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Alterations in Receptor Expression or Agonist Concentration Change the Pathways Gastrin-Releasing Peptide Receptor Uses to Regulate Extracellular Signal-Regulated Kinase

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ABSTRACT

G protein-coupled receptors activate extracellular signal-regulated kinases (ERKs) via different pathways in different cell types. In this study, we demonstrate that gastrin-releasing peptide receptor (GRPr) regulates ERK through multiple pathways in a single cell type depending upon receptor expression and agonist concentration. We examined stably transfected BALB/c 3T3 fibroblasts expressing GRPr constructs at different levels and treated the cells with several concentrations of bombesin (BN, a GRPr agonist) to activate a variable number of GRPr per cell. GRPr induced two waves of ERK activation and one wave of ERK inhibition. One wave of activation required an intact GRPr carboxyl-terminal domain (CTD). It peaked 6 min after addition of high BN concentration ([BN]) in cells with high GRPr expression. Another wave of activation was CTD-independent. It peaked 2 to 4 min after BN addition in cells when [BN] and/or GRPr expression were lower. The early wave of ERK activation was more sensitive than the later one to pretreatment with Bisindolylmaleimide I (GF 109203X) (a protein kinase C inhibitor) or hypertonic sucrose. Because these two waves of activation differ in time course, dose-response curve, requirement for GRPr CTD, and sensitivity to inhibitors, they result from different signaling pathways. A third pathway in these cells inhibited ERK phosphorylation 2 min after addition of high [BN] in cells with high GRPr expression. Furthermore, a GRPr-expressing human duodenal cancer cell line showed differential sensitivity to GF 109203X throughout BN-induced ERK activation, indicating that GRPr may activate ERK via multiple pathways in cells expressing endogenous GRPr.

Mitogen-activated protein kinases (MAPKs) are a family of highly conserved serine/threonine kinases that are activated by a wide range of extracellular stimuli (Davis, 1993). The best characterized isoforms, extracellular signal-regulated kinases, ERK1 ($p44^{MAPK}$) and ERK2 ($p42^{MAPK}$), are directly activated on specific threonine and tyrosine residues by the dual specificity MAPK kinases (Payne et al., 1991; Crews et al., 1992). G protein-coupled receptors (GPCRs) regulate MAPK activity through multiple pathways requiring activation of effectors such as protein kinases A and C, ras, or receptor tyrosine kinases (Liebmann, 2001). Recent studies have revealed that the mechanisms by which GPCRs use to activate MAPK depend on cell context (Liebmann, 2001).

Bombesin (BN)-like peptides are potent mitogens for a variety of cells (Rozengurt and Sinnett-Smith, 1983; Cuttitta et al., 1985; Charlesworth et al., 1996) and have been implicated as autocrine/paracrine growth factors for small cell lung cancer and other human tumors (Cuttitta et al., 1985; Carney et al., 1987). BN-like peptides signal through a family of GPCRs, including gastrin-releasing peptide receptor (GRPr) (Kroog et al., 1995). The agonist-occupied GRPr activates G_q to induce phospholipase C-β-mediated hydrolysis of phosphatidylinositides that leads to Ca^{2+} mobilization and activation of PKC (Kroog et al., 1995). Evidence also suggests that GRPr couples to $G_{12/13}$ (Sinnett-Smith et al., 2000). GRPr activates MAPK in several cell lines such as Swiss 3T3 cells, Rat-1 cells, and STC-1 cells. MAPK activation is re-

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ABBREVIATIONS: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; BN, bombesin; GRP, gastrin-releasing peptide; GRPr, gastrin-releasing peptide receptor; PKC, protein kinase C; CTD, carboxyl-terminal domain; DMEM, Dulbecco's modified Eagle's medium; ME, methyl ester; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; GFP, green fluorescent protein; BSA, bovine serum albumin; EGFP, enhanced green fluorescent protein; TBST, Tris-buffered saline/Tween 20; PP, protein Ser/Thr phosphatase; PTP, protein tyrosine phosphatase; RTK, receptor tyrosine kinase; GF 109203X, 3-[1-[3-(dimethylaminopropyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride.

quired for the mitogenic effects of BN (Seufferlein et al., 1996; Charlesworth and Rozengurt, 1997; Nemoz-Gaillard et al., 1998; Koh et al., 1999). However, the structural elements of GRPr required for BN-induced MAPK activation have not been identified.

The carboxyl-terminal domain (CTD) of GPCRs, including GRPr, is involved in attenuating receptor signaling after continuous or repeated exposure to agonist, a process known as "desensitization" (Ally et al., 2003). The CTD of many GPCRs is also implicated in activation of signaling (Aquilla et al., 1996). Members of the arrestin family of CTD-interacting proteins contribute to GPCR-mediated MAPK activation by functioning as scaffolds for components of the MAPK cascade or by recruiting Src (Ahn et al., 1999; DeFea et al., 2000a; McDonald et al., 2000). In addition, receptor internalization is essential for activation of MAPK for some GPCRs (Daaka et al., 1998; Ignatova et al., 1999), and a CTD truncation mutant of GRPr is defective in receptor internalization (Benya et al., 1993). Therefore, we sought to determine whether GRPr CTD is required for BN-induced MAPK activation.

To evaluate the role of the GRPr CTD in BN-induced ERK activation, we used stably transfected BALB/c 3T3 fibroblast cell lines expressing wild-type or CTD mutants of GRPr. BALB fibroblasts are a mouse cell line similar to Swiss 3T3 cells, but they do not express endogenous GRPr. We found that in BALB fibroblasts, GRPr can induce two waves of MAPK activation through distinct pathways (CTD-dependent and -independent) and one inhibitory pathway that decreases ERK phosphorylation. The amplitude of each pathway depended on the concentration of BN ([BN]) added to the cells and the concentration of GRPr ([GRPr]) per cell. We also found evidence for GRPr-mediated MAPK activation via multiple pathways in HuTu 80, a duodenal cancer cell line expressing endogenous GRPr. Our data indicate that besides cell context, receptor number and agonist concentration determine the mechanisms by which GPCRs regulate MAPK activity, different signaling pathways may participate at different times after agonist addition, and multiple pathways may potentially be activated in a single cell type.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin/streptomycin, G-418, and LipofectAMINE reagent were from Invitrogen (Carlsbad, CA). BN was from Bachem California (Torrance, CA). [D-Phe^6]BN(6-13) methyl ester ([D-Phe^6]BN(6-13) ME) was a gift from Dr. David Coy (Tulane University, New Orleans, LA). ^{125}I-Labeled BN (^{125}I-BN; 2200 Ci/mmol) and HuTu 80 cells were obtained from Sam Mantey and Dr. Robert Jensen (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). TPA was purchased from Sigma-Aldrich (St. Louis, MO). GF 109203X was from Calbiochem (San Diego, CA) and sucrose was from Fisher Scientific (Fair Lawn, NJ). Anti-phospho-ERK and anti-total ERK polyclonal antibodies were from Promega (Madison, WI) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. pEGFP-N$_1$-β-arrestin1 (pEGFP-N$_1$-arrestin2) was a gift from Dr. Nigel Bunnet (University of California, San Francisco, CA). β-Arrestin2-GFP (arrestin3-GFP) was a gift from Dr. Marc Caron (Duke University, Durham, NC) (Barak et al., 1997).

Cell Culture

Untransfected BALB/c 3T3 fibroblasts and HuTu 80 cells were maintained at 37°C in DMEM supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum. Stably transfected BALB fibroblasts were maintained in the above-mentioned media plus 300 μg/ml G-418.

MAPK Assay

BALB cells and HuTu 80 cells were plated in 35-mm polystyrene dishes at 425,000 cells/dish or 450,000 cells/dish, respectively. The following day, cells were nearly confluent. Cells were washed twice with phosphate-buffered saline, and incubated in DMEM (without serum) for 18 to 24 h at 37°C. Next, cells were incubated with 2 ml of DMEM or DMEM plus dimethyl sulfoxide, GF 109203X, or 0.45 M sucrose for the times indicated at 37°C before BN and TPA stimulation. BN and TPA were added to the dishes in 100 μl of DMEM (prewarmed to 37°C), and media were mixed by gentle shaking. Cells were then incubated for various times in the incubator at 37°C as described in the figure legends. Then, the media were removed and cells were lysed at 4°C with 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 100 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin). Lysates were clarified by centrifugation at 13,700 rpm in a microcentrifuge for 15 min at 4°C, and protein concentration was determined with the bicinchoninic acid protein assay from Pierce Chemical (Rockford, IL) using the manufacturer's instructions. Thirteen to 19 μg of protein was resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes at 30 V overnight at 4°C. Activation of ERK2 was assessed by Western blotting using an anti-phospho-ERK antibody. Membranes were blocked in 1% BSA in Tris-buffered saline (150 mM NaCl and 20 mM Tris-HCl, pH 7.4) at 37°C for 1 h, incubated with an anti-phospho-ERK antibody diluted 1:5000 in 0.1% BSA in TBST (0.05% Tween 20 in Tris-buffered saline) at room temperature for 2 h, washed three times with TBST, incubated in donkey anti-rabbit antibody conjugated with horseradish peroxidase diluted 1:5000 in 0.1% BSA in TBST at room temperature for 1 h, and then washed three times with TBST. Proteins were visualized by enhanced chemiluminescence (Pierce Chemical). Band intensity was quantified using Kodak Digital Science Image Station 440CF with Kodak 1D 3.5 Image Analysis Software. Band intensity was linearly related to phospho-ERK levels throughout the range of the assay and could therefore be used to measure relative levels of phospho-ERK (data not shown). Data are reported as "fold-stimulation", which is defined as band intensity of BN-treated conditions divided by band intensity of vehicle control for the same time point. When band intensity in BN-treated samples was less than vehicle control, fold-stimulation was less than 1 (see *Results*).

Blots were stripped in 2% SDS and 0.1 M β-mercaptoethanol in 62.5 mM Tris-HCl, pH 6.8, at 55°C for 30 min, washed, and reprobed with antibody against total ERK to ensure equal loading. Western blotting using anti-total ERK antibody was performed following the manufacturer's instructions. In brief, membranes were incubated with antibody to total ERK (0.2 μg/ml for 1 h at room temperature), followed by donkey anti-rabbit antibody conjugated with horseradish peroxidase (1:8000 for 1 h at room temperature).

Binding and Internalization Assays

The number of GRPrs per cell in HuTu 80 cells were determined by ^{125}I-BN binding as described previously (Ally et al., 2003).

Internalization Assay Method A. GRPr internalization was monitored in disaggregated cells using a modification of a previously published procedure (Benya et al., 1994). In brief, confluent cells were washed, mechanically disaggregated, and suspended at a concentration of 3×10^5 cells/ml in binding solution (solution A: 98 mM NaCl, 59 mM KCl, 5 mM pyruvate, 6 mM fumarate, 5 mM gluta-

mate, 11 mM glucose, 25 mM HEPES, 2.2 mM KH_2PO_4, 0.1% BSA, 0.02% bacitracin, 1.5 mM $CaCl_2$, and 1 mM $MgCl_2$ adjusted to pH 7.4) or 0.45 M sucrose in solution A. Cells were incubated with 75 pM ^{125}I-BN (2200 Ci/mmol) and 0.5 nM nonradioactive BN for various times at 37°C. At the indicated times, 100-μl samples were removed and diluted in 1 ml of solution B (0.2 M acetic acid, pH 2.5, and 0.5 M NaCl), and then incubated for 5 min at 4°C (to remove surface radioligands). Parallel incubations were conducted in solution A to determine total binding at all time points. The samples were filtered through GF/F filters (Whatman, Maidstone, UK) presoaked in 4% BSA. The filters were washed three times with solution S (20 mM Tris-HCl, pH 8, 100 mM NaCl, and 25 mM $MgCl_2$), and then bound ^{125}I was measured with a gamma counter. Nonspecific binding, measured in the presence of 10^6-fold excess of nonradioactive BN was subtracted from all points to obtain specific binding. Results were expressed as the percentage of total specific binding.

Internalization Assay Method B. GRPr internalization was also monitored with adherent cells to mimic conditions in the MAPK assay. Myc-GRPr cells were plated at 20,000 cells/well on 24-well plates. The following day, cells were rinsed with solution A and incubated in either solution A or 0.45 M sucrose in solution A for 20 min at 37°C. Cells were then treated with 75 pM ^{125}I-BN and 1 nM nonradioactive BN for 8 min. Cells were washed once with 0.6 ml of solution A. 0.6 ml of solution A or solution B was added to the wells and incubated at 4°C for 5 min, and then all liquid was removed. Cells remained adherent after the 4°C incubation (data not shown). Adherent cells were lysed in 0.2 M NaOH and 1% SDS. ^{125}I in lysates was measured with a gamma counter. Experiments were performed in duplicate in both methods. Results were similar with both methods.

Confocal Microscopy

Myc-GRPr cells were plated in glass bottom 35-mm dishes (MatTek, Ashland, MA) at 150,000 cells/dish and cultured overnight. Cells were transfected with 0.3 μg of pEGFP-N_1-arrestin2 or arrestin3-GFP by LipofectAMINE method following the manufacturer's instructions. Twenty-four to 48 h after transfection, the media were replaced with solution A, and cells were stimulated with 100 nM BN and imaged in real time on the stage of a Bio-Rad Radiance 2000 laser scanning confocal microscope preheated to 37°C. The fluorescence signal was recorded using parameters published previously (Ally et al., 2003).

Statistical Analyses

Results are expressed as mean ± S.E. Differences between treatment and vehicle control or between two different treatment conditions were analyzed by Student's t test, with $p < 0.05$ considered to be significant.

Results

The Carboxyl-Terminal Domain of GRPr Is Required for Bombesin-Induced ERK Activation in BALB/c 3T3 Mouse Fibroblasts Expressing High Levels of GRPr. To determine whether the CTD of GRPr is involved in BN-induced ERK activation, we studied stably transfected BALB/c 3T3 fibroblast cell lines expressing high levels (240,000–950,000 receptors/cell) of either wild-type or CTD mutant GRPr (Ally et al., 2003). The cell lines studied were myc-GRPr, expressing myc-tagged wild-type GRPr; Δ346, expressing CTD truncation of GRPr afterLeu^{345}; and S/T-mut, expressing GRPr with 12 of 13 Ser and Thr residues distal to Leu^{345} (all except Thr^{371}) replaced with nonphosphorylatable residues. Confluent cells were serum starved in DMEM for 18 to 24 h, and then treated with or without saturating concentration of BN (100 nM) for various times and analyzed by Western blotting using an anti-phospho-ERK antibody to detect activated ERK. Stimulation of ERK phosphorylation by the PKC activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was used as a positive control. BN and TPA were dissolved in DMEM identical to incubation media and maintained at 37°C before use. DMEM alone was used in parallel as the vehicle control. Addition of DMEM alone induced reproducible ERK phosphorylation in transfected BALB cells with a peak at 4 to 6 min (DMEM in Fig. 1B). The response to addition of DMEM alone also occurred in untransfected BALB cells (data not shown), implying that it is not mediated through GRPr. Therefore, all experiments were performed using DMEM in parallel to other treatments to obtain the treatment-specific effect, and data are reported as fold-stimulation relative to DMEM (see *Materials and Methods*).

Addition of 100 nM BN to myc-GRPr cells induced an approximately 5-fold ERK activation above that induced by DMEM alone with a peak 6 min after addition of BN (Fig. 1A, ●, and B, top). In contrast, 100 nM BN failed to stimulate ERK above DMEM in Δ346 (Fig. 1A, ○, and B, middle) and S/T-mut cells (Fig. 1A, △, and B, bottom), indicating that with large numbers of agonist-occupied GRPr, BN activated ERK in a CTD-dependent manner. But, all three cell lines

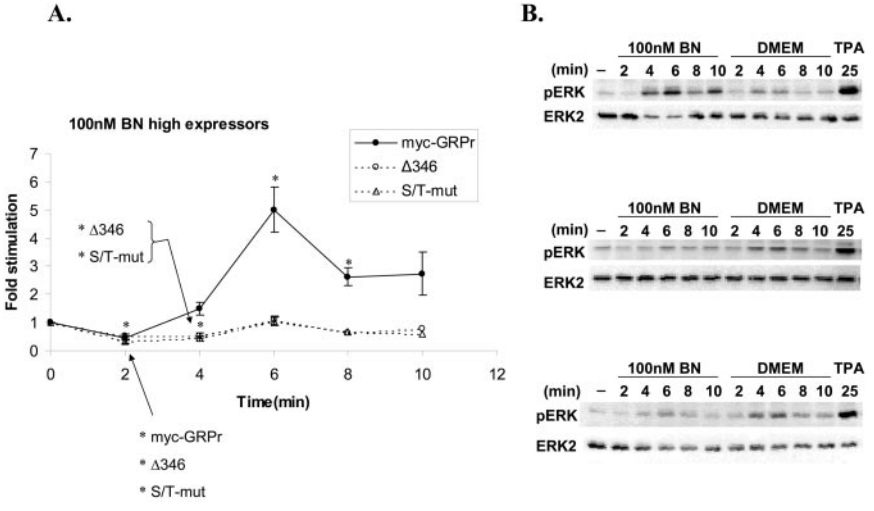

Fig. 1. BN-induced ERK activation in cells expressing high levels of GRPr requires an intact CTD. Serum-starved myc-GRPr cells (●), Δ346 cells (○), and S/T-mut cells (△) were incubated in fresh serum-free DMEM for 1 h before stimulation. Cells then received no treatment (–) or were treated with 100 nM BN, serum-free DMEM, or 100 nM TPA for various times as indicated. Cells were lysed and subjected to SDS-PAGE analysis, followed by Western blotting with anti-phospho-ERK antibody (pERK). ERK activity was measured as described under *Materials and Methods*. Equal loading was confirmed by anti-total ERK2 Western blotting (ERK2). A, results shown are the mean ± S.E. of at least three experiments except for time points 8 and 10 min in Δ346 and S/T-mut cells are the mean of two experiments. Data are expressed as fold-stimulation, which is defined as BN-induced/DMEM-induced ERK phosphorylation. *, $p < 0.05$ compared with DMEM treatment, $n \geq 3$. Mean maximum response (in myc-GRPr at 6 min) is 72% of TPA-induced ERK phosphorylation. B, representative Western blots for myc-GRPr (top), Δ346 (middle), and S/T-mut cells (bottom).

showed a modest decrease in ERK phosphorylation 2 and/or 4 min after BN addition. The failure of Δ346 and S/T-mut cells to show ERK activation was not due to the lack of myc tag because wt3, a BALB/c 3T3 cell line expressing wild-type GRPr at similar levels to myc-GRPr (Ally et al., 2003), showed similar BN-induced ERK phosphorylation to myc-GRPr (data not shown). BN did not activate ERK in untransfected BALB cells, demonstrating that BN-induced ERK activation in BALB cells is a GRPr-specific response (data not shown). In addition, GRPr-induced ERK phosphorylation required activation by agonist, because the pure GRPr antagonist [D-Phe^6]BN(6-13)ME had no effect on ERK phosphorylation (data not shown).

The Magnitude and Mechanism of ERK Activation by BN Are a Function of Both the Concentration of Added BN and the Number of GRPr per Cell. To begin to study GRPr-mediated ERK activation under more physiologically relevant conditions, we used stably transfected BALB/c 3T3 fibroblast cell lines expressing wild-type (wt-low) or CTD truncation mutant of GRPr (Δ346-low) at much lower levels (approximately 93,000 and 82,000 receptors/cell, respectively) (Tsuda et al., 1997; Ally et al., 2003). The expression of wild-type or CTD truncation mutant of GRPr in these low expressors is 3- to 5-fold less than that in equivalent high expressors and is comparable with some cancer cell lines expressing endogenous GRPr (Jensen et al., 2001). BN (100 nM) induced ERK activation in wt-low with small peaks at 2 and 6 min (Fig. 2A, ●). We were surprised to find that BN also induced ERK activation with a broad peak from 2 to 4 min in Δ346-low, indicating that ERK activation by BN did not always require an intact CTD in the BALB/c 3T3 fibroblast model system (Fig. 2A, ○). These results in Δ346-low were inconsistent with data from cell lines expressing high levels of GRPr CTD mutants (Fig. 1).

We next sought to determine whether the inconsistent results between high and low expressors of the CTD truncation mutant were caused by different levels of [GRPr]/cell (leading to different absolute numbers of agonist-occupied GRPr per cell when exposed to 100 nM BN) or by different BALB/c 3T3 cell backgrounds after clonal selection for GRPr expression. Lower concentration of agonist (0.3 nM BN) was used to stimulate myc-GRPr and Δ346 cells to lower the percentage of GRPr occupied in these cells. Because the K_d for BN binding to mouse GRPr is ~1 to 3 nM in transfected BALB/c cells (Kroog et al., 1995), 0.3 nM BN should lead to occupancy of ~1/4 to 1/10 of all GRPr in a cell as steady state is approached.[1] In contrast to results after addition of 100 nM BN, we observed that addition of 0.3 nM BN to high expressors resulted in a predominant ERK activation at 2 min in both cell lines (Fig. 2B). Consistent with this result, when 1 nM BN was added to wt-low and Δ346-low, ERK activation was detected at 2 to 4 min in both cell lines, and the 6-min response in wt-low disappeared (Fig. 2C). Therefore, the patterns of ERK activation in transfected BALB cells were functions of both [BN] and [GRPr]/cell but not the specific BALB/c 3T3 cell background.

There seemed to be two distinct waves of BN-induced ERK phosphorylation in transfected BALB fibroblasts. One occurred at an earlier time and did not require CTD. The other occurred later and required CTD. To further correlate the intensity of the early and late peaks in ERK phosphorylation with [BN] and [GRPr]/cell, we monitored ERK phosphorylation in myc-GRPr or Δ346 cells stimulated with various concentrations of BN for 2, 4, and 6 min. As shown in Fig. 3, [BN] dictated the intensity of each peak. In myc-GRPr cells, the shape of the dose-response curves for the early and late peaks were very different (Fig. 3, A and C). The early wave of ERK activation had a biphasic dose-response curve with maximal amplitude at ~0.3 nM BN, whereas the later wave of ERK activation had a sigmoidal dose-response curve and peaked at saturating concentration of BN. The dose-response curves in Δ346 at 2 and 4 min resembled the early wave of ERK

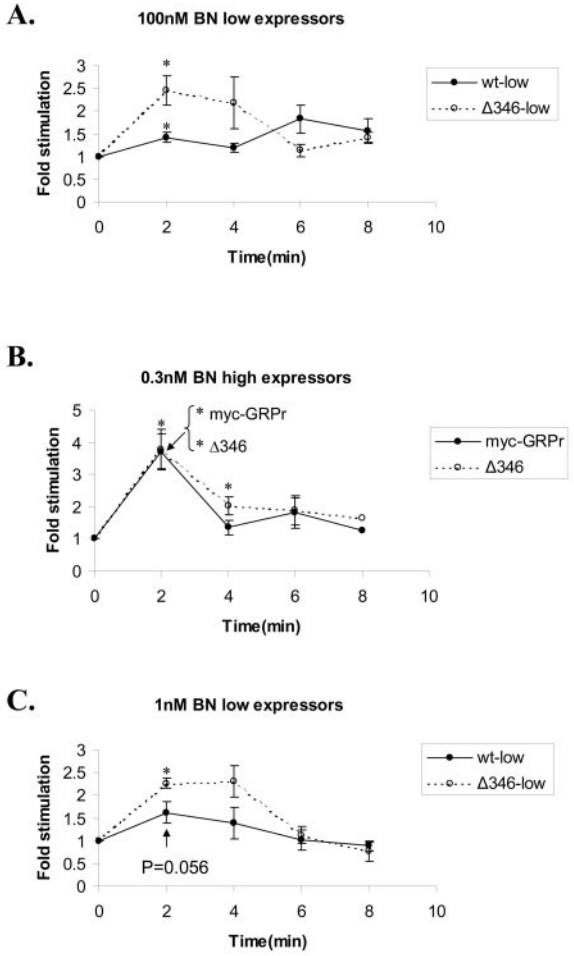

Fig. 2. BN activates ERK in a CTD-independent manner when GRPr expression or BN concentration are low. A and C, serum-starved wt-low cells (●) and Δ346-low cells (○) were incubated in fresh serum-free DMEM for 1 h before stimulation. Cells were treated with 100 nM BN (A), 1 nM BN (C), or serum-free DMEM for various times as indicated. Cells were lysed and ERK activity was measured as in Fig. 1. Data are the mean ± S.E. of at least three experiments. B, serum-starved myc-GRPr cells (●) and Δ346 cells (○) were handled as described above, except that cells were treated with 0.3 nM BN or serum-free DMEM. Results are the mean ± S.E. of five experiments for time points 0–6 min and two experiments for time points 8 min. *, $p < 0.05$ compared with DMEM treatment, $n \geq 3$.

[1] Although most agonists (which stimulate receptor internalization) do not reach binding equilibrium, receptor theory can provide a rough guide for the concentration of receptor-ligand complexes ([RL]) relative to free receptor ([R]) as steady state is approached during the first few minutes after agonist ([L]) addition. Receptor theory states: K_d = [L]/[RL]. Rearranging terms, [RL]/ = [L]/K_d. If [L] = 0.3 nM and K_d = 2 nM, [RL] = 0.3/2 = 0.15. Because [R_{TOT}] = [RL]+, [RL] = 0.13[R_{TOT}] or [RL] = ~1/8[R_{TOT}].

activation in myc-GRPr cells (compare Fig. 3, D and E, with A). Little or no activation of ERK 6 min after addition of BN was detected in Δ346 with all concentrations tested (Fig. 3F). Consistent with this result, when an excess amount of the specific GRPr antagonist [D-Phe^6]BN(6-13)ME (1 μM) was added with 100 nM BN to myc-GRPr cells as a competitor for GRPr binding sites, the pattern of ERK phosphorylation resembled the pattern induced by 1 nM BN in these cells because activation was detected at both 2 and 6 min (data not shown). Therefore, in a single cell context, different numbers of agonist-occupied receptors induced different patterns of ERK activation.

High Numbers of BN-Occupied GRPr Induce a Pathway That Decreases ERK Phosphorylation. The early wave of ERK activation (2 min) had a biphasic dose-response curve in both cell lines with high receptor expression (Fig. 3, A and D). There was no 2-min ERK activation after addition of 10 or 100 nM BN, suggesting that the first wave of ERK activation might be inhibited when very large numbers of GRPr are activated. Consistent with this idea, addition of 100 nM BN reduced the level of ERK phosphorylation relative to DMEM alone at early times (2 and/or 4 min) in all high expressors (Fig. 1). To confirm the existence of an inhibitory pathway that decreases ERK phosphorylation either through activation of phosphatases or inhibition of kinases, two methods were used to raise the basal level of ERK activity before BN addition. We either preincubated confluent and serum-starved myc-GRPr and Δ346 cells with the PKC activator TPA (Fig. 4, A and C) or grew myc-GRPr and Δ346 cells in media containing serum (Fig. 4, B and D). Addition of 100 nM BN for 2 min markedly decreased both TPA- (Fig. 4A, open column) and serum-induced (Fig. 4B, open column) ERK phosphorylation (BN-induced fold stimulation decreases to $\ll$1), whereas 0.3 nM BN had little or no effect on ERK phosphorylation (Fig. 4, A and B, closed columns). This demonstrated that a high [BN] in the setting of high [GRPr]/cell induced an inhibitory pathway which decreased ERK phosphorylation. Moreover, GRPr CTD was not required for this pathway because the decrease in ERK phosphorylation also occurred in Δ346 (Fig. 4, A and B). Therefore, the absence of the early wave of ERK activation when [GRPr]/cell and [BN] are both high may be explained by an induction of an inhibitory pathway under these circumstances. We also asked whether the inhibitory pathway was detectable 6 min after addition of BN. Myc-GRPr cells showed little or no decrease in TPA- or serum-stimulated ERK phosphorylation 6 min after addition of 100 or 0.3 nM BN (Fig. 4, C and D, left side). In contrast, 6 min of BN treatment decreased TPA-induced ERK phosphorylation in Δ346 (Fig. 4C, right side). This may result from either prolongation (via impaired desensitization) of the inhibitory pathway in Δ346 or its uncovering in the absence of the late stimulatory pathway (Figs. 1A and 4C). However, in the presence of serum, Δ346 showed a modest stimulation of ERK phosphorylation after 6 min of BN treatment at both concentrations (Fig. 4D). The inconsistency between the results from TPA and serum-preincubated cells suggested that the time course of ERK phosphorylation was determined not only by agonist and receptor concentrations but also by the signaling pathways that were coactivated.

High [BN] Mediates ERK Dephosphorylation through Protein Tyrosine Phosphatases. In cells with high [GRPr]/cell, high [BN] induced an inhibitory pathway that reduced ERK phosphorylation 2 min after BN addition in both myc-GRPr and Δ346 (Fig. 4, A and B). But, there was also BN-induced inhibition of ERK phosphorylation in Δ346 under some conditions 6 min after BN addition (Fig. 4C, right side). The latter result suggested that the late wave of BN-induced ERK

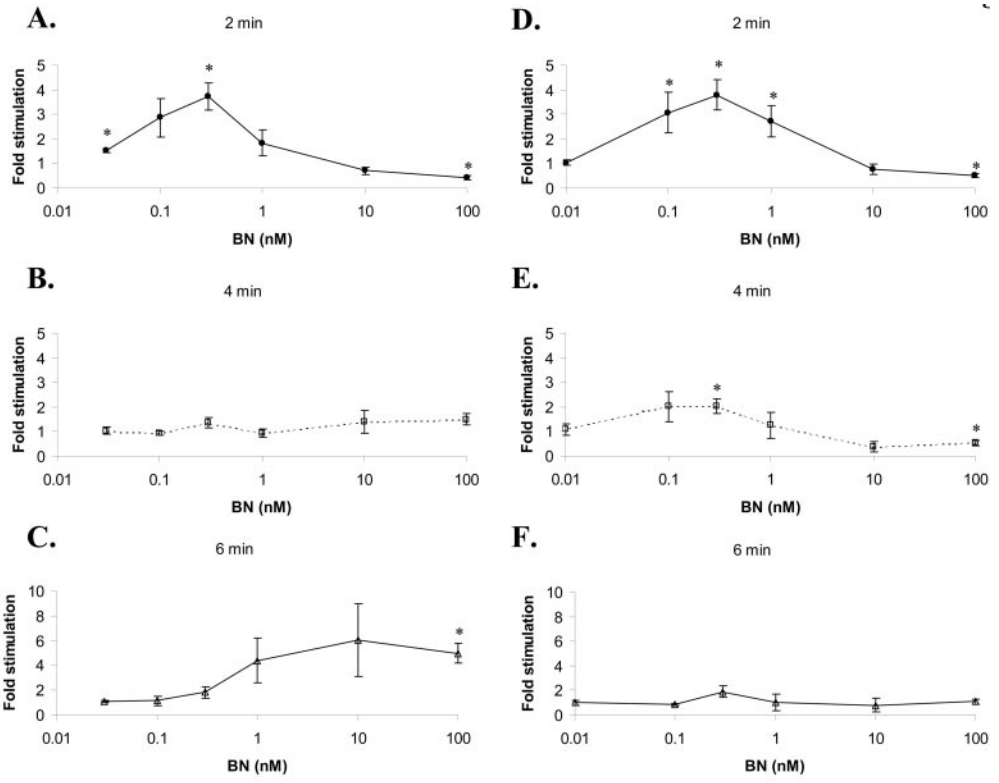

Fig. 3. Effects of BN concentration on the magnitude of early and late waves of ERK activation. Serum-starved myc-GRPr cells (A–C) and Δ346 cells (D–F) were incubated in fresh serum-free DMEM for 1 h, and then treated with various concentrations of BN or serum-free DMEM. ERK activity was measured 2 min (●), 4 min (□), or 6 min (△) after addition of BN. ERK activity was measured as in Fig. 1. Results shown are the mean ± S.E. of at least three experiments. *, $p < 0.05$ compared with DMEM treatment, $n \geq 3$.

phosphorylation was absent in CTD mutants (Fig. 1) because of a prolonged inhibitory pathway rather than an absence of a CTD-dependent late stimulatory pathway. To distinguish between these possibilities, we examined the inhibitory pathway further. We determined whether protein phosphatases mediate the inhibitory pathway and if so, which class(es) of protein phosphatases are responsible. We pretreated cells with 1 μM okadaic acid or 1 mM orthovanadate to test for involvement of protein Ser/Thr phosphatases PP1 and PP2A or protein tyrosine phosphatases (PTPs), respectively. Okadaic acid pretreatment did not prevent 100 nM BN-induced reduction of ERK phosphorylation, indicating that the inhibitory pathway was not mediated through PP1 and PP2A (data not shown). In contrast, orthovanadate pretreatment prevented most of the ERK dephosphorylation induced by 2-min exposure to 100 nM BN in both myc-GRPr and Δ346 cells (Fig. 5A), indicating the involvement of PTPs in high GRPr occupancy-induced ERK dephosphorylation.

If the lack of the late wave of ERK activation in Δ346 was caused by prolongation of the inhibitory pathway, blockage of PTPs should have revealed the late wave of ERK activation (at 6 min) in Δ346. However, orthovanadate pretreatment failed to bring out the second wave of ERK activation in Δ346 (Fig. 5B). Therefore, these data demonstrate that the absence of the late wave of ERK activation in Δ346 is caused by the impairment of the late stimulatory pathway, and the level of BN-induced ERK phosphorylation we observed at each time point is a combination of the relative activities of three pathways: two stimulatory pathways that increase ERK phosphorylation, and an inhibitory one that decreases ERK phosphorylation.

Two Waves of ERK Activation Show Differential Sensitivity to Sucrose and a PKC Inhibitor. The two waves of ERK activation differed in time course, dose-response curve, and requirement for CTD, suggesting that separate pathways had been activated. We next searched for molecules differentially involved in each wave of ERK phosphorylation. We first tested whether G protein signaling is required for either or both waves of ERK activation using a cell line expressing ~500,000 copies of a GRPr construct (R139G) with a point mutation in the highly conserved DRY motif required for G protein coupling in GRPr and other rhodopsin-like GPCRs (Benya et al., 1994). Neither wave of ERK activation nor the decreased ERK phosphorylation occurring with high numbers of BN-occupied receptors was present in R139G, indicating that G protein coupling is essential for all three responses (data not shown).

Receptor internalization has been shown to be a critical process for some GPCR-mediated MAPK activation, and Δ346 is defective in GRPr internalization (Benya et al., 1993; Daaka et al., 1998; Ignatova et al., 1999). Because the later wave of ERK activation by BN was also CTD-dependent, we tested whether GRPr internalization is required for this wave using hypertonic sucrose to block GRPr internalization. Sucrose (0.45 M) completely blocked GRPr internalization in disaggregated cells (Fig. 6A). Similar results were seen in two additional experiments with adherent cells and internalization measured at a single time point as described in

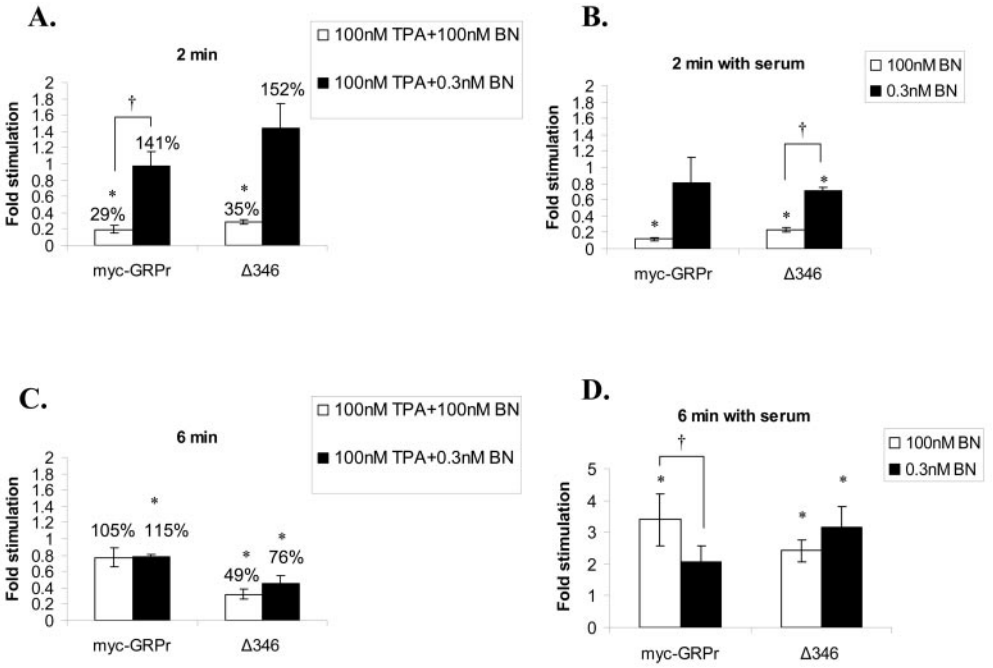

Fig. 4. Treatment of high expressors with 100 nM BN for 2 min inhibits ERK phosphorylation. A, serum-starved myc-GRPr cells and Δ346 cells were incubated in fresh serum-free DMEM for 1 h. Cells were then incubated with 100 nM TPA for 25 min and treated with 100 nM BN (TPA + 100 nM BN), 0.3 nM BN (TPA + 0.3 nM BN), or serum-free DMEM during the last 2 min of TPA incubation. B, myc-GRPr cells and Δ346 cells were grown in DMEM containing 10% serum to confluence, and then incubated in fresh DMEM + 10% serum for 1 h. Cells were treated with 100 nM BN, 0.3 nM BN, or DMEM with serum for 2 min. C and D, cells were treated as in A and B, respectively, except ERK phosphorylation was measured 6 min after addition of BN or DMEM. ERK activity was measured as in Fig. 1. Data shown are the mean ± S.E. of at least three experiments. Fold-stimulation in A and C is defined as (TPA + BN-induced ERK phosphorylation)/(TPA + DMEM-induced ERK phosphorylation). Numbers above the bars refer to TPA + BN-induced ERK phosphorylation as a percentage of ERK phosphorylation induced by 100 nM TPA alone in the same experiment and differ modestly from the values for fold stimulation because of the different reference value used. *, $p < 0.05$ compared with TPA + DMEM (in A and C) or compared with DMEM treatment (in B and D), $n \geq 3$. †, $p < 0.05$ comparison between 100 and 0.3 nM BN, $n \geq 3$.

method B under *Materials and Methods* (data not shown). But, the later (6-min) wave of ERK activation was not eliminated by 0.45 M sucrose pretreatment, suggesting that GRPr internalization is not required for this response. We were surprised to find that 0.45 M sucrose inhibited most of the early (2-min) wave of ERK activation (Fig. 6B). We also found that 0.45 M sucrose nearly abolished TPA-induced ERK activation in these transfected BALB cells (data not shown). These data were similar to the results found with TRH receptor (Smith et al., 2001) and suggested that besides blocking GRPr internalization, hypertonic sucrose interferes with other cellular functions that are important for the early (2-min) wave of GRPr-stimulated ERK phosphorylation and for TPA-stimulated ERK phosphorylation.

GRPr-mediated arrestin (arrestin2 and arrestin3) translocation to plasma membrane requires an intact GRPr CTD (Ally et al., 2003). In addition, arrestins have been implicated in MAPK activation for several GPCRs (Ahn et al., 1999; DeFea et al., 2000a; McDonald et al., 2000). If arrestin translocation is involved in the later wave of ERK activation by BN, hypertonic sucrose should not prevent arrestins from translocating to the plasma membrane because hypertonic sucrose pretreatment failed to eliminate the later wave of ERK activation (Fig. 6B). We transiently transfected GFP-tagged arrestin2 or arrestin3 into myc-GRPr and followed arrestin translocation in response to BN after 0.45 M sucrose pretreatment. Hypertonic sucrose did not prevent or slow arrestin translocation to the plasma membrane (data not shown). Hence, we cannot exclude the possibility that arrestin translocation plays a role in the later wave of ERK activation by BN.

GRPr can activate ERK through both PKC-dependent and -independent pathways (Seufferlein et al., 1996; Charlesworth and Rozengurt, 1997). Therefore, we examined whether PKC could participate in one wave of ERK activation but not the other using a specific PKC inhibitor GF 109203X to block PKC kinase activity. One-hour pretreatment with 0.75 μM GF 109203X blocked TPA-induced ERK activation, indicating that PKC activity was inhibited (Fig. 7A, right side). GF 109230X (0.75 μM) completely abolished the early wave of BN-induced ERK activation in myc-GRPr [compare 0.3 nM BN for 2 min (0.3–2) with DMEM for 2 min (DMEM-2) after GF 109203X pretreatment (+)], but only partially inhibited the later one [compare 100 nM (100–6) and 0.3 nM BN for 6 min (0.3–6) with DMEM for 6 min (DMEM-6) after GF 109203X pretreatment (+)]. Together, differential sensitivity of the early and late waves of ERK activation to hypertonic sucrose and GF 109203X support the idea that a different signaling pathway mediates each wave of ERK activation.

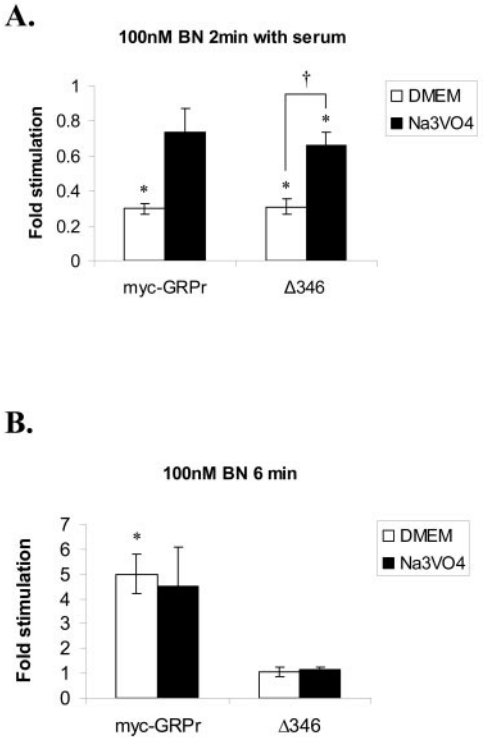

Fig. 5. Vanadate reverses the inhibition of ERK phosphorylation by high dose of BN. A, myc-GRPr and Δ346 cells were grown in DMEM containing 10% serum to confluence and then incubated with 1 mM orthovanadate in fresh DMEM + 10% serum (Na_3VO_4) or fresh DMEM + 10% serum (DMEM) for 30 min. After this pretreatment, cells were treated with 100 nM BN or DMEM with serum for 2 min. B, serum-starved myc-GRPr and Δ346 cells were incubated with 1 mM Na_3VO_4 in serum-free DMEM (Na_3VO_4) or serum-free DMEM (DMEM) for 30 min. Cells were subsequently stimulated with 100 nM BN or serum-free DMEM for 6 min. ERK activity was measured as in Fig. 1. Data shown are the mean ± S.E. of at least three experiments in each case. *, $p < 0.05$ when comparing BN with DMEM treatment for the times indicated, $n \geq 3$. †, $p < 0.05$ when comparing Na_3VO_4 with DMEM pretreatment, $n \geq 3$.

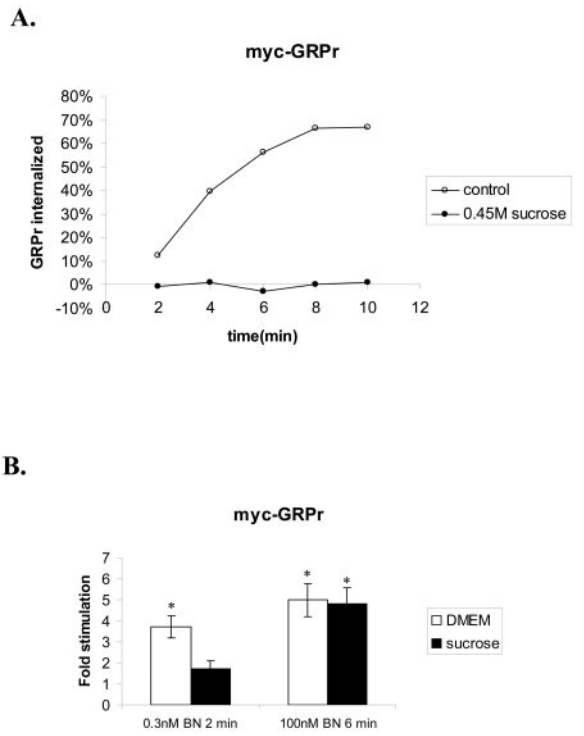

Fig. 6. Effects of hypertonic sucrose on the early and late waves of ERK activation. A, myc-GRPr cells were disaggregated then incubated with solution A (control) or 0.45 M sucrose in solution A at 37°C for 20 min. ^{125}I-BN (75 pM) and 0.5 nM nonradioactive BN were added and BN internalization measured as described in method A under *Materials and Methods*. Data represent the average of duplicate determinations for each time point. B, serum-starved myc-GRPr cells were incubated with 0.45 M sucrose in serum-free DMEM (sucrose) or serum-free DMEM (DMEM) for 20 min. Cells were subsequently stimulated with BN or serum-free DMEM for the times indicated. ERK activity was measured as in Fig. 1. Data shown are the mean ± S.E. of at least three experiments. *, $p < 0.05$ when comparing BN with DMEM treatment for the times indicated, $n \geq 3$.

HuTu 80 Duodenal Cancer Cells Have GRPr-Mediated ERK Activation Exhibiting Varying Sensitivity to PKC Inhibition. GRPr is capable of stimulating two distinct waves of ERK activation in transfected BALB fibroblasts. To determine whether this may occur in cells expressing endogenous GRPr, we studied the pattern of ERK activation in HuTu 80 cells, a duodenal cancer cell line. HuTu 80 cells express similar numbers of GRPr per cell (~38,000 GRPr/cell; data not shown) to the low expressors wt-low and Δ346-low. BN stimulated ERK phosphorylation in HuTu 80 cells, but DMEM alone did not (data not shown). The specific GRPr antagonist [D-Phe^6]BN(6-13)ME alone did not alter ERK phosphorylation, but it abolished BN-induced ERK phosphorylation in HuTu 80 cells, indicating that effects of BN are mediated through GRPr (data not shown). However, unlike the two distinct peaks of ERK activation in myc-GRPr, HuTu 80 cells showed ERK activation at all time points examined up to 8 min (Fig. 7B, ○), and the dose-response curves for ERK activation were sigmoidal when measured at both 2 and 6 min after addition of BN (data not shown). To determine whether ERK activation by BN in HuTu 80 cells may be a composite of several pathways with different time courses, we measured BN-stimulated ERK phosphorylation after sucrose or GF 109203X pretreatment. Sucrose pretreatment elicited strong and sustained ERK activation without BN to a level comparable with BN-induced ERK activation (without sucrose pretreatment). Therefore, sucrose was not a useful reagent in these cells (data not shown). GF 109203X (3 μM) was needed to suppress TPA-stimulated ERK phosphorylation in HuTu 80 (data not shown). As shown in Fig. 7B (●), 3 μM GF 109203X pretreatment completely inhibited BN-stimulated ERK phosphorylation at 2 min, but only partially inhibited ERK phosphorylation at 6 to 8 min. Therefore, like the difference found between the two waves of ERK activation in myc-GRPr, the early part of the sustained ERK activation in HuTu 80 cells was more sensitive to GF 109203X than the later part. Hence, these data demonstrate that the signaling pathways implicated in GPCR-induced MAPK activation can depend upon the time point examined and suggest that the two distinct waves of ERK activation observed in transfected BALB cells may exist in some cells expressing endogenous GRPr.

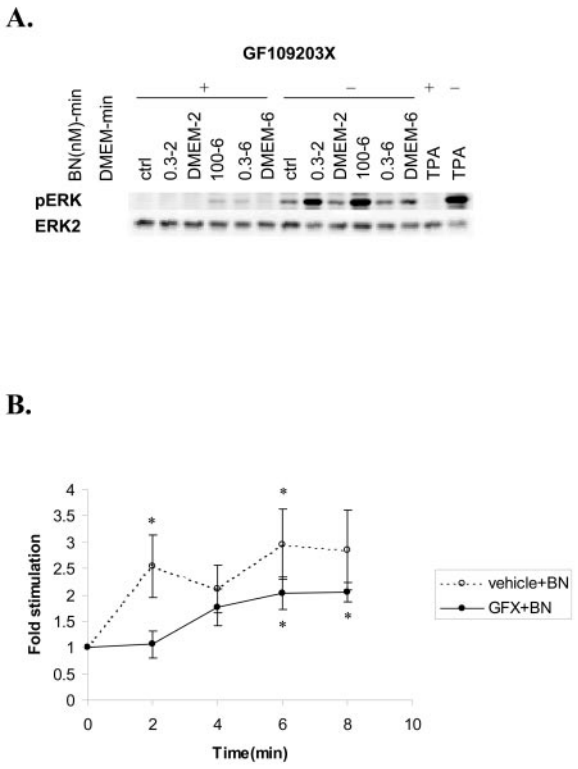

Fig. 7. PKC inhibition blocks BN-stimulated ERK activation to different degrees at different times after BN addition in HuTu 80 cancer cells. A, serum-starved myc-GRPr cells were incubated with 0.75 μM GF 109203X (+) or vehicle (−) for 1 h before stimulation. Cells received no treatment (ctrl) or were subsequently stimulated with TPA for 25 min (TPA), 100 nM BN, 0.3 nM BN, or serum-free DMEM for 2 or 6 min. Lanes are labeled as "additive-time" (e.g., 0.3 nM BN for 2 min is 0.3–2). Representative Western blots from three experiments with anti-phospho-ERK antibody (pERK) and anti-total ERK2 antibody (ERK2) are shown. B, serum-starved HuTu 80 cells were incubated with 3 μM GF 109203X (●) or vehicle (○) for 1 h. Cells then received no treatment (time 0) or were treated with 100 nM BN for various times as indicated or 100 nM TPA for 25 min (data not shown). ERK activity was measured as in Fig. 1. Data shown are the mean ± S.E. of four experiments. *, $p < 0.05$ compared with no treatment (time 0), $n = 4$. Maximum response is 38% of TPA induced ERK phosphorylation.

Discussion

GPCRs activate MAPK via cell type-specific mechanisms (Liebmann, 2001). In this study, we demonstrate that GRPr activation enhances or diminishes the level of ERK phosphorylation through three distinct pathways in a single cell type. The amplitude of each pathway was a function of both concentration of added BN and number of GRPr per cell. Evidence for two pathways that increase ERK phosphorylation was provided by differences in time course, dose-response curve, requirement for CTD, and differential sensitivity to GF 109203X and hypertonic sucrose. An inhibitory pathway that required tyrosine phosphatases was detected 2 min after addition of BN under conditions with high [BN] and [GRPr]/cell. In addition, evidence for two pathways that increase ERK phosphorylation was also found in a human cancer cell line expressing endogenous GRPr.

Our data indicate that a single cell type may use different pathways under different conditions (different receptor number per cell and agonist concentration) or more than one pathway after a single addition of agonist. Therefore, discrepancies in the literature as to the mechanism of ERK activation by GPCRs might be explained by several reasons in addition to cell-type specific factors. It has not been previously appreciated that the signaling pathways implicated in GPCR-activated MAPK phosphorylation depend upon receptor expression, agonist concentration, and time point evaluated. Consistent with our results, the amplitude of p38α MAPK phosphorylation was affected by the expression level of human cytomegalovirus US28 GPCR (US28) (Miller et al., 2003). At low expression, the carboxyl-terminal deletion mutant of US28 enhanced p38α MAPK phosphorylation to a similar extent as wild type. But at high expression, mutant US28 stimulated less p38α MAPK phosphorylation than wild type. Therefore, data obtained in overexpression systems must be validated in cells expressing physiologically relevant levels of receptors.

Our data are consistent with the results of Feldman et al. (1996) who found that addition of 100 nM GRP resulted in growth inhibition in BALB cells expressing ~10^6 GRPr/cell, but growth stimulation when 1 nM GRP was added to these

cells or when cells expressing ~46,000 GRPr/cell were stimulated with 100 nM GRP. Therefore, when GRPr number and agonist concentration are both very high, a pathway that inhibits ERK phosphorylation is induced and growth is inhibited.

In addition, the time after agonist addition that MAPK activation is examined may influence conclusions. It has been reported that different receptor constructs activate ERK through different mechanisms (DeFea et al., 2000b) and also that wild-type and truncated receptors activate ERK by the same pathway (Smith et al., 2001). We might have reached either of these conclusions had we limited our study to a single time point after BN addition.

In light of the existence of an inhibitory pathway, two apparent waves of ERK activation could be the result of one sustained ERK activation with an inhibitory pathway that decreases ERK phosphorylation transiently during the course of the activation. Our data strongly suggest two distinct stimulatory pathways because blockage of the inhibitory pathway by vanadate failed to reveal the later wave of ERK activation in Δ346, and two waves were still present in cells expressing GRPr with intact CTD even under conditions at which the inhibitory pathway was not detectable. The mechanism underlying the separation of two stimulatory pathways in time is not clear. One possibility is that the second wave of ERK activation requires transactivation of receptor tyrosine kinases (RTKs) or recruitment of signaling molecules to the activated receptor and therefore takes more time. However, the broad-spectrum tyrosine kinase inhibitor genistein inhibited both waves to a similar degree, making it less likely that an RTK is selectively involved in either wave (data not shown). In addition, although epidermal growth factor receptor and platelet-derived growth factor receptor are essential for MAPK activation induced by several GPCRs (Liebmann, 2001), we were unable to detect epidermal growth factor- or platelet-derived growth factor-induced ERK activation in BALB/c 3T3 cells (data not shown). Therefore, it is unlikely that epidermal growth factor receptor or platelet-derived growth factor receptor mediates either wave of GRPr-mediated ERK phosphorylation in these cells. Identification of the RTKs that are expressed in BALB cells and blockage of their activity are required to test whether a specific RTK is involved in one or both waves of ERK activation.

The initial hypothesis we tested in this study was that GRPr CTD might be required for BN-induced MAPK activation because receptor internalization and/or arrestins (CTD-interacting proteins) had been implicated in GPCR-induced MAPK activation in other systems. Agonist-induced GRPr internalization and arrestin (arrestin2 and arrestin3) translocation to the plasma membrane are CTD-dependent processes (Ally et al., 2003). Our data show that an intact CTD is required for the later wave of ERK phosphorylation (at 6 min), suggesting that CTD-interacting molecules or other CTD-dependent processes might be involved in the later stimulatory pathway. Using hypertonic sucrose, we found that GRPr internalization was not required for the later wave. But, hypertonic sucrose did not inhibit arrestin translocation. Therefore, roles of arrestins have not been definitively determined in this study and may be complex. Arrestins contribute to homologous receptor desensitization of GPCRs (Freedman and Lefkowitz, 1996; Ferguson, 2001) but also activate a second wave of GPCR-dependent signaling (Ahn et al., 1999; DeFea et al., 2000a; McDonald et al., 2000). Although arrestins have not been definitely implicated in acute desensitization of GRPr signaling, they may play a dual role in GRPr signaling in BALB/c 3T3 cells: terminating the early wave and initiating the later wave of ERK activation. Consistent with recent data demonstrating that GRPr CTD mutants showed a stronger and prolonged BN-stimulated inositol-(1,4,5)-trisphosphate generation and Ca^{2+} mobilization than wild type (Ally et al., 2003), the early wave of ERK activation (at 2 min) was also stronger and lasted longer in Δ346-low than wt-low, suggesting that CTD (and arrestins) may be involved in acute desensitization of the early wave of ERK activation. Further studies using techniques to alter arrestins expression or function will be required to test this hypothesis.

Our data indicate that regulation of ERK phosphorylation by GRPr is a function of [BN] and [GRPr]/cell. However, the molecular mechanisms that govern how [BN] and [GRPr]/cell determine the magnitude of each pathway altering ERK phosphorylation are not solved in this study. Classical receptor occupancy theory suggests that this efficacy would be dependent upon the number of agonist-receptor complexes (in our case, the number of BN-occupied GRPr/cell). On the other hand, rate theory suggests that efficacy is a function of the rate of agonist-receptor interactions (in our case, the rate of BN binding to GRPr). BN binding to GRPr does not reach a plateau for at least 10 min after addition of 1 nM BN to GRPr-expressing cells (Ally et al., 2003). Therefore, after addition of low [BN] (0.01–1 nM) as used in several experiments, the two waves of GRPr-stimulated ERK activation would be completed as binding was continuing. This is similar to what we have seen with the time course of GRPr-stimulated inositol-(1,4,5)-trisphosphate generation (Ally et al., 2003) and suggests that efficacy may be a function of the initial rate of ligand-receptor association. Rate theory, in which efficacy is a function of $\Delta[LR]/\Delta t$ not [LR], may therefore explain our data better than occupancy theory.

In contrast to our results in HuTu 80 cells, Qu et al. (2002) reported that BN did not induce ERK phosphorylation in these cells. The difference could be the result of different experimental conditions or a different subclone of HuTu 80. In fact, we found that HuTu 80 cells expressed ~38,000 GRPr/cell, which is a higher receptor expression than that has been reported for this cell line (~5900 GRPr/ cell) (Williams and Schonbrunn, 1994). This difference in GRPr expression may have resulted in different results for ERK activation. We also found that after being cultured for about 1 month, HuTu 80 cells showed a much weaker ERK activation in response to BN (data not shown). Whether GRPr are gradually lost in HuTu 80 cells over time in culture remains unclear, but changes in the cell line after prolonged time in culture may be responsible for the differences reported.

In addition, in a result unrelated to GRPr signaling, we observed that ERK was activated in BALB fibroblasts solely by addition of a small volume of media identical in composition and temperature to incubation media. It has been shown that fluid shear stress activated MAPK in several cells such as endothelial cells, smooth muscle cells, and osteoblasts (Jo et al., 1997; Li et al., 1999; Yan et al., 1999; Azuma et al., 2000; You et al., 2001). Addition of DMEM and subsequent agitation of the dish might create the shear stress necessary

for activating MAPK. HuTu 80 cells did not show DMEM-induced ERK activation, suggesting that molecule(s) or pathways(s) responsible for this effect were not present in HuTu 80 cells.

Finally, we do not know whether each wave of ERK activation has distinct functional consequences and under which conditions each wave of ERK activation is physiologically relevant. Future studies on identification of other molecules involved in these two waves of ERK activation will be required to address these questions.

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