Protein Kinase C Deficiency Blocks Recovery from Agonist-induced Desensitization*

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Protein phosphorylation is central to agonist-induced attenuation of the function of G-protein-linked receptors. Stable expression of RNA antisense to specific protein kinase mRNAs permitted analysis of loss-of-function mutants of A431 human epidermoid carcinoma cells, lacking protein kinase A, protein kinase C, or β-adrenergic receptor kinase. Deficiency of protein kinase C, but not the others, amplified rather than attenuated agonist-induced desensitization. In wild-type cells, the time for recovery from desensitization was -25 min following removal of agonist. In the protein kinase C-deficient cells, no resensitization was observed even 60 min after agonist removal. Like protein kinase C-deficiency, inhibition of protein kinase C with bisindolylmaleimide or calphostin C blocked resensitization. Resensitization was suppressed by FK506, an inhibitor of protein phophatase 2B, mimicking protein kinase C deficiency, but in a non-additive manner. The data reveal protein kinase C and protein phosphatase 2B to be critical elements of resensitization.

Chronic stimulation of G-protein-linked receptors (GPRs) provokes attenuation of the receptor-mediated signal, or desensitization (1). Protein phosphorylation is a critical element of agonist-induced desensitization involving at least three prominent kinase activities (2), cAMP-dependent protein kinase (protein kinase A), calcium and phospholipid-sensitive protein kinase (protein kinase C), and members of the G-protein-linked receptor kinase family, like the β-adrenergic receptor kinase. β2-Adrenergic receptors (β2AR) are substrates for protein kinase A, protein kinase C, and protein-dependent receptor kinase, as well as growth factor receptors with intrinsic tyrosine kinase activity (3, 4). Studies of the complex roles of protein kinases in the functional regulation of these multiply phosphorylated receptor substrates has been accelerated through the use of loss-of-function mutant cells in which target protein kinases have been suppressed by antisense oligodeoxynucleotides (2) and dominant negative mutant kinase (5), in addition to protein kinase inhibitors (6), receptor mutagenesis (7, 8), and reconstitution of purified elements in vitro (9).

Recently, we reported cell type-specific roles of various protein kinases in agonist-induced desensitization using oligodeoxynucleotides to suppress these enzymes transiently (2). Suppression of protein kinase C, but not protein kinase A or β-adrenergic receptor kinase, amplified rather than attenuated agonist-induced desensitization in a variety of cell types. In the current work we explore this novel role of protein kinase C. Protein kinase C is shown to be obligatory for resensitization of GPRs, its action blocked by protein kinase C deficiency, by the protein kinase C inhibitor bisindolylmaleimide, and mimicked by FK506, a protein phosphatase 2B inhibitor.

EXPERIMENTAL PROCEDURES

Cell Culture—Human epidermoid carcinoma cells (A431) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), penicillin (100 U/ml), and streptomycin (100 μg/ml). Stably transfected A431 cells expressing antisense RNA to protein kinase A, protein kinase C, and β-adrenergic receptor kinase were maintained in medium containing geneticin (0.5 mg/ml, Life Technologies, Inc., Gaithersburg, MD).

Construction of the pLNC Retroviral Vectors—The antisense sequences for protein kinase A-α, –β, and –γ isoforms, G-protein-linked receptor kinase-1 (11), and human protein kinase C-β isoform (12), respectively, were engineered into the HindIII/ClaI sites of the pLNCx retroviral vector using standard recombinant DNA techniques (13). The pLNCx vector contains the gene to confer neomycin resistance under the control of the 5' and 3' long terminal repeats of the mouse Moloney virus, and the expression of the antisense RNA is under the control of the cytomegalovirus promoter. Antisense RNA to protein kinase A targets both α- and β-isoforms of the catalytic subunit of protein kinase A (10, 14). RNA antisense to β-adrenergic receptor kinase targets both α- and β-adrenergic receptor kinase-1 and -2 (11, 15), but not rhodopsin kinase or G-protein-linked receptor kinase-4 and -5 (16). The α, β, and γ-isoforms of protein kinase C are targeted by RNA antisense to protein kinase C (12, 17, 18).

Stable Transfection—A431 cells were transfected with the pLNCx plasmid using LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) reagent according to the manufacturer's protocol. Positive transfectants were obtained by selection with the neomycin analog geneticin (0.5 mg/ml). The ability of the antisense RNAs to suppress the expression of protein kinases in the transfected donets was determined by immuno blotting analysis and assay of residual enzyme activity.

Immunoblotting Analysis—Cells were harvested and homogenized in 10 mM Hepes buffer, pH 7.4, 2 mM MgCl2, 2 mM EDTA containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride. Nuclei were precipitated by low speed centrifugation. Fifty micrograms of post-nuclear fraction protein was subjected to 10% SDS-polyacrylamide gel electrophoresis and the separated proteins were transferred onto a nitrocellulose membrane. Expression of protein kinase C was probed with antibody against the α-isoform of protein kinase C (Life Technologies, Inc., Gaithersburg, MD). β-Adrenergic receptor kinase levels were probed with polyclonal antibodies raised against the peptide sequence 389-402 of β-adrenergic receptor kinase-1.

Protein Kinase A and C Assays—Protein kinase A and C activities were assessed by using pre-made assay kits from Life Technologies, Inc. The manufacturer's protocol was followed. Protein kinase A activity is defined as the amount of phosphate incorporated into a substrate peptide, Kemptide, in the presence of 10 μM cyclic AMP minus...
that incorporated in the presence of the protein kinase A inhibitor peptide (1 μM). The specific activity of protein kinase C is the difference between phosphorylation of an acetylated peptide derived from myelin basic protein in the presence of 10 μM phenol 12-myristate 13-acetate to that in the presence of 20 μM protein kinase C inhibitory peptide (19–36).

Radioligand Binding Studies—The number of β2-adrenergic receptors was determined by radioligand binding to intact cells incubated with 0.5 nM [125I]iodocyanopindolol (ICYP) (DuPont NEN, Boston, MA) in the presence or absence of 10 μM propranolol at 23 °C for 60 min. The incubation buffer contained 50 mM Tris-HCl, pH 7.5, 10 μM MgCl₂, and 150 mM NaCl. The affinity constants for ICYP and isoprosteral (Sigma) binding were determined using crude membrane fractions incubated with or without 0.1 mM GTP-γ-S. The dissociation constant for ICYP was calculated by Scatchard plot analysis with ICYP concentrations ranging from 0 to 1 nM. The high (Kₐ) and low (Kᵢ) affinities for isoprosteral binding to β2AR were determined by isoprosteral (0–1 μM) displacement of ICYP (0.25 nM). The Kᵢ values were from a two-site curve fitting (GraphPad Software, San Diego, CA) to the data obtained in the absence of GTP-γ-S. The Kᵢ values were from a single-site curve fitting to the data obtained in the presence of 100 μM GTP-γ-S.

Suppression via Antisense Oligodeoxynucleotides—Specific antisense and sense oligodeoxynucleotides were synthesized and purified to cell culture grade (Operon Tech., Alameda, CA), as described (2). Wild-type A431 cells were treated with 30 μM oligodeoxynucleotides for 2 days prior to the analysis of desensitization.

Desensitization and Resensitization of β2AR—Two days prior to the desensitization analysis, cells were seeded in 96-well plates at a density of 25,000–50,000 cells/well. Cells were washed and challenged with or without 1 μM isoproterenol for 30 min at 37 °C. At the end of the first challenge, cells were washed three times, and incubated in Heps buffer containing Ro-20–1724 (0.1 μM, Calbiochem, San Diego, CA) and adenosine deaminase (0.5 unit/ml) for 5 min prior to (and included in) the second challenge with 1 μM agonist. For resensitization, isoprosteral-treated cells were washed free of agonist and maintained in buffer containing no phosphodiesterase inhibitor for 60 min after the first challenge. Five minutes before the second challenge of the agonist, cells were incubated again in the presence of Ro-20–1724 and adenosine deaminase. The agonist-induced cAMP production within 5 min incubation in the second challenge of agonist was measured. cAMP accumulation was determined as described (19). The data are calculated as “% desensitization” in which “100% desensitization” reflects no cAMP accumulation in response to a second challenge with the agonist. “0% desensitization” refers to a cAMP response to a second challenge equivalent to that obtained in response to the first challenge.

Effect of Protein Kinase C or Protein Phosphatase Inhibitors on Receptor Resensitization—Cells were preincubated in the presence or absence of protein kinase C inhibitors, such as bisindolylmaleimide and calphostin C (Calbiochem), or protein phosphatase inhibitors, such as FK506 (Fujisawa USA, Deerfield, IL), cyclosporin A (Signal Transduction, San Diego, CA), fenvalerate (LC Laboratories, Woburn, MA), okadaic acid, and calyculin A (Boehringer Mannheim, Indianapolis, IN) for 15 min before experiments. Each inhibitor was included throughout the incubation for desensitization and resensitization as described before.

RESULTS

Stable Suppression of Protein Kinases by Antisense RNA—Antisense sequences of 29–33 bases in length were engineered into the retroviral expression vector pLNCx (20) targeting protein kinase A, protein kinase C, and β2-adrenergic receptor kinase, and used to stably transfect A431 cells. After transfection, neomycin-resistant clones were selected and tested for expression of kinase by immunoblotting (Fig. 1) and by measurement of enzyme activity (not shown). As shown by immunoblotting, the expression of β-adrenergic receptor kinase (Fig. 1A, molecular mass — 79 kDa) and protein kinase C (Fig. 1B, molecular mass — 80 kDa) was suppressed effectively in the clones stably expressing the RNA antisense to each. Assay of total protein kinase C activity agreed well with the immunoblotting, displaying >80% reduction. The expression of PKCα and PKCγ was suppressed by the antisense, whereas PKCβ and PKCε isoforms were not detected (not shown). As previously reported (21), the assay for β-adrenergic receptor kinase activity was of insufficient sensitivity to accurately detect endogenous β-adrenergic receptor kinase levels in the extracts of wild-type cells. Likewise, immunoblotting of protein kinase A in wild-type cells was not sensitive enough to detect endogenous levels of the enzyme in whole cell extracts. Total protein kinase A activity, however, was directly assayed and found to be reduced 70–85% in clones at early passages.

Suppression of Agonist-induced Desensitization: Comparison of Oligodeoxynucleotide Treatment to Stable Expression of Antisense RNA—The initial observations of unique roles of protein kinase A, protein kinase C, and β-adrenergic receptor kinase in agonist-induced desensitization were obtained using treatment with oligodeoxynucleotides antisense and sense to mRNA of each kinase (2). Creating stably transfected clones expressing RNA antisense to each protein kinase permitted studies preceded by the small-scale required for oligodeoxynucleotide-induced suppression. Stable transfectants expressing RNA antisense to protein kinase A, protein kinase C, and β-adrenergic receptor kinase were analyzed for agonist-induced desensitization and the results were compared to those obtained by oligodeoxynucleotide-induced suppression (Fig. 2). The data demonstrate a sharp reduction in agonist-induced desensitization in the A431 clones deficient in either protein kinase A or β-adrenergic receptor kinase. In the protein kinase C-deficient clones, in contrast, agonist-induced desensitization was markedly potentiated. Stable transfection with the empty expression vector itself has no effect. The extent of isoprosteral-induced desensitization was nearly doubled in cells made deficient in protein kinase C. Both wild-type and empty vector transfected cells reached the maximal desensitization (~30% reduction in Vₘₐₓ at 10 min following challenge with the agonist. The extent of desensitization plateaued from 10 to 40 min. In contrast, protein kinase C-deficient cells displayed a progressive increase in desensitization (30–50%) from 10 to 40 min following the challenge (not shown). These data agreed well with those obtained with oligodeoxynucleotide-treated cells. The unexpected potentiation of desensitization by protein kinase C-deficiency (2) was confirmed by these studies. The availability of stable transfectants lacking protein kinase C permitted further analysis of the role of this kinase in desensitization.

β2-adrenergic Receptor Properties in Protein Kinase C-deficient Cells—The protein kinase C-deficiency provoked a small, but not significant, reduction in the level of β2AR expression (Table I). A similar reduction was observed with cells trans-
Protein Kinase C and Receptor Recovery

**Fig. 2. Suppression of protein kinases by antisense oligodeoxynucleotides and stable production of antisense RNA.** A, A431 cells were grown in the presence or absence of 30 μM oligodeoxynucleotides either sense or antisense to the protein kinase targeted, for 2 days prior to analysis of desensitization. Desensitization was performed as described under “Experimental Procedures.” β2AR in wild-type, non-treated cells displayed ~30% desensitization under the condition. The results are expressed as mean ± S.E. (n = 3). For cells treated with sense oligodeoxynucleotides, the results of a single experiment expressed as mean ± S.D. are displayed. B, for each antisense sequence, two clones were selected for analysis and the results were combined for calculation. Clones stably transfected with empty vector displayed 30% desensitization under this condition. The results are expressed as mean ± S.E. (n = 4–7). PKA, protein kinase A; βARK, β-adrenergic receptor kinase.

**Table I**

|          | ICYP | Isoproterenol |
|----------|------|---------------|
| clones   |       |               |
|          |      |               |
| WT       | 73,000 ± 4000 | 146 ± 24 |
| PKC-deficient | 56,000 ± 9000 | 164 ± 34 |
|          | 2.5 (21%) | 5.9 (21%) |

*Statistically, not different from WT (p > 0.05).

**Table II**

|          | Isoproterenol-stimulated cAMP response |
|----------|---------------------------------------|
|          | Basal (pM/5 min/10⁶ cells) | EC₅₀ |
| WT       | 1.2 ± 0.4 | 20.0 ± 1.1 |
| PKC-deficient | 0.9 ± 0.3a | 16.4 ± 2.6a |

*Statistically, not different from WT (p < 0.05).

i.e. resensitization. The V₅₀ for resensitization in the control clones was ~25 min. Within 50 min of washout of agonist, resensitization was completed in these clones. The protein kinase C-deficient cells, in sharp contrast, displayed a rather static level of desensitization. Desensitization persisted for more than 60 min following the washout of the agonist in the protein kinase C-deficient cells.

A second approach, treating cells with inhibitors of protein kinase C was explored. Wild-type cells treated with either bisindolylmaleimide (0.3 μM) (22) or calphostin C (0.3 μM) (23) displayed a blunted resensitization, retaining ~70% of agonist-induced desensitization at 60 min following washout of the agonist (not shown). Taken together, the data from the loss-of-function mutant clones and from wild-type clones treated with either inhibitor of protein kinase C provide an explanation for the potentiation of desensitization observed in the protein kinase C-deficient cells, i.e. protein kinase C activity is critical to the recovery phase of agonist-induced desensitization.

Role of Protein Phosphatase Activities in Receptor Resensitization—The loss of receptor resensitization in protein kinase C-deficient cells and the central role of protein phosphorylation in agonist-induced desensitization prompted consideration of protein phosphatases. A series of well characterized protein phosphatase inhibitors were screened for effects on resensitization of β2AR, following a 30-min agonist-induced desensitization (Table III). Inhibition of protein phosphatase 1 and 2A by okadaic acid (100 nM) and calyculin A (10 nM) failed to alter significantly receptor resensitization, when measured at 60
inhibitor vanadate (100 nM), also failed to alter the desensitization. Noticeably, neither okadaic acid nor calyculin A enhanced desensitization, measured at 5 min post the first challenge of agonist.

Most striking were the effects obtained with the inhibitor of calcium/calmodulin-dependent protein phosphatase (protein phosphatase 2B), FK506. FK506 associates with immunophilin FKBP12 and specifically inhibits protein phosphatase 2B activity (25, 26). Treatment with FK506 potentiated agonist-induced desensitization by more than 2-fold (27 versus 75% desensitization) measured at 5 min post washout. FK506-treated cells displayed a persistent desensitization at 60 min postchallenge of agonist, much like deficiency in protein kinase C (Fig. 3). Cells treated with inhibitor of protein phosphatase 2B, either cyclosporin A (100 ng/ml) or fenvalerate (10 nM) (25, 27), also displayed blunted resensitization (not shown). Protein phosphatase 2B expression was readily detected in these cells via immunoblotting (not shown).

The effects of FK506 treatment on resensitization were dose-dependent (Table IV). Since FK506 and protein kinase C deficiency both suppressed the ability of the cells to recover from agonist-induced desensitization, it was of interest to explore whether their effects were additive. Although both FK506 and protein kinase C-deficiency abolished recovery of cells from agonist-induced desensitization, the effects of the two, in combination, were not additive. Treatment of protein kinase C-deficient cells with FK506 did not suppress resensitization further than protein kinase C-deficiency alone. As expected, treatment of protein kinase C-deficient cells with phorbol 12-myristate 13-acetate failed to change either the extent of desensitization or resensitization (not shown).

### DISCUSSION

The attenuation that accompanies chronic stimulation is a fundamental feature of cell signaling. For GPLR, agonist-induced desensitization (1) and down-regulation (28) are nearly universally observed. Protein phosphorylation is central to agonist-induced desensitization, with roles implicated for several protein kinases including protein kinase A (7, 29), protein kinase C (9, 30), and G-protein-linked receptor kinases (31). In our earlier studies of protein kinases and agonist-induced desensitization, we created loss-of-function mutants by treating cells with oligodeoxynucleotides antisense to specific kinases (2). Elimination of protein kinase A and β-adrenergic receptor kinase resulted in a profound loss of attenuation in response to β-adrenergic stimulation of cAMP accumulation in a variety of cell types. Loss of protein kinase C, in contrast, potentiated rather than blunted agonist-induced desensitization.

In the current work, use of stably transfected clones permitted further analysis of the effects of protein kinase C-deficiency on desensitization. The hypothesis that protein kinase C acts to suppress desensitization and/or enhance resensitization was
tested in loss-of-function mutants, deficient in protein kinase C. Elimination of protein kinase C potentiated desensitization, but was shown not to influence the onset of the process, prompting analysis of the resensitization process. Protein kinase C-deficiency was found not only to influence the recovery of cells from agonist-induced desensitization, but also to block the recovery phase entirely. Loss-of-function mutant cells failed to recover from desensitization for more than an hour following agonist washout. Empty vector transfected cells displayed a recovery process with a t1/2 ~ 25 min. This critical observation was confirmed using an independent approach, treatment of wild-type cells with either bisindolylmaleimide or calphostin C, inhibitors of protein kinase C.

The central role of protein phosphorylation in agonist-induced desensitization for GPLR prompted an analysis of protein phosphatase activity in the recovery phase. The role and character of phosphatases in the resensitization process were established using well known phosphatase inhibitors, specific for protein phosphatase 1, protein phosphatase 2A, protein phosphatase 2B, and protein phosphotyrosine phosphatase activities. Although protein phosphatase 2A has been shown to dephosphorylate β-adrenergic receptor kinase-phosphorylated GPLR in the reconstituted system (32), neither okadaic acid nor calyculin A, inhibitors of protein phosphatase 2A altered significantly the recovery of agonist-induced desensitization. These observations indicate no major role of protein phosphatase 2A in resensitization in these cells, and further suggest that the reversal of β-adrenergic receptor kinase-mediated phosphorylation is not essential for the recovery of receptor activity. Likewise, the inability of vanadate ions to alter agonist-induced desensitization argues against a role of protein phosphotyrosine phosphatase in resensitization of GPLR. Although shown recently to be substrates for tyrosine kinases both in vivo (3) and in vitro (4), β2AR are cross-regulated by hormones like insulin and insulin-like growth factor-I, whereas activation of tyrosine kinases is not known to occur in homologous desensitization.

In association with immunophilin FKBP12, FK506 is known to inhibit protein phosphatase 2B activity (25, 26). Treating cells with FK506 potentiated agonist-induced desensitization of β2AR-mediated cAMP accumulation and attenuated sharply the recovery from desensitization that typically follows wash-out of the agonist. These effects were dose-dependent and suggest a central role of protein phosphatase 2B in the recovery phase from agonist-induced desensitization of GPLRs. The action of FK506 observed in wild-type cells was absent in the protein kinase C-deficient mutants. Taken together these data provide a compelling case for a central role of protein phosphatase 2B in protein kinase C-dependent recovery of cells from agonist-induced desensitization (Fig. 4). In view of the data obtained in the loss-of-function mutant clones lacking protein kinase C, activation of protein kinase C with phorbol esters might be expected to reduce desensitization overall, by accelerating the recovery phase. In the loss-of-function mutants treatment with phorbol 12-myristate 13-acetate had no effect, as expected. Paradoxically, treating wild-type cells with phorbol 12-myristate 13-acetate enhanced desensitization (not shown). The basis for this effect was revealed recently in studies reported by Chuang et al. (33) demonstrating that protein kinase C potentiates the activity of β-adrenergic receptor kinase (Fig. 4). Thus, protein kinase C appears to play a pivotal role in regulating GPLR desensitization. In the forward direction, activation of protein kinase C leads to increased β-adrenergic receptor kinase activity promoting greater desensitization. In the reverse direction, protein kinase C is obligatory and central to the recovery from desensitization. The action of protein kinase C on the recovery phase appears to require protein phosphatase 2B. As determined through metabolic labeling of the cells with 32P, protein phosphatase 2B was phosphorylated in response to phorbol 12-myristate 13-acetate treatment of the cells (not shown). This novel role for protein kinase C highlights a new dimension in our understanding of cross-talk between GPLR-mediated responses. In view of the ability of catecholamines to activate both α-adrenergic receptors (linked to phospholipase C and protein kinase C activation) and β-adrenergic (linked to adenylcyclase activation) receptors co-expressed in many cells, such cross-talk would seem integral in cell signaling.

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