Extracellular Signal-Regulated Kinase 1/2-Mediated Transcriptional Regulation of G-Protein-Coupled Receptor Kinase 3 Expression in Neuronal Cells

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ABSTRACT

Relatively small changes in G-protein-coupled receptor kinase (GRK) 3 expression (~2-fold) profoundly affect α_2-adrenergic receptor (AR) function and preferentially regulate neuronal α_{2A}- and α_{2B}-AR signaling. In the present study, we provide evidence that epinephrine (EPI)-induced up-regulation of GRK3 protein expression in two neuronal cell lines, BE(2)-C cells (endogenously express α_{2A}- and β_2-AR) and BN17 cells [endogenously express α_{2B} (NG108) and transfected to express β_2-AR] is due in part to increased GRK3 gene expression. In both cell lines, the increase in GRK3 transcription occurred via an extracellular signal-regulated kinase (ERK) 1/2-dependent mechanism because the increase in GRK3 mRNA is eliminated in the presence of the mitogen-activated protein kinase/ERK kinase 1/2 inhibitor, U0126 [1,4-diamino-2,3-dicyano-1,4-bis (2-amino phenylthiobutadiene)]. EPI-induced GRK3 mRNA up-regulation also is prevented in the presence of propranolol or phentolamine. Moreover, GRK3 mRNA did not increase in response to EPI treatment in NG108 cells (endogenously express α_{2B}-AR with no β_2-AR). Both these results suggest that simultaneous activation of α_2- and β_2-AR by EPI is required for the ERK1/2-dependent increase in GRK3 mRNA. The EPI-induced increase in GRK3 mRNA was unaffected in the presence of the protein kinase C inhibitor, chelerythrine chloride. Finally, EPI treatment resulted in increased nuclear translocation and accumulation of the transcription factors, Sp-1 and Ap-2, in BE(2)-C cells. Taken together, our results demonstrate the involvement of the ERK1/2 pathway in selective up-regulation of GRK3 mRNA expression, possibly via activation of Sp-1 and Ap-2 transcription factors in neuronal cells.

G-protein-coupled receptor kinases (GRKs) specifically interact with agonist-occupied G-protein-coupled receptors (GPCRs) to mediate receptor phosphorylation. This phosphorylation increases the affinity of the receptor for arrestin, and receptor/G-protein interaction is inhibited even in the presence of agonist. The resulting rapid loss of receptor responsiveness is termed desensitization and in many instances is followed by the removal of the receptor from the plasma membrane via clathrin-mediated endocytosis (Penn et al., 2000; Penela et al., 2006). GRK3, a member of the

GRK2/3 subfamily of the GRK group of kinases, is modestly expressed in most cell types compared with GRK2. Hence, it has not been extensively studied. Traditionally, the genes for GRK2/3 were considered to be part of a family of housekeeping genes, without selective regulation. However, recent observations from our laboratory and others suggest otherwise.

Several previous studies have examined the regulation of GRK2 expression utilizing multiple approaches. For example, GRK2 mRNA levels were reported to be regulated in a variety of disease states and during differentiation (De Blasi et al., 1995; Penela et al., 2000, 2006; Sefton et al., 2000). An association between modest changes in GRK2 expression and impaired GPCR signaling is also observed in various pathophysiological situations, including experimental models of hypertension (Gros et al., 1997); heart failure (Ungerer et al., 1993); cardiac hypertrophy, myocardial ischemia, and infarction (Choi et al., 1997; Dorn et al., 2000; Yu et al., 2000);

ABBREVIATIONS: GRK, G-protein-coupled receptor kinase; GPCR, G-protein-coupled receptor; AR, adrenoceptor; CRF, corticotropin-releasing factor; NE, norepinephrine; ERK, extracellular signal-regulated kinase; EPI, epinephrine; DMEM, Dulbecco's modified Eagle's medium; MEK, mitogen-activated protein kinase/ERK kinase; U0126, 1,4-diamino-2,3-dicyano-1,4-bis (2-amino phenylthiobutadiene); RT, reverse transcriptase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; NG108-15 (NG108), neuroblastoma/glioma hybrid cell; BE(2)-C, human neuroblastoma cell(s); DAPI, 4',6-diamidino-2-phenylindole; CC, chelerythrine chloride; PBS, phosphate-buffered saline; PBSS, PBS containing 1.2% sucrose; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PKC, protein kinase C; BPD, bipolar disorder.

rheumatoid arthritis (Lombardi et al., 2001); ventricular volume overload disease (Dzimiri et al., 2004); and cystic fibrosis lung (Mak et al., 2002). Almost universally, these studies have reported increase in GRK2 expression with no changes in GRK3 expression. In an attempt to define the mechanisms responsible for changes in GRK2 expression, the promoter for the GRK2 gene has been identified. GRK2 promoter-luciferase reporter constructs have demonstrated that phorbol esters, α_1-AR agonists, and serum stimulation (Ramos-Ruiz et al., 2000; Theilade et al., 2005) increase GRK2 expression at the transcriptional level. However, there are relatively few reports of the selective increase in GRK3 expression (Dautzenberg and Hauger, 2001; Dautzenberg et al., 2002), and the GRK3 gene promoter has not been characterized but is predicted to lie in the genomic region immediately 5′ to exon 1 (Barrett et al., 2003).

Recent observations suggest that the regulation of GRK3 expression also warrants greater attention. For example, using a neuronal cell model, we observed that the stress hormone/neurotransmitter, EPI, selectively up-regulates GRK3 expression and that this increase renders the $\alpha_{2A/B}$-AR more sensitive to agonist-stimulated desensitization and down-regulation (Bawa et al., 2003; Desai et al., 2005). No changes in GRK2 expression were observed. Other reports suggest that κ-opioid agonists and corticotropin-releasing factor (CRF) selectively regulate GRK3 expression and that signaling through both the CRF_1 and κ-opioid receptor is preferentially regulated by GRK3 over GRK2 (Dautzenberg and Hauger, 2001; Dautzenberg et al., 2002; McLaughlin et al., 2004). Therefore, selective changes in GRK3 expression without changes in GRK2 transcription have been reported and have been implicated in selective alterations in GPCR signaling.

Given our observations of the selective up-regulation of GRK3 expression by EPI, but not NE, in two different neuronal cell lines, the present study was undertaken to begin to identify the mechanisms responsible for these changes in GRK3 expression. Our results reveal a transcriptional regulation of GRK3 expression that requires the simultaneous activation of both α_2- and β_2-adrenoceptors and the participation of the extracellular signal-regulated kinases (ERKs) 1/2.

Materials and Methods

Materials. The following were purchased from the indicated sources. (−)Epinephrine bitartrate (EPI), phenylmethylsulfonyl fluoride, Dulbecco's modified Eagle's medium (DMEM), F12-DMEM, HAT supplement (0.1 mM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine), sodium metabisulphite, theophylline, HEPES, bovine serum albumin, poly-L-lysine hydrobromide, MEK inhibitor U0126, propranolol, and phentolamine were from Sigma-Aldrich (St. Louis, MO). G418 sulfate (Geneticin) (Calbiochem, La Jolla, CA), fetal bovine serum, and penicillin-streptomycin were from Atlanta Biological (Norcross, GA). RNeasy Mini kit (catalog no. 74104) was purchased from QIAGEN (Valencia, CA), and Superscript one-step RT-PCR kit (catalog no. 11922-028) was purchased from Invitrogen (Carlsbad, CA). Ethidium bromide (catalog no. H5041) and loading dye (catalog no. G190A) were purchased from Promega Corp. (Madison, WI). Molecular ruler, 100 bp (catalog no. 170-8202), and prestained SDS-PAGE protein marker (catalog no. 161-0324) were purchased from Bio-Rad (Hercules, CA). Nuclear extraction NE-PER kit (no. 78833) was purchased from Pierce Chemical (Rockford, IL). Anti-Sp-1 (sc-59), Ap-2 (sc-184) antibodies, and horseradish peroxi-

dase-conjugated anti-rabbit secondary antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Phosphospecific ERK1/2 and total ERK1/2 were purchased from Cell Signaling, Inc. (Beverly, MA). NG108 and BN17 cells were obtained from Dr. Graeme Milligan (University of Glasgow, Glasgow, UK). BN17 cells are NG108 cells transfected to overexpress human β_2-AR (250–300 fmol/mg protein). BE(2)-C cells were generously provided by Dr. Robert Ross (Fordham University, Bronx, NY). Reagents used in immunofluorescence were purchased from the source indicated: anti-rabbit Alexa-488 (no. A11034; Invitrogen), VECTASHIELD mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, CA), paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), Mowiol 4–88 reagent (EMD Biosciences, San Diego, CA), poly-D-lysine (Sigma-Aldrich), L-lysine hydrochloride (JT Baker, Phillipsburg, NJ), sodium periodate (Fisher Scientific, Fair Lawn, NJ), and goat serum (Sigma-Aldrich). Coverslips used to grow cells were from VWR Scientific (West Chester, PA), and the Superfrost microscope slides used to mount the cover slips were purchased from Fisher Scientific (Pittsburgh, PA).

Cell Culture. The neuroblastoma/glioma hybrid NG108 cells were maintained in a humidified atmosphere (5% CO_2:95% air) in DMEM supplemented with 10% heat-inactivated fetal calf serum, penicillin, streptomycin, and HAT supplement. BN17 cells were maintained similarly except that the media contained G418 (0.4 mg/ml) to retain selection pressure. BE(2)-C cells were maintained in a humidified atmosphere (6% CO_2:94% air) in a 1:1 mixture of Eagle's minimal essential medium with nonessential amino acids and Ham's F12 medium containing 10% fetal bovine serum, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin sulfate. All cells were grown until 70 to 80% confluence before EPI treatment. α_2-AR levels were expressed at comparable levels in BN17 as well as BE(2)-C cells (40–60 fmol/mg). β_2-AR levels were very different, 250 fmol/mg in BN17 versus 20 fmol/mg in BE(2)-C cells. BE(2)-C cells endogenously express β_2-AR, whereas BN17 are NG108 cells transfected to express β_2-AR.

Pretreatment. BN17, NG108 and BE(2)-C cells were pretreated with vehicle (medium containing 0.1 mM ascorbate and 1 μM sodium metabisulphite) or vehicle containing 0.3 μM EPI for 5, 15, 30, or 60 min for RT-PCR experiments.

MEK1/2 Inhibitor, Chelerythrine Chloride, Propranolol, and Phentolamine Treatment. Cells, when ~70% confluent, were treated in serum-free media for 2 h in the presence of the MEK1/2-inhibitor, U0126 (10 μM), chelerythrine chloride (CC) (10 μM), propranolol (1 μM), or phentolamine (10 μM) at 37°C within an incubator in the appropriate environment. After pretreatment, the cells were treated with vehicle/EPI for the desired time points in the continued presence of the pretreatment agent.

Western Blot Analysis. Cells were washed once with 1× PBS buffer, pH 7.4, lysed immediately in 100 to 200 μl of hypotonic lysis buffer (50 mM Tris-HCl, pH 7.4, 4 mM EDTA, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin) followed by five to six passes through a 23-gauge needle and subsequently centrifuged at 1000 rpm for 10 min to remove cellular debris and nuclei (Salim et al., 2003). The lysates thus obtained were checked for their protein concentration using Pierce's protein detection kit (Smith et al., 1985). The cell lysates were diluted with 4× Laemmli buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.1 mg/ml bromphenol blue), resolved on SDS-PAGE (10% gel), and transferred to polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NJ). Levels of Sp-1 and Ap-2 were determined by immunoblotting using anti-Sp-1 and anti-Ap-2 antibody (1:250 and 1:200 dilution, respectively). Levels of phospho-ERK1/2 and total ERK1/2 were determined by using specific phospho-ERK1/2 and total ERK1/2 antibodies (1:1000 dilution). The immunoreactive bands were detected by a horseradish peroxidase-conjugated secondary antibody, and the blots were developed using chemiluminescence reagent made by adding p-coumaric acid and luminol (Sigma-Aldrich) in 1 M Tris-HCl and hydrogen peroxide

solution. Chemiluminescence was detected by an Alpha Innotech (San Leandro, CA) imaging system, and densitometry was quantified using Fluorchem FC8800 software (Alpha Innotech).

Protein Estimation. Protein concentrations were determined by the Pierce protein detection kit (catalog no. 232009; Pierce) using BCA protein assay reagent A (catalog no. 23223) and reagent B (catalog no. 23224) (Smith et al., 1985).

Semiquantitative RT-PCR. Total RNA was isolated from NG108, BN17, or BE(2)-C cells after vehicle/EPI treatment at the indicated time intervals using the RNeasy RNA isolation kit per manufacturer's guidelines. The purity of the RNA was determined by the ratio of spectrophotometric readings at 260 and 280 nm. The RNA used for RT-PCR had a ratio of 1.5 to 2.0. RT-PCR was carried out in a programmable thermal controller (iCycler; Bio-Rad) with the following oligonucleotide primers: forward primer for detecting human GRK3 in BE(2)-C cells, 5′-AGAGCACAGCTTTTTCAAAGGT-GTTG-3′, which corresponds to position 1344 to 1369 of human GRK3 cDNA (Dautzenberg et al., 2002); reverse primer for detecting human GRK3 in BE(2)-C cells, 5′-GTCAGAAATGGGTTTC-CCAGTTTCAG-3′, which corresponds to position 1721 to 1696 of human GRK3 cDNA (Dautzenberg et al., 2002); forward primer for detecting human β-actin in BE(2)-C cells, 5′-AAGATGACCCAGAT-CATGTTTGAGAC-3′, which corresponds to position 393 to 418 of human β-actin (Palchaudhuri et al., 1999); reverse primer for detecting human β-actin in BE(2)-C cells, 5′-CTGCTTGCTGATCCA-CATCTGCTGG-3′, which corresponds to position 1121 to 1096 of human β-actin (Palchaudhuri et al., 1999); forward primer for detecting GRK2, 5′-GTTGCTGCAGAGGGATGTCAACCG-3′, which corresponds to position 1388–1411 of human GRK2 cDNA (Dautzenberg et al., 2002); reverse primer for detecting GRK2, 5′-GTCA-GAAAGGGGTTGCCCATCTTGG-3′, which corresponds to position 1828–1804 of human GRK2 cDNA (Dautzenberg et al., 2002); forward primer for detecting GRK3 in BN17 cells, 5′-AATTGAGGC-CAGGAAGAAGGCTA-3′, which corresponds to position 1605–1627 of rat GRK3 cDNA (Arriza et al., 1992); reverse primer for detecting GRK3 in BN17 cells, 5′-TCAGAGGCCGCTGCTATTTCTGTGACA-3′, which corresponds to position 2041–2067 of rat GRK3 cDNA (Arriza et al., 1992); forward primer for detecting GAPDH in BN17 cells, 5′-TACTCCTTGGAGGCCATGTA-3 (Andresen et al., 2004); and reverse primer for detecting GAPDH in BN17 cells, 5′-CGTG-GAGTCTACTGGCGTCT-3′ (Andresen et al., 2004).

Both cDNA synthesis and PCR were performed using superscript RT-PCR kit in a single tube with gene-specific primers and total RNA isolated from NG108, BN17, or BE(2)-C cells using the RNeasy minikit. One cycle of 50°C for 30 min and 94°C for 2 min was carried out for cDNA synthesis and predenaturation, followed by 40 cycles of 94°C for 15 s (denature), 55°C for 30 s (anneal), and 72°C for 10 min for final extension. PCR products were separated and visualized in an ethidium bromide-stained agarose gel (2%) by electrophoresis.

Immunofluorescence. The cells were processed for immunoflu-orescence as described previously (Desai et al., 2006). In brief, BE(2)-C cells were grown on poly-D-lysine-coated 20- × 20-mm glass cover slips to 40 to 70% confluence. The cells were then exposed to vehicle/EPI (0.3 μM) for 5 and 15 min. Next, the cells were washed with PBS containing 1.2% sucrose (PBSS) and fixed with 4% para-formaldehyde in PBSS at 4°C for 15 min. The following steps were carried out at room temperature. The fixed cells were incubated in 0.034% L-lysine, 0.05% Na-m-periodate for 20 min, washed, and permeabilized with 0.2% Triton X-100 for 10 min. After further wash, the cells were blocked with 10% normal goat serum for 15 min. Primary (anti-Sp-1 or anti-Ap-2 antibody) and secondary (anti-rabbit Alexa488 conjugated antibody) antibodies were diluted in PBSS with 0.2% goat serum and 0.05% Triton X-100. The cells were incubated with anti-Sp-1 or anti-Ap-2 antibody for 1 h at room temperature or overnight at 4°C, followed by Alexa 488-conjugated secondary anti-body for 1 h in complete darkness. The cells were washed three times with PBSS before and after incubation with secondary antibody. The cover slips were then mounted on slides with a drop of a 1:1 mixture

of Mowiol solution and VECTASHIELD mounting medium with DAPI. Fluorescence examination of at least six fields on the same slide was performed under an oil immersion objective (×60, 1.4 numerical aperture) using a filter selective for Alexa 488 or DAPI using an Olympus IX81 Fluorescence Deconvolution Microscope Sys-tem (Olympus America, Center Valley, PA). DAPI staining enabled us to determine the area occupied by the nucleus in the BE(2)-C cells for us to monitor movement of Sp-1 and/or Ap-2 into the nucleus from the cytosol. At each time point, a representative group of cells was assessed for the extent of nuclear translocation. As a negative con-trol, we stained the cells either with primary (anti-Sp-1/Ap-2) or secondary (anti-rabbit Alexa 488 conjugated) antibody alone to de-termine the specificity of the fluorescence signal. Images were opti-mized using AutoDeblur and Autovisualization deconvolution soft-ware (Media Cybernetics, Silver Spring, MD) and transferred to Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA) for the produc-tion of final figures.

Data Analysis. For comparison between groups, the values were expressed as mean ± S.E.M. Comparisons between groups were made either by Student's t test or one-way analysis of variance followed by Tukey's post hoc test where appropriate (GraphPad Software, Inc. San Diego, CA), and groups were considered signifi-cantly different if $p < 0.05$.

Results

The effect of EPI (0.3 μM) treatment on the level of GRK3 mRNA expression in BE(2)-C and BN17 cell lines was deter-mined using RT-PCR. This concentration of EPI is the min-imum required to produce up-regulation of GRK3 protein expression (Desai et al., 2004). In BE(2)-C cells that endog-enously express α_{2A}-AR and β_2-AR, acute (5–60 min) expo-sure to 0.3 μM EPI produced an increase in GRK3 mRNA within 5 min; maximal expression was observed between 15 and 60 min (Fig. 1A). Preincubation of the cells with the MEK1/2 inhibitor, U0126 (10 μM; 2 h), before EPI treatment prevented the increase in GRK3 mRNA. In BN17 cells that endogenously express α_{2B}-AR and are transfected to express β_2-AR, EPI produced an increase in GRK3 mRNA similar to that observed in BE(2)-C cells, and this also was prevented by U0126 (10 μM; 2 h) pretreatment (Fig. 1B). Incubation of BE(2)-C or BN17 cells with 0.3 μM EPI for 5 min activates ERK1/2 phosphorylation in both cell lines (Fig. 1C; data shown for BN17 cells only), and pretreatment with U0126 (10 μM, 2 h) is sufficient to block ERK1/2 phosphorylation as detected by western blotting (Fig. 1C; data shown for BN17 cells only). MEK1/2 inhibitor treatment by itself had no effect on GRK3 mRNA in either the BE(2)-C or BN17 cells (data not shown). GRK2 mRNA expression did not change upon EPI treatment in either BE(2)-C (Fig. 2A) or BN17 (Fig. 2B) cells.

In our previous studies, we had observed that up-regula-tion of GRK3 expression required simultaneous activation of both α_2-AR and β_2-AR. Therefore, we investigated the role of these receptors in the increase in GRK3 mRNA and activa-tion of ERK1/2 by EPI. In both BE(2)-C and BN17 cells, blockade of β_2-AR with propranolol (1 μM) eliminated the EPI-induced increase in GRK3 mRNA (Fig. 3, A and B). Likewise, blockade of α_2-AR with phentolamine (10 μM) pre-vents the EPI-induced increase in GRK3 mRNA expression in both BE(2)-C and BN17 cells (Fig. 3, A and B). Moreover, in NG108 cells that endogenously express only α_2-AR, we did not observe an increase in GRK3 mRNA expression in re-sponse to treatment with 0.3 μM EPI (Fig. 4). These results suggest that, as previously observed for GRK3 protein up-
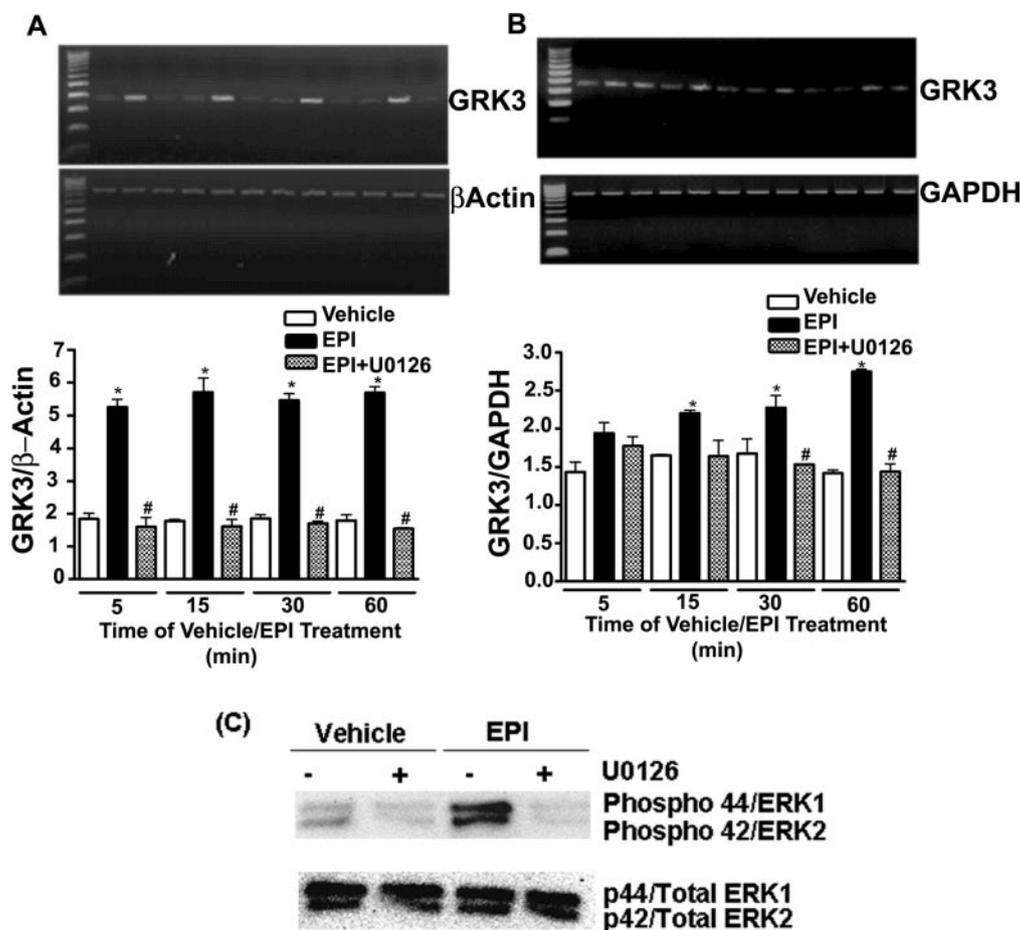
Fig. 1. Quantitation of mRNA levels for GRK3 in BE(2)-C and BN17 cells exposed to EPI (0.3 μM) treatment. BE(2)-C cells (A) or BN17 cells (B) were treated with vehicle/EPI (0.3 μM) for the indicated times in the absence/presence of the MEK1/2 inhibitor, U0126 (10 μM). Then, total RNA isolated from treated cells was reverse transcribed and amplified using primers for human GRK3 and β-actin [BE(2)-C cells, A] or rat GRK3 and GAPDH (BN17 cells, B). PCR products were electrophoresed through a 2% agarose gel, stained with 0.5 μg/ml ethidium bromide, and visualized under UV light. A molecular ruler (100–3000 bp) on the left-hand side of the gel is used for reference. ∗, significantly different from vehicle at $p < 0.05$; #, significantly different from EPI at $p < 0.05$. Shown is a representative semiquantitative RT-PCR gel depicting up-regulation of GRK3 mRNA expression. Each bar represents mean ± S.E.M. values for the densitometric quantitation ratio of GRK3 mRNA normalized to β-actin or GAPDH mRNA (internal loading controls) obtained from three independent experiments. C, BN17 cells were treated with vehicle/EPI (0.3 μM) for 5 min in the presence/absence of the MEK1/2 inhibitor U0126 (10 μM). The cells were harvested in hypotonic lysis buffer as described previously (Salim et al., 2003). The whole cell lysate was subjected to SDS-PAGE and immunoblotted with anti-phospho-p44/42 (ERK1/2) antibody first. The immuoblot was stripped of the bound antibodies and reprobed for total p44/p42 (ERK1/2).

regulation, EPI-induced increase in GRK3 mRNA requires simultaneous activation of both α_2-AR and β_2-AR.

Since there have been previous reports that GRK2 expression is regulated by PKC (Ramos-Ruiz et al., 2000), we also examined the effect of EPI on GRK3 mRNA in the presence of the PKC inhibitor, chelerythrine chloride. Pretreatment of BE(2)-C cells or BN17 cells with chelerythrine chloride (10 μM) had no effect on the response to EPI (0.3 μM) treatment (Fig. 5, A and B).

Recent reports describing the putative promoter region of the GRK3 gene have suggested the presence of binding sites for the transcription factors, Sp-1 and Ap-2, in the GRK3 promoter region (Barrett et al., 2003). Therefore, we examined the nuclear translocation of these transcription factors in response to EPI (0.3 μM) treatment. We detected increased Sp-1 and Ap-2 protein accumulation in nuclear extracts of BE(2)-C cells treated with EPI (0.3 μM) for 5 and 15 min compared with vehicle-treated cells (Fig. 6, A and B). Nuclear accumulation of Sp-1 protein in the BE(2)-C cells was increased by ~13 and 35% (expressed as percentage of

vehicle) after 5 and 15 min of EPI treatment, respectively. Nuclear accumulation of Ap-2 protein was increased ~36 and 40% after 5 and 15 min, respectively. Nuclear translocation of Sp-1 and Ap-2 in response to EPI treatment also was investigated by immunofluorescence (Fig. 7, A and B). BE(2)-C cells were treated with EPI (0.3 μM) or vehicle for 5 and 15 min. After the treatment, cells were processed for immunofluorescence as described previously (Desai et al., 2006). In separate samples, Sp-1 or Ap-2 were visualized with Alexa-488, whereas cell nuclei were stained with DAPI for visualization (blue). In vehicle-treated cells, Sp-1 (red) appeared as a punctate signal dispersed throughout the cytosol with limited nuclear localization (blue) (Fig. 7A). In cells treated with EPI, the intensity of the Sp-1 signal in the nuclei increased, indicating increased nuclear localization with increasing time (5–15 min). Moreover, as nuclear localization of Sp-1 increased, the Sp-1 signal disappeared from the cytosol. Similar results depicting increased nuclear translocation upon EPI treatment were obtained for the transcription factor, Ap-2 (Fig. 7B).
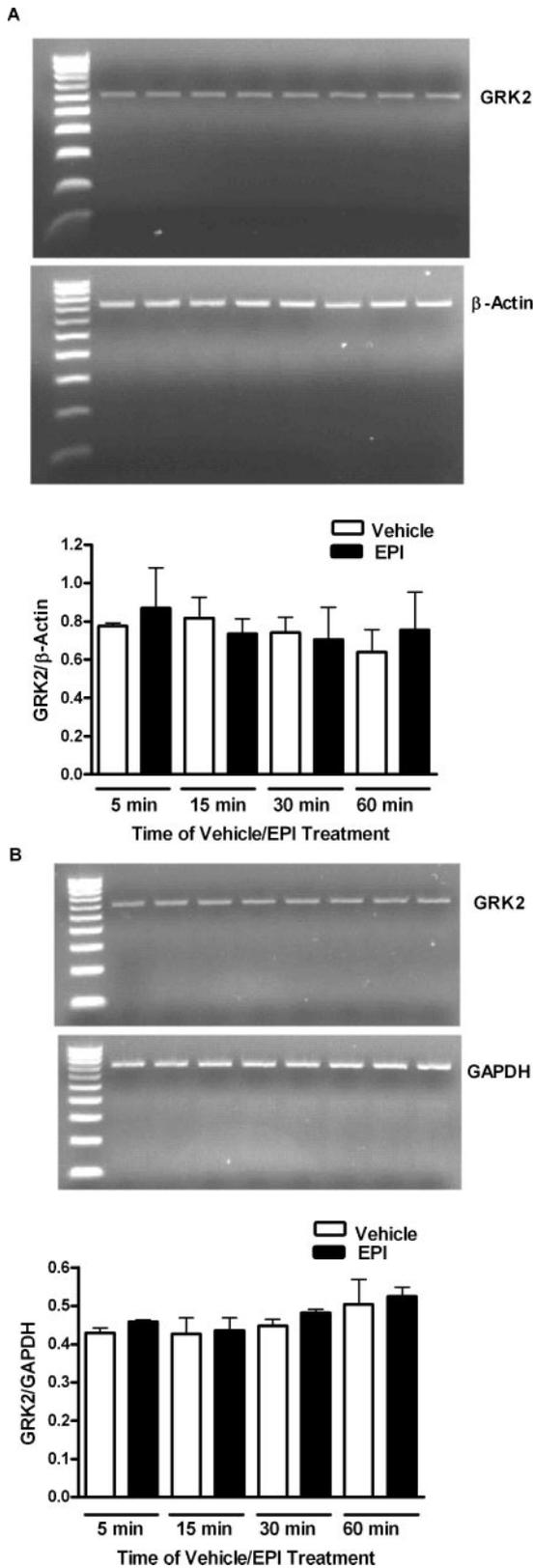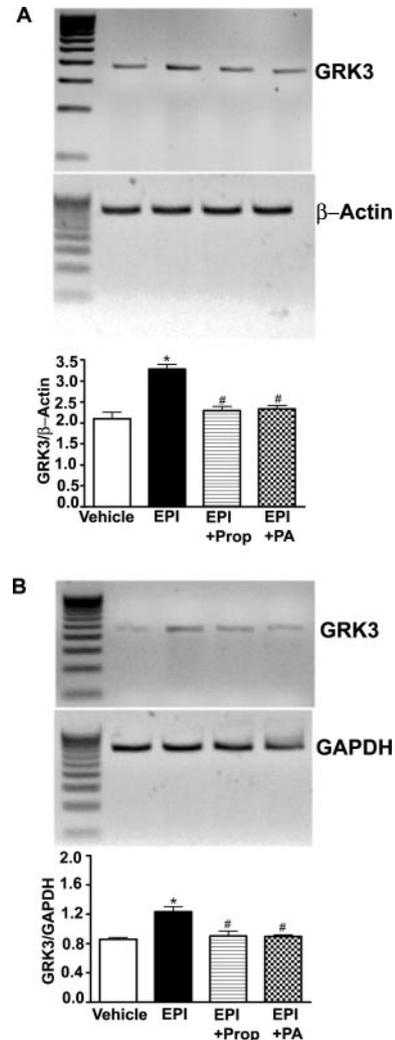
Fig. 3. Quantitation of mRNA levels for GRK3 in BE(2)-C and BN17 cells exposed to EPI in the presence of propranolol or phentolamine. Cells were treated with EPI (0.3 μM for 30 min) in the absence/presence of propranolol (1 μM) or phentolamine (10 μM). Total RNA isolated from treated cells was reverse transcribed and amplified using primers for human GRK3 and β-actin [BE(2)-C cells, A] or rat GRK3 and GAPDH (BN17 cells, B). PCR products were electrophoresed through a 2% agarose gel, stained with 0.5 μg/ml ethidium bromide, and visualized under UV light. A molecular ruler (100–3000 bp) on the left-hand side of the gel is used for reference. ∗, significantly different from vehicle at $p < 0.05$; #, significantly different from EPI at $p < 0.05$. Shown is a representative semiquantitative RT-PCR gel depicting GRK3 mRNA expression. Each bar represents mean ± S.E.M. values for the densitometric ratio of GRK3 mRNA normalized to the β-actin or GAPDH mRNA (internal loading control) obtained from three independent experiments.

Discussion

We have demonstrated previously that EPI selectively up-regulates GRK3 protein expression and sensitizes α_2-AR to desensitization and down-regulation in neuronal cells (Bawa et al., 2003; Desai et al., 2005). The results of the present study suggest that this increase in GRK3 expression is the result of an ERK1/2-dependent increase in GRK3 mRNA by

Fig. 2. Quantitation of mRNA levels for GRK2 in BE(2)-C and BN17 cells exposed to EPI (0.3 μM) treatment. BE(2)-C cells (A) and BN17 cells (B) were treated with vehicle/EPI (0.3 μM) for the indicated times. Then, total RNA isolated from treated cells was reverse transcribed and amplified using primers for human GRK2 and β-actin [BE(2)-C cells, A] or rat GRK2 and GAPDH (BN17 cells, B). PCR products were electrophoresed through a 2% agarose gel, stained with 0.5 μg/ml ethidium bromide, and visualized under UV light. A molecular ruler (100–3000 bp) on the

left-hand side of the gel is used for reference. Shown is a representative semiquantitative RT-PCR gel depicting no up-regulation of GRK2 mRNA expression. Each bar represents mean ± S.E.M. values for the densitometric quantitation ratio of GRK2 mRNA normalized to β-actin or GAPDH mRNA (internal loading controls) obtained from three independent experiments.
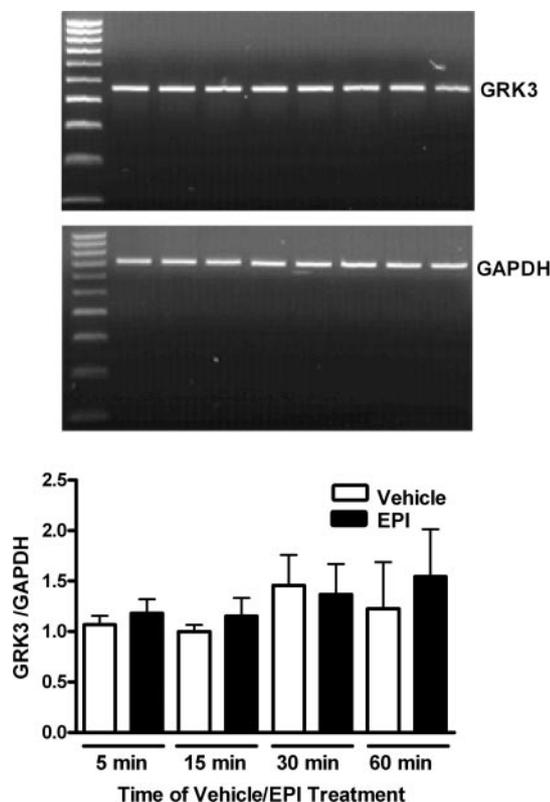
Fig. 4. Quantitation of mRNA levels for GRK3 in NG108 cells exposed to EPI treatment. NG108 cells were treated with vehicle/EPI (0.3 μM) for the indicated times. Then, total RNA isolated from treated cells was reverse transcribed and amplified using primers for rat GRK3 and GAPDH. PCR products were electrophoresed through a 2% agarose gel, stained with 0.5 μg/ml ethidium bromide, and visualized under UV light. A molecular ruler (100–3000 bp) on the left-hand side of the gel is used for reference. Shown is a representative semiquantitative RT-PCR gel depicting GRK3 mRNA expression. Each bar represents mean $\pm$ S.E.M. values for the densitometric quantitation ratio of GRK3 mRNA normalized to GAPDH (internal loading control) obtained from three independent experiments.

Fig. 5. CC does not affect the level of GRK3 mRNA in BE(2)-C or BN17 cells. BE(2)-C (A) and BN17 (B) cells were treated with vehicle/EPI (0.3 μM) for the indicated times in the absence/presence of the PKC inhibitor, CC (10 μM). Then, total RNA isolated from treated cells was reverse transcribed and amplified using primers for human GRK3 and β-actin [BE(2)-C cells, A] or rat GRK3 and GAPDH (BN17 cells, B). PCR products were electrophoresed through a 2% agarose gel, stained with 0.5 μg/ml ethidium bromide, and visualized under UV light. A molecular ruler (100–3000 bp) on the left-hand side of the gel is used for reference. *, significantly different from vehicle at $p < 0.05$. Shown is a representative semiquantitative RT-PCR gel depicting GRK3 mRNA expression. Each bar represents mean $\pm$ S.E.M. values for the densitometric quantitation ratio of GRK3 mRNA normalized to the β-actin or GAPDH mRNA (internal loading control) obtained from three independent experiments.

EPI. Moreover, this transcriptional activation seems to require the simultaneous activation of α_2- and β_2-AR. Finally, we provide evidence that activation of GRK3 transcription by EPI is accompanied by increased nuclear localization of the transcription factors Ap-1 and Sp-2, both of which have been suggested to bind to the putative GRK3 promoter.

The results presented herein provide additional evidence that the expression of GRK3 is regulated independently from GRK2 and that GRK3 expression can be up-regulated without changes in GRK2 expression. We had reported previously that EPI can selectively increase GRK3 protein expression in neuronal cells without changes in the cellular content of GRK2 protein. We now have demonstrated that EPI also selectively increases GRK3 mRNA without increasing GRK2 mRNA in both BE(2)-C and BN17 cells. These results are consistent with those of Hauger and coworkers, who also have reported selective increases in GRK3 mRNA in response to agonist treatment. In Y79 retinoblastoma cells, treatment with CRF increased GRK3 mRNA, whereas there was no change in GRK2 mRNA (Dautzenberg et al., 2002). In the same cell line, this group reported that the pituitary adenylyl cyclase-activating polypeptide receptor type 1 agonist, pituitary adenylyl cyclase-activating polypeptide 38, also produced a selective increase in GRK3 mRNA with no
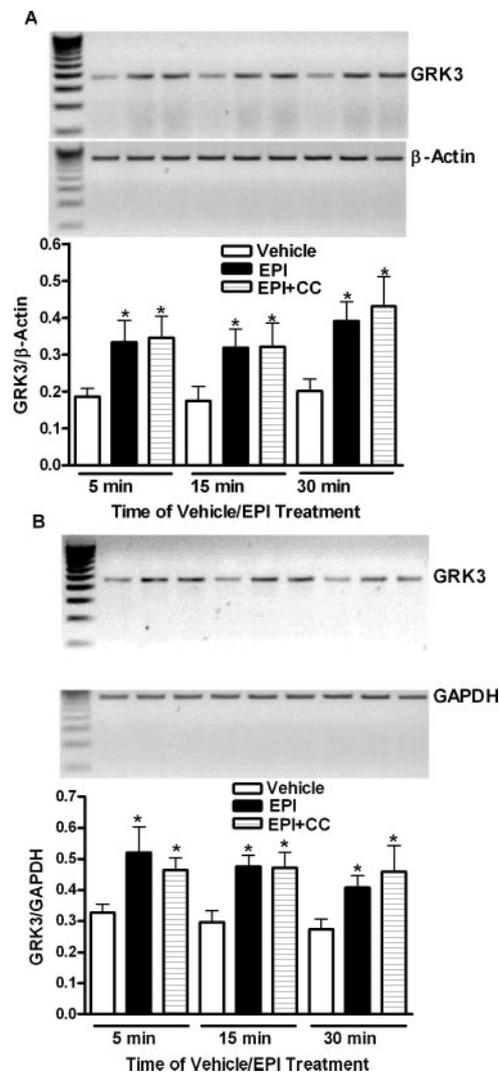
change in GRK2 mRNA (Dautzenberg and Hauger, 2001). Similarly, numerous reports of increases in GRK2 mRNA without increases in GRK3 mRNA have been reported. For example, in ventricular tissue from patients with dilated cardiomyopathy, GRK2 levels were significantly elevated, whereas GRK3 levels were unchanged (Dzimiri et al., 2004). Likewise, GRK2 but not GRK3 mRNA was found to be elevated in peripheral lung tissue samples from patients with cystic fibrosis (Mak et al., 2002). Therefore, several different approaches have provided evidence of the differential regulation of GRK2 versus GRK3 expression in various cell types.

Another objective of the present study was to determine the receptors and the signaling pathways responsible for the
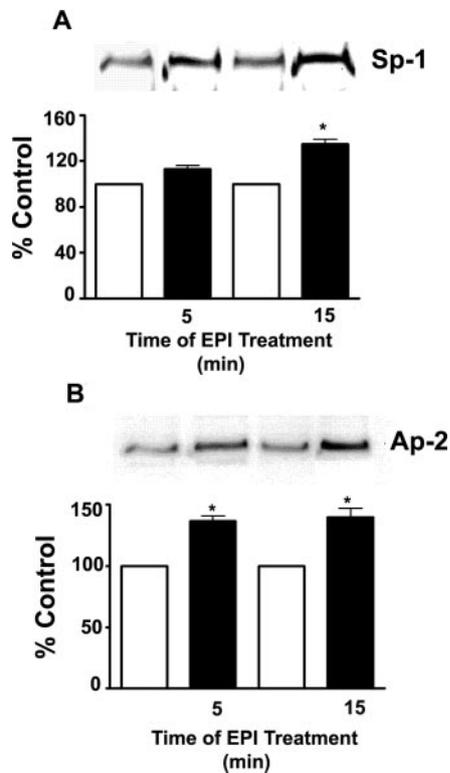
A

B

Fig. 6. EPI treatment causes increased Sp-1 and Ap-2 accumulation in the nuclei of BE(2)-C cells. BE(2)-C cells were treated with EPI (0.3 μM)/vehicle for 5 and 15 min. The treatment was terminated, cells were washed once with PBS, harvested, and processed for nuclear extract preparation using an NE-PER kit. Equal amounts of protein from the nuclear extracts were subjected to SDS-PAGE and western blotting. Sp-1 (A) and Ap-2 (B) proteins were detected using anti-Sp-1 and Ap-2 antibodies, and immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence. *, significantly different from control at $p < 0.05$.

Fig. 7. EPI treatment causes nuclear translocation of Sp-1 and Ap-2 in BE(2)-C cells. Cells were treated with EPI (0.3 μM)/vehicle for 5 and 15 min and processed for immunofluorescence as described under *Materials and Methods*. Samples were incubated with rabbit anti-Sp-1/Ap-2 antibody followed by addition of anti-rabbit Alexa-488. Cells also were stained with DAPI for visualization of cell nuclei. Shown are representative images and overlays of at least five to six fields on the same slide of Sp-1/Ap-2 (visualized by selective Alexa filter, pseudocolored red), DAPI (staining for cell nuclei visualized by selective DAPI filter), and overlay of the two images under an oil immersion objective ($\times$60, 1.4 numerical aperture) on an Olympus 1X81 Fluorescence Deconvolution Microscope System.

EPI-induced increase in GRK3 expression. We had previously observed in both BE(2)-C and BN17 cells that up-regulation of GRK3 protein expression required simultaneous activation of both α_2-AR and β_2-AR. Therefore, we investigated whether this was true for the increase in GRK3 mRNA expression. Pretreatment with either the nonselective α-adrenoceptor antagonist, phentolamine, or the nonselective β-adrenoceptor antagonist, propranolol, was sufficient to eliminate the EPI-induced increase in GRK3 mRNA expression. Our results also indicate that ERK1/2 activation is required for the EPI-induced increase in GRK3 mRNA expression because this increase is eliminated in the presence of the MEK1/2 inhibitor, U0126. These two observations, when combined, suggest the interesting possibility that activation of either α_2- or β_2-ARs alone does not produce sufficient activation of ERK1/2 to increase GRK3 transcription. This is somewhat surprising because in other cells, several investigators have reported β_2-adrenoceptor-dependent activation of ERK1/2 (Schmitt and Stork, 2000). Even α_2-AR-dependent activation of ERK1/2 has been reported (Della Rocca et al., 1997). It is possible that, at the EPI concentrations studied, activation of either receptor individually does not sufficiently activate ERK1/2 to produce an increase in GRK3 mRNA, whereas the summation of the activation by both receptors is sufficient. Although this possibility is not ruled out by our results, one observation suggests that this is unlikely. In NG108 cells, from which BN17 cells are derived
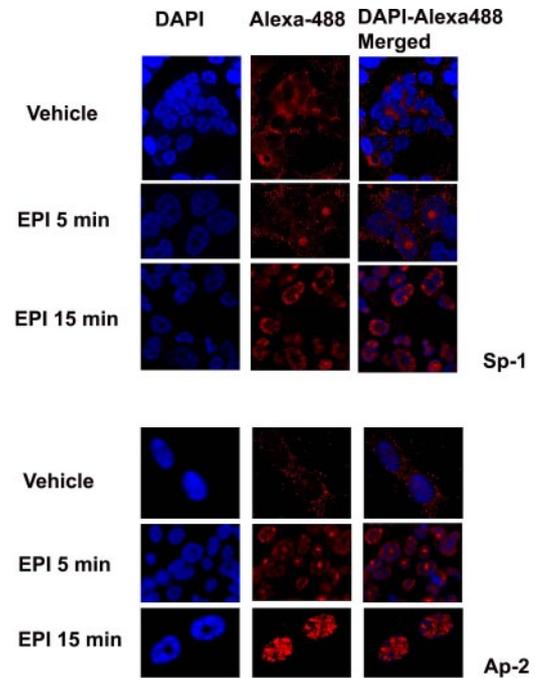
by transfection with the β_2-AR, concentrations of EPI up to 10 μM do not produce an increase in GRK3 mRNA expression. Another possibility is that activation of α_2-AR and β_2-AR simultaneously stimulates a unique signaling pathway not activated by either receptor individually. An example of such signaling has been reported recently for the dopamine D1 and D2 receptors (Lee et al., 2004). When either receptor is activated individually, no intracellular calcium signal is generated, but simultaneous activation of the two receptors results in a PKC-dependent increase in intracellular calcium. Coincidentally, the D1 and D2 receptors, when activated independently, activate G_i/G_o- and G_s-mediated signaling pathways, respectively, neither of which contributes to the G_q-mediated activation of PKC when the D1 and D2 receptors are activated simultaneously. Furthermore, it has been demonstrated that this unique signaling pathway is the result of heterodimerization of the D1 and D2 receptors (Lee et al., 2004). Heterodimerization of α_{2C}-AR and β_2-AR has been reported recently in human embryonic kidney 293 cells (Prinster et al., 2006). However, the relevance of this observation to the present study may be limited due to the significant differences in signaling/trafficking between the α_{2C}-AR and the α_{2A}- and α_{2B}-AR present in our neuronal cells. Future studies will examine whether a similar $\alpha_{2A/B}$-/β_2-AR heterodimer in our neuronal cells contributes to EPI-induced activation of ERK1/2 and increased GRK3 mRNA expression.

A final factor considered in the present investigation is

the identity of the transcription factor(s) that might be activated to increase GRK3 gene expression in response to EPI. Unlike the GRK2 gene, where the promoter region has been cloned and expressed as a promoter/reporter construct in vascular smooth muscle cells, the GRK3 gene promoter has not been characterized. The promoter is proposed to be located in the 2-kb region of DNA just 5′ to the GRK3 gene transcription start site, and within this region are the consensus-binding sites for several transcription factors (Barrett et al., 2003). Given the fact that ERK1/2 activation was required to increase GRK3 mRNA expression, we considered two transcription factors known to be activated by ERK1/2 and whose binding sites are present in the putative GRK3 promoter region, namely Sp-1 and Ap-2. EPI treatment increased Sp-1 and Ap-2 protein accumulation in nuclear extracts. This suggests the possible involvement of Sp-1/Ap-2 in the EPI-induced increase in GRK3 gene expression. An increase in the DNA binding activity of Sp-1 and Ap-2 following ERK1/2 activation was previously reported (Milanini et al., 1998).

The regulation of GRK3 expression may have important implications in bipolar and other affective disorders based upon recent observations. A genome-wide linkage study suggested that the chromosome 22q11 region contained a susceptibility locus for bipolar disorder (BPD) in families of northern European Caucasian ancestry (Lachman et al., 1997; Kelsoe et al., 2001). The gene for GRK3 is found in this region, and sequence analysis identified a single nucleotide polymorphism in the putative promoter region of the GRK3 gene (Barrett et al., 2003). A subset of patients with BPD express this polymorph, and lymphocytic cell lines derived from these BPD patients exhibit a selective decrease in GRK3 expression; the reduced levels of GRK3 were inversely related to the severity of psychosis and mania (Niculescu et al., 2000). A significant increase in GRK3 mRNA in the prefrontal cortex following amphetamine administration in rats also has been reported (Barrett et al., 2003), suggesting that GRK3 plays an important role in the brain's homeostatic response to dopamine and possibly other neurotransmitters. These observations suggest that control of GRK3 expression may have significant implications in mood disorders.

In conclusion, the present study demonstrates that simultaneous activation of α_2 and β_2-adrenoceptors results in an ERK1/2-dependent increase in GRK3 mRNA expression accompanied by the translocation of Sp-1 and Ap-2 to the nucleus. Future studies will examine the possible contribution of receptor heterodimers in the activation of ERK1/2 as well as characterization of the promoter for the GRK3 gene.

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