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Agonist- and Protein Kinase C-Induced Phosphorylation Have Similar Functional Consequences for Gastrin-Releasing Peptide Receptor Signaling via G_q

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ABSTRACT

Acute desensitization of many guanine nucleotide-binding protein-coupled receptors (GPCRs) requires receptor phosphorylation and subsequent binding of an arrestin. GPCRs are substrates for phosphorylation by several classes of kinases. Gastrin-releasing peptide receptor (GRPr) is phosphorylated by a kinase other than protein kinase C (PKC) after exposure to agonist and is also a substrate for PKC-dependent phosphorylation after treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Using GRPr mutants, we examined receptor domains required for agonist- and TPA-induced phosphorylation of GRPr and consequences of these phosphorylation events on GRPr signaling via G_q. Agonist- and TPA-stimulated GRPr phosphorylation in cells require an intact carboxyl terminal domain (CTD). GRPr is phosphorylated in vitro by GPCR kinase 2 (GRK2) and multiple PKC isoforms. An intact DRY motif is required for agonist-stimulated phosphorylation in cells, and agonist-dependent GRK2 phosphorylation in vitro. Although GRPr CTD mutants do not show enhanced in vitro coupling to G_q relative to intact GRPr, CTD mutants have more potent G_q-dependent signaling in cells. Acute desensitization involves CTD-independent processes because desensitization can precede ligand binding in intact GRPr and CTD mutants. TPA-mediated impairment of GRPr-G_q signaling in cells also requires an intact CTD. Similar to GRK2 phosphorylation, PKC phosphorylation reduces GRPr-G_q coupling by approximately 80% in vitro. Arrestin translocation to plasma membrane requires agonist, an intact DRY motif, and GRPr phosphorylation. Therefore, agonist- and PKC-induced GRPr phosphorylation sites are in nearby regions of the receptor, and phosphorylation at both sites has similar functional consequences for G_q signaling.

An activated GPCR catalyzes guanine nucleotide exchange on the α subunit of a heterotrimeric G protein, leading to the formation of Gα-GTP. The subsequent dissociation of the heterotrimeric G protein leads to stimulation of signal transduction cascades mediated by the free Gα-GTP and G$\beta\gamma$ subunits. Mechanisms have evolved that limit the amplitude and/or duration of signal transduction cascades and are collectively referred to as "desensitization". At the molecular level, desensitization may result from the degradation of ligand, changes in receptor availability or activity, or changes in the availability or activity of downstream effector molecules. The most extensively studied GPCRs have been rhodopsin, which signals through transducin (G_t), and the β_2-adrenergic receptor (β_2AR), which signals through G_s. In both cases, rapid agonist-induced receptor phosphorylation, along with subsequent binding of an arrestin to the receptor, play critical roles in receptor deactivation (Krupnick and Benovic, 1998).

Arrestins directly interact with phosphorylated, agonist-occupied GPCRs. Visual arrestin uncouples rhodopsin from transducin and desensitizes rhodopsin signaling. The two mammalian nonvisual arrestins, arrestin2 (β-arrestin1) and

R.A.A. and K.L.I. contributed equally to this work.

ABBREVIATIONS: GPCR, G protein-coupled receptor; β_2AR, β_2-adrenergic receptor; GRPr, gastrin-releasing peptide-preferring receptor; PLCβ, phospholipase C-β; Ins(1,4,5)P_3, inositol-(1,4,5)-trisphosphate; D-Phe^6-697, [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]BN(6–14); PKC, protein kinase C; PKA, protein kinase A; GRK, G protein-coupled receptor kinase; βARK, β-adrenergic receptor kinase; BN, bombesin; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; ME, [D-Phe^6]BN(6-13) methyl ester; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; BSA, bovine serum albumin; DTT, dithiothreitol; RIPA, radioimmunoprecipitation assay; MOPS, 3-(*N*-morpholino)propanesulfonic acid; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; KRH, Krebs-Ringer-HEPES; $[Ca^{2+}]_i$, intracellular calcium concentration; CTD, carboxyl terminal domain; GF 109203X, bisindolylmaleimide I; mAChR, muscarinic acetylcholine receptor.

arrestin3 (β-arrestin2), uncouple GPCRs such as the β_2AR from G proteins and are required for β_2AR desensitization (Krupnick and Benovic, 1998). In addition, recent studies have demonstrated roles for β-arrestins in GPCR internalization and the activation of kinases, including Src and extracellular signal-regulated kinase (Pierce and Lefkowitz, 2001).

Bombesin-like peptides elicit a variety of effects, including mitogenesis, hormone secretion, and modulation of neuron firing rate (Lebacq-Verheyden et al., 1990). These effects are transduced through a family of GPCRs, including the gastrin-releasing peptide receptor (GRPr) (Kroog et al., 1995a). An activated GRPr catalyzes guanine nucleotide exchange on $G\alpha_q$ and $G_{12/13}$ (Hellmich et al., 1997; Sinnett-Smith et al., 2000). $G\alpha_q$-GTP activates phospholipase C-β (PLCβ), which catalyzes hydrolysis of phosphatidylinositol bisphosphate into inositol (1,4,5) trisphosphate [Ins(1,4,5)P_3] and diacylglycerol, second messengers that activate protein kinase C (PKC) and increase the concentration of free intracellular calcium concentration, respectively.

In addition to rhodopsin and the β_2AR, GRPr and other GPCRs are also rapidly phosphorylated after addition of agonist (Kroog et al., 1995b). Several classes of protein kinase have been implicated in GPCR phosphorylation: 1) second messenger-dependent protein kinases A (PKA) and C; 2) second messenger-independent kinases, called G protein-coupled receptor kinases (GRKs), which include rhodopsin kinase (GRK1) and β-adrenergic receptor kinase 1 (GRK2); and 3) casein kinase 1α (Premont et al., 1995; Tobin et al., 1997; Krupnick and Benovic, 1998). Many GPCRs, including GRPr, are known to be substrates for phosphorylation by GRKs in vitro (Krupnick and Benovic, 1998; Kroog et al., 1999). Several are also proven substrates for phosphorylation by second messenger-dependent kinases (Kelleher and Johnson, 1986; Richardson et al., 1992). Most study has focused on the role of GRKs in GPCR signaling, but the relative contributions of each class of kinase may vary under different conditions (Udovichenko et al., 1997). Receptors phosphorylated by different classes of kinases may have distinct affinities for arrestin and unique functions (Lohse et al., 1992; Lefkowitz et al., 2002).

The molecular mechanisms governing acute desensitization of GRPr are not well characterized. GRPr undergoes rapid, agonist-induced desensitization to G_q-mediated signaling (Kroog et al., 1995a) and phorbol ester pretreatment also inhibits GRPr-G_q signaling, although agonist-induced desensitization is not mediated by PKC (Plevin et al., 1990; Walsh et al., 1993). Similarly, GRPr is rapidly phosphorylated in vivo by a protein kinase other than PKC after exposure to bombesin (BN), and GRPr is also a substrate for phosphorylation after activation of PKC by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Kroog et al., 1995b). In vitro, GRPr is a substrate for phosphorylation by GRK2. Both in vitro GRK2-phosphorylated GRPr and agonist-phosphorylated GRPr prepared from intact cells show approximately 75 to 80% reduction in the rate of catalysis of guanine nucleotide exchange on G_q in an in vitro reconstitution assay in the absence of arrestin (Kroog et al., 1999). However, the importance of these findings for GRPr desensitization in cells is unknown.

This study was undertaken with several goals: 1) determine the roles of the DRY motif and putative phosphorylation sites in the carboxyl terminal domain in agonist- and TPA-stimulated GRPr phosphorylation in cells; 2) evaluate GRPr as an in vitro substrate for phosphorylation by PKC; 3) characterize the G_q signaling properties of phosphorylation-deficient GRPr mutants in vivo and in vitro; and 4) determine whether arrestins may play a role in GRPr signaling and agonist- and TPA-dependent desensitization by examining whether GRPr activation and/or phosphorylation stimulate the translocation of arrestin to the plasma membrane.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), G418, penicillin/streptomycin, LipofectAMINE reagent, and 4 to 20% Tris-glycine polyacrylamide gels were from Invitrogen (Carlsbad, CA). BN was from Bachem (Torrance, CA). Ins(1,4,5)P_3 and TPA were purchased from Sigma-Aldrich (St. Louis, MO). Bisindolylmaleimide I (GF 109203X) was from Calbiochem (La Jolla, CA). [D-Phe^6]BN(6-13) methyl ester (ME) was a gift from Dr. David Coy (Tulane University, New Orleans, LA). [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]BN(6-14) (D-Phe^6-697) was a gift from Biomeasure (Milford, MA). ^{125}I-Labeled bombesin (^{125}I-bombesin; 2,200 Ci/mmol), ^{125}I-labeled [Tyr^6]-697 (^{125}I-697; 2,200 Ci/mmol), and ^{125}I-labeled [D-Tyr^6]BN(6-13) methyl ester (^{125}I-ME; 2,200 Ci/mmol) were generous gifts from Sam Mantey and Dr. Robert Jensen, (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). ^{125}I-ME, [γ-^{32}P]ATP (3,000 Ci/mmol), guanosine 5′-*O*-(3-thio)triphosphate ([^{35}S]GTPγS; 1,250 Ci/mmol), and inositol-1,4,5-trisphosphate, D-inositol-1-^{3}H(N) ([^{3}H]Ins(1,4,5)P_3) (15–30 Ci/mmol) were from PerkinElmer Life Sciences (Boston, MA). [^{3}H]Ins(1,4,5)P_3 was also purchased from Amersham Biosciences Inc. (Piscataway, NJ). Enucleated frozen eyes from *Sepia officinalis* were from the National Resource for Cephalopods (Galveston, TX). PKC isoforms were a gift from Dr. Peter Blumberg (National Cancer Institute, Bethesda, MD). PKCα for coupling studies was purchased from Sigma-Aldrich. GRK2 (βARK1) was a gift from Drs. Jeffery Benovic and Tapan Som (Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA). Fresh bovine adrenal glands were bought from Max Insel Cohen, Inc. (Livingston, NJ). The pan-arrestin antibody F4C1 was a gift from Dr. Larry Donoso (Wills Eye Hospital, Philadelphia, PA). β-Arrestin2 (arrestin3) antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and β-arrestin1 (arrestin2) antibody was from BD Transduction Laboratories (San Diego, CA). EGFP-N_1-β-arrestin1 was a gift from Dr. Nigel Bunnett (University of California, San Francisco, San Francisco, CA). β-Arrestin2-GFP was a gift from Dr. Marc Caron (Duke University, Durham, NC).

Cell Culture. Stably transfected Balb/c 3T3 mouse fibroblasts were maintained at 37°C in DMEM containing 300 μg/ml G418, 50 units/ml penicillin, and 50 μg/ml streptomycin supplemented with 10% FBS.

Stable Expression of Receptor Constructs in Balb/c 3T3 Fibroblasts. Cells were transfected with 2 μg of recombinant pcDNA3 plasmid using 6 μl of LipofectAMINE reagent as per the manufacturer's instructions. Selection of clones expressing wild-type and mutant GRPr (Fig. 1) was performed as described previously (Kroog et al., 1995b).

Membrane Preparation and Urea Extraction. Published methods (Hellmich et al., 1997) were used to prepare GRPr containing postnuclear (P2) membranes from cells expressing high levels of GRPr. 7M urea-extracted membranes were prepared as described previously (Kroog et al., 1999).

Quantitation of Ligand Binding Sites. GRPr ligand binding sites in membrane preparations were quantitated by analysis of the displacement of the specific GRPr antagonist ^{125}I-ME by ME or displacement of the GRPr agonist ^{125}I-697 by D-Phe^6-697 as de-

scribed previously for ^{125}I-ME (Kroog et al., 1999). Results were similar with both radioligands (data not shown).

GRPr ligand binding sites in intact cells were quantitated by analysis of the displacement of ^{125}I-ME by ME or displacement of ^{125}I-bombesin by bombesin using previously published protocols (Kroog et al., 1995b, 1998). Results were similar with both protocols (data not shown).

Agonist Binding Progress Curves. Disaggregated cells were resuspended in binding solution (solution B; 98 mM NaCl, 59 mM KCl, 5 mM pyruvate, 6 mM fumarate, 5 mM glutamate, 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) and warmed to 37°C. ^{125}I-Bombesin (250,000 cpm/ml) in a final concentration of 1 nM bombesin was added and aliquots removed at various time points. The aliquots were filtered over GF/F filters presoaked in 3% BSA. Soaking filters in BSA decreased nonspecific binding of radioligands (data not shown). The filters were washed four times with stop solution (solution S; 20 mM Tris, pH 8, 100 mM NaCl, 25 mM $MgCl_2$) and then bound ^{125}I measured with a gamma-counter.

Intact Cell Phosphorylation. GRPr phosphorylation assays in whole cells were performed using published procedures (Kroog et al., 1999). Briefly, cells were incubated for 3 h at 37°C in phosphate-free media with 5% dialyzed FBS and 50 μCi of $^{32}PO_4$ per milliliter (PF media) and then treated as described in the figure legends. Reactions were stopped by removing PF media, washing the cells twice with ice-cold PBS, and adding 0.5% SDS/50 mM Tris, pH 8.0, with 1 mM DTT. Phosphorylated GRPrs were collected by immunoprecipitation overnight in RIPA buffer (0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM Tris-HCl, pH 8, 150 mM NaCl, 10 mM NaF, and 2 mM EDTA) using 5 μl of anti-GRPr serum (antibody 3) and 30 μl of protein A/G. Immune complexes were collected by centrifugation and washed four times with RIPA buffer. Laemmli buffer was added to the complexes to elute protein, and the supernatant run on a 4 to 20% gradient SDS-polyacrylamide gel. X-ray film and a PhosphorImager (Amersham Biosciences) were used for detection and quantitation.

Purification of G Proteins. Cuttlefish (*Sepia officinalis*) retinal $G\alpha_q$ and bovine brain $\beta\gamma$ (brain $\beta\gamma$) were purified using published procedures (Sternweis and Robishaw, 1984; Kroog et al., 1999).

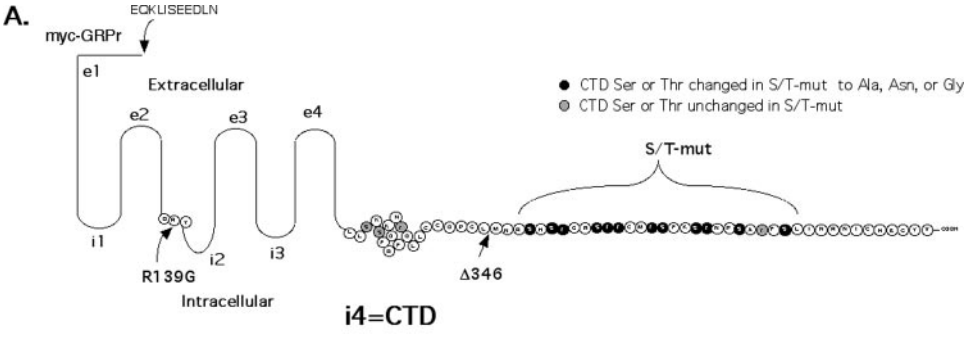
In Vitro Phosphorylation. Samples were incubated in a solution with 20 to 50 mM MOPS, pH 7.5, 3 mM $MgSO_4$, 1 mM EDTA, and 100 μM AEBSF. Other reagents were added as indicated in the text and figures. Reactions were incubated at 30°C for 30 to 45 min and stopped as described in the figure legends unless being used for two-step phosphorylation-coupling assays (see next section). GRPr protein was collected by immunoprecipitation using polyclonal anti-GRPr serum as described previously (Kroog et al., 1999). Immunoprecipitated proteins were resolved on a 4 to 20% Tris-glycine polyacrylamide gel and then incorporation of ^{32}P assessed by exposing dried gels to X-ray film.

In Vitro Coupling Assay (GDP/GTPγS Exchange Assay). The GRPr catalyzed exchange of GDP for GTPγS on $G\alpha_q$ was determined using previously published procedures (Kroog et al., 1999). GTPγS assay solution (solution G) for all experiments contained 50 mM MOPS, pH 7.5, 3 mM $MgSO_4$, 1 mM EDTA, 1 μM GDP, 1 mM thymidine 5′-monophosphate, 0.3% BSA, 1 mM DTT, and 100 mM NaCl. [^{35}S]GTPγS (8–10 nM; 0.01–0.0125 μCi/μl) was added to solution G as indicated. Reaction volumes were 20 to 30 μl. Reaction velocities were determined by incubating samples at 30°C and removing 6-μl aliquots at timed intervals or processing the entire sample at a single time point during the linear portion of the assay. Both methods yielded equivalent results. Aliquots were added to 4 ml of ice-cold solution S to terminate the reaction and then samples processed and radioactivity quantitated as described previously (Hellmich et al., 1997). Determination of background binding was performed for each time point by incubating membranes, $\beta\gamma$, and 1 μM bombesin in solution G without $G\alpha_q$. No difference was found in background, with or without bombesin (data not shown).

For two-step phosphorylation/coupling assays, phosphorylation reaction was performed in solution G (without GDP) supplemented as indicated in the figure. Coupling assay was initiated by adding GRPr-containing membranes (treated with or without PKCα in the phosphorylation assay) directly to the other components without any processing.

Preparation of Ins(1,4,5)P_3 Binding Protein. Ins(1,4,5)P_3 binding protein was prepared using modifications of published procedures (Baukal et al., 1985; Palmer et al., 1989). Fat and connective tissue were dissected away from adrenal glands and then adrenal cortices dissected free from medullary tissue. Adrenal cortices were homogenized with a blender until smooth in ice-cold microsome solution (solution M; 20 mM Tris-HCl, pH 8, 1 mM EGTA, 1 mM DTT, and 100 μM AEBSF). The homogenate was filtered through coarse cheesecloth. The filtrate was further homogenized using a Potter-Elvehjem homogenizer and then centrifuged at 1,000*g* for 10 min at 4°C. The supernatant was centrifuged at 25,000*g* for 30 min at 4°C to pellet microsomes. The resulting pellet was resuspended in solution M, Dounce-homogenized (tight pestle), and centrifuged at 25,000*g* for 30 min at 4°C. The supernatant was aspirated and the pellet resuspended in solution M and recentrifuged at 25,000*g* for 30 min at 4°C. The final pellet was resuspended in solution M, snap-frozen on dry ice, and stored at −70°C until use.

Measurement of Ins(1,4,5)P_3 Mass. Ins(1,4,5)P_3 accumulation was measured using a method based on published procedures (Baukal et al., 1985; Palmer et al., 1989). Transfected Balb fibroblasts were grown to 70 to 90% confluence on 150-mm dishes. Cells were harvested by scraping, disaggregated, and placed into KRH



B.

Construct	Myc epitope	DRY motif	Carboxyl Terminal Domain (CTD)
GRPr	no	DRY	LLSKSFRKQFNTQLLCCQPGLMNRSHSTGRSTTCMTSFKSTNPSATFSLINRNICHEGYV
Myc-GRPr	**yes**	DRY	LLSKSFRKQFNTQLLCCQPGLMNRSHSTGRSTTCMTSFKSTNPSATFSLINRNICHEGYV
Δ346	no	DRY	LLSKSFRKQFNTQLLCCQPGL
S/T-mut	no	DRY	LLSKSFRKQFNTQLLCCQPGLMNR**AHGA**GR**NAA**CM**AAA**FK**GA**N**PAA**TFGLINRNICHEGYV
R139G	no	D**G**Y	LLSKSFRKQFNTQLLCCQPGLMNRSHSTGRSTTCMTSFKSTNPSATFSLINRNICHEGYV

Fig. 1. Wild-type GRPr and mutant GRPr constructs. A, snake-like plot of the murine GRPr, indicating the extracellular (e1–e4) and intracellular domains (i1–i4) and the locations of the myc-epitope (EQKLISEEDLN), DRY motif, and CTD mutations. Arrow at Δ346 indicates the carboxyl terminus of the Δ346 mutant. B, primary sequences for the DRY motif and carboxyl terminal domain in GRPr constructs. Changes from wild-type are shown in bold.

buffer (25 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose, 100 μM AEBSF). Cells were washed twice in KRH, resuspended in KRH supplemented with 0.1% (w/v) BSA at a concentration of 4.5×10^6 cells/ml (high expressors) or 7×10^6 cells/ml (low expressors), and incubated at 37°C for 10 min. Bombesin was warmed to 37°C and 0.5 volumes added to cells to generate the indicated final bombesin concentrations. Aliquots (300 μl) were removed at the times specified, lysed with 0.2 volumes of ice-cold 20% (v/v) $HClO_4$, and then incubated on ice for 20 min. Samples were centrifuged at 2,000*g* at 4°C for 25 min to pellet insoluble material. The supernatants were neutralized with ice-cold 5 M or 1.5 M KOH, and then the $KClO_4$ precipitate was removed by centrifugation. The supernatants were frozen and stored at −70°C until use.

Ins(1,4,5)P_3 was measured using a radioreceptor assay. Fifty-five microliters of each sample was assayed in a final volume of 320 μl in Ins(1,4,5)P_3 assay solution (solution I; 47 mM Tris-HCl, pH 7.5, 18.75 mM NaCl, 94 mM KCl, 0.94 mM EDTA, 0.94 mg/ml BSA, 0.38 mM 2,3 diphosphoglycerate) with 75 μl of binding protein and ~6,000 cpm of [^{3}H]Ins(1,4,5)P_3 per tube. Samples were vortexed and then placed on ice for 8 min and centrifuged at maximum speed at 4°C for 3 min to pellet the binding protein. After removal of the supernatant, samples were placed at room temperature, 1 ml of 2% SDS was added to denature the proteins, and bound radioactivity measured using liquid scintillation. Ins(1,4,5)P_3 mass was calculated based on a standard curve generated using nonradioactive Ins(1,4,5)P_3 assayed simultaneously with the experimental samples. To achieve the same assay conditions for the standards as for the samples, the standards were supplemented with 55 μl of a mock cell lysate solution prepared in a similar manner to the experimental samples, but without any cells added to KRH + BSA. Nonspecific binding was determined in the presence of 4 μM Ins(1,4,5)P_3.

Western Blotting. Samples were resolved on 4 to 20% or 10% Tris-glycine polyacrylamide gels and transferred overnight onto nitrocellulose. Western blot analysis was performed using F4C1 at a dilution of 1:250 or anti-GRPr serum (antibody 3) at a dilution of 1:300 using published methods (Kroog et al., 1995b). The anti-GRPr serum can recognize all GRPr constructs (Fig. 2, B and D). Western blotting with β-arrestin1 (arrestin2) antibody at a dilution of 1:250 and β-arrestin2 (arrestin3) antibody at a dilution of 1:100 was performed per the manufacturers' instructions. Quantification was done using a Kodak ImageStation with Kodak 1D software.

Confocal Microscopy. Stably transfected Balb 3T3 fibroblasts (expressing high levels of GRPr constructs) were plated in MatTek glass-bottomed 35-mm dishes at 150 to 200,000 cells/dish. The next day, each dish was transfected with 0.1 to 1 μg of EGFP-N_1-β-arrestin1 (arr2) or 1 μg β-arrestin2-GFP (arr3) plasmid using 6 μl of LipofectAMINE reagent as per the manufacturer's instructions. Twenty-four hours after transfection, the medium was removed and solution B added. Bombesin was added to achieve a final concentration of 100 nM or 1 μM as indicated and cells imaged in real time on the stage of a Radiance 2000 laser scanning confocal microscope

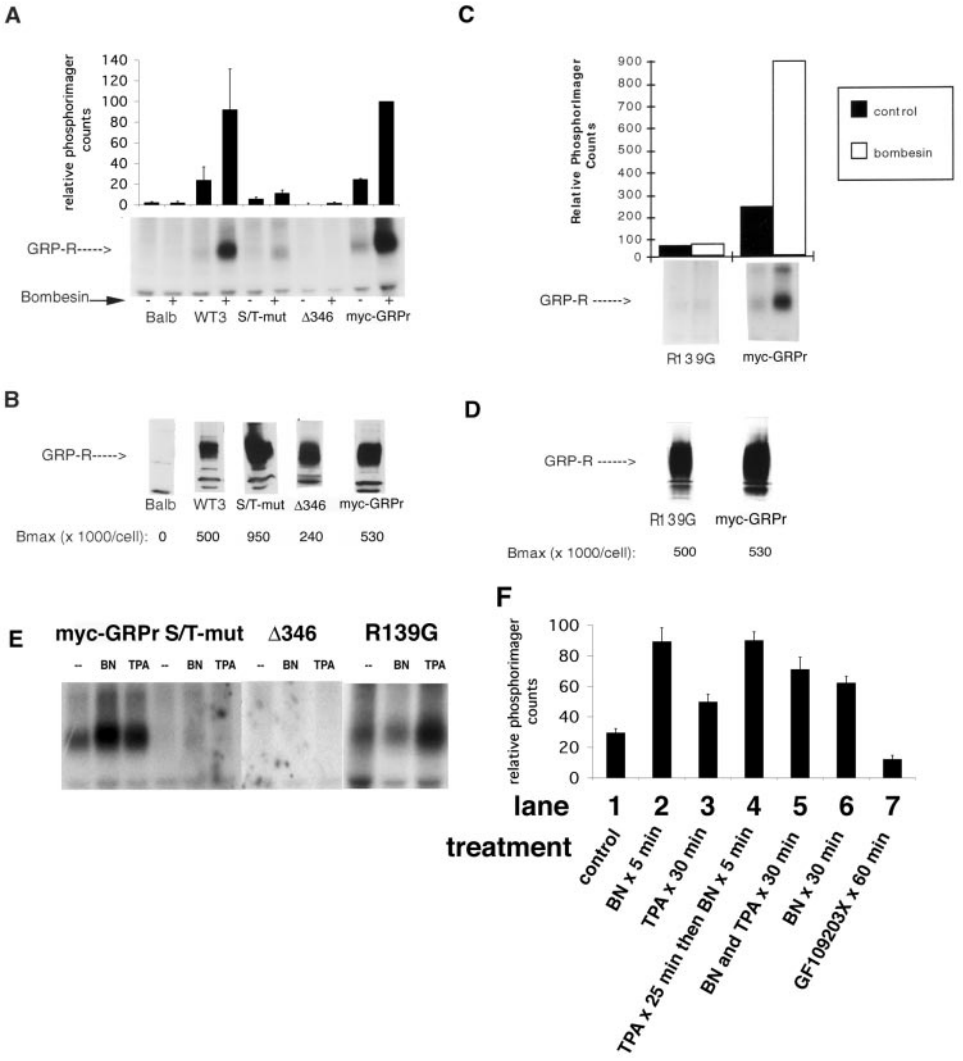


Fig. 2. Molecular requirements for agonist- and TPA-induced GRPr phosphorylation in cells. A, C, and E, phosphorylation assay, cells were labeled for 3 h with PO_4-free DMEM with 5% dialyzed fetal calf serum and 50 μCi/μl $^{32}PO_4$. The following cell lines were stimulated with or without 100 nM BN for 5 min or 100 nM TPA for 25 min: Balb fibroblasts (Balb) or stably transfected Balb fibroblasts expressing wild-type (WT3), myc-tagged wild-type (myc-GRPr), or mutant GRPrs (S/T-mut, Δ346, or R139G) as described in the text. Immunoprecipitation of GRPr was performed as described under *Materials and Methods*. X-ray film and a PhosphorImager were used for detection and quantitation of phospho-GRPr. Graph in A is the mean ± S.E.M. of three to seven experiments with the value for myc-GRPr + 100 nM bombesin set at 100 in each experiment. F, phosphorylation assay was performed with myc-GRPr cells as described above. Cells were stimulated with 100 nM BN, 300 nM TPA, or 1.75 μM GF 109203X as indicated. Graph (using arbitrary units) is the mean ± S.E.M. of five experiments for lanes 1 to 6 and three experiments for lane 7. B and D, immunoblotting was performed as described under *Materials and Methods* using an anti-GRPr serum at a dilution of 1:300 to analyze the relative level of wild-type and mutant GRPr. In B, two separate blots were used. B_{max} was determined in a separate experiment using whole cell equilibrium binding with a pure receptor antagonist (ME) as described under *Materials and Methods*.

(Bio-Rad, Hercules, CA) preheated to 37°C. The fluorescence signal was recorded at a scanning rate of 166 lines/s with a Kr/Ar laser for excitation at 488 and 568 nm. The optics was a Nikon Eclipse 200 with a 60× numerical aperture 1.4 PlanApo objective. In cells with an intact GRPr, bombesin added to a final concentration of 100 nM or 1 μM elicited similar arrestin translocation (data not shown).

Calcium Imaging. Real-time recording of $[Ca^{2+}]_i$ was performed in single transfected Balb cells using published procedures (Hellmich et al., 1999) in the presence of 2 mM extracellular Ca^{2+} or 1 mM EGTA as described in the text and figures. Other reagents were added as indicated. Briefly, cells were plated on glass coverslips, loaded with 2 μM fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) and incubated in KRH buffer plus 0.1% BSA. Cells were imaged using a Diaphot inverted microscope (Nikon, Garden City, NY) and the microscope coupled to a dual monochromater system via a fiber optic cable (Photon Technology International, South Brunswick, NJ). Fluorescence was detected using an intensified charged coupled device camera (Dage-MTI, Michigan City, IN) and the images processed using ImageMaster software from Photon Technology International. Data are reported as the 340/380-nm ratio.

Results

Molecular Requirements for Agonist- and TPA-Induced GRPr Phosphorylation in Cells. To study the molecular mechanisms of GRPr acute desensitization, we first examined whether an intact carboxyl terminal domain (CTD) and an intact DRY motif (near the cytoplasmic end of the third transmembrane helix) of the GRPr are required for agonist- and PKC-induced GRPr phosphorylation. We used stably transfected Balb/c 3T3 mouse fibroblast cell lines expressing one of several murine GRPr constructs at high levels (Fig. 1). Wild-type GRPr is expressed at ~500,000 receptors/cell in WT3. Wild-type GRPr with an amino terminal myc-epitope tag is expressed at ~530,000 receptors/cell in myc-GRPr (referred to as 5′ET4 in previous publications; Kroog et al., 1995b, 1999). myc-tagged GRPr performs similarly to wild-type in all assays to date (Kroog et al., 1995b). These cell lines were compared with other cell lines expressing mutant GRPr constructs (also at high levels) that showed severely impaired agonist-induced receptor internalization (Benya et al., 1993; Benya et al., 1994) and agonist-induced GRPr down-regulation and long-term desensitization (Benya et al., 1995). CTD truncation mutant Δ346 (referred to as T^{346} in previous publications) deletes all residues in the distal CTD (after Leu^{345}) and is expressed at ~240,000 receptors/cell in cell line Δ346. CTD mutant S/T-mut (referred to as JF1 in previous publications) has most Ser and Thr residues in the distal CTD converted to residues, which are not substrates for phosphorylation and is expressed at ~950,000 receptors/cell in cell line S/T-mut. Sequencing analysis of this construct from the S/T-mut cell line determined that it eliminated 12 of 13 Ser and Thr residues distal to Cys^{341}, with only Thr^{371} remaining (data not shown). R139G is a point mutant with Arg^{139} changed to Gly and is expressed at ~500,000 receptors/cell in cell line R139G. This mutation alters the highly conserved (D/E)R(Y/W) motif in rhodopsin-like G protein-coupled receptors (such as GRPr). Residues in the ERY motif of rhodopsin participate in several hydrogen bonds with surrounding residues (Palczewski et al., 2000). In GRPr, the DRY motif is required for normal coupling to heterotrimeric G proteins (Benya et al., 1994).

Figure 2 shows the agonist- and PKC-induced phosphorylation of GRPr in intact cells. To stimulate PKC-regulated phosphorylation of GRPr, we used the PKC activator TPA because TPA-induced GRPr phosphorylation is prevented by pretreatment with a PKC inhibitor (Kroog et al., 1995b). Both CTD mutants (Fig. 2, A and E) and R139G (Fig. 2, C and E) are markedly impaired in agonist-induced GRPr phosphorylation. PKC-stimulated phosphorylation in cells proceeds only in receptors with an intact CTD but does not require the ability to couple to G proteins because R139G can undergo TPA-stimulated phosphorylation (Fig. 2E). Bombesin- and TPA-stimulated phosphorylation are mutually inhibitory because the combination of TPA and bombesin does not increase the level of phosphorylation above that of bombesin alone (Fig. 2F, compare lanes 2 and 4 or lanes 5 and 6). Most of GRPr phosphorylation at baseline is probably caused by PKC or a kinase regulated by PKC, because bisindolylmaleimide I (GF 109203X, a PKC inhibitor) eliminates most of the basal phosphorylation (Fig. 2F, compare lanes 1 and 7).

We also examined GRPr phosphorylation in vitro to provide further evidence that TPA-stimulated GRPr phosphorylation is mediated by PKC and to explore the reason uncoupled receptor R139G does not undergo agonist-induced phosphorylation. For these experiments, urea-extracted GRPr-containing membranes were incubated in vitro with various kinases and cofactors. Using this system, we previously determined that urea-extracted membranes have no intrinsic GRPr kinase activity and that GRPr is a substrate for phosphorylation in vitro by the G protein-coupled receptor kinase GRK2 in an agonist- and G$\beta\gamma$-dependent manner (Kroog et al., 1999). In contrast, R139G is not a substrate for phosphorylation by GRK2 in vitro (Fig. 3C), suggesting that R139G is unable to interact normally with this kinase. We also found that multiple PKC isozymes stimulate phosphorylation of a broad 70- to 90-kDa band with similar shape and mobility to the one phosphorylated by GRK2 (Fig. 3, compare A and B, with C). To observe phosphorylation of this band, GRPr must be included in the kinase assay and not just added subsequently to the immunoprecipitation (data not shown). Therefore, the 70-to 90-kDa band represents PKC-mediated GRPr phosphorylation, not PKC autophosphorylation.

Carboxyl-Terminal Domain Mutants Are Not Intrinsically More Active than Wild-Type GRPr. To determine whether the CTD is involved in acute desensitization, we compared the intrinsic rate of guanine nucleotide exchange on $G\alpha_q$ catalyzed by the myc-tagged wild-type and CTD mutant GRPr constructs. Differences in acute desensitization can only be inferred from experiments on downstream signaling pathways if the intrinsic catalytic activity of different receptor constructs is similar. 7M urea-extracted membranes were prepared from myc-GRPr, S/T-mut, and Δ346 cells to provide GRPr constructs in a biologically relevant membrane environment (without associated functional G proteins). Receptor-containing membranes were combined in vitro with [^{35}S]GTPγS, purified cuttlefish retinal $G\alpha_q$, and bovine brain G$\beta\gamma$, with or without 1 μM bombesin. S/T-mut (Fig. 4, A and C) and Δ346-low (Figs. 4, B and C) did not show enhanced coupling relative to myc-GRPr either in the absence or presence of bombesin in vitro (and seemed to be somewhat less active). Therefore, neither CTD mutant is constitutively active (in the absence of agonist) or intrinsically more active (in the presence of agonist) than wild type. Any enhancement in signaling seen with CTD mutants in intact cells must be

caused by the presence of regulatory molecules that are absent in the in vitro assay.

Agonist-Induced Acute Desensitization of Inositol Trisphosphate Generation Involves CTD-Dependent and -Independent Processes. PLCβ catalyzed generation of inositol Ins(1,4,5)P_3 and diacylglycerol from phosphatidylinositol bisphosphate is one of the earliest events in G_q signaling. To study the molecular mechanisms of acute desensitization of GRPr-G_q signaling in intact cells, we attempted to generate stably transfected Balb/c 3T3 mouse fibroblast cell lines expressing equal numbers of GRPr constructs at much lower levels than those used for the phosphorylation studies. wt-low expresses the wild-type GRPr at 93,000 ± 15,000 (mean ± S.E) receptors/cell. Δ346-low expresses the truncation mutant Δ346 at 82,000 ± 14,000 receptors/cell. S/T-mut-low expresses the phosphorylation-deficient mutant S/T-mut at 65,000 ± 12,000 receptors/cell. wt-low undergoes agonist-induced phosphorylation to a level commensurate with its level of GRPr expression (data not shown). Because total inositol phosphate generation stimulated by bombesin is very sensitive to receptor expression (Tsuda et al., 1997), we compared wt-low with Δ346-low (which expresses a more similar number of receptors/cell). In contrast to the diminished catalytic activity seen in vitro with Δ346, bombesin-stimulated Ins(1,4,5)P_3 generation has a greater amplitude and duration in Δ346-low than in wt-low after stimulation with saturating bombesin (Fig. 5A). As has been seen with endogenous GRPr in Swiss 3T3 fibroblasts (Plevin et al., 1990), the response peaked rapidly (within 12–18 s) and then returned toward baseline, even in the continued presence of agonist. The shape of the response curve is similar in both cell lines, but the response is enhanced in Δ346-low. The rapid decrease in Ins(1,4,5)P_3 mass in both cell lines suggests that there are at least two components to the desensitization: a CTD-dependent process affecting the overall amplitude of the response and a CTD-independent process modulating the shape [and at least partially responsible for the rapid decline in Ins(1,4,5)P_3 mass]. To examine the CTD-independent process further, we stimulated the high-expressing myc-GRPr, Δ346, and S/T-mut cell lines with 1 nM bombesin and compared the Ins(1,4,5)P_3 mass to the on-rate for binding of 1 nM bombesin to these receptors. Because these cells express different numbers of receptors per cell, we cannot directly compare the amplitude of the Ins(1,4,5)P_3 response between cells. However, when normalized for receptor number, the CTD mutants had a larger amplitude and duration of response than myc-GRPr (data not shown). The shape of the response curve is similar between the high expressors stimulated with 1 nM bombesin and the low expressors stimulated with 100 nM bombesin (Fig. 5, B–D). In all high-expressing cells, the major decrease in Ins(1,4,5)P_3 mass not only occurs in the presence of continued bombesin (as in experiments with saturating bombesin) but also precedes the binding of ~70% of bombesin to receptors, indicating that this component of acute desensitization is able to prevent signaling from unoccupied receptors *before* they have bound ligand. After the addition of 1 nM bombesin to wt-low and Δ346-low, the CTD-dependent difference in amplitude is still present (Fig. 5E), but the response in wt-low continues to slowly increase and more closely resembles the binding on-rate (Fig. 5E, inset).

GRPr CTD Mutants Have Enhanced Ca^{2+} Mobilization at Low Bombesin Concentrations. Because Ins(1,4,5)P_3 stimulates release of Ca^{2+} from intracellular stores, we evaluated Ca^{2+} mobilization in the low-expressing cells. Experiments were initially performed in the presence of extracellular Ca^{2+}. Full dose-response curves showed similar increases in $[Ca^{2+}]_i$ in wt-low and Δ346-low after addition of high concentrations of bombesin, but an increased amplitude and duration in $[Ca^{2+}]_i$ in Δ346-low relative to wt-low after addition of lower bombesin concentrations (Fig. 6A). The peak response to bombesin differed starting at concentrations less than 1 nM (Fig. 6B), and because of the change in the duration of the response, the total area under the $[Ca^{2+}]_i$ curve was much greater in Δ346-low than wt-low at sub-nanomolar concentrations (Fig. 6C). Under these assay condi-

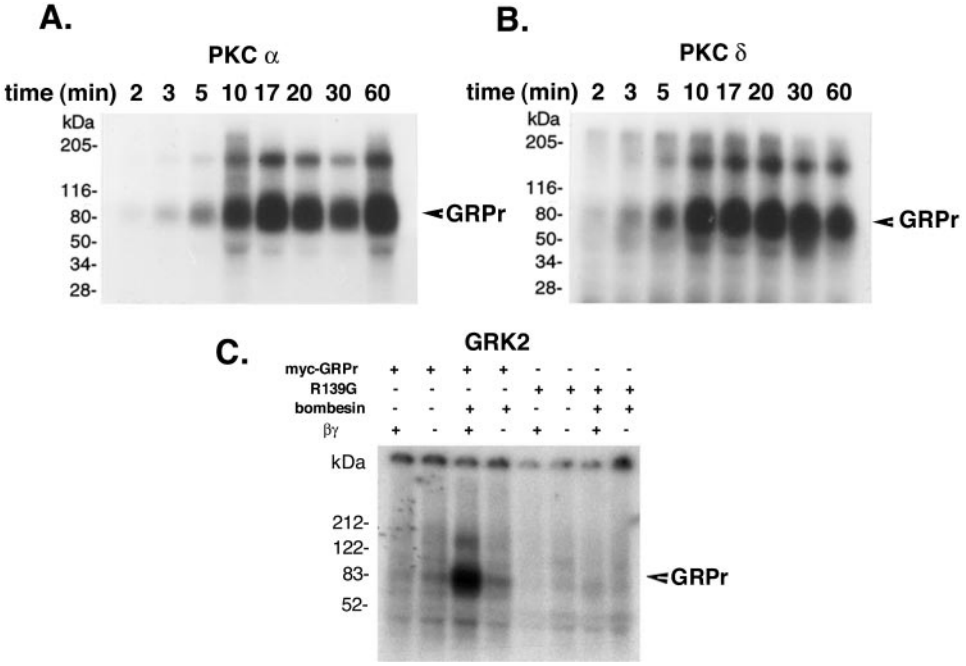


Fig. 3. In vitro phosphorylation of GRPr and R139G. Urea (7 M)-extracted myc-GRPr and R139G membranes were prepared and receptor number determined as described under *Materials and Methods*. A and B, 20 pM myc-GRPr membranes were incubated at 30°C with 10 μM PKC α (A) or δ (B) in a reaction mixture containing 50 mM HEPES, pH 7.4, 1 mM EDTA, 7.5 mM $MgCl_2$, 10 μM ATP, 0.1 μCi of [^{32}P]ATP, 100 nM TPA, and 10 mM NaF. At the indicated times, an aliquot was removed and the reaction stopped by the addition of 300 μl of RIPA buffer. Immunoprecipitation was performed as described under *Materials and Methods*. C, 7.2 nM myc-GRPr or R139G membranes was combined with 100 nM GRK2, 1 mM thymidine 5′-monophosphate, 100 μM ATP, 0.3 μCi/μl [^{32}P]ATP, 20 mM MOPS, pH 7.5, 1 mM EDTA, 3 mM $MgSO_4$, 100 μM AEBSF with (+) or without (−) 1 μM bombesin and 100 nM bovine brain $\beta\gamma$ ($\beta\gamma$). Samples were incubated at 30°C for 45 min and the reactions stopped with 2.5% SDS, 50 mM Tris, pH 8. Immunoprecipitation was performed as described under *Materials and Methods*.

tions, there is Ca^{2+}-dependent Ca^{2+} influx that provides a second component of Ca^{2+} mobilization and is referred to as capacitative Ca^{2+} entry (Putney et al., 2001). To remove the capacitative Ca^{2+} response and only examine the $Ins(1,4,5)P_3$-stimulated increase in $[Ca^{2+}]_i$, we performed experiments under Ca^{2+}-free conditions (with extracellular EGTA and without extracellular Ca^{2+}). Without extracellular Ca^{2+}, both CTD mutants demonstrated enhanced bombesin-stimulated Ca^{2+} mobilization relative to wild-type GRPr at several doses of bombesin (Fig. 6D). To measure the size of the residual intracellular Ca^{2+} pool after addition of saturating bombesin, we added the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin after bombesin. Experiments (with wt-low and Δ346-low) showed that <20% of the intracellular Ca^{2+} was still thapsigargin-sensitive 2 min after stimulation with 100 nM bombesin (data not shown). This suggests that 100 nM bombesin empties the intracellular Ca^{2+} pools in both cell lines and may explain why the increases in $[Ca^{2+}]_i$ in both cell lines are similar after addition of high concentrations of bombesin.

PKC-Induced Inhibition of GRPr Signaling Requires an Intact CTD. Activation of PKC by phorbol ester before addition of bombesin inhibits bombesin-stimulated $Ins(1,4,5)P_3$ generation and Ca^{2+} mobilization in Swiss 3T3 fibroblasts (Plevin et al., 1990; Walsh et al., 1993). However, PKC stimulated inhibition of GRPr signaling does not involve receptor down-regulation because short-term activation of PKC (for ≥1 h) does not stimulate GRPr internalization in either Swiss 3T3 fibroblasts (Brown et al., 1987) or myc-GRPr cells (data not shown) in the absence of agonist. TPA pretreatment does not alter the affinity of GRPr for agonist in Swiss 3T3 cells (Brown et al., 1987) or myc-GRPr cells (data not shown). Because in vitro GRK2-mediated GRPr phosphorylation and in vivo agonist-induced phosphor-

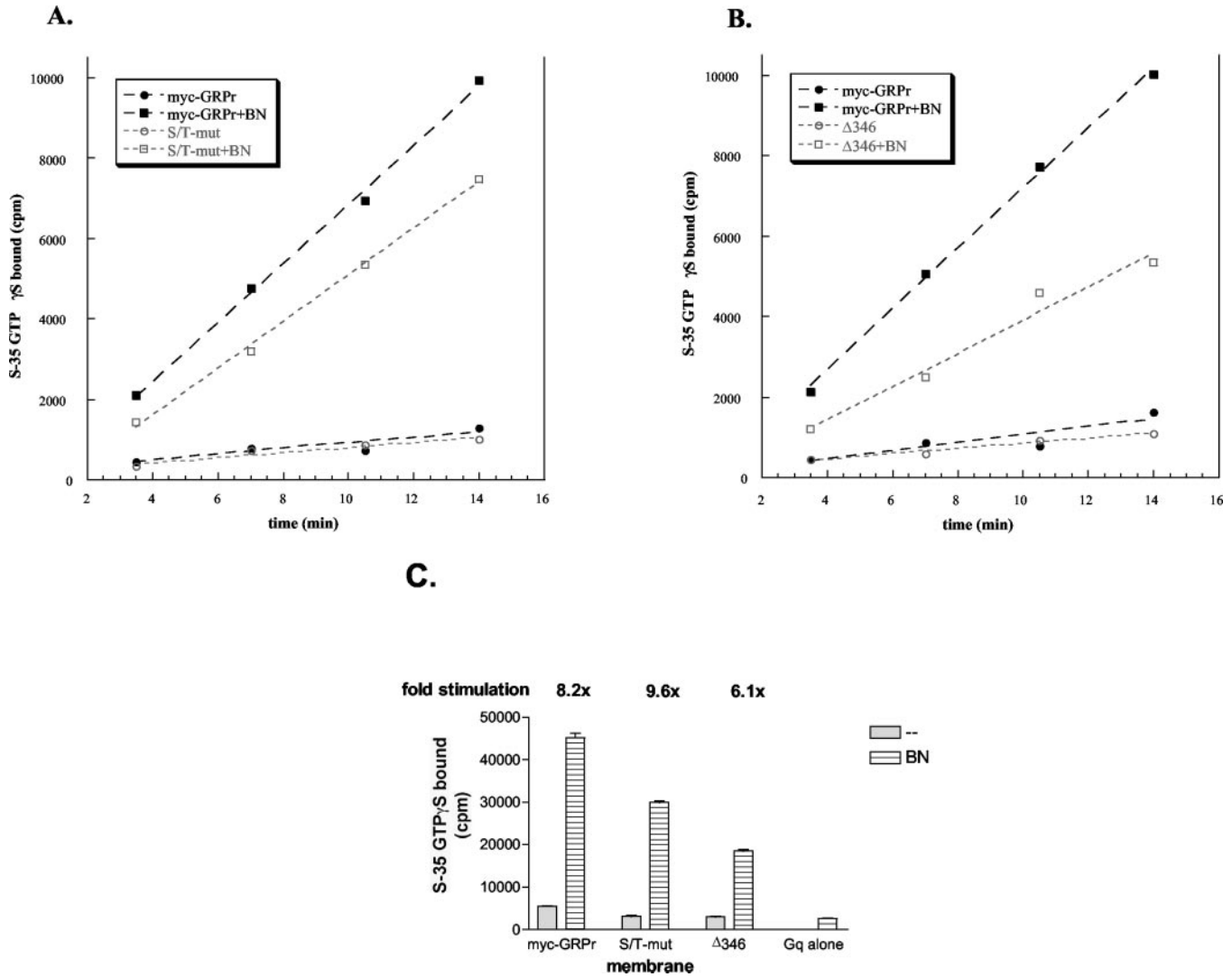


Fig. 4. In vitro coupling of GRPr and GRPr CTD mutants: phosphorylation-deficient mutants are not constitutively active or more potent than wild-type GRPr. 7M urea-extracted myc-GRPr, S/T-mut, and Δ346 membranes were prepared and receptor number determined as described under *Materials and Methods*. For each receptor, 1.6 nM was combined in solution G with 8 nM (0.01 μCi/μl) [^{35}S]GTPγS, 90 nM $G\alpha_q$ and 100 nM (A and B), or 90 nM (C) bovine brain βγ. A and B, myc-GRPr (●), S/T-mut (○, □ in A), and Δ346 (○, □ in B) membranes were incubated at 30°C with (■, □) or without (●, ○) 1 μM bombesin. At the indicated times, aliquots were processed as described under *Materials and Methods*. Curves are best linear fit. C, membranes were incubated in solution G as in A and B with (striped columns) or without (solid columns) 1 μM bombesin at 30°C. Binding reactions proceeded for 15 min. Results are the mean ± S.D. from an experiment performed in triplicate. Data are presented with a background subtracted from each time point consisting of membranes, βγ, and 1 μM bombesin in solution G without $G\alpha_q$.

ylation both reduce GRPr-stimulated catalysis of guanine nucleotide exchange on G_q by approximately ~75% (in the absence of arrestin) (Kroog et al., 1999), PKC-mediated inhibition of GRPr signaling may also involve phosphorylation-induced uncoupling. PKC can phosphorylate GRPr in vitro (Fig. 3), and TPA-induced GRPr phosphorylation in cells requires an intact CTD. However, because agonist and TPA stimulate GRPr phosphorylation at distinct sites (Williams et al., 1996), agonist- and PKC-induced phosphorylation may have different functional consequences. To evaluate the mechanism for TPA-mediated inhibition of GRPr-catalyzed G_q signaling, we examined the effect of PKC pretreatment on in vitro GRPr coupling to G_q. PKCα pretreatment reduced myc-GRPr catalyzed guanine nucleotide exchange on G_q in vitro by approximately 80%, but only reduced S/T-mut coupling by ~10% (Fig. 7A). This suggests that in vitro PKC-induced GRPr phosphorylation has a similar effect on coupling to G_q as does phosphorylation induced in vitro by GRK2 or in vivo by agonist. Consistent with the requirement for an intact CTD for PKC-mediated reduction in the rate of guanine nucleotide exchange on G_q, TPA pretreatment also inhibits bombesin-stimulated Ins(1,4,5)P_3 generation in wt-low to a greater degree than in Δ346-low. Peak response in the presence and absence of TPA pretreatment occurred 12 to 24 s after addition of 100 nM bombesin (Fig. 7B). TPA inhibited wt-low by ~80% and Δ346-low by only ~23% in this period (Fig. 7C). TPA pretreatment also inhibited the bombesin-stimulated increase in $[Ca^{2+}]_i$ to a similar degree. The area under the Ca^{2+} curve was inhibited ~77% in wt-low, but only 6% in Δ346-low and 19% in S/T-mut-low (Fig. 7D). Similar results were seen with other clones expressing low levels of wild-type GRPr and Δ346 (data not shown).

Bombesin-Induced Arrestin Translocation by GRPr Requires Both Receptor Phosphorylation and an Intact DRY Motif. Arrestins are required for acute desensitization of certain types of G protein signaling, internalization of GPCRs, and stimulation of GPCR signaling by acting as adapter proteins. To ascertain whether arrestins might be

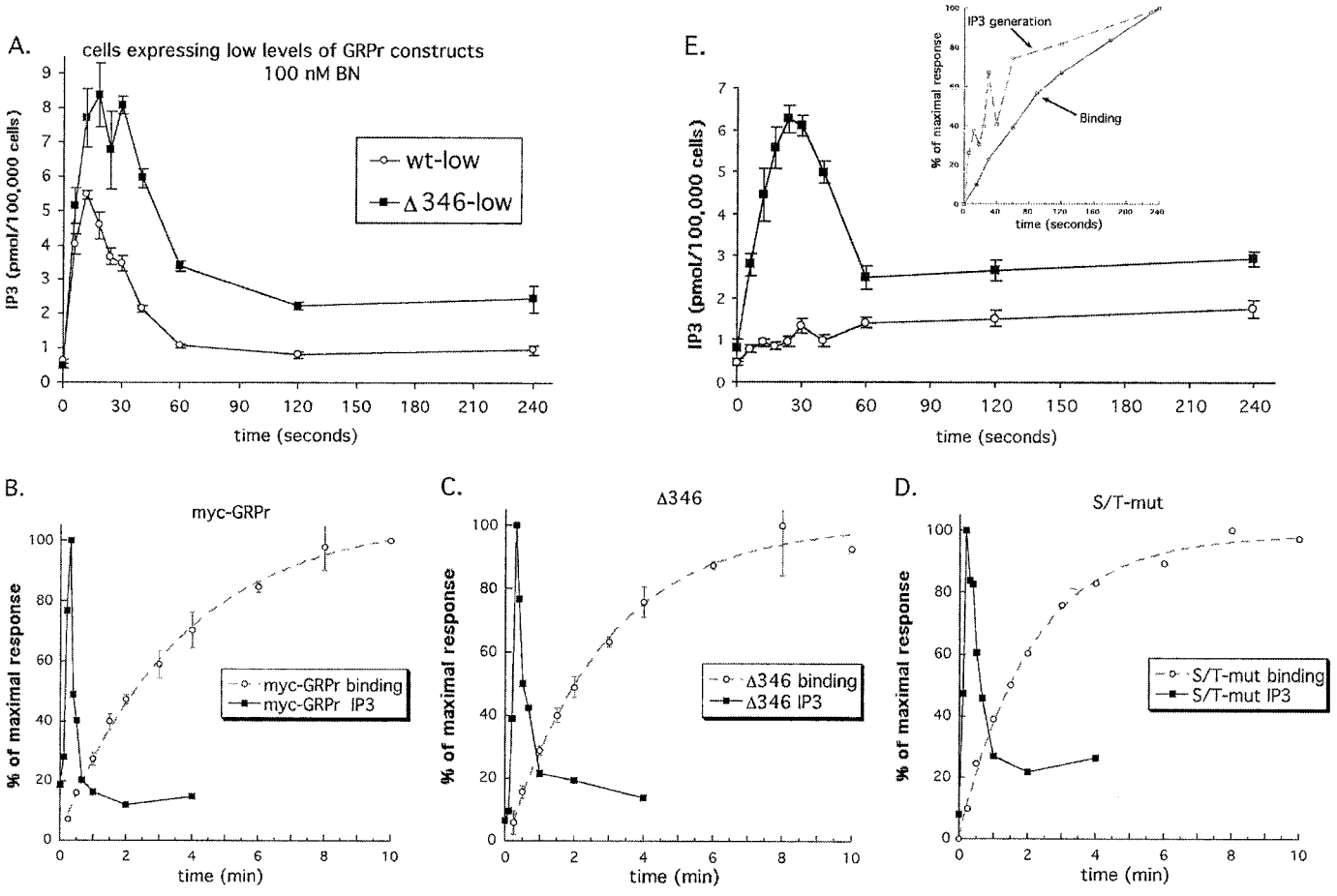


Fig. 5. Inositol trisphosphate generation in response to bombesin in cells expressing low or high levels of GRPr constructs and comparison with binding time course. A and E, wt-low (○) and Δ346-low (■) cells were grown on 150-mm dishes and then scraped, disaggregated, and placed into KRH buffer. The cells were warmed to 37°C and bombesin added to a final concentration of 100 nM (A) or 1 nM (B–E). At the indicated times, an aliquot of cells was removed and the cells lysed with 20% (v/v) $HClO_4$. Insoluble material was removed and the samples neutralized as described under *Materials and Methods*. The next day, an Ins(1,4,5)P_3 radioreceptor assay was performed using a crude Ins(1,4,5)P_3 receptor preparation from bovine adrenal cortex and [^{3}H]Ins(1,4,5)P_3 as described under *Materials and Methods*. Samples were counted by liquid scintillation and Ins(1,4,5)P_3 mass determined. The Ins(1,4,5)P_3 values in A and E are the average ± S.E.M of 6 to 10 data points generated from three to four separate experiments with each value determined in duplicate. B–D, Ins(1,4,5)P_3 assay was performed as in A and E using high-expressing myc-GRPr, Δ346, and S/T-mut cells. The Ins(1,4,5)P_3 experiments shown are representative of at least four experiments (B and C) or two experiments (D). Ins(1,4,5)P_3 values are from duplicate determinations and are expressed as percentage of maximal response. Agonist binding progress curves were performed with 1 nM bombesin with 250,000 cpm/ml of ^{125}I-bombesin as described under *Materials and Methods*. Data are expressed as percentage of maximum binding (mean ± S.D.) from three similar experiments (B and C) or mean of two similar experiments (D). E, inset, comparison of 1 nM bombesin binding progress curve of myc-GRPr with 1 nM bombesin Ins(1,4,5)P_3 generation from wt-low. Data are shown as percentage of maximal response at 4 min.

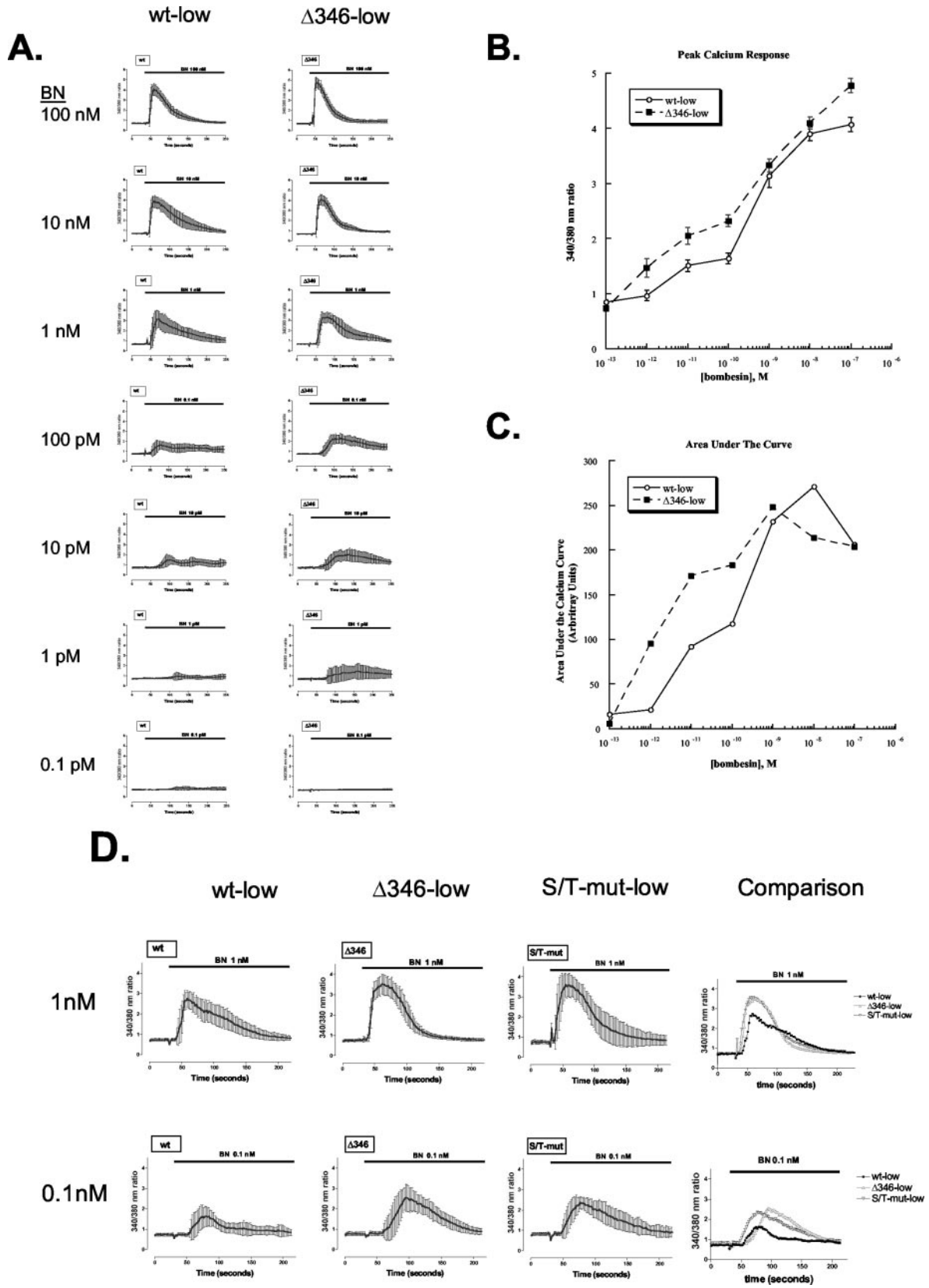


Fig. 6. Calcium mobilization in response to bombesin in the presence or absence of extracellular Ca^{2+}: GRPr carboxyl terminal domain mutants have enhanced Ca^{2+} mobilization at low bombesin concentration. A and D, real-time recording of $[Ca^{2+}]_i$ was performed in single wt-low, Δ346-low, and S/T-mut-low cells loaded with 2 μM fura-2 acetoxymethyl ester as described under *Materials and Methods* in the presence of 2 mM extracellular Ca^{2+} (A–C) or 1 mM EGTA and no extracellular Ca^{2+}(D). KRH buffer containing various concentrations of bombesin from 100 nM to 0.1 pM were added at the indicated times. Data represent the average ± S.D. for 40 individual cells per condition and are representative of three similar experiments (A) or a single experiment in duplicate (D). B, peak Ca^{2+} mobilized at each dose of BN in A. C, total Ca^{2+} mobilized at each dose of BN in A as measured by the area under the Ca^{2+} curve.

involved in acute desensitization of GRPr signaling and/or inhibition of GRPr signaling by PKC, we sought to determine whether phosphorylation and/or agonist activation of GRPr stimulates translocation of arrestin isoforms to the plasma membrane. We examined arrestin2 and arrestin3, the major nonvisual arrestins. Agonist activation of GRPr stimulated the rapid translocation of GFP-tagged arrestin2 and arrestin3 from the cytoplasm to the plasma membrane in cells with intact GRPr (myc-GRPr and WT3; Fig. 8, left). Decrease in cytoplasm GFP signal was detectable within 30 s in both myc-GRPr and WT3 cells, and GRPr was able to decrease the GFP signal in the cytoplasm to a greater degree in cells transfected with arrestin3 than arrestin2 (Fig. 8, bottom left). When examined up to 10 min after addition of bombesin, GFP signal was rarely detected within endocytic vesicles (data not shown). GPCRs with selectivity for arrestin3 over arrestin2 and transient association with arrestins at the plasma membrane have been referred to as "class A" receptors (Oakley et al., 2000).

Little translocation of either arrestin was seen in Δ346,

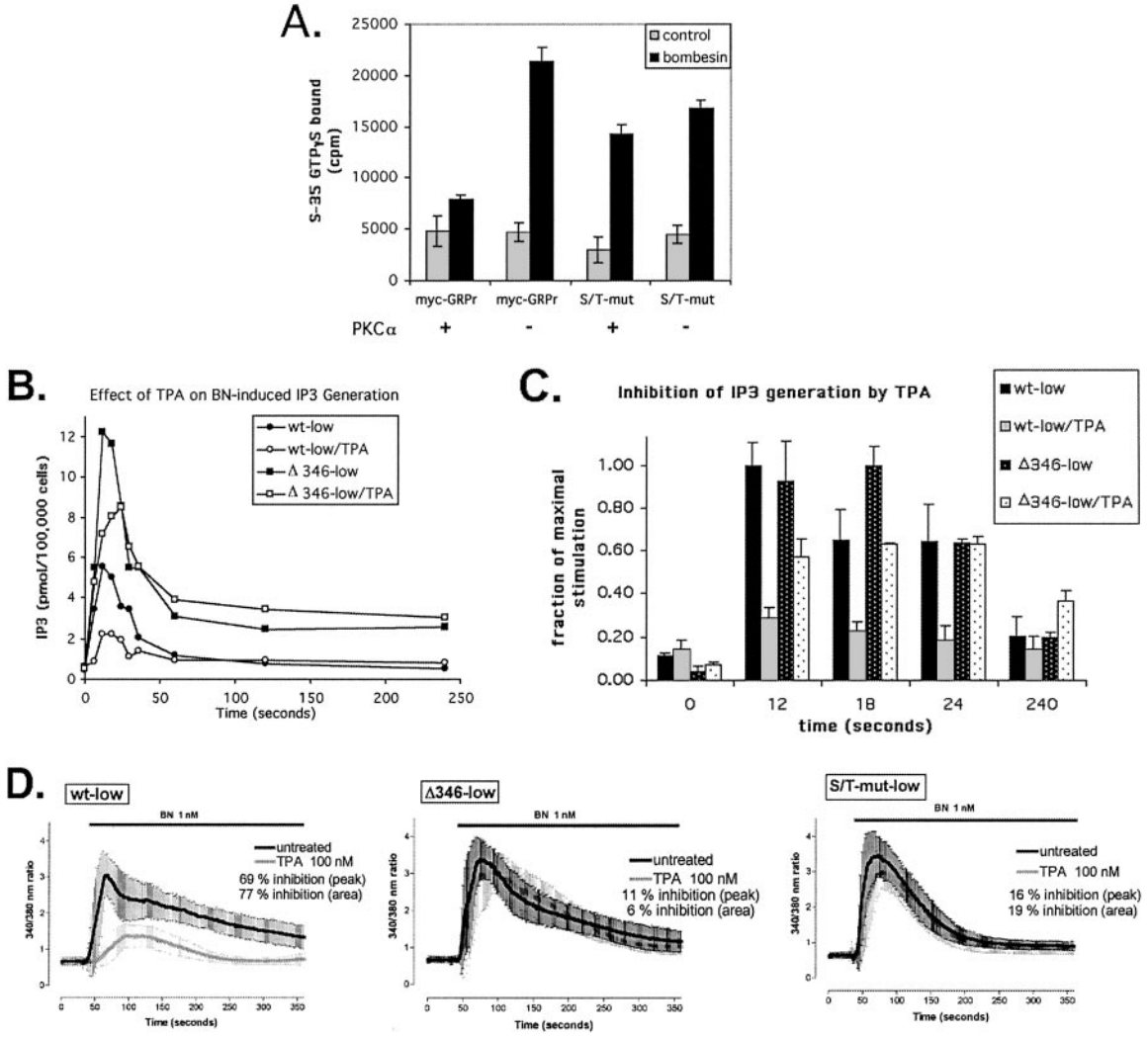


Fig. 7. PKC-induced inhibition of GRPr signaling requires an intact CTD. A, two-step phosphorylation-coupling assay. Urea (7 M)-extracted myc-GRPr and S/T-mut membranes were prepared and receptor number determined as described under *Materials and Methods*. For phosphorylation, 4 nM of each membrane preparation was combined in solution G (without GDP) with 100 μM ATP, 100 nM TPA, and 1.4 mM $CaCl_2$ with (+) or without (−) 0.8 mU/μl PKCα (Sigma-Aldrich). Reactions were incubated at 30°C for 30 min and then put on ice. For coupling, after the initial incubation, 1.8 nM of each receptor was combined in solution G with 10 nM (0.0125 μCi/μl) [^{35}S]GTPγS, 90 nM $G\alpha_q$ and 90 nM bovine brain βγ with (black columns) or without (gray columns) 1 μM bombesin. Binding reactions proceeded for 18 min. Data are presented with a background subtracted from each time point consisting of membranes, βγ, and 1 μM bombesin in solution G without $G\alpha_q$. Results are the mean ± S.D. from an experiment performed in triplicate. B, wt-low (●, ○) and Δ346-low (■, □) cells were prepared as described in Fig. 5 and then placed into KRH buffer. The cells were warmed to 37°C and 0.001% dimethyl sulfoxide (DMSO) (●, ■) or 100 nM TPA (○, □) added for 30 min. Bombesin was added to a final concentration of 100 nM. At the indicated times, an aliquot of cells was removed, the cells lysed, and $Ins(1,4,5)P_3$ generation determined as described in Fig. 5 and under *Materials and Methods*. The data are the average from an experiment with duplicate determinations. C, wt-low (solid columns) and Δ346-low (stippled columns) cells were prepared as in B and pretreated with 0.001% DMSO (black and black stippled columns) or 100 nM TPA (gray and white stippled columns) for 30 min. Bombesin was added to a final concentration of 100 nM. At the indicated times, an aliquot of cells was removed and $Ins(1,4,5)P_3$ generation determined as in B. The data are the average ± S.E. from three experiments with duplicate determinations of each data value. D, real-time recording of $[Ca^{2+}]_i$ was performed in single wt-low, Δ346-low, and S/T-mut-low cells as described under *Materials and Methods* in the presence of 2 mM extracellular Ca^{2+}. Control cells (black lines) or cells pretreated with 100 nM TPA for 30 min (gray lines) were stimulated with 1 nM bombesin at the indicated time. Data represent the average ± S.E. for 40 individual cells per condition and are representative of two similar experiments with these cell lines and one experiment with other cell lines expressing low levels of either wt-GRPr or Δ346.

S/T-mut, or R139G cells (Fig. 8, right). This is consistent with in vitro data from studies with other GPCRs that interaction of arrestins with GPCRs requires receptor phosphorylation (Gurevich et al., 1995). Similar results were seen when endogenous arrestins were examined in P2 membrane fractions from each cell line after 2-min stimulation with bombesin (Fig. 9). Therefore, arrestin interaction with phosphorylated GRPr occurs very quickly after agonist activation and may contribute to agonist-induced desensitization.

To further explore the mechanism of PKC-induced inhibition of GRPr signaling, we studied the ability of TPA-pretreated cells to undergo arrestin translocation in response to bombesin. Activation of PKC does not prevent bombesin-induced translocation of arrestin2 or arrestin3 to the plasma membrane in myc-GRPr cells (Fig. 10, top), nor does it greatly facilitate translocation of arrestin in response to bombesin by R139G (Fig. 9, far right; and Fig. 10, bottom). Because (like wild-type GRPr), R139G undergoes phosphorylation after TPA treatment, both receptor phosphorylation and an intact DRY motif are required for normal interaction with arrestin.

Discussion

In this study, we have examined the molecular requirements for GRPr phosphorylation, bombesin-induced arrestin translocation, and acute desensitization of GRPr-G_q signaling. We demonstrate that an intact CTD is required for phosphorylation of GRPr in cells by either agonist stimulation or activation of PKC. An intact DRY motif near the cytoplasmic end of the third transmembrane helix is required for agonist- but not PKC-stimulated GRPr phosphorylation

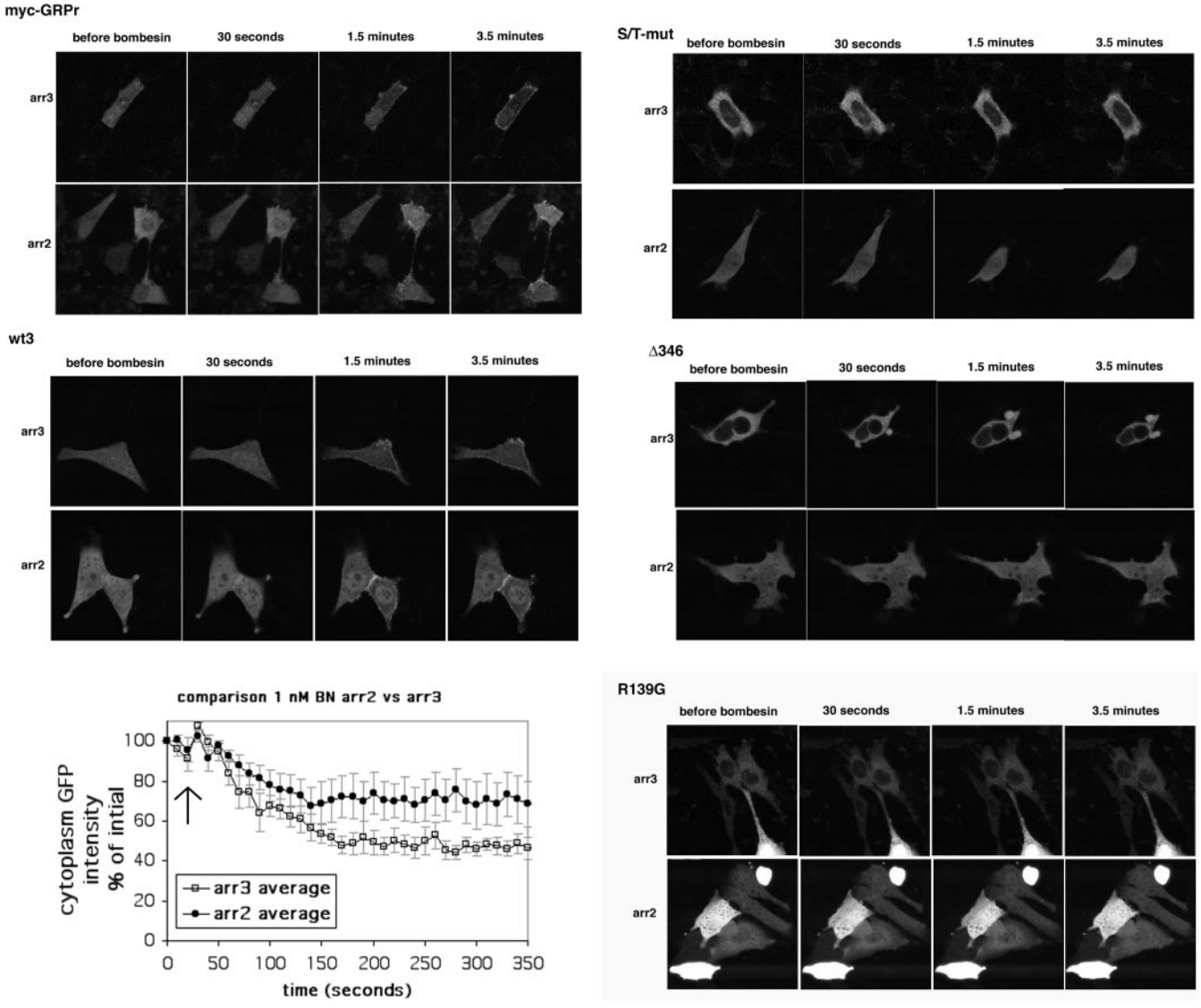


Fig. 8. Bombesin-induced arrestin translocation by GRPr requires receptor phosphorylation. Myc-GRPr, WT3, S/T-mut, Δ346, and R139G cells (expressing high levels of GRPr constructs) were plated in MatTek glass-bottomed 35-mm dishes at 150 to 200,000 cells/dish. The next day, each dish was transfected with either 0.3 μg of EGFP-N_1-β-arrestin1 (arr2) or 1 μg of β-arrestin2-GFP (arr3) plasmid using 6 μl of LipofectAMINE reagent as per the manufacturer's instructions. Twenty-four hours after transfection, the medium was removed and binding buffer was added. Bombesin was added to a final concentration of 100 nM (Myc-GRPr, WT3, S/T-mut, and Δ346 cells) or 1 μM (R139G cells) and cells imaged in real time as described under *Materials and Methods*. Bottom left, myc-GRPr cells were stimulated with bombesin to a final concentration of 1 nM and images taken every 10 s. Arrow indicates time of bombesin addition. Results are mean ± S.E. for three cells for each treatment in the same experiment and representative of four additional experiments each (myc-GRPr or WT3) with bombesin added to a final concentration of 100 nM and images taken every minute.

in cells. Phosphorylation-deficient CTD mutants do not exchange guanine nucleotide on G_q in vitro with greater efficacy than wild-type GRPr in the presence or absence of agonist. However, in an intact cell, CTD mutants show greater amplitude and duration of bombesin-stimulated $Ins(1,4,5)P_3$ generation and Ca^{2+} mobilization than wild type, suggesting that the CTD is involved in acute desensitization of G_q signaling. The difference between wild-type and CTD mutants is more pronounced at low receptor occupancy. At higher levels of receptor occupancy, other processes play large roles in desensitizing G_q signaling. In cells expressing very high numbers of GRPr constructs, $Ins(1,4,5)P_3$ mass declines toward basal levels well before maximal bombesin binding to GRPr. In addition, in cells expressing lower levels of GRPr, the size of intracellular Ca^{2+} pools limits GRPr-dependent Ca^{2+} mobilization. Similar to acute desensitization of agonist-induced signaling, inhibition of signaling by activation of PKC requires GRPr phosphorylation and is associated with a decrease in bombesin-stimulated catalysis of guanine nucleotide exchange in vitro. An intact CTD and DRY motif are also required for agonist-stimulated translocation of arrestin2 and arrestin3 to the plasma membrane. Arrestin translocation is detectable within 30 s of addition of agonist. The agonist-dependent association between arrestin and GRPr is not detectably altered by phosphorylation caused by activated PKC (before the addition of agonist).

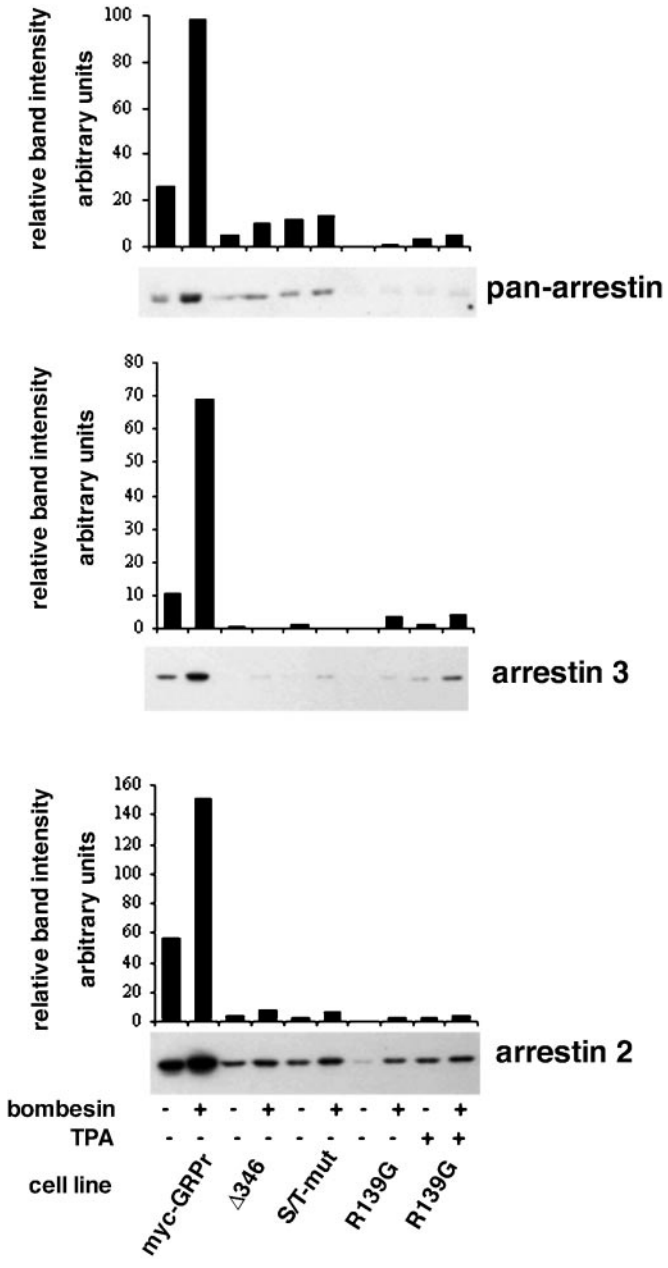


Fig. 9. Bombesin-induced translocation of endogenous arrestins to a membrane fraction by GRPr requires both receptor phosphorylation and an intact DRY motif. Various cell lines expressing high levels of GRPr constructs were grown in 150-mm^2 dishes and pretreated with (+) or without (−) 100 nM TPA for 30 min at 37°C for 30 min then for 2 min with (+) or without (−) 1 μM bombesin. The cells were harvested, and P2 membranes prepared as described under *Materials and Methods*. P2 membrane (26 μg) from each cell line were resolved on a 10% SDS-polyacrylamide gel electrophoresis gel; transferred onto nitrocellulose; probed with a 1:250 dilution of the pan-arrestin antibody F4C1, 1:250 dilution of arrestin2 antibody, or 1:100 dilution of arrestin3 antibody; developed by chemiluminescence; and quantitated using a Kodak ImageStation.

Our data on acute desensitization after addition of agonist are consistent with the role of an intact CTD in rhodopsin shut off in vivo (Chen et al., 1995) and in acute desensitization of several G_q-coupled receptors in cells, including the neurokinin-1 receptor (Li et al., 1997). Our data on the rapid bombesin-induced translocation of arrestin to the plasma membrane are consistent with a role for arrestin family member(s) in CTD-dependent GRPr desensitization, although they do not prove the involvement of arrestins. Further studies using techniques to alter arrestin function or arrestin protein expression will be required to define arrestins' role in phosphorylation-induced uncoupling of GRPr from G_q. Additional molecules that directly uncouple phosphorylated GRPr (such as arrestins) are likely to be required for acute GRPr desensitization because phosphorylation alone is insufficient to completely uncouple GRPr from G_q.

We found one major difference between GRPr and the reported behavior of some other GPCRs. For the β_2AR, (second messenger-independent) GRK phosphorylation sites are in the CTD, whereas (second messenger-dependent) PKA phosphorylation sites are in the third intracellular domain (Hausdorff et al., 1989). As a consequence, distinct pathways of desensitization exist for β_2AR phosphorylated by GRK versus second messenger-dependent kinases (Pitcher et al., 1992) and PKA phosphorylation induces a switch in the G protein specificity for β_2AR (Lefkowitz et al., 2002). Additionally, GRK2 and PKC can additively phosphorylate the m1 muscarinic acetylcholine receptor (mAChR), suggesting the sites are spatially and functionally separate (Haga et al., 1996). PKC phosphorylation decreases mAChR coupling to G_o by 35 to 40% (Richardson et al., 1992). In contrast, GRPr phosphorylation induced by agonist or activation of PKC are mutually exclusive and seem to have similar functional consequences, including decreasing GRPr-G_q coupling by ~75 to 80%. Although agonist and TPA stimulate GRPr phosphorylation at distinct sites (Williams et al., 1996), these sites are likely to be very close to one another within the CTD. Little or no phosphorylation is detected in the CTD mutants with either stimulus, and the addition of agonist and TPA together leads to a level of phosphorylation similar to that of agonist alone. Finally, the functional effect of phosphorylation by both mechanisms is similar in all assays to date, including the ability to stimulate translocation of arrestins

and facilitate the internalization of agonist-occupied GRPr (data not shown). These findings suggest GRPr may be more functionally similar to rhodopsin than β_2AR or mAChR. TPA in intact retinas and PKC in vitro stimulate rhodopsin phosphorylation in the proximal CTD at sites distinct from GRK1 phosphorylation (Kelleher and Johnson, 1986; Greene et al., 1997). PKC phosphorylation also uncouples rhodopsin from transducin (Kelleher and Johnson, 1986). The presence of nearby sites of phosphorylation in GRPr for different kinases may explain why phosphorylation by GRK2 and PKC inhibits GRPr signaling to a similar degree. This may also explain why others interpreted their data to suggest that GRPr internalization and long-term desensitization are mediated by PKC (Benya et al., 1993, 1994, 1995). Point mutation in the distal CTD intended to implicate PKC in certain processes may have had the unintended consequence of inhibiting phosphorylation by many kinases. The only way to confirm where agonist and TPA stimulate GRPr phosphorylation will be to identify the sites by biochemical means, as has been done for rhodopsin.

Similar consequences for GRPr phosphorylation mediated by different kinases may be the result of structural limitations. Hydropathy analysis (Kroog et al., 1995a) and modeling based on the crystal structure of rhodopsin (data not shown) predict that like rhodopsin, GRPr has very short third intracellular and carboxyl terminal domains. Unlike

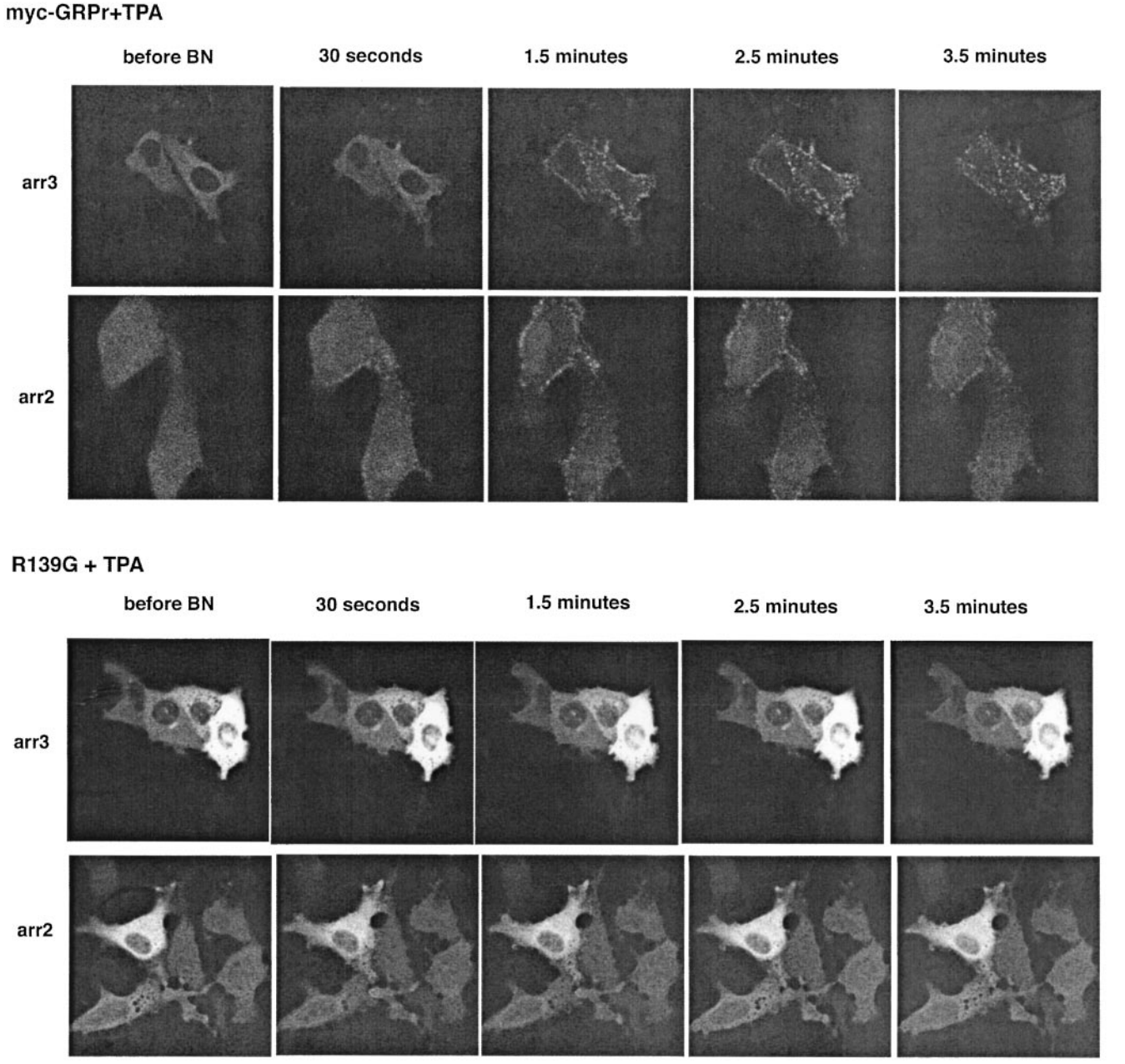


Fig. 10. Bombesin-induced arrestin translocation can occur after TPA-induced receptor phosphorylation in myc-GRPr but not R139G. myc-GRPr and R139G cells were transfected with 0.3 μg of EGFP-N_1-β-arrestin1 (arr2) or 1 μg β-arrestin2-GFP (arr3) plasmid as in Fig. 8. Cells were treated with 100 nM TPA for 30 min before addition of bombesin to a final concentration of 100 nM (myc-GRPr) or 1 μM (R139G). Cells were imaged in real time as described under *Materials and Methods*.

other GPCRs, such as β_2AR, GRPr may not have sufficiently distinct phosphorylation sites to provide for major differences in functional consequences, although more subtle differences may emerge with further study. Furthermore, because we have not examined the ability of phospho-GRPr to couple to other G proteins (such as $G_{12/13}$), we cannot rule out the possibility that one or both phosphorylation events allow for coupling to alternate G proteins or alternate signaling pathways. However, because sequential addition of TPA and agonist stimulates arrestin translocation with a similar time course as agonist alone, TPA-phosphorylated GRPr is unlikely to couple to other G proteins in cells.

After treatment with TPA, GRPr remains on the cell surface but is uncoupled from G_q. Agonist "activation" of TPA-induced phospho-GRPr would then stimulate receptor internalization with little or no generation of $Ins(1,4,5)P_3$ or increase in $[Ca^{2+}]_i$. Potentially, receptor internalization is a mechanism whereby coupling to G_q can be restored if internalization allows for dephosphorylation. However, this "uncoupled" cell surface receptor may still be able to undergo agonist-activated coupling to non-G protein-mediated pathways. For example, arrestin-dependent signaling may be maintained because the receptor is still able to stimulate the recruitment of arrestin to the plasma membrane.

PKC may be activated by any number of stimuli, all of which may result in impaired GRPr signal transduction. GRPr-stimulated PKC activation may play a role in GRPr desensitization under some circumstances. GRPr undergoes heterologous desensitization (Vinayek et al., 1990), but it is not known whether heterologous desensitization of GRPr signaling is mediated by PKC under any circumstances or TPA-mediated GRPr desensitization mimics physiologically relevant desensitization.

Another finding of the study is that the conserved (D/E)R(Y/W) motif is required for GRPr interaction with several signaling partners in addition to G_q. This motif has been implicated in the activation of rhodopsin-like GPCRs (Oliveira et al., 1994). R139G is uncoupled from G_q in vitro (X. Jian, personal communication) and agonist binding to R139G does not stimulate Ca^{2+} mobilization in cells (Benya et al., 1994). Additionally, R139G is not a substrate for phosphorylation by GRK2 in vitro and stimulates little translocation of arrestin to the plasma membrane in cells, even in the presence of agonist and PKC-stimulated phosphorylation. Therefore, the DRY motif and the surrounding interface of the third transmembrane helix with the second intracellular loop may be either a site of interaction with G_q, GRK2, and arrestin, or DRY may be needed for an agonist-stimulated conformational change that is recognized by each partner. Finally, because R139G internalizes with little detectable translocation of arrestin, GRPr internalization may proceed independently from arrestin. Whether this is a physiologically relevant mechanism for GRPr internalization is not known.

Finally, the mechanism of CTD-independent acute desensitization of $Ins(1,4,5)P_3$ generation seen at high receptor occupancy is not known. The decrease in $Ins(1,4,5)P_3$ mass must be the result of rapid changes in the rate of either $Ins(1,4,5)P_3$ generation or $Ins(1,4,5)P_3$ degradation. We did not examine PLCβ activity, $Ins(1,4,5)P_3$ kinase activity, or $Ins(1,4,5)P_3$ phosphatase activity. Further studies will be needed to determine the mechanism for this process and in which situations it is physiologically relevant.

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