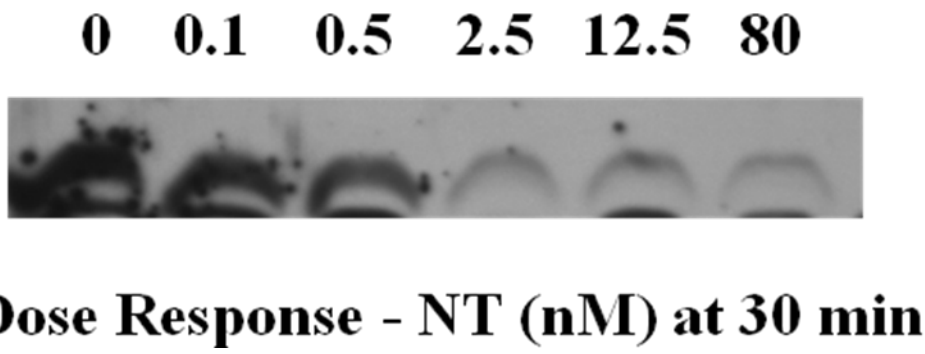

Figure 11 – Dose response for the effect of NT on the level of NT are in caveolae. PC3 cells were treated with doses of NT for 30 minutes. Cellular membranes were separated by density centrifugation and western blotting for NTR was performed on the caveolar fraction. Note that the IC50 for the displacement of NTR was about 1 nM.

Our next goal was to determine if the ability of NT to displace NTR from caveolae was subject to antagonism. SR48692 is a highly specific NTR antagonist that binds to the NT binding site but does not activate the receptor. PMA is a PKC activator that reduces the affinity of the receptor, most likely through phosphorylation events. Cells were pretreated with SR48692 or PMA for 30 min and then stimulated with NT for an additional 30 min. **Fig 12** shows that the effect of 3 nM NT to displace NTR from the caveolar fraction was totally inhibited by 1μM SR48692 and diminished by 100 nM PMA. These results are in keeping with the known ability of these compounds to inhibit NT binding and NTR function.

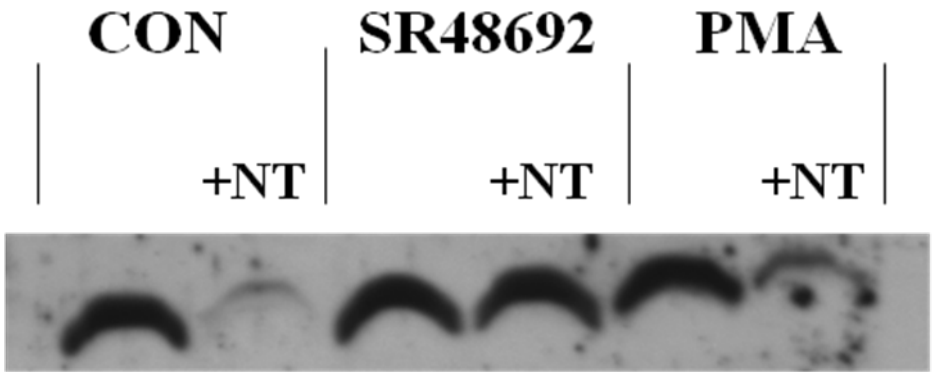

Figure 12 – The effect of NT on the distribution of NTR in caveolae was blocked by SR48692 and diminished by PMA. Cells were pretreated with the agents shown and then exposed to 100 nM NT for 30 minutes. Membranes were separated and NTR was measured in the caveolar fraction.

Our final goal was to examine the effects of FLAVs on this response to NT. As before, cells were pretreated with the agents for 30 min and then stimulated with 3 nM NT for an additional 30 min. The results in Fig 13 confirm the effect of NT to displace NTR from the caveolar fraction and the ability of SR48692 to block this effect. Although the effects seen for the agents examined suggest that they antagonized the response to NT, we have not done a sufficient number of these studies to draw a definite conclusion. Difficulties have arisen in regards to the stability of NTR in some of our recent experiments, in that the western blots show variable amounts of lower molecular weight bands. Since the various agents might affect the stability of NTR, we have to be cautious in interpreting the results. We are investigating a number of factors that might input into this problem, such as incubator contamination, failure of the protease inhibitors, temperature of the samples during work-up and non-specific effects of the agents on protease action. Despite these reservations however, our preliminary conclusion from the studies performed to date is that some FLAVs can inhibit the ability of NT to displace NTR from the caveolar fraction.

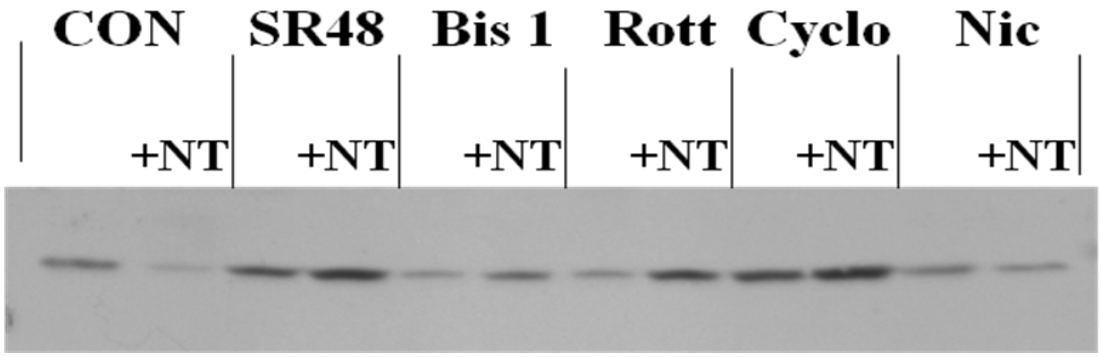

Figure 13 – The inhibitory effect of various agents on the displacement of NTR from caveolae by NT. C ells were pretreated with SR48692, Bis 1, rottlerin, methyl β - cyclodextrin or micardipine for 30 minutes and then with 3 nM NT for an additional 30 minutes. Membranes were separated and NTR levels were measured in the caveolar fraction by western blotting. Note that the agents shown inhibited the effect of NT.

One of the perplexing things about the effects of FLAVs is that they enhance (by 3-fold) the binding of NT to its receptor, whereas they inhibit NTR signaling and the cellular responses to NT. Although this seems counter intuitive, there is precedence for it in that EGFR binding is elevated 5-fold by agents that inhibit its intrinsic tyrosine kinase activity. In the case of EGFR, the tyrosine kinase inhibitor prevents EGFR from phosphorylating its own tyrosine residues, perhaps then preventing receptor desensitization upon binding. The end result is an apparent increase in binding in the face of inhibited responses. Based on our results, one might suggest that FLAVs inhibit RTKs that regulate NTR function and also participate in its desensitization. The loss of NTR from the caveolar signaling module may be one aspect of this desensitization. Nevertheless, by blocking the receptor signaling and the subsequent desensitization, FLAVs would increase the apparent binding.

C- Effect of FLAV on cellular ATP levels.

It is conceivable that inhibition of PKC and RTKs might involve changes in cellular ATP levels. In our manuscript provided in earlier reports (Carraway et al, Regulatory Peptides 141, 140-153, 2007) we showed that NT receptor binding and signaling were sensitive to metabolic inhibitors. Glycolytic and mitochondrial inhibitors, which reduced cellular ATP levels and activated AMP kinase, produced the same effects on NT growth signaling that are seen in the presence of FLAVs. Therefore, we tested the effects of quercetin and BIS-1 on cellular ATP levels. The results in **Fig 14** indicate that quercetin and BIS-1, tested across the concentration range that altered NT receptor signaling, did not affect cellular ATP levels. In contrast, antimycin A (which served as the positive control) dramatically reduced cellular ATP levels across the concentration range that altered NT receptor binding and signaling.

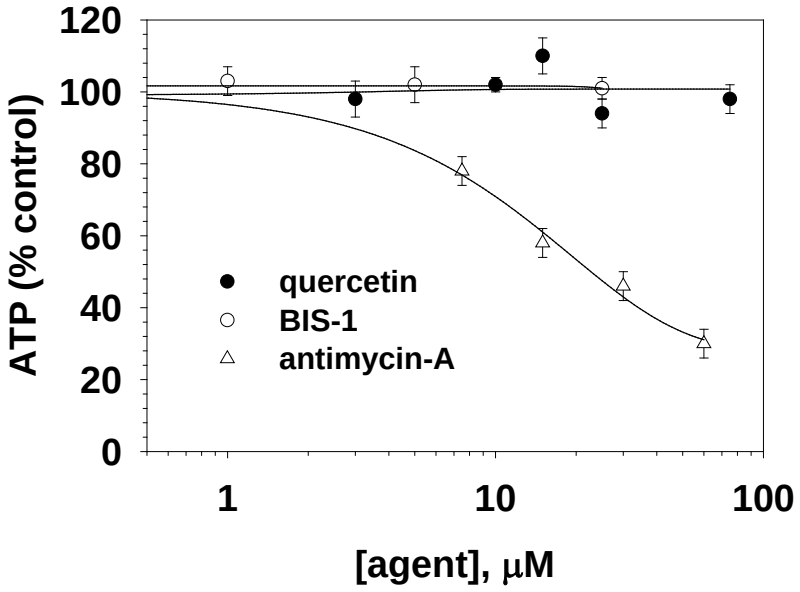

Figure 14 - Quercetin and BIS-1 do not alter cellular ATP levels in PC3 cells. Quiescent cells were incubated with the indicated concentrations of quercetin, BIS-1 or antimycin A for 45 min. Cells were extracted with TCA and cellular levels of ATP were measured using a luciferase based assay. Results are representative of at least 3 experiments.

However, it is possible that PLAVs exert more subtle effects on metabolism, which are not reflected as a global decrease in ATP levels. In order to address this idea, we examined the effects of FLAVs in cells deprived of glucose. Quiescent cells were withdrawn from glucose for 1.5 hrs in order to put more demand on the metabolic systems. However, this did not compromise their ability to maintain cellular ATP levels under control conditions throughout the experiment. Cells were then treated with FLAVs or control for 45 min in the absence of glucose and cellular ATP levels were measured. Fig 15 shows that FLAVs dose-dependently decreased cellular ATP levels relative to the control. In addition, the potency order (galangin > luteolin > apigenin > quercetin > 5-OH flavone) was similar to the activity order for the effects on NTR. These results suggest that the effect of FLAVs on kinase activity in live cells may be due to metabolic stress. In other words, the metabolic effects of FLAVs could be subtle and localized such that an effect on total cellular ATP levels can only be seen under stressful conditions. Nevertheless, local effects on metabolism could limit availability of substrates for kinases such as RTK and PKC.

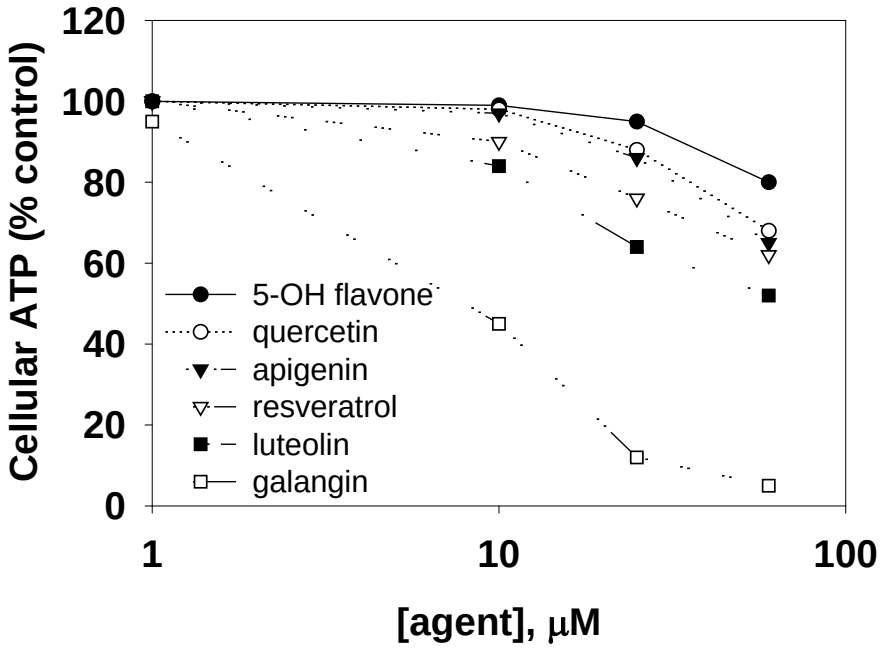

Figure 15 – FLAVs diminish cellular levels of ATP in PC3 cells in the absence of glucose. Cells were withdrawn from glucose for 1.5 hours and then treated with the agents shown for 45 minutes at 37 C. ATP levels were measured using the luciferase assay. Note that galangin and luteolin were the most potent FLAVs as was shown previously for the effect on NT receptor binding.

2. KEY RESEARCH ACCOMPLISHMENTS-

- Determined the EC50 for the effects of 22 FLAVs on NT receptor binding.
- Related the FLAV effects on NT receptor binding to those for inhibition of various kinases, showing most similarity to the effects on receptor tyrosine kinases.
- Demonstrated ability of FLAVs to inhibit tyrosine kinase-mediated effect on MAPK activation.
- Demonstrated effect of tyrosine phosphatase inhibitor peroxovanadate on NT receptor binding.
- Demonstrated effect of tyrosine phosphatase inhibitor peroxovanadate on MAPK activation.
- Demonstrated effect of tyrosine phosphatase inhibitor zinc pyrithione on NT receptor binding.
- Demonstrated effect of tyrosine phosphatase inhibitor zinc pyrithione on MAPK activation.
- Demonstrated effect of zinc chelators on NT receptor binding
- Demonstrated that NTR was localized to caveolae in PC3 cells and could be displaced by removing cholesterol from the membrane using β-methyl cyclodextrin.

- Demonstrated that NT displaced NTR from the caveolar fraction and that NT antagonist SR48692 and PKC activator PMA inhibited this effect
- Demonstrated that FLAVs were not metabolic inhibitors in that they reduced cellular ATP levels in the absence of glucose.

4. REPORTABLE OUTCOMES-

A. Regulatory Peptides 133:105-114, 2006

B. Peptides 27:2445-2460, 2006

C. **Prostaglandins, Leukotrienes and Essential Fatty Acids 74:93-107, 2006**

D. Regulatory Peptides 141:140-153, 2007

E. **Regulatory Peptides 147:96-109, 2008**

5. CONCLUSIONS-

Our earlier results showed that the NT receptor is subject to homologous regulation by PKC. The present results suggest that NT receptor is also subject to regulation by protein tyrosine kinases (PTKs). Since we also demonstrate that FLAVs are able to inhibit PTK-mediated effects, our results suggest that the ability of FLAVs to inhibit NT receptor function could involve PTKs as well as PKC.

Other results indicate that NT receptor is localized to membrane caveolae where the receptor presumably associates with effectors to induce its effects. Our results clearly indicate that NT causes a decrease in the level of NTR in these signaling modules, presumably by activating the receptor and initiating a desensitization response. Since our preliminary findings support the notion that FLAVs inhibit the effect of NT to displace the receptor from caveolae, we propose that this may be the mechanism by which FLAVs inhibit NT receptor function. It is possible that an inhibitory effect of FLAV on PTKs underlies the inhibition of this NT response. It is possible that the movement of NTR represents a component of the desensitization response and if so, this may explain how FLAVs increase NT receptor binding.

The relevance of this work to the regulation of PC growth and to the development of useful therapeutics is shown by the fact that the effects of various FLAVs on NT receptor binding correlate to

those on DNA synthesis in cultured PC cells. Thus, the mechanism by which FLAVs alter NT receptor function may be similar to that by which FLAVs inhibit the growth of PC cells. Our studies point towards a mechanism involving inhibition PTK and PKC activity, perhaps leading to the disruption of growth signaling modules in caveolae.

6. REFERENCES-

The references are listed in the body of this and earlier reports, and in the various articles that were included in the APPENDIX of these reports.

7. APPENDIX-

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Protein kinase C inhibitors alter neurotensin receptor binding and function in prostate cancer PC3 cells

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Abstract

Prostate cancer PC3 cells expressed constitutive protein kinase C (PKC) activity that under basal conditions suppressed neurotensin (NT) receptor function. The endogenous PKC activity, assessed using a cell-based PKC substrate phosphorylation assay, was diminished by PKC inhibitors and enhanced by phorbol myristic acid (PMA). Accordingly, PKC inhibitors (staurosporine, Go-6976, Go-6983, Ro-318220, BIS-1, chelerythrine, rottlerin, quercetin) enhanced NT receptor binding and NT-induced inositol phosphate (IP) formation. In contrast, PMA inhibited these functions. The cells expressed conventional PKCs (α, βI) and novel PKCs (δ, ε), and the effects of PKC inhibitors on NT binding were blocked by PKC downregulation. The inhibition of NT binding by PMA was enhanced by okadaic acid and blocked by PKC inhibitors. However, when some PKC inhibitors (rottlerin, BIS-1, Ro-318220, Go-69830, quercetin) were used at higher concentrations (>2 μM), they had a different effect characterized by a dramatic increase in NT binding and an inhibition of NT-induced IP formation. The specificity of the agents implicated novel PKCs in this response and indeed, the inhibition of NT-induced IP formation was reproduced by PKCδ or PKCε knockdown. The inhibition of IP formation appeared to be specific to NT since it was not observed in response to bombesin. Scatchard analyses indicated that the PKC-directed agents modulated NT receptor affinity, not receptor number or receptor internalization. These findings suggest that PKC participates in heterologous regulation of NT receptor function by two mechanisms: a) — conventional PKCs inhibit NT receptor binding and signaling; and b) — novel PKCs maintain the ability of NT to stimulate PLC. Since NT can activate PKC upon binding to its receptor, it is possible that NT receptor is also subject to homologous regulation by PKC.
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Keywords: Neurotensin; Prostate cancer; Protein kinase C; G protein receptor binding; G protein receptor signaling

1. Introduction

Neurotensin (NT), a regulatory peptide found in the nervous system and in endocrine cells of the intestinal mucosa [1, 2], has multiple roles as a neurotransmitter and hormone [3–5]. Considerable evidence suggests that NT could contribute to the growth of normal and neoplastic cells [6,7]. The high affinity G protein-coupled NT receptor NTS1 is overexpressed in many primary human tumors and cell lines, including human prostate cancer PC3 cells [8]. We showed that stimulation of PC3 cells with NT induced a growth response that involved protein kinase C (PKC)-dependent transactivation of the EGF receptor and activation of downstream mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase [9]. The growth effects of NT in other systems and its ability to activate MAPK [10], as well as to enhance cyclic AMP formation [11] and to phosphorylate GSK-3α/β [12] were also PKC-dependent. In a number of these cells, NT was shown to activate phospholipase C (PLC) [13,14], stimulating the formation of inositol phosphate (IP) and the mobilization of intracellular Ca^{2+} [15]. Since PKC activity is dependent on the levels of Ca^{2+} and DAG, NT could activate both conventional and novel PKCs, and our recent work indicates that NT enhanced PKC isotype phosphorylation assessed by western blotting in PC3 cells (S Hassan, unpublished results). Not only

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might PKC mediate some of the effects of NT, but the activation of PKC could possibly exert feedback effects on NT receptor binding and signaling. Despite these findings, there is a paucity of information concerning the relationships between PKC activity and NT receptor function.

The responsiveness of cells to signaling inputs is regulated by a variety of mechanisms that serve to maintain homeostasis and coordinate signaling events. Some of the effects of NT have been shown to display homologous desensitization, such that there is a diminished response to a second stimulus given after the initial one. Heterologous desensitization has also been noted using stimuli that do not activate NT receptors [16]. These findings suggest that NT receptors could be subject to regulation by second messenger-dependent kinases, which could alter the availability of or the activity of the receptor. For the G protein-coupled receptors that have been studied extensively, such as the rhodopsin receptor and the β2-adrenergic receptor, there is considerable evidence suggesting that receptor phosphorylation is an important mechanism for regulating receptor responsiveness [17,18]. One generality that has emerged from the work in the laboratory of Robert Levkowitz and others is that G protein receptor kinases usually mediate phosphorylation events that are associated with agonist-induced (homologous) receptor desensitization, whereas signaling kinases such as PKC and PKA usually promote receptor phosphorylations involved in heterologous desensitization [18,19]. Endocytosis by β-arrestin and clathrin dependent mechanisms can also be part of the desensitization process [20]. In regards to NTS1, however, these mechanisms have not been well studied. Agonist dependent phosphorylation of HA-tagged NTS1 has been demonstrated in HEK-293 cells and the importance of certain C-terminal serine residues has been studied [21]. However, the kinases involved and the relationship to the desensitization of NT-induced signaling have not been studied. In addition, the effects on the parameters of receptor binding and internalization have not been defined.

In order to better understand the potential role of PKC in regulating NT receptor function, we set out to determine which PKC isotypes were expressed in PC3 cells and to study the effects of PKC activation and inhibition on NT receptor binding and signaling. The PKC family of serine/threonine kinases phosphorylate proteins at sites resembling the consensus motif RXXS/TXR [22,23] and phospho-specific antibodies towards similar motifs have been used to measure PKC substrate phosphorylation in cells [24,25]. Subcategories of PKC isotypes have been defined, including the conventional PKCs (PKCα, βI, βII and γ), the novel PKCs (PKCδ, ε, θ and η) and the atypical PKCs (PKCζ, μ and ι) based on their activity requirements [26,27]. Conventional PKCs are activated by Ca^{2+}, phosphatidylserine (PS) and diacylglycerol (DAG), whereas novel PKCs respond to PS and DAG, and atypical PKCs respond only to PS. Phorbol 12-myristate 13-acetate (PMA), which can mimic the effects of DAG, has been used to activate and/or downregulate conventional and novel isoforms of PKC. The phosphorylation of PKCs and their translocation from cytosol to membrane compartments that occur following acute treatment with PMA have been accepted as indicators of PKC activation. Chronic treatment with PMA on the other hand has generally been found to downregulate PKC expression. Many of the PKC inhibitors that have been used to investigate the involvement of PKC in cellular processes act by blocking the ATP binding site [23]. The most potent of these include the structurally related compounds staurosporine, bisindolylmaleimide I (BIS-1), Ro-328220, Go-6983 and Go-6976 [28]. These compounds exhibit a rather broad isotype specificity [29], except for Go-6976 which preferentially inhibits conventional PKCs, and Go-6983 which does not inhibit PKCμ [30]. Although rottlerin was originally described as a specific PKCδ inhibitor [31], further study indicated that it did not inhibit the enzyme directly [29] and that it could act as a mitochondrial uncoupler [32]. Less potent PKC inhibitors that act by mechanisms that are not well defined include chelerythrine [33] and quercetin [34].

In the work reported here, we first examined the effects of PKC-directed agents on PKC substrate phosphorylation in PC3 cells, establishing that these cells displayed a high level of constitutive PKC activity that responded to PKC activation and inhibition. Then, we investigated the effects of PKC activation, PKC inhibition and PKC isotype knockdown on NT receptor function. Two categories of PKC inhibitors were defined which had dramatically different effects on NT receptor binding and signaling. Based on the selectivity of these inhibitors and the effects of PKC isotype knockdown, we concluded that conventional and novel PKCs played opposite roles in regulating NT receptor function.

2. Materials and methods

2.1. Materials

[^{125}I]-sodium iodide (2000 Ci/mmol), [1,2-^{3}H(N)]-myo-inositol (60 mCi/mmol) and [methyl-^{3}H]-thymidine were obtained from Perkin Elmer Life Science (Boston, MA). The phospho-specific antiserum to the PKC substrate motif (#2261) was from Cell Signaling Technology (Beverly, MA). The antibodies towards PKCα, PKCβI, PKCβII, PKCδ, PKCε, PKCγ and HRP-linked secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). SuperSignal chemiluminescent substrate was obtained from Pierce (Rockford, IL). Staurosporine was from Biomol (Plymouth Meeting PA). Bisindolylmaleimide I (BIS-1), bisindolylmaleimide V (BIS-V), chelerythrine chloride, Go-6976, Go-6983 and Ro-318220 were from Calbiochem (San Diego, CA). Phorbol-12 myristate 13-acetate (PMA), rottlerin, quercetin and all other chemicals were from Sigma (St. Louis, MO).

2.2. Tissue culture

PC3, PC3M, DU145, MatLyLu and HT29 cells, obtained from American Type Culture Collection (Manassas, VA), were maintained in F12K medium (PC3, PC3M) and DMEM medium (DU145, MatLyLu, HT29), supplemented with 10% fetal bovine serum [8]. PC3, PC3M (its highly metastatic clone), and DU145 are androgen-independent cell lines derived from

human prostate adenocarcinomas. MatLyLu was derived from a rat prostate carcinoma and HT29 from a human colon carcinoma. LNCaP^{CS} cells, a clone derived from the human prostate LNCaP cell line, were a gift from Shuk-mei Ho, Department of Environmental Health, University of Cincinnati Medical School. The conditions for growing these cells have been described by us [35]. For western blots, cells were grown in 60 mm dishes; for all other studies, cells were grown in 24-well culture plates. For IP studies, cells were labeled with ^{3}H-inositol using medium 199 (Difco), which has a low inositol content.

2.3. Binding to cultured cells

HPLC-purified monoiodinated NT (^{125}I-NT) at 2000 Ci/mmol was prepared and binding was performed as described by us [13]. In brief, cells in 24-well plates (80–90% confluent) were washed with 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. Stock solutions (2–10 mM) of the PKC inhibitors in dimethyl sulfoxide (DMSO) were stored at −20°C, except for quercetin (prepared just before use), and were diluted in Locke to give ≤1% DMSO. Equilibrium binding at 37 °C was performed for 30 min using 10^5cpm/ml ^{125}I-NT in 1.0 ml Locke. The reaction was stopped on ice, the medium was aspirated and cells were washed in ice-cold saline. Cellular binding was determined as radioactivity per μg protein in cells extracted in 0.3 M NaOH. Specific binding, displaceable by 1 μM NT (20,000-fold excess), was 95% of total binding. Binding displacement curves were constructed and binding parameters were determined as described [35]. Cell-surface binding and internalization were assessed by washing cells at room temperature for 2 min with 1 ml of 0.2 M acetic acid, 0.5 M NaCl.

The binding parameters for the ligands used to measure bombesin (BOM) receptor binding and EGF receptor binding in PC3 cells were described by us [13]. In brief, equilibrium binding was performed in a manner identical to that for NT using HPLC-purified ^{125}I-[Nle^{14}]-BOM (10^5 cpm, 50 pM) and ^{125}I-EGF (10^5 cpm, 50 pM). Specific binding was defined as that displaceable by 1 μM BOM (20,000-fold excess) or 0.1 μM EGF (2000-fold excess).

2.4. IP formation

Formation of [^{3}H]-IP was measured as described by us [13]. Briefly, PC3 cells in 24-well plates were incubated 48 h with myo-[^{3}H]-inositol (2.5 μCi/ml) in medium 199, 1% fetal calf serum. Cells were pretreated with Locke, 15 mM LiCl for 10 min to inhibit phosphatases. In some cases, they were concomitantly pretreated with test agents or vehicle (DMSO) in Locke, 15 mM LiCl as indicated. The reaction was initiated by adding NT, BOM or vehicle (Locke). After 30 min at 37 °C, the medium was aspirated, ice-cold 0.1 M formic acid in methanol was added and the plates were placed at −20 °C overnight. IP was adsorbed to AG-1X8, which was washed in 5 mM myo-inositol and eluted in 1.5 M ammonium formate. Scintillation counting was performed in Ecoscint (National Diagnostics).

2.5. Inhibition of PKCδ and PKCε in PC3 cells through stable transfection of shRNA expression plasmids

Plasmid constructs (Open Biosystems, Huntsville, AL) designed to constitutively express shRNAs that specifically target either PKCδ (Open Biosystems catalog number RHS1764-9492328 or RHS1764-9098879) or PKCε (RHS1764-9493023) or a control plasmid encoding a non-silencing shRNA (RHS1707) were purchased from the UMASS shRNA Core Facility. The parent plasmid (pSM2a) contains the bacterial puromycin resistance gene under the control of the PGK promoter and the mir-30 gene containing gene-specific synthetic microRNA sequences under the control of the U6 promoter to drive microRNA production [36]. PC3 cells were transfected with individual plasmids on 100 mm tissue culture dishes using FuGene transfection reagent (Roche) according to the manufacturer's instructions, and stably transfected cells were selected by the addition of puromycin (1.0 μg/ml) to the culture medium. Visible clones were isolated after 2–3 weeks of selection using cloning cylinders, propagated, and screened by western blot analysis to examine PKCδ and PKCε expression levels. Clones displaying significant inhibition of PKCδ or PKCε were grown under continued puromycin selection for use in NT binding and other experiments. PKCδ was significantly inhibited by only one of two shRNAmir constructs tested (RHS1764-9098879).

2.6. Western blotting

Western blot analysis was performed as described by us [9]. In brief, PC3 cells in 60-mm dishes were withdrawn from serum for 24 h. Cells, washed in Locke, were exposed to stimuli at 37 °C for times indicated. After washing with ice-cold PBS containing phosphatase inhibitors, cells were placed on ice and scraped into 2× SDS buffer (plus inhibitors) and sonicated. Equal amounts of protein were separated by SDS-PAGE using polyacrylamide minigels and electroeluted onto PVDF (Immobilon P, Millipore). Blots were incubated with primary antibodies in blocking buffer for 18 h at 4 °C. After washing, blots were incubated with HRP-linked secondary antibodies for 1 h at 20 °C, and ECL was performed using supersignal west pico reagent (Pierce, Rockford, IL) and films were scanned for computerized densitometric analysis. After staining with one antibody, blots were stripped and re-probed using different antibodies for comparison and normalization.

3. Results

3.1. PC3 cells displayed constitutive PKC activity and PMA-induced PKC activity

The growth-promoting effects of NT in PC3 cells are PKC-dependent. In order to assess the importance of PKC for NT receptor binding and signaling, we first determined the basal level and stimulated level of PKC activity in the cells by

performing western blotting using a phospho-specific antiserum directed towards PKC substrates exhibiting the motif R/K-X-S-Hyd-R/K (where Hyd = hydrophobic amino acid). PKC isotype specificity considerations predicted that this assay would primarily detect substrates phosphorylated by conventional PKCs (α, βI, βII, γ), and might respond to novel PKCε substrates but would react poorly with novel PKCδ substrates [37].

Untreated cells (results not shown), and cells incubated with 0.1% DMSO (vehicle control) for 30 min, exhibited at least ten positive bands with molecular sizes ranging from 40 kDa to 140 kDa (Fig. 1A, control). In contrast, cells pretreated for 30 min with 1 μM concentrations of staurosporine, BIS-1, Go-6983 or Ro-318220 displayed less intense labeling of as many as eight of these bands, which was most evident for the 40 kDa, 50 kDa and 140 kDa bands (Fig. 1A). The inhibitory effects of Go-6976 and chelerythrine were seen at higher concentrations (5–10 μM), whereas rottlerin (10 μM) and quercetin (15–40 μM) gave only a slight inhibition (Fig. 1A and C). That these effects were due to the inhibition of constitutive PKC activity was shown by stimulating the cells with PKC activator PMA, which further enhanced the labeling of these bands as well as others not seen under basal conditions (Fig. 1B, control). As expected, the effects of PMA were attenuated (Fig. 1B and D) by pretreating the cells with staurosporine (1 μM), BIS-1 (1 μM), Ro-318220 (1 μM), Go-6976 (1–10 μM), Go-6983 (1–10 μM) or chelerythrine (5 μM). In contrast, rottlerin (1–10 μM) and quercetin (15–40 μM) inhibited only slightly (Fig. 1B and D). These findings suggested that the identified bands represented PKC substrates which were constitutively phosphorylated by PKCs that were active under basal conditions and that could be further activated by PMA. The effects of the inhibitors were consistent with the prediction that this assay detected primarily substrates that could have been phosphorylated by conventional PKCs (α, βI, βII, γ) or novel PKCε but not novel PKCδ.

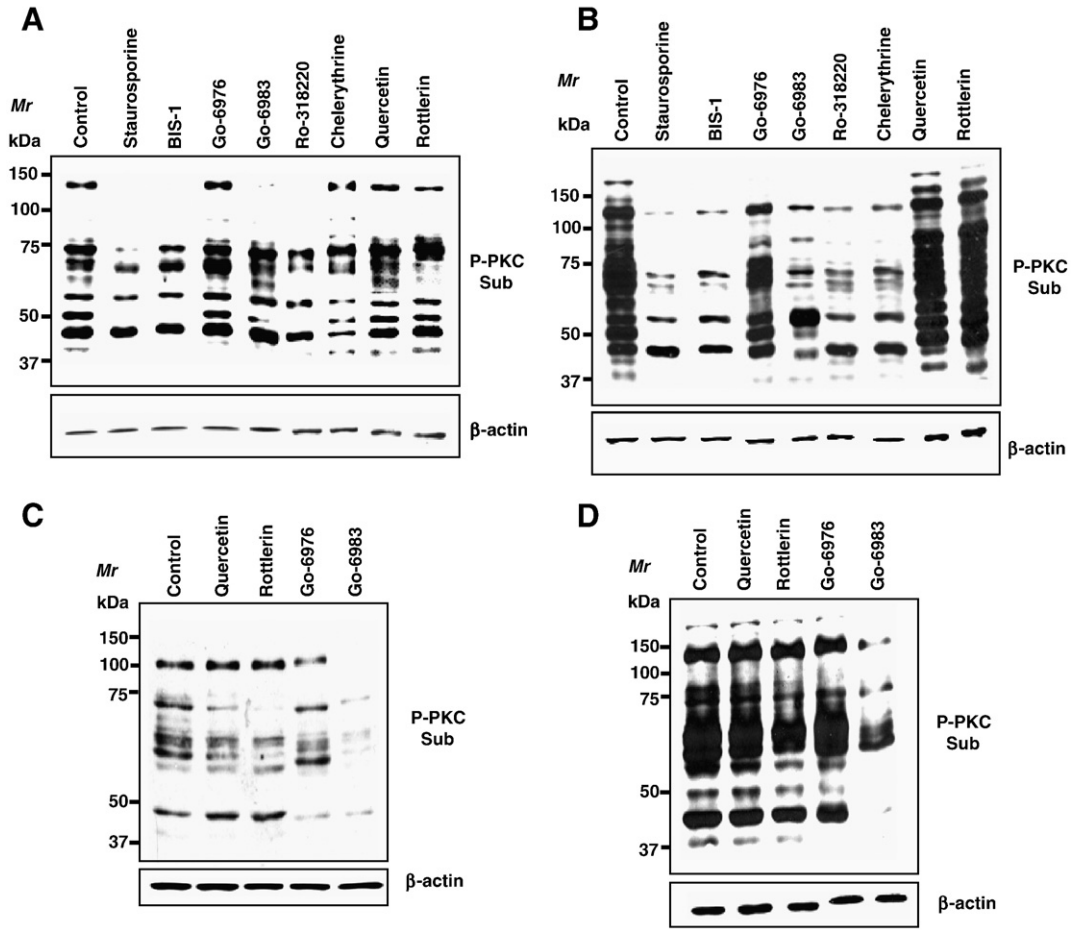

Fig. 1. PC3 cells displayed constitutive PKC activity (A and C) and PMA-induced PKC activity (B and D) that was sensitive to PKC inhibitors. Endogenous PKC substrate phosphorylation was assessed by western blotting using a phopho-specific antiserum to the motif R/K-X-S-Hyd-R/K. In A, quiescent cells were incubated with staurosporine (1 μM), BIS-1 (1 μM), Go-6976 (1 μM), Go-6983 (1 μM), Ro-318220 (1 μM), chelerythrine (5 μM), quercetin (15 μM) rottlerin (1 μM), or 0.1% DMSO (control) for 30 min. In B, cells pretreated as in A were then stimulated with 100 nM PMA for 5 min. In C, quiescent cells were incubated with quercetin (40 μM), rottlerin (10 μM), Go-6976 (10 μM), Go-6983 (10 μM), or 0.1% DMSO (control) for 30 min. In D, cell pretreated as in C were then stimulated with 100 nM PMA for 5 min. Cell extracts containing equal amounts of protein were subjected to western blotting using the PKC substrate-specific antiserum. Shown are typical results that are representative of at least three experiments each.

3.2. PKC activator PMA inhibited NT binding and NT-induced IP formation in PC3 cells

To determine whether PKC activity could regulate NT receptor function, we first tested the effect of PKC activator PMA on NT binding in intact PC3 cells. Specific binding of ^{125}I-NT (10^5 cpm/ml) to the cells, measured at equilibrium (30 min), was 21.5±1.9 cpm/μg protein (n=9), which was consistent with our prior work [13]. Incubation of the cells with PMA for 15 min decreased NT binding by as much as 42% with an IC50≅1 nM (Fig. 2A). Pretreating the cells with the inhibitor of protein phosphatases (okadaic acid) enhanced the effect of PMA on NT binding (Fig. 2B). Pretreating the cells with PKC inhibitors (staurosporine, BIS-1, Go-6983 and Go-6976), using the dose (1 μM) shown in Section 3.1 to block the PMA-induced phosphorylation of PKC substrates, prevented the effect of PMA on NT binding (Fig. 2C). These results indicated that PMA-induced PKC activity decreased NT binding by an effect involving protein phosphorylation.

Next, we tested the effect of PMA on NT-stimulated PLC activity in PC3 cells. Stimulation of the cells with 30 nM NT (a maximal dose) increased IP formation 3.9±0.4 fold (mean± SEM, n=8) in keeping with our prior work [13]. As could be predicted from its inhibitory effect on NT binding, PMA diminished NT-induced IP formation and exhibited an IC50≅ 1 nM (Fig. 4A). Taken together, these results indicated that PMA-induced PKC activity inhibited both NT receptor binding and NT receptor-mediated activation of PLC.

3.3. PKC inhibitors enhanced NT binding and NT-induced IP formation in PC3 cells

The studies in Section 3.1 indicated that PC3 cells displayed constitutive PKC activity. To determine whether this constitutive PKC activity regulated NT receptor function, we tested the effects of PKC inhibitors on NT binding and NT-induced IP formation. Six PKC inhibitors (staurosporine, Go-6976, Go-6983, chelerythrine, BIS-1, and Ro-328220) elevated NT binding when used at the low micromolar concentrations shown in Section 3.1 to inhibit PKC substrate phosphorylation. Thus, at concentrations ≤2 μM, these agents elevated NT binding by 20–40% (Fig. 3A and B). NT binding was also elevated 20–40% by rottlerin (0.5–2 μM) and quercetin (5–20 μM), which inhibited PKC substrate phosphorylation to a lesser degree (Fig. 3B and 1C).

In keeping with their ability to enhance NT binding, the PKC inhibitors also enhanced NT-induced IP formation by 15–30% when used in the same concentration range (Fig. 4B). The negative control compound for PKC inhibitors (BIS-V) had little effect on NT binding or NT-induced IP formation at concentrations up to 10 μM (results not shown). Taken together, these findings suggested that the ≅30% elevation in NT binding and NT-induced IP formation involved an inhibition of the constitutive PKC activity displayed by these cells. In keeping with this, NT binding was decreased by ≅20% by protein phosphatase inhibitor okadaic acid, which would be expected to enhance the effects of the constitutive PKC activity (Fig. 2B).

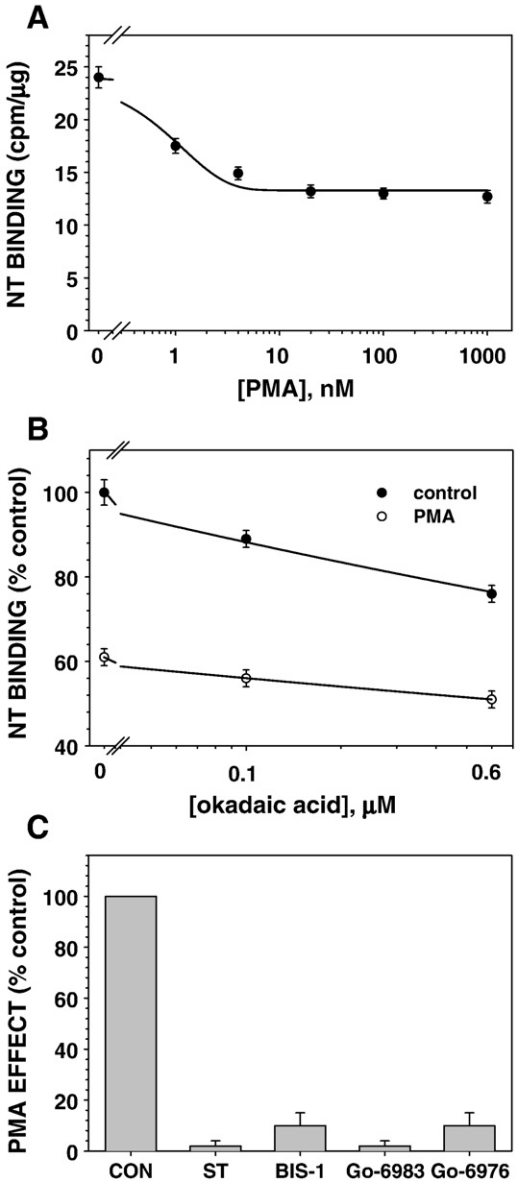

Fig. 2. PKC activator PMA decreased NT binding to PC3 cells (A), and its effect was enhanced by protein phosphatase inhibitor okadaic acid (B) and inhibited by pretreatment with PKC inhibitors (C). Specific NT binding was measured to intact cells at 37° C. In A, cells were pretreated with indicated concentrations of PMA for 15 min prior to measuring NT binding. In B, cells were pretreated with okadaic acid in the presence and absence of 100 nM PMA for 20 min prior to measuring NT binding. In C, cells were pretreated with staurosporine (1 μM), BIS-1 (1 μM), Go-6983 (1 μM), Go6976 (1 μM) or 0.05% DMSO (vehicle control) for 20 min. Then, 10 nM PMA was added for 10 min and NT binding was measured. For each experiment, the data (mean±SEM) are representative of at least three experiments.

3.4. Higher doses of some PKC inhibitors further enhanced NT binding and inhibited NT-induced IP formation

At concentrations >2 μM, five PKC inhibitors (Go-6983, BIS-1, rottlerin, Ro-318220 and quercetin) dramatically enhanced NT binding (Fig. 3A and B). Thus, these agents displayed the ability to increase NT binding by as much as 200%. Associated with this response was a dose-dependent inhibition

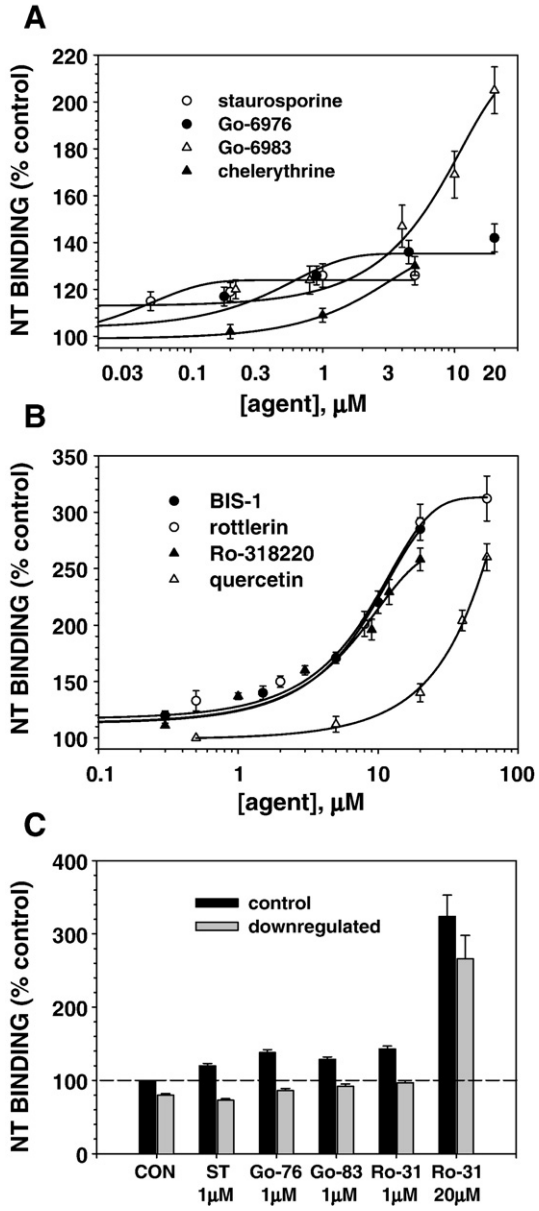

Fig. 3. PKC inhibitors enhanced NT binding to PC3 cells (A, B), and the response to low doses of PKC inhibitors was blocked by downregulation of PKC (C). Specific NT binding was measured to intact cells. In A and B, sub-confluent cells were pretreated with the indicated concentrations of agents for 15 min prior to measuring NT binding. The data (mean±SEM) were from at least 4 experiments. In C, sub-confluent cells were pretreated with 1 μM PMA (downregulated) or 0.01% ethanol (control) for 24 h. After washing in Locke, cells were pretreated with staurosporine (ST), Go-6976 (Go-76), Go-6983 (Go-83), Ro-318220 (Ro-31) or 0.05% DMSO (CON) for 15 min prior to measuring NT binding. The data (mean±SEM; $n=4$) from two experiments were expressed as % control. Note that PKC downregulation blocked the response to the low dose (1 μM) of each inhibitor and diminished the response to the high dose (20 μM) of Ro-31220.

of NT-induced IP formation by as much as 70% (Fig. 4C). In contrast, three PKC inhibitors (staurosporine, Go-6976 and chelerythrine) were unable to further elevate NT binding beyond ≅40%, even at higher doses (Fig. 3A). In addition, staurosporine and Go-6976 did not inhibit NT-induced IP formation (Fig. 4C).

These results indicated that we had defined two categories of PKC inhibitors differing in their ability to modulate NT receptor function. The agents in category I (staurosporine, Go-6976 and chelerythrine) produced only response I (a modest enhancement of NT binding and NT-induced IP formation). In contrast, the agents in category II (Go-6983, BIS-1, rottlerin, Ro-318220 and quercetin) produced two responses in a dose-dependent manner. At low concentrations response I was obtained, whereas at higher concentrations response II (a dramatic increase in NT

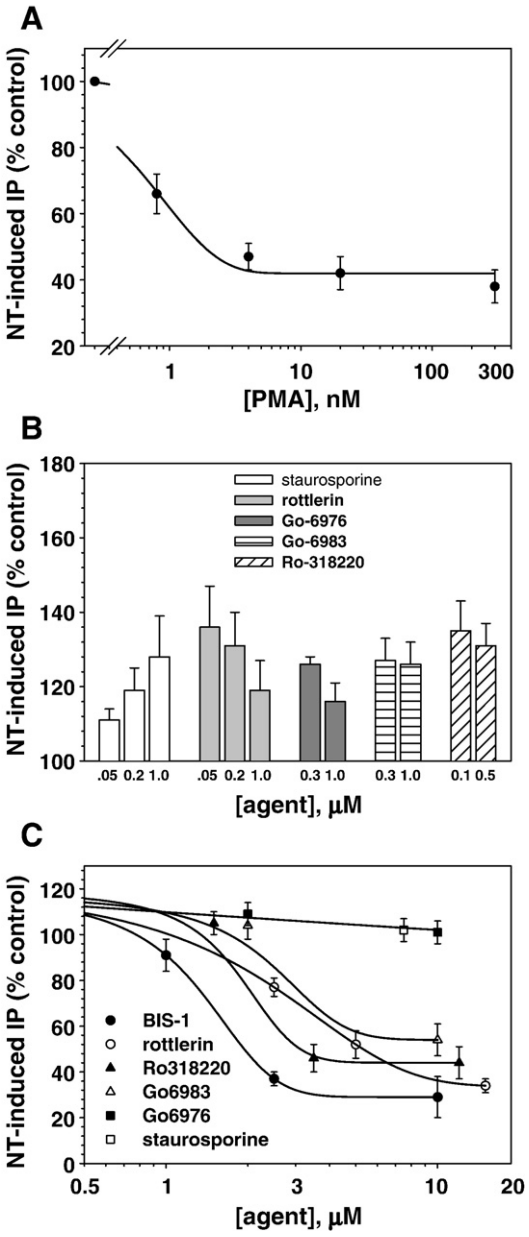

Fig. 4. NT-induced IP formation was inhibited by PKC activator PMA (A), whereas it was enhanced by low doses (B) and inhibited by high doses (C) of PKC inhibitors. IP formation was measured in response to 30 nM NT. The effect of NT (≅4-fold elevation) was calculated as the increment above the appropriate control. In A, cells were pretreated with indicated concentrations of PMA for 15 min prior to stimulation with NT. In B and C, cells were pretreated with the indicated agents for 15 min prior to stimulation with NT. For each panel, the data (mean±SEM) are representative of at least three experiments.

binding and an inhibition of NT-induced IP formation) was seen. Three of the agents in category II were known to inhibit conventional PKCs more than novel PKCs when used at low concentrations [30,38]. This suggested that our findings might be attributed to a disproportionate inhibition of PKC isotype activity, and we postulated that inhibition of conventional PKCs induced response I, whereas an inhibition of novel PKCs brought about response II. However, not all of the data fit this model and it was still possible that other targets were involved, especially in response II.

3.5. PKC isotype expression and downregulation

PC3 cells expressed conventional PKC subtypes (α, βI) as well as novel PKC subtypes (δ, ε) as determined by western blotting (Fig. 5). Pretreating the cells with 1 μM PMA for 24 h downregulated the expression of conventional PKCα and PKCβI more than novel PKCδ and PKCε (Fig. 5). Using this method to downregulate PKC expression, we then tested the effects of PKC inhibitors on NT binding. The results in Fig. 3C show that PKC downregulation blocked the modest elevation in NT binding in response to 1 μM doses of staurosporine, Go-6976, Go-6983 and Ro-318220, but only reduced (by 25%) the dramatic response to 20 μM Ro-318220. These results provided further support for the idea that low doses of PKC inhibitors caused a modest increase in NT binding by inhibiting conventional PKCα and PKCβI, whereas high doses of PKC inhibitors (from category II) caused a dramatic increase in NT binding by inhibiting novel PKCδ and PKCε. Thus, the partial inhibition of the response to 20 μM Ro-318220 (Fig. 3C) was consistent with the partial downregulation of PKCδ and PKCε (Fig. 5). However, it was also possible that targets other than PKC mediated part of this response.

3.6. Effect of PKCδ and PKCε knockdown on NT binding

To determine if the effects of category II inhibitors on NT binding were mediated by PKCδ or PKCε, RNAi technology

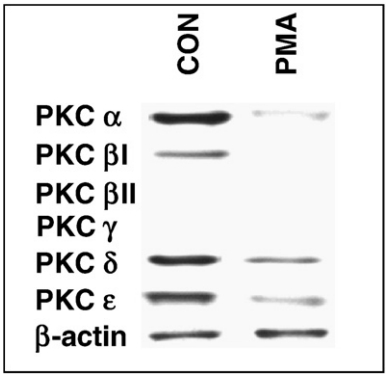

Fig. 5. Western blot assessing the expression of PKC isotypes in control PC3 cells and in cells pretreated with PMA. Cells grown in 60 mm dishes were withdrawn from serum for 24 h and then pretreated with 0.1% DMSO or 1 μM PMA for 24 h. Whole cell extracts were prepared, 40 μg protein was subjected to SDS-PAGE and western blotting was performed using antisera specific for PKCα, PKCβI, PKCβII, PKCγ, PKCδ and PKCε. β-actin was used as the loading control. The results shown are representative of two experiments.

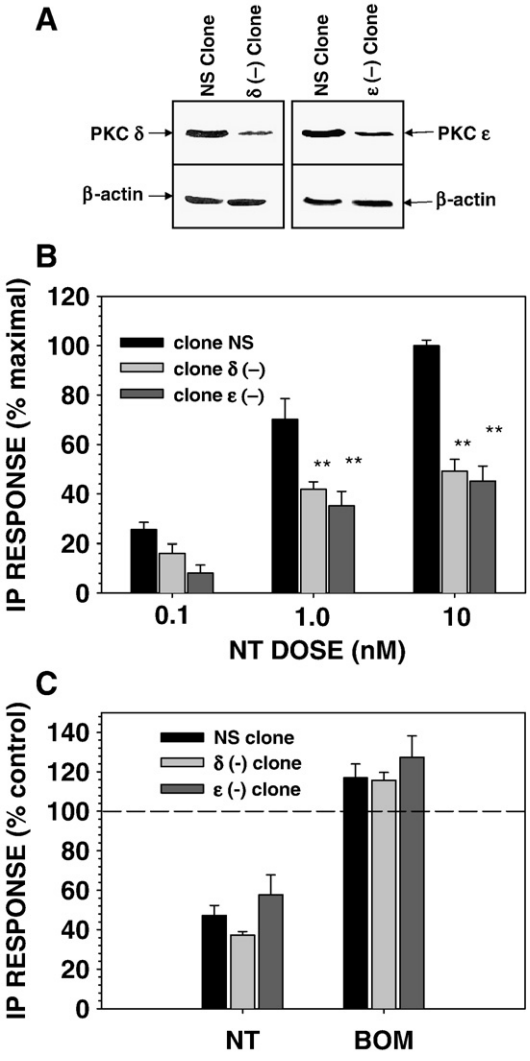

Fig. 6. Effect of PKCδ and PKCε knockdown on PKC expression (A), NT-induced IP formation (B) and on the ability of BIS-1 to alter NT-induced and BOM-induced IP formation (C). In A, an equal number of cells from each clone was extracted, 40 μg protein was subjected to SDS-PAGE and western blotting was performed for PKCδ and PKCε. β-actin was the loading control. The results, representative of multiple experiments using 2 to 5 clones each, indicate >50% knockdown of PKCδ and PKCε. In B, equal numbers of cells from each clone were stimulated with the indicated doses of NT. IP formation was measured and the responses were calculated as % maximal. In C, cells pretreated 15 min with 2.5 μM BIS-1 or vehicle control, were stimulated with 10 nM NT or 3 nM BOM. The IP responses were calculated as % control, which gave ≅4-fold elevation (NT) and ≅10-fold elevation (BOM). In B and C, the data (mean± SEM) are representative of at least three experiments. ** $p<0.01$.

was used to individually knockdown these novel PKC isotypes. A puromycin resistance gene was incorporated into the DNA construct to facilitate clone selection, and several clones were obtained that displayed >50% knockdown of PKCδ and PKCε (Fig. 6A). Based on results from western blotting, one clone representing each treatment was selected for further analysis: nonsense (NS clone); PKCδ(−) clone; and PKCε(−) clone. Basal NT binding, determined when the cells were ≅90% confluent and expressed as cpm/μg protein (mean±SEM, $n=4$), did not differ for the 3 clones (NS clone, 18.0±1.4; PKCδ(−) clone, 15.8±1.2; and PKCε(−) clone, 15.4±1.1). Assessing the

effects of BIS-1 and rottlerin on cellular NT binding in these clones indicated that they were also not significantly different (Table 1). These results indicated that PKCδ knockdown or PKCε knockdown by itself was not sufficient to alter basal NT binding or the effects of BIS-1 and rottlerin on NT binding. One possible explanation was that knockdown of both PKCδ and PKCε (and perhaps PKCα and PKCβI) was necessary for the effect.

3.7. Effect of PKCδ and PKCε knockdown on NT-induced IP formation

NT was less effective in elevating IP formation in the PKCδ(−) and the PKCε(−) clones as compared to the NS clone (Fig. 6B). This was not due to a general effect on PLC activity or the level of phospholipid substrate since the IP response to 3 nM BOM was not inhibited in the PKCδ(−) clone (% control response, 95±2; $n=6$) and was enhanced in the PKCε(−) clone (% control response, 148±13; $n=6$). Consistent with the importance of both PKCδ and PKCε in this regulation, BIS-1 further inhibited the NT response and further enhanced the BOM response, even in the PKCδ(−) and PKCε(−) clones (Fig. 6C). These results were in keeping with the hypothesis that PKCδ and PKCε activity maintained the ability of NT receptor to stimulate PLC.

3.8. PMA decreased and PKC inhibitors increased both cell-surface NT binding and internalization in PC3 cells

Since internalization of the NT receptor occurs during NT binding to PC3 cells [13], we sought to determine if activation or inhibition of PKC altered this process. NT receptor internalization, determined as the percentage of NT binding that resisted acid washing of the cells, was 69±3% (mean±SEM, $n=5$) under control conditions. Pretreatment of the cells with PKC activator PMA decreased both cell-surface NT binding and internalized NT binding by ≅50% (Fig. 7A). Although there was a tendency for PMA to increase the percentage of internalization (Fig. 7A legend), the results did not differ significantly (e.g., control, 68±2% vs 25 nM PMA, 72±2%; $p>0.1$).

Table 1
Effect of BIS-1 and rottlerin on NT receptor binding in PKC knockdown and control clones of PC3 cells

Cell clone	NT binding (% DMSO control)			
	BIS-1		Rottlerin	
	0.5 μM	2.5 μM	0.5 μM	2.5 μM
NS control	155±14	216±20	150±16	181±17
PKCδ(−)	168±13	254±17	145±13	222±18
NS control	140±13	225±19	140±14	236±19
PKCε(−)	167±12	268±21	177±15	244±18

NT receptor binding to each PC3 cell clone was measured in the presence of BIS-1 or rottlerin at the indicated concentrations as compared to the vehicle (DMSO) control. The cells (80–90% confluent) were pre-incubated for 15 min with the agents in Locke prior to the binding reaction. Control NT binding for each clone was similar when expressed as cpm/μg protein: NS control, 18.0±1.4; PKCδ(−); 15.8±1.2; PKCε(−), 15.5±1.1 ($n=4$). For each clone, the effects of BIS-1 and rottlerin were expressed as % control NT binding (mean±SEM; $n=3$ experiments). The results for the different clones were not significantly different.

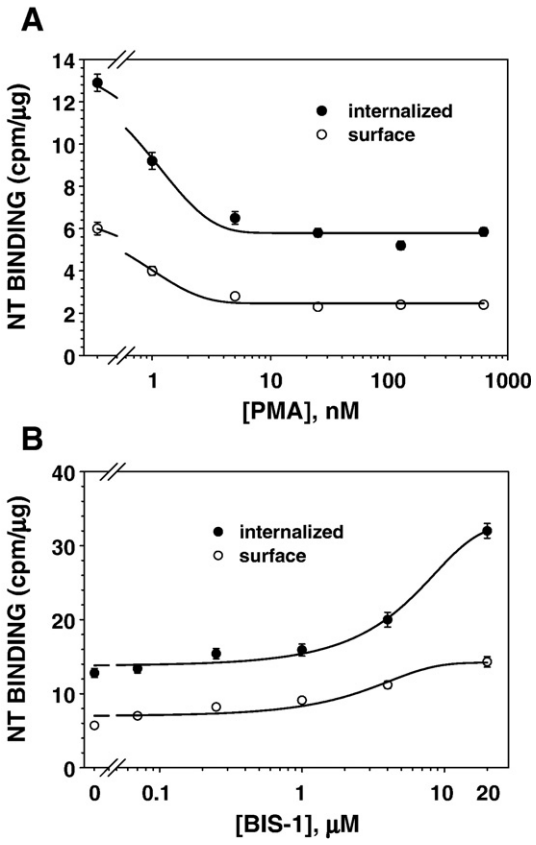

Fig. 7. NT binding to PC3 cells was diminished by PMA (A) and enhanced by BIS-1 (B), without an effect on the percentage of ^{125}I-NT internalized by the cells. Cells were pretreated 10 min with indicated concentrations of PMA, BIS-1 or vehicle control. After the binding reaction, cell-surface and internalized binding were determined by acid washing. The data (mean±SEM) were pooled from three experiments. In A, internalization expressed as percentage of total binding (mean±SEM) was: 68±2, 70±2, 70±2, 72±2, 68±2 and 71±2 at the 0, 1, 5, 25, 125 and 625 nM doses of PMA. In B, % internalization was 69±3, 66±2, 65±2, 64±2, 64±2 and 69±2 at the 0, 0.07, 0.25, 1, 4 and 20 μM doses of BIS-1.

Pretreatment of the cells with PKC inhibitor BIS-1 increased both cell-surface NT binding and internalized NT binding by as much as 2.5-fold (Fig. 7B). There was a tendency for BIS-1 to decrease the percentage of internalization (Fig. 7B legend); however, the results did not differ significantly (e.g., control, 69±3% vs 1 μM BIS-1, 64±2%; $p>0.05$). These results indicated that the changes in NT binding induced by PKC activation or PKC inhibition could not be attributed solely to any effects on NT receptor internalization.

3.9. Clathrin-coated vesicle-mediated endocytosis

To assess the importance of clathrin-coated vesicle-mediated endocytosis in the effects of PKC inhibitors, cellular NT binding was measured in hypertonic medium (Locke containing sucrose), which is known to block clathrin-coated vesicle-mediated endocytosis of ligands and receptors [39]. Sucrose

dose-dependently inhibited NT binding to the cells, with 0.33 M sucrose giving about 45% inhibition (Fig. 8A). However, sucrose unexpectedly inhibited both NT binding to the cell-surface and NT internalization, such that the internalized percentage was only slightly decreased (Fig. 8B). This was in contrast to the results for BOM receptor which was used as the positive control [40], where sucrose inhibited BOM internalization, enhanced BOM cell-surface binding and decreased the internalized percentage (Fig. 8B). In addition, when the NT binding reaction was done in 0.33 M sucrose, the response to BIS-1 was enhanced and the effect of PMA was diminished (Table 2). These findings suggested that hyperosmolar sucrose stimulated PKC activity and indeed, we found that cellular PKC substrate phosphorylation was enhanced by exposing the cells to 0.33 M sucrose for 15 min (Fig. 8C).

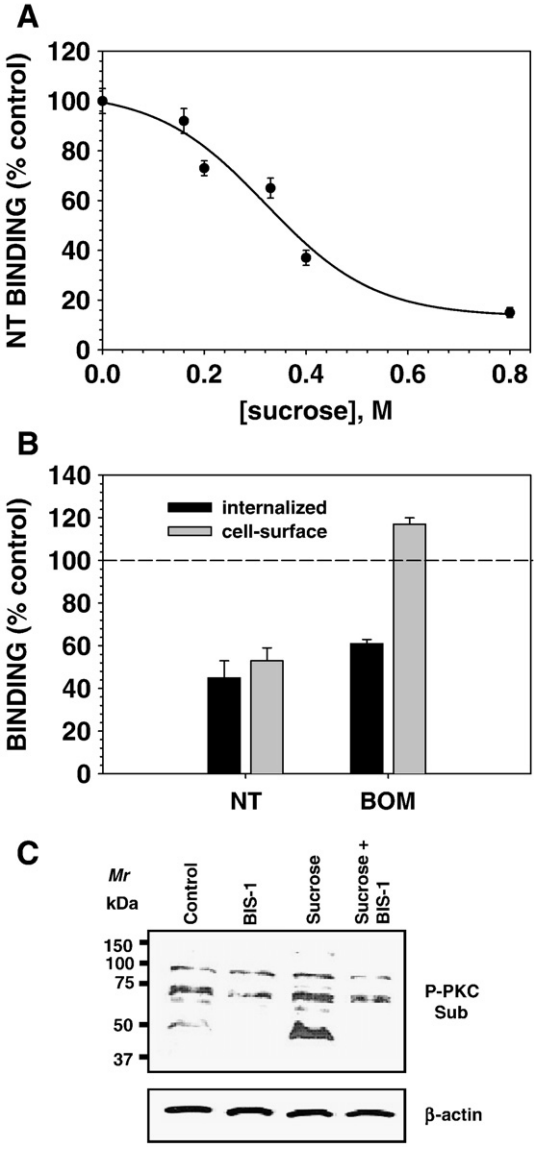

Fig. 8. Effect of hypertonic sucrose on NT receptor binding (A), NT receptor and BOM receptor internalization (B) and PKC substrate phosphorylation (C) in PC3 cells. In A, total cellular NT binding was measured in Locke containing varying concentrations of sucrose and the results were expressed relative to control. In B, the effect of 0.33 M sucrose on cell-surface and internalized binding for the NT receptor and the BOM receptor is shown. After binding was performed with ^{125}I-NT or ^{125}I-BOM, internalized and cell-surface radioactivity was measured by acid washing. The data (mean±SEM) show % binding obtained in sucrose relative to Locke control. Internalization of NT receptor expressed as percentage of total binding was 70±2 (Locke) and 65±2 (sucrose), while that for the BOM receptor was 68±1 (Locke) and 54±1 (sucrose). In C, quiescent cells, pretreated with 1 μM BIS-1 or vehicle control for 30 min, were stimulated with Locke or 0.33 M sucrose for 15 min. Cell lysates were subjected to western blotting using the PKC substrate specific antiserum. β-actin was used as loading control. Shown are typical results representing 3 experiments.

Table 2
Effects of PKC-directed agents on internalized and cell-surface NT binding in Locke and hypertonic sucrose

Agent	Concentration (μM)	Internalized NT binding (% control)[a]		Cell-surface NT binding (%control)[a]	
		Locke	Sucrose	Locke	Sucrose
DMSO	(0.1%)	100	100	100	100
BIS-1	0.6	126±4	153±5[b]	117±4	141±4[b]
	3.0	157±13	216±18[b]	145±6	194±10[b]
PMA	1	70±6	95±5[b]	48±6	77±11[b]

[a] The effect of each agent on NT receptor binding was tested in Locke or in Locke containing 0.33 M sucrose. The cells (80–90% confluent) were pre-incubated for 15 min with the agents or the vehicle control (0.1% DMSO) in Locke prior to the binding reaction. The medium was changed to Locke or sucrose containing the agents, and ^{125}I-NT was added. After 30 min, the cells were placed on ice and NT internalization and NT binding to the cell-surface were measured using the acid washing technique. The data (mean±SEM; $n=4$ experiments) were expressed relative to the appropriate vehicle control. For the control condition, sucrose reduced internalized NT binding and cell-surface NT binding to a similar extent (45–55%).

[b] Results in sucrose differed significantly from those in Locke ($p<0.05$).

We interpreted these findings to indicate that clathrin-coated vesicle-mediated endocytosis participated in NT receptor internalization, but the effect of BIS-1 on NT receptor binding persisted despite inhibition of this process with sucrose. Furthermore, our results suggested that sucrose decreased cellular NT binding at least partly by activating PKC, which is consistent with work in NIH/3T3 cells showing that hyperosmolality increased PKC activity within 10 min [41].

3.10. PMA increased and PKC inhibitor decreased the IC50 for NT binding in PC3 cells

To determine whether the effects of PKC-directed agents on NT binding reflected changes in receptor affinity or receptor number, NT displacement data were analyzed. The PKC activator PMA shifted the NT displacement curve to the right (Fig. 9A). The average IC50 (mean±SEM) was increased from 1.07±0.05 nM (control) to 2.17±0.17 nM (0.5 μM PMA; $p<0.01$) in 3 experiments. In contrast, the PKC inhibitor Ro-318220 shifted the NT displacement curve to the left (Fig. 9B). The average IC50 was decreased from 1.35±0.22 nM (control) to 0.55±0.09 nM (1 μM Ro-318220; $p<0.01$) in 3 experiments. Scatchard analyses (Fig. 9C and D) indicated that the changes in NT binding were due to the demonstrated changes in receptor affinity and could not be attributed to changes in receptor

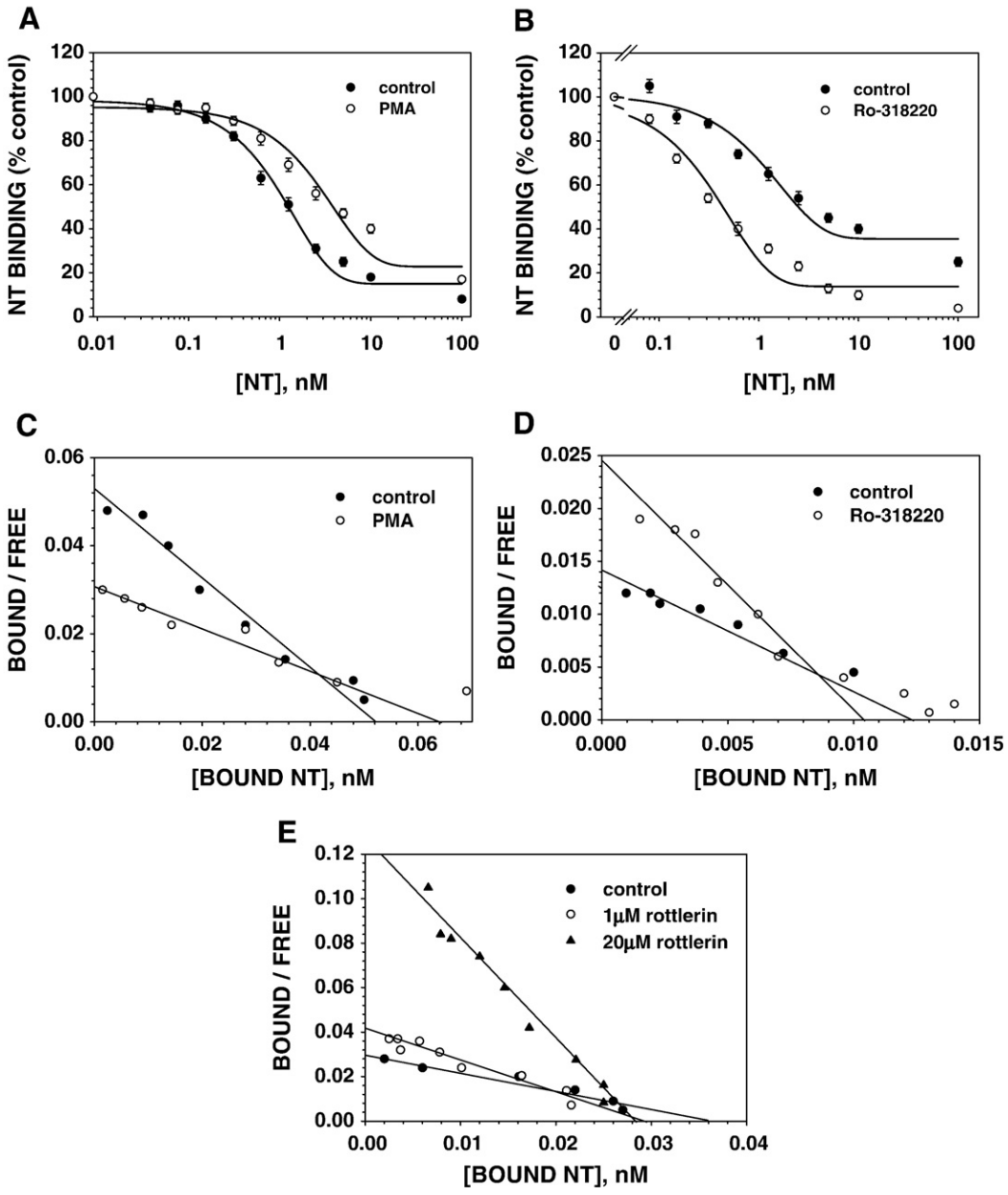

Fig. 9. Binding displacement curves (A, B) and Scatchard plots (C, D, E) for NT binding to PC3 cells in the presence and absence of PKC-directed agents. Cells were pretreated with agents indicated or control for 30 min. Then, specific NT binding was measured in the presence of indicated concentrations of NT at equilibrium (30 min). NT binding was decreased 35% by 100 nM PMA, whereas it was increased 54% by 1 μM Ro-318220. In A and B, log dose–response plots are shown in which NT binding was expressed as percentage of control. The IC50 was shifted to the right by PMA (A) and to the left by Ro-318220 and rottlerin (B). Results are from typical experiments that were repeated twice. C, D and E are Scatchard plots for typical experiments, showing that the changes in NT binding were due to shifts in receptor affinity (slope of line) rather than receptor number (intercept at *x*-axis). In C, note that two components were seen for PMA and the line drawn estimates only the high affinity sites. In C, Ki was 1.0 nM (control) and 2.1 nM (PMA); Bmax was 260 fmol/mg (control) and 320 fmol/mg (PMA). In D, Ki was 1.0 nM (control) and 0.42 nM (1 μM Ro-318220); Bmax was 203 fmol/mg (control) and 186 fmol/mg (1 μM Ro-31882). In E, Ki was 1.2 nM (control), 0.69 nM (1 μM rottlerin) and 0.23 nM (20 μM rottlerin); Bmax was 246 fmol/mg (control), 219 fmol/mg (1 μM rottlerin) and 199 fmol/mg (20 μM rottlerin).

number. Studies comparing the effects of inhibitors from categories I and II showed that 1 μM Go-6976 gave results that were similar to those for 1 μM Ro-318220 (results not shown). In addition, Fig. 9E shows that the effect of the high dose (20 μM) of the category II inhibitor rottlerin on the binding parameters was similar to that of the low dose (1 μM), except that there was a greater shift in the Ki to even higher affinity, without an increase in receptor number.

3.11. Receptor specificity

To determine if these responses were specific to the NT receptor, we tested PKC inhibitors for effects on BOM receptor binding and EGF receptor binding in PC3 cells [13]. Since the BOM [42] and EGF receptors [43] are known to be regulated by PKC, this provided another test regarding the specificity of these agents. Under control conditions, BOM receptor binding

Table 3
Effects of PKC-directed agents on BOM-induced IP formation in PC3 cells

Agent	Dose (μM)	IP formation[a] (% control)
PMA	0.001	43±4[c]
	0.04	25±3[c]
	0.1	18±2[c]
Staurosporine	0.2	129±7[b]
	0.8	120±5
	3.2	117±7
Go-6976	0.4	138±5[b]
	2	107±5
	10	109±8
Go-6983	0.4	129±6[b]
	2	129±6[b]
	10	119±5
BIS-1	0.1	117±5
	0.5	124±5[b]
	2.5	138±5[b]
Ro-318220	0.2	125±6
	0.8	129±6[b]
	3.2	78±6
Rottlerin	0.3	146±9[b]
	1	81±6
	10	45±6[c]

[a] Cells were pretreated with the indicated concentrations of each agent or vehicle control for 15 min, then stimulated with 2 nM BOM for 30 min, and IP formation was measured. The increment in IP formation was expressed as % control (mean±SEM) for at least three experiments.
[b] BOM-induced IP formation was significantly increased ($p<0.05$).
[c] BOM-induced IP formation was significantly decreased ($p<0.01$).

was 112±14 cpm/μg ($n=6$) and EGF receptor binding was 35.7±2.7 cpm/μg ($n=6$) in agreement with our prior work. Pretreatment of the cells with PKC inhibitors from category I (staurosporine) and category II (BIS-1 and rottlerin) modestly elevated BOM receptor binding (<20% increase) and EGF receptor binding (<30% increase). These agents were effective in the low micromolar range and their effects did not increase dramatically at higher doses. These results indicated that the robust elevation (>200% increase) in NT receptor binding caused by category II PKC inhibitors was specific to the NT receptor, whereas the smaller elevation (<30%) was also seen for the BOM and EGF receptors.

The PKC-directed agents were also tested for the ability to alter IP formation in response to BOM since it was known to stimulate PLC [13]. IP formation in PC3 cells was enhanced 4.7±1.1 fold by 1 nM BOM and 14.5±2.5 by 10 nM BOM. Therefore, we used 2 nM BOM as the stimulus to test the effects of the agents. As expected, the PKC activator PMA inhibited the response to BOM, exhibiting an IC50 near 1 nM (Table 3). When used at nanomolar and low micromolar doses, the PKC inhibitors (staurosporine, Go-6976, Go-6983, BIS-1, Ro318220 and rottlerin) enhanced BOM-induced IP formation by 20–40% (Table 3). These results, which were similar to those for NT, suggested that the constitutive PKC activity in PC3 cells inhibited PLC activation in response to both NT and BOM. This interpretation is consistent with work showing that BOM receptor function is subject to PKC regulation [44].

Although the enhancement of the IP response to BOM fell off at higher doses of rottlerin (Table 3) as was seen for NT (Fig. 4C), in general the results for BOM differed from those for NT. For example, doses of BIS-1, Ro-318220 and Go-6983 that inhibited NT-induced IP formation by >40% (Fig. 4C) did not inhibit BOM-induced IP formation (Table 3). These results indicated that the inhibition of NT-induced IP formation by the PKC inhibitors in category II was relatively specific to NT.

3.12. Cell specificity

To determine if the effects of PKC inhibitors were specific to PC3 cells, we assessed the effect of BIS-1 on NT binding in other cancer cell lines, including prostate carcinomas (PC3M, DU145, LNCaP^{CS}, MatLyLu) and a colon carcinoma (HT29). The level of NT receptor expression in these cell lines was previously described by us [35]. As compared to the vehicle control (0.06% DMSO), specific binding in the presence of 6 μM BIS-1 (mean±SEM; $n=3$ experiments) was: PC3 (186±11); HT29 (188±12); MatLyLu (217±13); PC3M (227±12); LNCaP^{CS} (162±15) and DU145 (276±18). These results indicate that 6 μM BIS-1 enhanced NT binding similarly in a number of cancer cell lines.

4. Discussion

Here, we report for the first time that NT receptor function in a number of prostate cancer cell lines is regulated by endogenous PKC activity. Focusing on PC3 cells, we demonstrated the expression of constitutive PKC activity that, under basal conditions, suppressed NT receptor binding and NT-induced IP formation. Thus, eight different PKC inhibitors (staurosporine, BIS-1, Ro-318220, Go-6976, Go-6983, chelerythrine, rottlerin and quercetin) enhanced NT binding and NT-induced IP formation by 20–40% at low micromolar concentrations that for most of these agents were shown to inhibit conventional PKC substrate phosphorylation in the cells. Not only was NT receptor function inhibited in a tonic manner by the basal PKC activity but pharmacologic activation of PKC was also shown to further inhibit NT receptor function. Thus, PKC activator PMA further inhibited NT binding and NT-induced IP formation. Our results established that NT receptor function was regulated by a heterologous desensitization process that appeared to involve primarily conventional PKC activity. However, our studies also uncovered a second mechanism by which novel PKC activity appeared to maintain (perhaps via effects on G protein coupling) the ability of NT receptor to activate PLC. Overall, our findings imply that, depending on the predominant PKC isotypes involved, heterologous regulation of NT receptor function can serve to either inhibit or maintain NT receptor function. Since NT-induced growth signaling in PC3 cells is PKC-dependent [9] and is associated with enhanced PKC isotype phosphorylation (S Hassan, unpublished data), our findings are also compatible with the existence of homologous regulation of NT receptor function via feedback effects of the PKC isotypes activated by NT. However, further studies are needed before a definitive conclusion can be drawn.

Although we have not yet identified all of the PKC isotypes involved, our studies point towards roles for both conventional (Ca^{2+}-sensitive) PKCs and novel (Ca^{2+}-insensitive) PKCs, with the latter exerting more striking effects. This was suggested initially by the fact that rottlerin, a PKCδ (novel)-specific inhibitor, was one of the most potent and efficacious agents to enhance NT receptor binding (Fig. 3B), whereas the conventional-specific inhibitor Go-6976 was much less effective (Fig. 3A). However, the results were puzzling since some of the inhibitors with broad specificity (BIS-1, Ro-318220, Go-6983) were quite effective while others (staurosporine, cherlerythrine) were rather ineffective (Fig. 3A and B). Furthermore, the effects of some of the agents on NT-induced IP formation were dose-dependently biphasic, while others were monophasic (Fig. 4B and C). Careful analysis finally led us to conclude that there were two different responses: response I was characterized by a modest enhancement (≅40%) in NT receptor binding and NT-induced IP formation; response II involved a dramatic enhancement (≅200%) in NT receptor binding associated with an inhibition of NT-induced IP formation. Response I was brought about by most of the PKC inhibitors at the low micromolar concentrations that were shown to inhibit conventional PKC substrate phosphorylation. On the other hand, response II could only be induced by inhibitors in category II (rottlerin, BIS-1, Ro-318220, Go-6983 and quercetin) and only at higher concentrations (>2 μM).

It seems probable that PKC inhibition was involved in both responses I and II, and that the outcome depended on the differential effects on specific PKC isotypes. Western blotting demonstrated that the major isotypes present were the conventional PKCs (α, βI) and the novel PKCs (δ, ε). The results for Go-6976 (specific for conventional PKCs) were consistent with the idea that inhibition of PKCα and/or PKCβI produced response I. The results for BIS-1 and Ro-318220 (known to require 5–16-fold higher concentrations to inhibit novel PKCs) and for rottlerin (putative PKCδ inhibitor) suggested that response II involved an inhibition of PKCδ and/or PKCε. To explain the results for the other inhibitors (which had broad specificity), one might propose that staurosporine and chelerythrine were unable to gain access to or sufficiently inhibit PKCδ and/or PKCε and thus, did not produce response II. Consistent with this interpretation, we found that knockdown of PKCδ or PKCε reproduced at least one aspect of response II, the inhibition of NT-induced IP formation. Although the PKCδ(−) and PKCε(−) clones did not exhibit the enhanced NT binding that is characteristic of response II, this apparent difference in PKC isotype dependence could have been due to differences in the assay conditions. For example, the NT binding assay (in contrast to the IP assay) did not involve stimulating the cells with high levels of NT that might have induced homologous desensitization. Another explanation is that knockdown of both PKCδ and PKCε (and possibly PKCα and PKCβI) might have been required to reproduce the effects of category II inhibitors on NT binding. Finally, it might have been that some of the effects of category II inhibitors on NT binding involved targets other than PKC.

The simplest interpretation of our results at this time is that inhibition of conventional PKCα and PKCβI produced response I, whereas the additional inhibition of novel PKCδ and PKCε produced response II. Thus, when BIS-1 was used at low doses that preferentially inhibited conventional PKCs, NT binding and IP formation were modestly enhanced. It seems likely that the increase in NT receptor binding led to the enhanced IP response. Therefore, a reasonable model is that conventional PKCs modulate NT binding to regulate receptor function. On the other hand, when BIS-1 was used at higher doses that inhibited novel as well as conventional PKCs, NT binding was dramatically enhanced and IP formation was inhibited. Since the inhibition of IP formation was specific to the NT receptor, it seems likely that this involved some form of G protein uncoupling. For example, novel PKC activity might maintain NT receptor–effector function by keeping G protein receptor kinases in check. Whatever the mechanism, this model is consistent with most of the data. For example, knockdown of PKCδ or PKCε would be expected to inhibit NT-induced IP formation but it would not necessarily cause the dramatic increase in NT receptor binding. This is because the uncoupled receptor would still be subject to binding inhibition imposed by constitutive PKCα and PKCβI activity.

The mechanistic studies reported here show that the modulation of NT receptor binding by the PKC-directed agents could not be attributed to changes in receptor number or receptor internalization. However, PKC activation or inhibition induced a remarkable change in receptor affinity that was consistent with the idea that PKC activity shifted the NT receptor to a low affinity state. Thus by blocking the constitutive PKC activity of the cells, PKC inhibitors shifted the NT receptor to a higher affinity state resulting in increased binding activity. Consistent with this, NT binding to the cell-surface and NT internalization were increased similarly and dose-responsively by BIS-1. The enhancing effects of BIS-1 persisted despite the inhibition of clathrin-coated vesicle-mediated endocytosis by sucrose, indicating that the mechanism was not likely to involve a change in receptor trafficking. Although it seems clear that a shift in NT receptor affinity is the basis for the regulation of NT binding by PKC, we do not know if the effects of PKC are direct or indirect. NT has been shown to induce phosphorylation of HA-tagged NT receptor in HEK-293 cells [21], but it is not known whether PKC participated in this or if PKC can under any conditions phosphorylate NT receptor. Since PKC inhibitors had only modest effects on BOM receptor binding and EGF receptor binding, the dramatic effects on the behavior of the NT receptor appeared to be receptor-specific. We demonstrated the effect of BIS-1 on NT receptor binding in a number of cancer cell lines, indicating that this behavior may apply generally to cells expressing the NT receptor.

It is interesting to note that the ability to alter NT receptor function was related to the chemical structures of the PKC inhibitors that were analogs of staurosporine. The agents in category II that induced dramatic effects on NT binding (BIS-1, Go-6983 and Ro-318220) were derivatives of maleimide, whereas the agents in category I that induced moderate effects on NT binding (staurosporine and Go-6976) were derivatives of carbazole. It is tempting to speculate about how the chemical nature of these derivatives might relate to their differing effects on

NT receptor function. In comparison to the carbazoles, the maleimides offer a more highly conjugated system that could promote redox reactions, one possibility involving donation of the central imide hydrogen. Interestingly, the maleimide derivative with a CH_3 group instead of hydrogen at this position (BIS-V) was inactive as a PKC inhibitor [45] and unable to modulate NT receptor function. This suggested that an antioxidative redox effect could play a role in these effects. We found that antioxidative polyphenols that can accumulate in membranes (and might possibly inhibit PKC) induced the type II response in PC3 cells [46]. BIS-1 is known to accumulate in mitochondrial membranes [47]; PKCδ was shown to translocate to mitochondrial membranes during apoptosis [48,49]; and Ro-318220 was found to induce mitochondrial apoptosis [50]. We showed that inhibitors of mitochondrial oxidative phosphorylation induced the type II response in PC3 cells [45]. Superoxide and hydrogen peroxide, which are produced by mitochondria, were found to activate PKC and this effect was reversed by antioxidants [51]. These findings suggest that the type II response could involve antioxidative effects of these agents on PKCδ and/or PKCε within mitochondria or other targets in PC3 cells.

Given that PKC activity can vary depending on cell type, stage of development and environmental inputs, these findings underline the importance of controlling these conditions when assessing NT receptor function in different systems. Since NT is an important mitogen in cancer cells, our findings suggest that the PKC-directed agents in category II, which inhibit NT receptor signaling, could be useful lead compounds for the development of new anticancer drugs. If these compounds produced their effects on NT receptor function by targeting PKCδ and/or PKCε, then these NT assays might be used to screen for substances that specifically inhibit or activate these novel PKCs. This could be useful since PKCδ and PKCε play important roles in cell cycle regulation [52], growth stimulation [53], growth inhibition [54] and apoptosis [55]. It should be noted that androgens can regulate the activity of the NT system [56, 57] as well as the expression of PKCδ in prostate LNCaP cells [58].

In conclusion, constitutive PKC activity was demonstrated in PC3 cells, which under basal conditions caused an inhibition of NT receptor function. Accordingly, low micromolar doses of PKC inhibitors released NT receptor from this inhibitory influence, enhancing NT binding and NT-induced IP formation moderately. This type I response appeared to involve inhibition of the conventional PKCs (α, βI). In addition, a second response was identified, which was induced by higher concentrations of a select group of PKC inhibitors. This type II response, characterized by a dramatic increase in NT binding and a pronounced inhibition of NT-induced IP formation, appeared to involve the novel PKCs (δ, ε), although other targets were possible. Overall, the results were consistent with the hypothesis that NT receptor function was subject to heterologous regulation by PKC and that two distinct mechanisms were involved.

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