0026-895X/05/6706-2025–2031$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 67:2025–2031, 2005

Vol. 67, No. 6
10801/3033284
Printed in U.S.A.

Neurotensin Stimulates Interleukin-8 Expression through Modulation of IκBα Phosphorylation and p65 Transcriptional Activity: Involvement of Protein Kinase Cα

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Received December 23, 2004; accepted March 3, 2005

ABSTRACT

Neurotensin (NT) is released in the gastrointestinal tract and participates in the pathophysiology of colonic inflammation. We have shown that NT mediates acute intestinal inflammation in vivo and stimulates nuclear factor-κB–dependent interleukin (IL)-8 expression in nontransformed human colonocytes in vitro. However, the exact mechanisms by which NT induces IL-8 expression have not been elucidated. In this study, we first show that NT stimulates IκBα phosphorylation and degradation and p65 phosphorylation and transcriptional activity. Inhibition of protein kinase C (PKC) activation significantly attenuates NT-induced IL-8 expression. This effect seems to be mediated through inhibition of IκBα phosphorylation and degradation and by p65 phosphorylation and transcriptional activity. We also show that intracellular calcium mobilization is necessary for NT-induced phosphorylation of IκBα and p65, suggesting that a conventional PKC is involved. Furthermore, transfection of a dominant-negative form of PKCα significantly reduces NT-induced IL-8 promoter activity. These results indicate that the conventional PKCα is an important mediator in the proinflammatory signaling pathway elicited by NT at the colonocyte level.

Neurotensin (NT) is a 13 amino acid neuropeptide that is highly expressed in the brain and the gastrointestinal tract (Carraway and Leeman, 1976). In the small intestine, NT is produced and secreted by specific endocrine cells (Polak et al., 1977) in response to several stimuli (Walsh, 1987). NT is involved in motility changes in the stomach and small and large intestine; stimulates ileal, pancreatic, and biliary secretion (Thor and Rosell, 1986; Walsh, 1987); and elicits Cl^- secretion in human colonic mucosa (Riegler et al., 2000). NT is also associated with growth of the intestinal mucosa, and stimulates proliferation of intestinal epithelial cells in vivo and in vitro (Wood et al., 1988; Maoret et al., 1999). NT binds to two different G-protein–coupled receptors, the high-affinity NTR1 and the low-affinity NTR2 (Vincent et al., 1999). Animal studies using receptor antagonists indicate that NTR1 mediates important colonic responses related to stress and inflammation. Thus, administration of the specific nonpeptide NTR1 antagonist SR 48692 to rats inhibits colonic mucin and prostaglandin E2 release and mast cell degranulation in response to immobilization stress (Castagliuolo et al., 1996), as well as colonic secretion and inflammation mediated by *Clostridium difficile* toxin A (Castagliuolo et al., 1999b). A dramatic up-regulation of NTR1 is evident in the colonic mucosa in patients with inflammatory bowel disease (Castagliuolo et al., 1999a) and in an animal model of *C. difficile* toxin A-induced colitis (Castagliuolo et al., 1999b). Several cell types may be involved in the proinflammatory responses to NT, including mast cells (Miller et al., 1995; Castagliuolo et al., 1996, 1999b), leukocytes (Goldman et al., 1983; Castagliuolo et al., 1999b) endothelial cells (Schaeffer et al., 1995), and macrophages (Lemaire, 1988).

We recently demonstrated that nontransformed human colonic epithelial NCM460 cells express functional NTR1. We also showed that in NCM460 cells overexpressing NTR1, NT stimulates the release of the potent neutrophil chemoattrac-

This study was supported by National Institutes of Health grant DK060729 (to C.P.). D.Z. and H.Z. are recipients of National Institutes of Health Career Development Awards (K01-DK064920 and K01-CA098581, respectively).
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.010801.

ABBREVIATIONS: NT, neurotensin; NF-κB, nuclear factor-κB; PKC, protein kinase C; BAPTA/AM, 1,2-bis(o-aminophenooxy) ethane-*N,N,N′,N′*-tetraacetic acid, acetoxymethyl ester; PMA, phorbol 12-myristate 13-acetate; IL, interleukin; IKK, IκB kinase; DAG, diacylglycerol; MAP, mitogen-activated protein; DN, dominant negative; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride; GF 109203X, 3-[1-[3-(dimethylaminopropyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride; SR 48692, 2-((1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3-yl)carbonylamino)tricyclo(3.3.1.1.(3.7))decan-2-carboxylic acid.

tant interleukin-8 (IL-8) (Zhao et al., 2001), indicating that NT may mediate colonic inflammation by acting directly on human colonocytes. The mechanism of NT-mediated stimulation of IL-8 gene expression involves the activation of nuclear factor-κB (NF-κB) (Zhao et al., 2001), a critical regulator for the expression of genes involved in intestinal inflammation (Schmid et al., 1998). It consists of homo- and heterodimers of the Rel family proteins, such as p65 and p50, retained in an inactive form in the cytoplasm by IκB inhibitory proteins. After exposure to a proinflammatory stimulus, IκBs become phosphorylated, ubiquitinated, and subsequently degraded, resulting in the translocation of NF-κB into the nucleus and activation of NF-κB–associated genes. NF-κB p65 transcriptional activity is also regulated by its phosphorylation at multiple sites by different serine/threonine kinases. Tumor necrosis factor-α induces rapid p65 phosphorylation at Ser536, located within the carboxyl-terminal transactivation domain though IκB kinases (IKKs), resulting in increased transcriptional activity of p65 (Sakurai et al., 1999; Madrid et al., 2001). Full transactivation of p65 in response to tumor necrosis factor-α also requires its phosphorylation at Ser529 by casein kinase II (Wang et al., 2000). Phosphorylation of Ser276 by the catalytic subunit of protein kinase A in response to lipopolysaccharide stimulates p65 transcriptional activity by promoting its interaction with the coactivator CREB-binding protein/p300 (Zhong et al., 1997, 1998). Numerous studies have indicated that the protein kinase C (PKC) family of proteins also regulates the NF-κB pathway by either activating IKKs or increasing p65 transactivation activity, or both (St-Denis et al., 1998; Vertegaal et al., 2000; Trushin et al., 2003). However, the particular PKC isoenzyme involved in this process depends on the particular stimulus and/or cell type.

To date, 11 closely related PKC isozymes have been described and classified into three subfamilies on the basis of their domain structure and their ability to respond to Ca^{2+} and DAG (Newton, 1995; Newton and Johnson, 1998). The "conventional" PKC isoforms (α, β1, βII, and ε) are regulated by DAG, which binds the C1 domain, and by Ca^{2+}, which binds the C2 domain. In contrast, the "novel" PKC isoforms (δ, ε, η, and σ) are not regulated by Ca^{2+} but do respond to DAG. The molecular structure of the novel PKC isoforms is similar to that of the conventional isoforms except for differences in the Ca^{2+}-binding domain. The third group of PKC isoforms includes the "atypical" PKC isoforms (ξ, λ/ι, and μ), which are not regulated by DAG or Ca^{2+}. Elevated concentrations of intracellular Ca^{2+} and DAG after stimulation can potentially activate either conventional or novel or both PKC isoforms, which mediate NF-κB activation (Szamel et al., 1998). A number of studies suggest that protein kinase Cs may also be involved in NT signaling because NT stimulates the formation of inositol (1,4,5) trisphosphate and increases intracellular calcium (Amar et al., 1986; Bozou et al., 1989). In addition, NT-induced MAP kinase activation can be attenuated by the PKC inhibitor GF 109203X (Poinot-Chazel et al., 1996). However the functional role of the protein kinase C family of proteins in NT proinflammatory signaling is still unknown.

We used nontransformed human colonic NCM460 cells overexpressing NTR1 to investigate whether PKCs are involved in IL-8 gene expression in response to NT and studied the signaling pathways involved in this process. Here, we show that PKC activation is involved in NT-NTR1-induced IL-8 expression through inhibition of IκBα phosphorylation and degradation and through p65 phosphorylation and transcriptional activity. Using a dominant-negative approach, we also present novel evidence that the conventional PKCα is a critical mediator involved the signaling pathway by which NT mediates IL-8 gene expression in human colonocytes.

Materials and Methods

Reagents. NT was purchased from Phoenix Pharmaceuticals (Belmont, CA). BAPTA/AM and GF 109203X were obtained from Calbiochem (San Diego, CA). Phospho-IκBα (Ser33/Ser36) monoclonal antibody, phosphor-NF-κB p65 (Ser536) antibody, and p65 antibody were from Cell Signaling Technology Inc. (Beverly, MA). PKCα antibody and β-actin antibody were from BD Biosciences (San Jose, CA) and Sigma-Aldrich (St. Louis, MO), respectively. IκBα polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Dominant-negative PKCα construct was kindly provided by Dr. Alex Toker at our hospital. NCM460 cells and modified three-dimensional culture medium were obtained from INCELL Corporation (San Antonio, TX). Bicinchoninic acid reagents for the measurement of protein concentration were purchased from Pierce Chemical (Rockford, IL).

Western Blot Analysis. Cells were washed twice with ice-cold phosphate-buffered saline and then incubated in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) for 10 min. Cell lysates were centrifuged at 1000*g* for 10 min. Equal amounts of cell extracts were separated by SDS-polyacrylamide gel electrophoresis (10%), and proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) at 100 V for 1 h at 4°C. Membranes were blocked in 5% nonfat dried milk in 50 mM Tris, pH 7.5, 0.15 M NaCl, and 0.05% Tween 20 and then incubated with phosphospecific antibodies (0.2 μg/ml) directed against IκBα or p65 or antibodies against IκBα or β-actin. Horseradish peroxidase-labeled antibodies were detected by SuperSignal Chemiluminescent Substrate (Pierce).

Transcriptional Activity of p65. A one-hybrid system was used to determine the transcriptional activity of p65 independent of its DNA binding as originally described by Schmitz and Baeuerle (1991). In this assay, p65 sequences was fused to DNA binding domain of the yeast GAL4 transcription factor (pGal4-p65), and luciferase reporter gene expression was controlled by two Gal4-binding sites [p(Gal4)2-luc], therefore allowing detection of the principal transactivation domain of p65. The two plasmids pGal4-p65 and p(Gal4)2-luc were described previously (Vanden Berghe et al., 1998).

IL-8 Measurement. IL-8 protein levels in colonic epithelial cell-conditioned media were determined by a double-ligand enzyme-linked immunosorbent assay using goat anti-human IL-8 (R&D Systems, Minneapolis, MN) as described previously (Zhao et al., 2001). Results were expressed as mean ± S.E. (in nanograms per milliliter). At least three independent experiments were performed for each experimental condition, each with triplicate measurements.

IL-8 Promoter Activity. A reporter construct containing 1521 base pairs (nucleotides −1481 to +40) of the promoter region of human IL-8 gene was used to determine transcriptional activity of the IL-8 gene as described previously (Zhao et al., 2001). In brief, cells were seeded in 12-well plates (0.2×10^6 cells/well) overnight and transiently transfected using Effectene Transfection Reagent (QIAGEN, Valencia, CA) with IL-8 promoter luciferase constructs or a control luciferase construct pRL-TK (Promega, Madison, WI) or other DNA constructs as indicated. Transfected cells were serum-starved for 24 h followed by exposure to NT for 4 h. Firefly and *Renilla reniformis* luciferase activities in cell extracts were measured using Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity was then calculated by normalizing IL-8

promoter-driven firefly luciferase activity to control *R. reniformis* luciferase activity. Data from all experiments are presented as the relative luciferase activity (mean ± S.E.) from at least two independent sets of experiments, each with triplicate measurements.

Statistical Analyses. Results were expressed as means ± S.E.M. Data were analyzed using the SIGMA-STAT professional statistics software program (SPSS Inc., Chicago, IL). Analyses of variance with protected *t* test were used for intergroup comparison.

Results

NT Stimulates Phosphorylation and Degradation of IκBα. We have shown previously that NT stimulates DNA binding activity of NF-κB in human colonic epithelial cells transfected with NTR1 (NCM460-NTR1 cells). To examine whether NT also induces IκBα phosphorylation and degradation in the parental NCM460 cells and NCM460-NTR1 cells, cells were treated with NT for various period of times. Equal amounts of cell protein were then subjected to Western blotting using a phospho-IκBα (Ser32/Ser36) monoclonal antibody or an IκBα antibody. As shown in Fig. 1, NT induced IκBα phosphorylation (Fig. 1a) and degradation (Fig. 1b) only in NCM460-NTR1 cells (Fig. 1a, left) but not in nontransfected NCM460 cells (Fig. 1a,. right), which are known to express low levels of NTR1 (Zhao et al., 2001). This is also consistent with our prior observation that NT stimulates NF-κB DNA binding and IL-8 gene expression only in NCM460-NTR1 cells (Zhao et al., 2001).

NT-Induced IκBα Phosphorylation Is Dependent on Intracellular Calcium Mobilization and Protein Kinase C Activation. Because NT was shown previously to induce intracellular calcium release and potentially activate protein kinase C (Amar et al., 1986; Bozou et al., 1989; Poinot-Chazel et al., 1996), we examined whether these signaling molecules are involved in NT-induced activation of the NF-κB pathway. To do this, NCM460-NTR1 cells were pretreated with BAPTA/AM, an intracellular calcium chelator, or GF 109203X, a PKC inhibitor specific for PKCα-, β_I-, β_{II}-, γ-, δ-, and ϵ-isozymes and then stimulated with NT for 15 min. IκBα phosphorylation was measured as in Fig. 1. The results indicated that pretreatment with either BAPTA/AM or GF 109203X alone blocked NT-induced IκBα phosphorylation in a dose-dependent fashion (Fig. 2a). To further examine the importance of PKC in this NT response, cells were pretreated with the PKC activator PMA overnight to deplete PKC expression and then were exposed to NT for 15 min. The data show that down-regulation of PKC also blocked NT-induced IκBα phosphorylation (Fig. 2b). Together, these results suggest that a calcium-dependent PKC may mediate this NT response.

NT Stimulates p65 Phosphorylation and Increases Its Transcriptional Activity. Because, as discussed above, phosphorylation of NF-κB p65 is an important step for its transcriptional activity, we next examined whether NT induces p65 phosphorylation in NCM460-NTR1 cells. Cells were treated with NT for various times, and equal amounts of cell protein were subjected to Western blotting using a phospho-NF-κB (Ser536) antibody. The results indicate that NT strongly induced p65 Ser536 phosphorylation (Fig. 3a). Next, we determined whether NT also increases p65 transcriptional activity. Cells were transiently transfected with pGal4-p65 and p(Gal4)2-luc as described in under *Materials and Methods* and then treated with NT for 6 h. Our results show that NT significantly increased p65 transcriptional activity (Fig. 3b).

NT-Induced p65 Phosphorylation and Transcriptional Activity Require Intracellular Calcium-Dependent Protein Kinase C Activity. To determine whether NT-induced p65 Ser536 phosphorylation involves intracellular calcium release and protein kinase C, cells were pretreated with BAPTA/AM or GF 109203X for 30 min or PMA overnight and then stimulated with NT for 10 min. p65 Ser536 phosphorylation was then examined as in Fig. 3. As shown in Fig. 3c, pretreatment with either GF 109203X, prolonged PMA treatment, or BAPTA/AM significantly inhibited NT-induced p65 Ser536 phosphorylation (Fig. 3c). To further study whether NT-induced p65 transcriptional activity involves protein kinase C, cells were transfected with

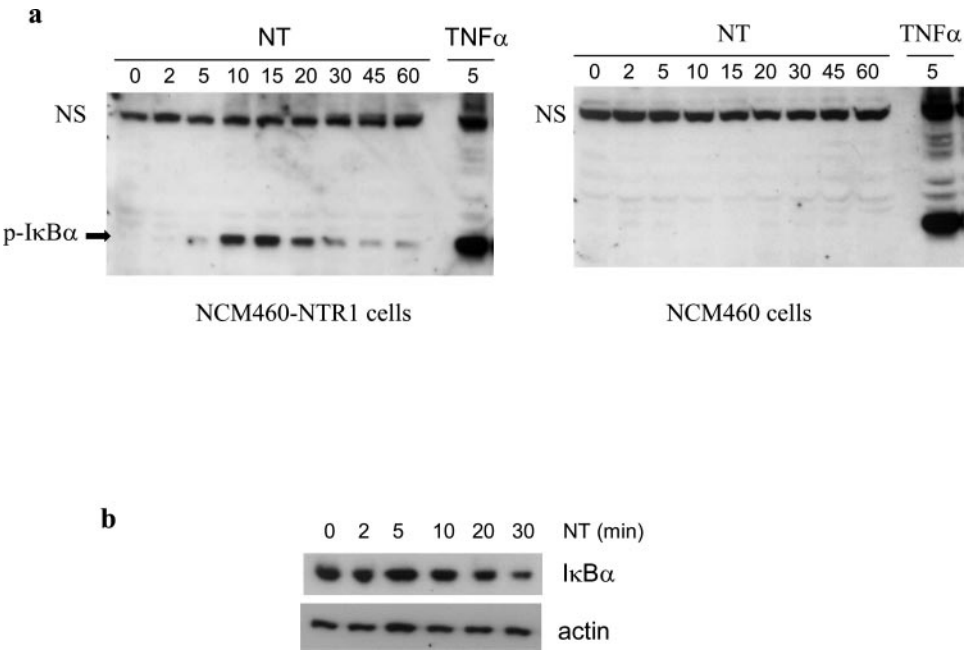

Fig. 1. NT stimulates phosphorylation and degradation of IκBα. NCM460 and NCM460-NTR1 cells were treated with NT (10^{-7} M) for various time points. Equal amounts of cell protein were subjected to Western blotting using a phospho-IκBα (Ser32/Ser36) monoclonal antibody (NS, nonspecific band) (a) or an IκBα antibody and actin antibody to control equal protein loading (b). Results are representative of three separate experiments.

pGal4-p65 and p(Gal4)2-luc as in Fig. 3 and were pretreated with GF 109203X followed by NT exposure. Figure 3d shows that inhibition of protein kinase C significantly reduced NT-induced p65 transcriptional activity.

Inhibition of Protein Kinase C Attenuates NT-Induced NF-κB–Dependent Reporter Expression. We have shown previously that NT increased NF-κB–driven luciferase reporter gene expression, which is a general readout for the NF-κB pathway (Zhao et al., 2003). Thus, we next examined whether inhibition of PKCs has any effect on this response. To do this, cells were pretreated with GF 109203X (1 μM) for 30 min and then stimulated with NT (10^{-7} M) or PMA (1 μM) for 6 h. We found that pretreatment with GF 109203X (Fig. 4a) significantly inhibited NT-induced NF-κB–driven reporter gene expression. To confirm the role of PKCs in the NT-induced NF-κB transcriptional activity, we also pretreated cells with PMA (1 μM) for 16 h, followed by 6 h of NT exposure. The results indicate that the NF-κB transcriptional activity induced by either NT or PMA (6-h treatment) was blocked by overnight pretreatment with PMA after considering that this pretreatment alone dramatically induced intracellular luciferase reporter expression driven by NF-κB (Fig. 4b).

Inhibition of Protein Kinase C Attenuates NT-Induced IL-8 Gene Expression. Because NF-κB plays a critical role in NT-induced IL-8 gene expression (Zhao et al., 2001) and PKCs mediate NF-κB activation in response to several stimuli, including T-cell receptor activation (Trushin et al., 2003), 12-*O*-tetradecanoylphorbol-13-acetate (Vertegaal et al., 2000), and lipopolysaccharide (St-Denis et al., 1998), we next examined whether NT-induced IL-8 gene expression involves protein kinase Cs. To do this, cells were pretreated with GF 109203X (1 μM) for 30 min or with PMA (1 μM) for 16 h and then stimulated with NT for 6 h. IL-8 production in the conditioned media was measured. The data show that pretreatment with GF 109203X significantly inhibited NT-induced IL-8 secretion (Fig. 5a). We next determined whether GF 109203X- or PMA-prolonged incubation inhibited NT-induced IL-8 promoter activity. Cells were transfected with an IL-8 promoter construct and then treated with GF 109203X for 30 min or PMA for 16 h before NT exposure for 6 h. These data indicate that GF 109203X (Fig. 5b) or prolonged PMA incubation (Fig. 5c) significantly inhibited NT-induced IL-8 promoter activity.

Involvement of PKCα in NF-κB–Dependent IL-8 Gene Expression in Response to NT. Our results presented above showed that NT-induced activation of the NF-κB pathway requires mobilization of intracellular calcium that can be inhibited by the PKC inhibitor GF 109203X, which selectively targets PKCα-, β_I-, β_{II}-, γ-, δ-, and

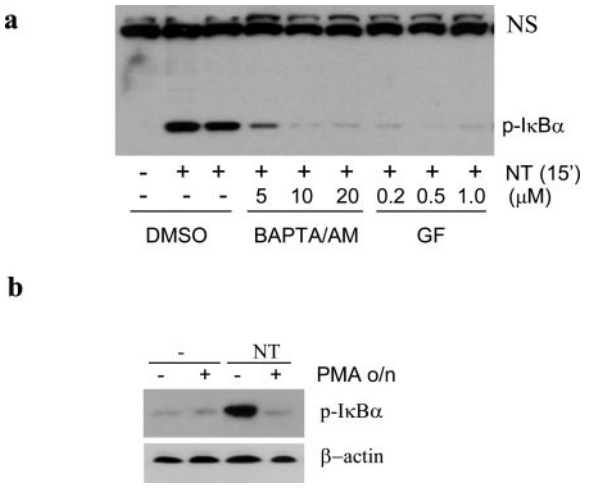

Fig. 2. Effect of BAPTA/AM and GF 109203X on NT-induced IκBα phosphorylation. NCM460-NTR1 cells were pretreated with BAPTA/AM (25 μM) or GF 109203X (1 μM) or vehicle control for 30 min (a) or PMA (1 μM) overnight (o/n) (b) and then stimulated with NT (10^{-7} M) for 15 min. IκBα phosphorylation was measured as described in Fig. 1. The NS band indicates a nonspecific protein. Results are representative of three separate experiments. NS, nonspecific band.

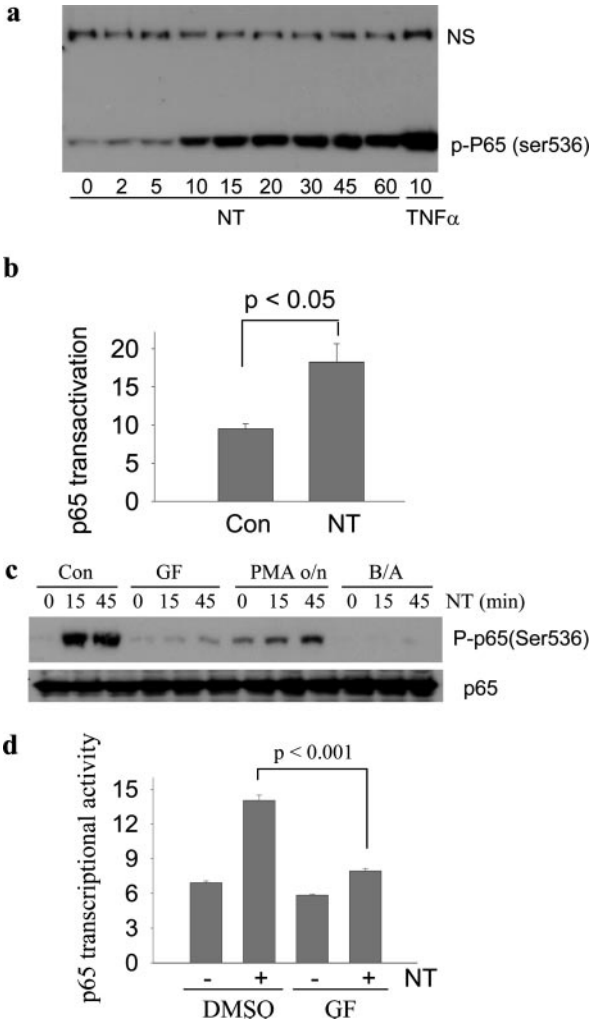

Fig. 3. Effect of BAPTA/AM or PKC inhibition on NT-induced p65 phosphorylation and transcriptional activity. a, cells were treated with NT (10^{-7} M) for various times. Equal amounts of cell protein were subjected to Western blotting using a phospho-NF-κB (Ser536) antibody. NS, nonspecific protein. b, cells were transiently transfected with pGal4-p65 and p(Gal4)2-luc as described under *Materials and Methods* and then treated with NT for 6 h. Results are representative of three individual experiments, each with triplicate determinations. c, cells were pretreated with BAPTA/AM (25 μM) or GF 109203X (1 μM) for 30 min or PMA (1 μM) overnight (o/n) and then stimulated with NT (10^{-7} M) for 15 or 45 min. p65 Ser536 phosphorylation and total p65 levels were examined as described in Fig. 3. Results are representative of three separate experiments. d, cells were transfected with pGal4-p65 and p(Gal4)2-luc as described in Fig. 3 and were pretreated with GF 109203X (1 μM) followed by 6 h of NT (10^{-7} M) exposure. The data show that inhibition of protein kinase C significantly reduced NT-induced p65 transcriptional activity. Results are representative of three individual experiments, each with triplicate determinations.

ϵ-isozymes, and by prolonged treatment with PMA, which down-regulates PKC expression. From this evidence, we hypothesized that a calcium-dependent PKCα pathway might be involved in the response, because it is highly expressed in NCM460 cells (data not shown). To test our hypothesis, we used a dominant-negative form of PKCα (pPKCα-DN). Overexpression of pPKCα-DN was first confirmed by immunoblotting with a PKCα-specific antibody after NCM460-NTR1 cells were transiently transfected with either pPKCα-DN or a control plasmid (Fig. 6a). Cells were transiently transfected with either pPKCα-DN or a control plasmid, along with an IL-8 promoter construct (Fig. 6b) or NF-κB reporter construct (Fig. 6c), as well as an internal control plasmid. Cells were then starved and treated with NT for 6 h. Luciferase reporter activities in an equal amount of cell extracts were measured. The results show that transfection of pPKCα-DN, but not its control plasmid, significantly inhibited both NT-induced IL-8 promoter activity (Fig. 6b) and NF-κB–dependent reporter gene expression (Fig. 6c).

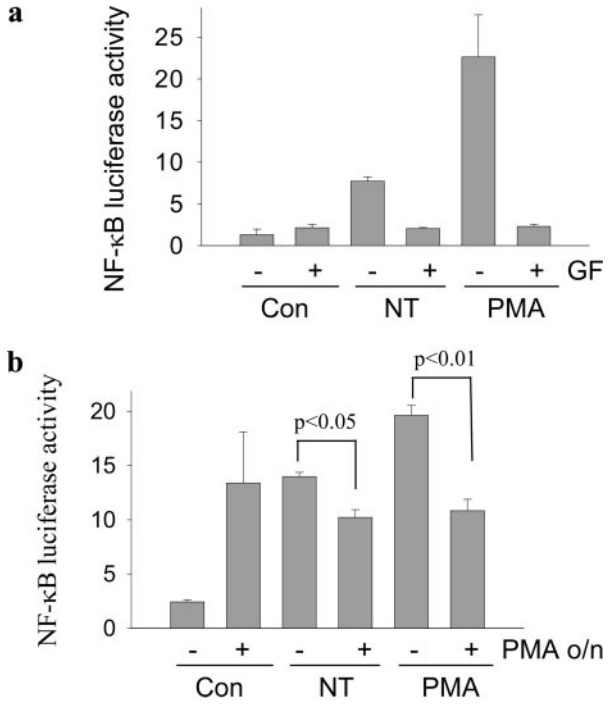

Fig. 4. Effect of PKC inhibition on NT-induced NF-κB–dependent reporter expression. Cells were transfected with an NF-κB–luciferase reporter construct and were pretreated with GF 109203X (1 μM) for 30 min (a) or PMA (1 μM) overnight (o/n) (b) followed by 6 h of NT (10^{-7} M) or PMA (1 μM) stimulation. Luciferase activity in equal cell lysates was measured. Results are representative of three separate experiments, each with triplicate determinations.

Discussion

Our laboratory has reported previously that NTR1 is dramatically up-regulated during colonic inflammation on various cell types, including colonic epithelial cells, and that antagonism of NTR1 activation significantly inhibited colonic inflammation in response to *C. difficile* toxin A (Castagliuolo et al., 1999b). Our recent study also showed that NCM460 human colonic epithelial cells express the high-affinity NT receptor that mediates extracellular signal-regulated kinase activation in response to NT (Zhao et al., 2001). Moreover, exposure of NCM460 cells overexpressing NTR1 to NT caused NF-κB–dependent expression of IL-8 and NT-mediated NF-κB DNA binding activity, and IL-8 expression was mediated by intracellular calcium release (Zhao et al., 2001). In this study, we further investigated the mechanism by which NT activates NF-κB–mediated IL-8 gene expression. Our results show that NT activates the NF-κB pathway by regulating IκBα phosphorylation and degradation and phosphorylation-dependent transcriptional activity of NF-κB p65. Moreover, NT-induced phosphorylation of both IκBα and p65 is mediated by calcium-dependent protein kinase C. In addition, we show that expression of dominant-negative mutant of protein kinase Cα significantly inhibited NT-induced IL-8 promoter activity and NF-κB–dependent reporter expression, suggesting that PKCα mediates the NF-κB pathway in response to NT.

Our observations that blockade of intracellular calcium by

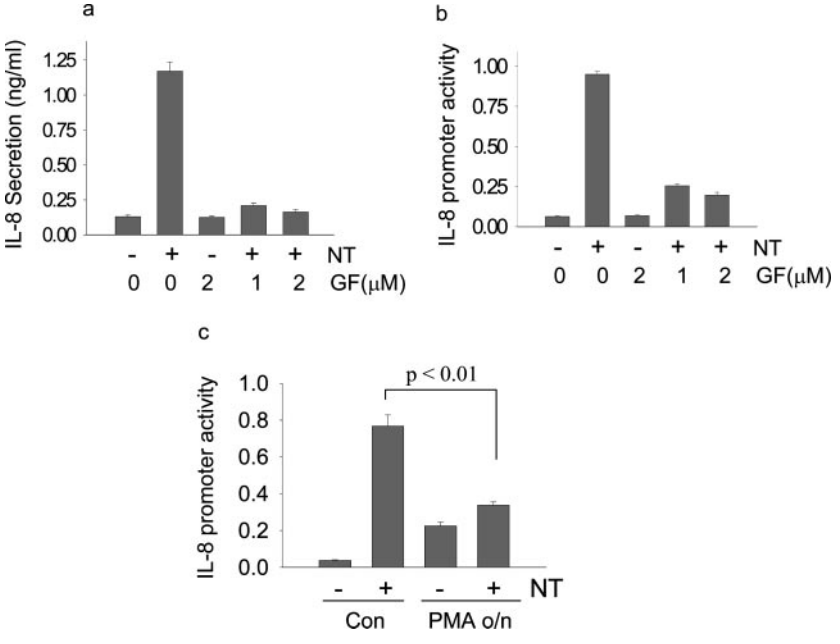

Fig. 5. Inhibition of protein kinase C attenuates NT-induced IL-8 gene expression. Cells were pretreated with GF 109203X (1 or 2 μM) for 30 min (a) and then stimulated with NT (10^{-7} M) for 6 h. IL-8 production in the conditioned media was measured. Cells were transfected with IL-8 promoter construct and then treated with GF 109203X (1 or 2 μM) for 30 min (b) or PMA (1 μM) for 16 h (c) before 6 h of NT exposure; o/n, overnight. Results are representative of three individual experiments, each with triplicate determinations.

BAPTA/AM diminished NT-induced phosphorylation of both IκBα and p65 are consistent with our previous finding that BAPTA/AM inhibited NT-induced NF-κB DNA binding activity and IL-8 gene expression (Zhao et al., 2001). Two mechanisms could explain how elevated intracellular calcium levels may regulate the NF-κB pathway. The first possibility is that increased intracellular calcium induces IκB phosphorylation by a calcium-calmodulin–dependent pathway because W7, a calmodulin antagonist, can inhibit IκB degradation (Mattila et al., 1990; Hughes et al., 1998). In addition, the calcium-dependent protein phosphatase calcineurin may synergize with protein kinase C to activate NF-κB by a yet-unknown mechanism (Mattila et al., 1990; Steffan et al., 1995; Trushin et al., 1999). Our previous observation that W7 or cyclosporin A partially inhibited NT-induced IL-8 secretion suggests that these calcium-dependent pathways may be partially involved in NF-κB activation after NT exposure (Zhao et al., 2001). Second, calcium may regulate the NF-κB pathway through calcium-dependent PKC, particularly PKCα, as demonstrated in many studies, including this study. PKCα has been shown previously to mediate activation of IKKs and subsequent IκBα phosphorylation and degradation, resulting in nuclear translocation and DNA binding of NF-κB in response to several extracellular stimuli, including T-cell receptor activation (Trushin et al., 2003), 12-*O*-tetradecanoylphorbol-13-acetate (Vertegaal et al., 2000), and LPS (St-Denis et al., 1998).

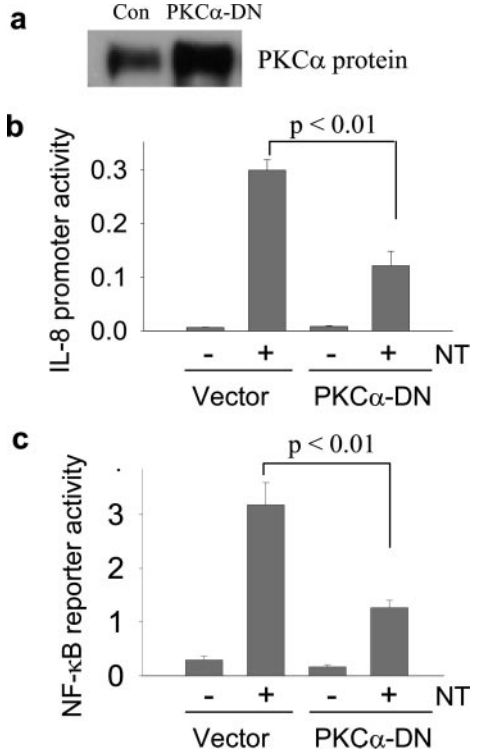

Fig. 6. Effect of dominant-negative PKCα on NF-κB–dependent IL-8 gene expression in response to NT. a, cells were transiently transfected with either pPKCα-DN or a control plasmid, and expression of PKCα was determined by Western blotting. b, cells were transiently transfected with either pPKCα-DN or a control plasmid and expression of PKCα along with IL-8 promoter construct (b) or NF-κB reporter construct (c), as well as an internal control plasmid. Cells were then starved and treated with NT (10^{-7} M) for 6 h. Luciferase reporter activities in equal amounts of cell extracts were measured. Results are representative of three individual experiments, each with triplicate determinations.

Previous studies have suggested that protein kinase Cs might be involved in NT signaling. For example, Poinot-Chazel et al. (1996) indicated that protein kinase Cs may be involved in NT-induced MAP kinase activation because the PKC inhibitor GF 109203X inhibited MAP kinase extracellular signal-regulated kinase phosphorylation in Chinese hamster ovary cells stably transfected with human NTR1. However, these results are different from results reported by us (Zhao et al., 2004) and Hassan et al. (2004), in which epidermal growth factor receptor transactivation is primarily responsible for NT-induced MAP kinase activation in human colonic epithelial cells and androgen-independent PC3 cells, respectively. Our current study provides the first direct evidence for the importance of PKC, and particularly PKCα, in NTR1 signaling to the NF-κB pathways. Therefore, NT may stimulate growth-related responses primarily through transactivation of the epidermal growth factor receptor, whereas it induces the expression of inflammatory cytokines by the calcium- and protein kinase C-mediated NF-κB pathway. Our results also provide insights to therapeutic approaches related to the NTR1 signaling pathway blockade that specifically targets proinflammatory signaling without affecting NT-related healing processes in the colonic mucosa during colitis.

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