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Oligodeoxynucleotides antisense to mRNA encoding protein kinase A, protein kinase C, and β-adrenergic receptor kinase reveal distinctive cell-type-specific roles in agonist-induced desensitization

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ABSTRACT **The roles of three protein kinases, cyclic AMP-dependent protein kinase (protein kinase A), protein kinase C, and β-adrenergic receptor kinase (βARK), implicated in agonist-induced desensitization of guanine nucleotide-binding protein (G-protein)-coupled receptors were explored in four different cell lines after 48 hr of incubation with oligodeoxynucleotides antisense to the mRNA encoding each kinase. Desensitization of β_2-adrenergic receptors was analyzed in cell types in which the activities of the endogenous complement of protein kinases A and C and βARK were distinctly different. Protein kinase A was necessary for desensitization of rat osteosarcoma cells (ROS 17/2.8), whereas the contribution of βARK to desensitization was insignificant. In Chinese hamster ovary cells that stably express β_2-adrenergic receptors and in smooth muscle cells (DDT_1MF-2), oligodeoxynucleotides antisense to βARK mRNA nearly abolished desensitization, whereas oligodeoxynucleotides antisense to protein kinase A mRNA attenuated desensitization to a lesser extent. In human epidermoid carcinoma cells (A-431), oligodeoxynucleotides antisense to either protein kinase A mRNA or βARK mRNA attenuated agonist-induced desensitization, providing a third scenario in which two kinases constitute the basis for agonist-induced desensitization. In sharp contrast, oligodeoxynucleotides antisense to protein kinase C mRNA were found to enhance rather than attenuate desensitization in DDT_1MF-2 and A-431 cell lines, demonstrating counterregulation between prominent protein kinases in desensitization. Using antisense oligodeoxynucleotides to "knock out" target protein kinases *in vivo*, we reveal distinctive cell-type-specific roles of protein kinase A, protein kinase C, and βARK in agonist-induced desensitization.**

Desensitization, a rapid decrease in responsiveness following an initial stimulation, is a widely observed phenomenon in signal transduction. For guanine nucleotide-binding protein (G protein)-coupled hormone action, β_2-adrenergic stimulation of adenylylcyclase remains a primary target for analysis (1). Protein phosphorylation is central to desensitization and reflects the activity of two distinct classes of kinases—second-message-dependent protein kinases and receptor-specific protein kinases. Protein kinase A (PKA), activated by the product of adenylylcyclase, cAMP, and protein kinase C (PKC), regulated by Ca^{2+} and phospholipids, exemplify the former class. Receptor-specific kinases, in contrast, phosphorylate only the agonist-occupied receptor and include rhodopsin kinase (2), β-adrenergic receptor kinases 1 (βARK1) (3) and 2 (βARK2) (4), muscarinic receptor specific kinase (5), and recently cloned G-protein-coupled receptor kinases 4 and 5 (6, 7). *In vitro* β_2-adrenergic receptors (β_2-ARs), rhodopsin, and muscarinic receptors are all substrates for phosphorylation by most members of this kinase family (8, 9).

The central role of phosphorylation in desensitization was revealed by several experimental approaches, including reconstitution studies *in vitro* (10), use of protein kinase inhibitors (11), receptor mutagenesis (12, 13), and overexpression of protein kinases (14). Remarkably, the extent to which the activities of PKA, PKC, and βARK vary among cells and the extent to which these kinases contribute to desensitization *in vivo* remain unanswered questions. Antisense RNA technology provides a powerful approach to suppressing specific protein targets in cells. The oncogene product c-myc (15), transforming growth factor β_3 (16), nicotinic acetylcholine receptor subunits (17), cyclins (18), and the α (19) and γ (20) subunits of G protein are but a few recent examples of target proteins suppressed by antisense oligodeoxynucleotides. In the present study we adopt this strategy to explore *in vivo* the roles of PKA, PKC, and βARK in a critical feature of molecular signaling via G-protein-coupled pathways—namely, desensitization.

MATERIALS AND METHODS

Cell Culture. Rat osteosarcoma cells (17/2.8) and hamster smooth muscle cells (DDT_1MF-2) derived from the vas deferens were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO/BRL) supplemented with 5% (vol/vol) fetal bovine serum (HyClone), penicillin (60 mg/ml), and streptomycin (100 mg/ml). Stably transfected Chinese hamster ovary cells (CHO) expressing hamster β_2-AR (13d) and human epidermoid carcinoma cells (A-431) were grown in DMEM containing 10% fetal bovine serum and antibiotics. Geneticin (0.5 mg/ml; GIBCO/BRL) was included in the medium of CHO 13d cells.

Desensitization of β_2-AR. Cells were seeded in 96-well plates at the density of 20,000 cells per well for 2 days prior to challenge with the β-adrenergic agonist (−)-isoproterenol. The monolayers were rinsed with Hepes-buffered medium (HBM, ref. 21; 10 mM Hepes, pH 7.5/13.4 mM NaCl/4.7 mM KCl/1.2 mM $MgSO_4$/2.5 mM $NaHCO_3$/5 mM glucose) and were incubated with or without 1 μM isoproterenol for 30 min at 37°C. At the end of the challenge, cells were washed three times and incubated in HBM containing 0.1 mM Ro-20-1724 (Calbiochem) and 0.5 unit of adenosine deaminase (Sigma) per ml for 5 min prior to and during a second challenge with isoproterenol. The concentration of agonist in the second challenge varied as indicated in the individual experiments

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Abbreviations: β_2-AR, β_2-adrenergic receptor; PKA, protein kinase A (cAMP-dependent protein kinase); βARK, β-adrenergic receptor kinase; PKC, protein kinase C; $oligo_{anti\text{-}PKA}$, oligodeoxynucleotides antisense to PKA mRNA; $oligo_{anti\text{-}\beta ARK}$, oligodeoxynucleotides antisense to βARK mRNA; $oligo_{anti\text{-}PKC}$, oligodeoxynucleotides antisense to PKC mRNA; G protein, guanine nucleotide-binding protein; Ac-MBP, acetylated myosin basic protein.

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shown in Fig. 1. The incubation was terminated at 15 min by the addition of 2 volumes of ethanol. An aliquot of the ethanolic solution was used for cAMP determination. The dose–response curves were analyzed by a nonlinear curve-fitting program, GRAPHPAD (GraphPad Software, San Diego).

cAMP Determination. cAMP accumulation was measured by competition binding (22). Briefly, aliquots of samples were dried under vacuum and reconstituted with 20 mM phosphate buffer (pH 6.0). [^{3}H]cAMP (0.5 pmol) was added to each sample as an internal standard to compete with unlabeled cAMP for the binding to bovine adrenal binding protein. The unbound cAMP was then removed by the addition of activated charcoal. The radioactivity in the protein-bound cAMP was measured by liquid scintillation counting. A standard curve was generated by plotting the radioactivity vs. the $\log_{10}$ concentration of cAMP. cAMP levels in samples were extrapolated from the standard curve.

PKA Activity Assay. PKA assay kits were purchased from GIBCO/BRL. The manufacturer's protocol was followed. Cell homogenate was incubated with the reaction mixture containing 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP, 0.25 mg of bovine serum albumin per ml, and 50 μM substrate peptide, Kemptide (23), for 5 min at 30°C. The reaction was terminated by spotting the mixture on phosphocellulose membrane followed by three washes with 1% phosphoric acid. The radioactivity was determined by liquid scintillation counting. PKA activity was defined as the amount of phosphate incorporated into substrate peptide in the presence of 10 μM cAMP minus that incorporated in the presence of 1 μM PKA inhibitory peptide, PKI (24).

βARK Activity Assay. βARK activity was measured by using urea-treated bovine rod outer segments as substrate as described by Benovic *et al.* (25). The reaction was performed at 30°C for 30 min in the presence or absence of normal laboratory illumination and terminated by the addition of 2× Laemmli sample loading buffer. The reaction mixtures were resolved by SDS/10% polyacrylamide gel electrophoresis. Gels were fixed and dried before they were subjected to autoradiography. The phosphorylation of rhodopsin was quantified by densitometric analysis of the autoradiogram.

PKC Activity Assay. PKC assay kits were purchased from GIBCO/BRL. The assay protocol developed by Yasuda *et al.* (26) was followed with minimal modification. Briefly, cells were extracted with 20 mM Tris buffer (pH 7.5) containing 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 25 μg of aprotinin per ml, and 25 μg of leupeptin per ml, and the extract was fractionated on a DEAE column (Whatman DEAE 52). Fractions eluted at 200 mM NaCl were used for activity assay. A synthetic, acetylated peptide derived from myelin basic protein (Ac-MBP) was used as substrate to assess the PKC activity. The reaction mixture contained 20 mM Tris·HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM $CaCl_2$, 20 μM [γ-^{32}P]ATP, and 50 μM Ac-MBP, and the reaction proceeded for 5 min at 30°C. At the end of the reaction, the mixture was spotted onto a phosphocellulose membrane, which was extensively washed, and the radioactivity was determined by liquid scintillation counting. The specific activity of PKC is the difference between phosphorylation of Ac-MBP in the presence of 10 μM phorbol 12-myristate 13-acetate and that in the presence of 20 μM inhibitory peptide, PKC (19–36).

Antisense Oligodeoxynucleotides. All of the oligodeoxynucleotides were synthesized and purified to cell culture grade by Operon Technologies (Alameda, CA). The antisense oligonucleotides designated oligo$_{\text{anti-PKA}\alpha}$, CTGCTCGCTGCCCTTCTTGG; oligo$_{\text{anti-PKA}\beta}$, GCGATCGCAGTGTTCCCCAT; oligo$_{\text{anti-}\beta\text{ARK}}$, ACCGCCTCCAGGTCCGCCAT; and oligo$_{\text{anti-PKC}}$, AAGGTGGGCTGCTTGAAGAA, are derived from the complementary DNA sequences encoding mouse PKA catalytic α subunit (28), mouse PKA catalytic β subunit (29), human βARK1 (30), and human PKC α isoform (31), respectively. Oligo$_{\text{anti-}\beta\text{ARK}}$ targets both βARK1 and βARK2 but not rhodopsin kinase or G-protein-coupled receptor kinases 4 and 5. Oligo$_{\text{anti-PKC}}$ targets α, β, γ, and δ isoforms of PKC. The antisense oligonucleotides were dissolved in DMEM to 300 μM as stock solutions and diluted 1:9 in experiments. In all of the experiments unless stated otherwise, oligo$_{\text{anti-PKA}\alpha}$ and oligo$_{\text{anti-PKA}\beta}$ were used in combination, 15 μM each.

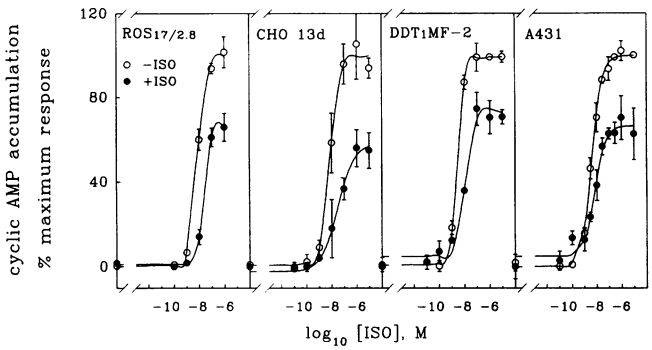

FIG. 1. Agonist-induced desensitization of G-protein-coupled receptors: analysis of β-adrenergic stimulation of cAMP accumulation. Cells were grown in 96-well plates and preincubated with (●) or without (○) 1 μM isoproterenol (ISO) for 30 min, followed by extensive washing (5 min). Cells were then challenged with increasing concentrations (from 0.01 nM to 10 μM) of this β-adrenergic agonist. cAMP accumulation in response to the second challenge was determined. Maximal accumulation of cAMP (pmol per 15 min per well) in response to 1 μM isoproterenol without and with desensitization was 44.8 and 22.9 in ROS 17/2.8 cells, 11.4 and 6.9 CHO cells in 13d cells, 5.1 and 3.7 in DDT$_1$MF-2 cells, and 68.2 to 46.2 in A-431 cells, respectively. For each experiment, "100%" represents cAMP accumulation in response to a maximal concentration of isoproterenol (minus basal cAMP accumulation) in naive, control cells. The data are presented as mean values ± SEM (n = 4).

Incubation with Oligodeoxynucleotides. After harvest, cells were suspended in serum-free DMEM at the density of 5×10^5 cells per ml. Forty microliters of cell suspension and 5 μl of 300 μM oligodeoxynucleotide stock solution were added to each well of a 96-well plate and incubated at 37°C. After 30 min, cells were replenished with 5 μl of fetal bovine serum, and the incubation was continued for 2 days. A 2-day incubation did not change the apparent cell growth and viability. Nearly equal cell densities and protein contents were detected in non-treated, antisense, and sense oligodeoxynucleotide-treated

Table 1. PKA, PKC, and βARK activities and β_2-AR expression

Cell line	Activity*			β_2-ARs per cell
	PKA	PKC	βARK	
CHO 13d	20.3 ± 0.3	2.5 ± 0.3	2.6	65,000 ± 8000
ROS 17/2.8	12.0 ± 2.1	1.6 ± 0.2	0.9 ± 0.03	13,000 ± 3000
DDT$_1$MF-2	12.8 ± 0.5	0.6 ± 0.1	0.9 ± 0.2	15,000 ± 1000
A-431	5.8 ± 1.0	1.3 ± 0.2	1.0 ± 0.5	48,000 ± 2000

PKA activity is defined as the difference in phosphorylation of 50 μM Kemptide in the presence of 10 μM cAMP and 1 μM protein kinase inhibitory peptide. PKC activity is defined as the difference in phosphorylation of 50 μM Ac-MBP-(4–14) peptide in the presence of 10 μM phorbol myristate and 20 μM peptide inhibitor of PKC. βARK activity is defined as the amount of light-dependent phosphorylation of dark-adapted rhodopsin catalyzed by whole-cell homogenates. The β_2-AR number was determined by radioligand binding to intact cells incubated with the high-affinity, β-adrenergic antagonist ligand [^{125}I]-iodocyanopindolol (0.5 nM) in the presence or absence of 10 μM propranolol. The results are expressed as mean values ± SEM (n = 3).

*Protein kinase activities are reported on a "per cell" basis: PKA, nmol/5 min per 10^7 cells; PKC, nmol/5 min per 10^7 cells; βARK, arbitrary units/30 min per 10^7 cells.

cells. However, extending the incubation to 4 days reduced cell viability of A-431 cells to 30% in the presence of 15 μM $oligo_{anti\text{-}PKA\alpha}$/15 μM $oligo_{anti\text{-}PKA\beta}$.

Suppression of Protein Kinase Activities by Antisense Oligodeoxynucleotides. The effects of antisense oligodeoxynucleotides on the expression of protein kinases were sampled by activity measurements or immunoblotting in several cell lines. For PKA, activity measured in CHO cells treated with 30 μM $oligo_{anti\text{-}PKA\alpha}$ was reduced by 40% and 80% on days 2 and 4, respectively. The combination of 15 μM $oligo_{anti\text{-}PKA\alpha}$ and 15 μM $oligo_{anti\text{-}PKA\beta}$ reduced activity in DDT_1MF-2 cells by >70% on day 2. The sense oligodeoxynucleotide for PKA used under the same conditions failed to suppress the kinase activity (data not shown). Immunoblotting analysis with anti-PKC α isoform antibody (GIBCO/BRL) showed suppression of PKC expression in A-431 cells treated with $oligo_{anti\text{-}PKC}$ but not with the sense oligodeoxynucleotide for PKC. Measurement of PKC activity showed a lesser reduction in cells treated with $oligo_{anti\text{-}PKC}$, reflecting suppression of only a subset of this kinase family. Initially we attempted to measure βARK activity by using bovine rhodopsin in urea-stripped rod outer segments as substrate, an assay system proven useful in measuring βARK activity in extracts from cells in which βARK has been overexpressed (14). The relatively low abundance precluded measurement of βARK in crude extracts of cell lines treated with $oligo_{anti\text{-}\beta ARK}$ as previously noted (14). Limited by the expense of the oligodeoxynucleotides for mass cultures, we expressed the constructs stably in one of the cell lines (A-431) and demonstrated the elimination of both βARK and PKC isoforms by immunoblotting analysis (data not shown).

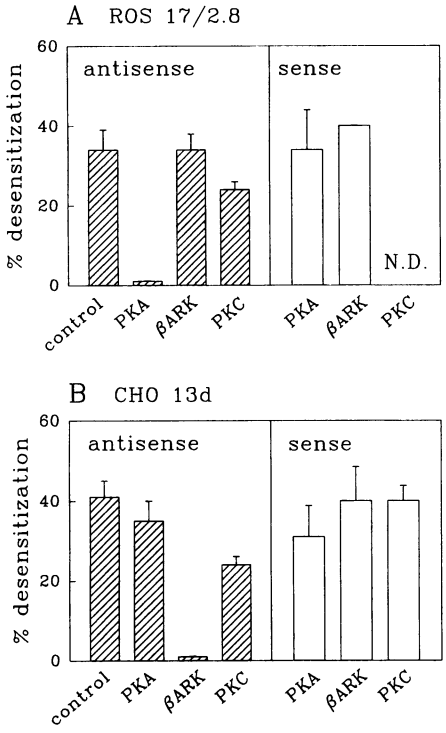

FIG. 2. Role of PKA, PKC, and βARK in agonist-induced desensitization of β_2-ARs: analysis by treatment of cells with oligodeoxynucleotides antisense to each kinase mRNA. Rat osteosarcoma 17/2.8 cells (*A*) and Chinese hamster ovary cells stably expressing β_2-ARs (clone 13d) (*B*) were grown in the presence or absence of 30 μM oligodeoxynucleotides either sense or antisense to the mRNA encoding the protein kinase targeted for 2 days prior to analysis of desensitization. Cells were then challenged for 30 min either with or without 1 μM isoproterenol followed by extensive washing. The washed cells were then challenged again either with or without 1 μM isoproterenol for 15 min, and the cAMP response was measured. The data are expressed as "% desensitization" in which "100% desensitization" would indicate no cAMP response on second challenge with agonist and "0% desensitization" would indicate a cAMP response in the second challenge equivalent to that obtained in the first challenge. For antisense oligodeoxynucleotide-treated cells, the results are expressed as the mean ± SEM from three separate experiments for each cell type. For cells treated with oligodeoxynucleotides, the results of a single experiment expressed as the mean ± SD are displayed. N.D., not determined.

RESULTS

Cell lines ROS 17/2.8, CHO 13d, DDT_1MF-2, and A-431, widely used to study agonist-induced desensitization of G-protein-coupled receptors (11, 32–35), were investigated. After β-adrenergic stimulation with 1 μM isoproterenol, each cell line displayed desensitization of cAMP accumulation in response to a second stimulation with a graded dose of isoproterenol (Fig. 1). Control cells displayed cAMP accumulation when concentrations of agonist exceeded 0.1 nM, with maximal accumulation at 100 nM. Desensitization was characterized by a reduction in the cAMP response of those cells previously challenged with agonist (1 μM isoproterenol for 30 min). At submaximal concentrations of agonist, desensitized cells displayed a 50–80% decline in their cAMP responses. With 1 μM isoproterenol, desensitized cells showed a reduction in cAMP response ranging from 25% to 40%. Additionally, ROS 17/2.8 and CHO 13d cells desensitized with isoproterenol displayed a 3- to 4-fold increase in the EC_{50} for agonist. The properties of agonist-induced desensitization are comparable to those reported from adenylylcyclase activity assays with membranes prepared from desensitized cells (11, 13, 36). The rapid-wash method developed for studying short-term regulation of receptors in intact cells offers excellent sensitivity, reproducibility, and avoids cell disruption. For the present study, desensitization was assayed *in vivo* as described above.

The activities of PKA, PKC, and βARK were established in each cell line, as was the level of expression of β_2-ARs (Table 1). Protein kinase activities were measured on a "per cell" basis under assay conditions providing full activation. CHO 13d cells displayed the greatest PKA activity, and A431 cells showed the least activity; ROS 17/2.8 cells and DDT_1MF-2 cells displayed approximately 60% of the PKA

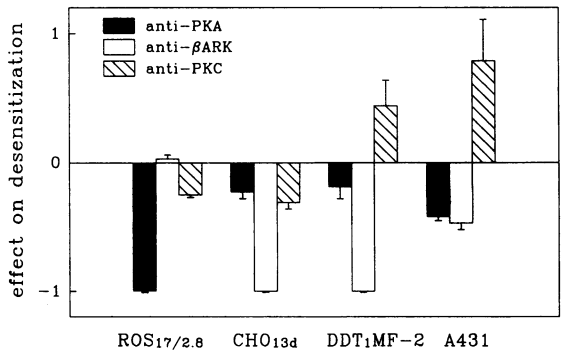

FIG. 3. Role of PKA, PKC, and βARK in agonist-induced desensitization of β_2-ARs: analysis by antisense RNA technology in several model cell lines. Cell growth and analysis of agonist-induced desensitization were as described in the legend to Fig. 2. The effect of antisense oligonucleotides ($oligo_{anti}$) is calculated as the % desensitization (see Fig. 2) in $oligo_{anti}$-treated cells minus that in control cells/% desensitization in control cells. Oligodeoxynucleotide-induced attenuation of desensitization provides a negative value, whereas potentiation of desensitization by prior treatment with oligodeoxynucleotides results in a positive value (*y* axis). Abolition of desensitization is equal to −1; a 100% increase in the magnitude of desensitization is equal to +1. The data are presented as mean values ± SEM from three to five independent experiments.

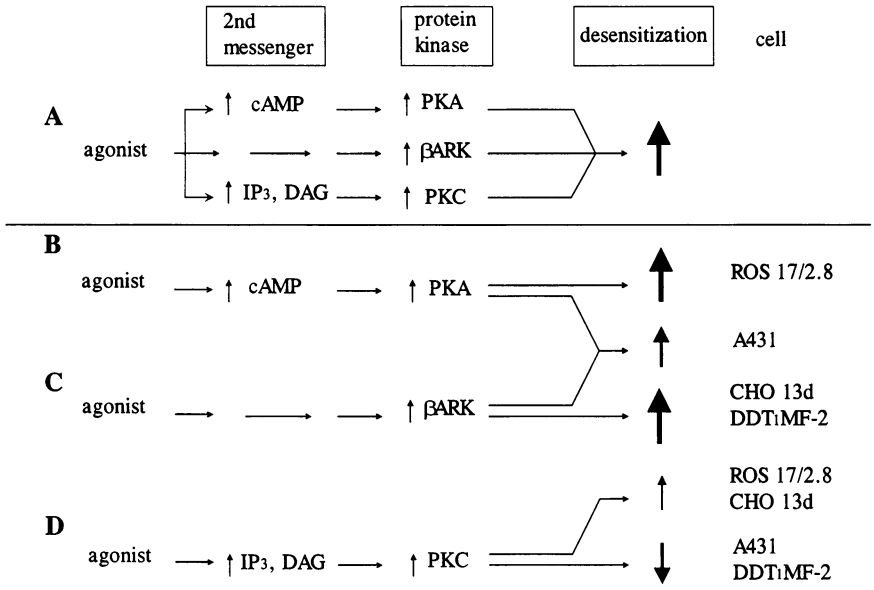

FIG. 4. Oligodeoxynucleotides antisense to protein kinase mRNAs reveal cell-type-specific roles for PKA, PKC, and βARK in agonist-induced desensitization. IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol.

activity observed in the CHO cells. PKC activities were highest in CHO 13d, least in DDT_1MF-2 cells, and intermediate (≈50% of CHO cell levels) in A-431 and ROS cell lines. βARK activity was equivalent in ROS, DDT_1MF-2, and A-431 cells, whereas it was about 2.5-fold greater in the CHO 13d cells. The rank order for expression of β_2-ARs (from highest to lowest) in the cell lines tested was CHO 13d > A-431 >> DDT_1MF-2 = ROS 17/2.8. No correlation between receptor expression and protein kinase activity was obvious.

The effects of treatment of cells with oligodeoxynucleotides for 48 hr, both sense and antisense to protein kinase mRNAs, on agonist-induced desensitization were explored. Treating ROS 17/2.8 cells with $oligo_{anti\text{-}PKA}$ completely blocked the agonist-induced desensitization, whereas $oligo_{anti\text{-}\beta ARK}$ and $oligo_{anti\text{-}PKC}$ failed to affect desensitization (Fig. 2*A*). By contrast, the treatment of CHO 13d cells with $oligo_{anti\text{-}\beta ARK}$ completely inhibited the desensitization, whereas treatment with $oligo_{anti\text{-}PKA}$ or $oligo_{anti\text{-}PKC}$ only slightly attenuated desensitization (Fig. 2*B*). Cells treated with sense oligodeoxynucleotides rather than antisense oligonucleotides to PKA, PKC, or βARK mRNA did not alter agonist-induced desensitization. Similarly, both agonist-stimulated and forskolin-stimulated cAMP accumulation was normal in cells treated with either sense or antisense oligodeoxynucleotides (data not shown).

The effects of antisense oligodeoxynucleotides to the mRNAs encoding the three protein kinases were explored to define *in vivo* the contribution of each to agonist-induced desensitization (Fig. 3). Most notable regarding the role of PKA, $oligo_{anti\text{-}PKA}$ was found to abolish desensitization in ROS 17/2.8 cells. For CHO 13d and DDT_1MF-2 cells, treatment with $oligo_{anti\text{-}PKA}$ resulted in only slight attenuation of desensitization. In contrast, $oligo_{anti\text{-}\beta ARK}$ abolished agonist-induced desensitization in both of these cell lines in which suppression of PKA activity had only a minor effect. $Oligo_{anti\text{-}\beta ARK}$ failed to affect agonist-induced desensitization in ROS 17/2.8 cells in which desensitization was abolished via suppression of PKA. In A-431 epidermoid carcinoma cells, oligodeoxynucleotides antisense to the mRNA encoding either PKA or βARK reduced desensitization by 40–50%.

Treating cells with $oligo_{anti\text{-}PKC}$ provided several new insights into desensitization. Since stimulation of cells with catecholamines would activate adenylylcyclase (via β_2-ARs) and PKC (via α-adrenergic receptors) as well, possible cross-regulation between these two G-protein-linked pathways was evaluated. In A-431 epidermoid carcinoma and DDT_1MF-2 hamster smooth muscle cells, $oligo_{anti\text{-}PKC}$ was found to enhance, rather than attenuate, β-agonist-induced desensitization. Sense oligodeoxynucleotides for this kinase had no effect on desensitization (data not shown). Suppression of PKC activity did not alter the ability of agonist to stimulate cAMP accumulation in naive cells [e.g., stimulation by 1 μM isoproterenol (or 50 μM forskolin) was identical for untreated cells and cells treated with oligodeoxynucleotides antisense to PKC mRNA]. Suppression of PKC did reduce, however, agonist-induced desensitization in ROS 17/2.8 and CHO 13d cells, suggesting that in some cells phosphorylation by PKC may contribute to agonist-induced desensitization.

DISCUSSION

By applying antisense technology in combination with the measurement of cAMP in intact cells, we explored agonist-induced desensitization *in vivo*. The different roles of protein kinases in modulation of G-protein-coupled receptor pathways were defined through specific suppression of each kinase. In contrast to the *in vitro* phosphorylation (10), overexpression of protein kinases (14), and mutagenesis of receptors (13), the current approach explores directly the involvement of a particular kinase in receptor regulation *in vivo*. In addition, two unique properties of antisense technology highlight advantages over the use of "selective" inhibitors of protein kinases. First, oligodeoxynucleotides can be used with intact, living cells, whereas kinase inhibitors, such as PKA inhibitory peptide and heparin, require cell permeabilization (11). Second, antisense oligodeoxynucleotides offer far greater specificity. In the absence of a selective inhibitor (37), antisense oligodeoxynucleotides provide a unique tool for specific suppression of βARK.

The current model for agonist-induced desensitization proposes a summation of the dominant roles of PKA and βARK (Fig. 4*A*) and a lesser but supporting role for PKC (10–14). The results shown for the A-431 cells fulfill the basic features of this model, demonstrating that suppression of either PKA or βARK attenuates desensitization by about 50%. However, the results derived from study of ROS 17/2.8, CHO, and DDT_1MF-2 cells clearly demonstrate that the "knockout" of a single protein kinase abolishes agonist-induced desensitization. One kinase, PKA in ROS 17/2.8 cells and βARK in CHO

and DDT_1MF-2 cells, is essential for agonist-induced desensitization. Accordingly, a new, more comprehensive scheme of desensitization emerges (Fig. 4 *B* and *C*). Agonist activation of cells leading to generation of a second messenger (cAMP) or the activation of a G-protein receptor kinase like βARK provides the dominant role in desensitization. A-431 cells display a hybrid pathway in which PKA and βARK converge.

A unique role for PKC is illuminated by the present study—i.e., counterregulation of agonist-induced desensitization (Fig. 4*D*). The failure for the $oligo_{anti\text{-}PKC}$ to alter stimulation of the adenylylcyclase by forskolin focuses attention on the receptor or G-protein levels, or on both levels. PKC has been shown to modulate the function of several G-protein-coupled receptors (38), and consensus sites for phosphorylation have been identified in many of these receptors. In the visual pathway, PKC has been shown to phosphorylate rhodopsin in the C-terminal region of the receptor (39). Phosphorylation of rhodopsin by PKC precludes further phosphorylation of this visual receptor by its receptor-specific kinase, rhodopsin kinase (39). Thus, phosphorylation of this visual receptor by PKC modulates the desensitization of receptor function catalyzed by rhodopsin kinase. Suppression of PKC reported in the present study promoted an enhanced desensitization by β-adrenergic agonists, suggesting a situation analogous to that in the visual pathway. However, these results do not preclude G proteins as other possible loci for PKC action in desensitization (40). We speculate that the different patterns of desensitization (Fig. 4) reflect multisite phosphorylation of the β_2-AR by tyrosine kinases (41) and by PKA, PKC, and βARK protein kinases, highlighting differences in the patterns of activation of those kinases in both the absence (basal) and presence of stimulation by β-adrenergic agonist.

The current study tests *in vivo* the linkage between βARK and β_2-AR-mediated responses. Oligodeoxynucleotides antisense to mRNAs encoding βARK 1 and βARK 2 "knocked out" desensitization induced by β-adrenergic agonists in CHO and DDT_1MF-2 cells. Molecular cloning has identified several new members of this family of G-protein–coupled receptor kinases (i.e., GRK4, GRK5, plus rhodopsin kinase) that have been shown to phosphorylate multiple receptors when reconstituted *in vitro* (42, 43). If the distribution of these receptor kinases were broad, our data would argue against redundancy at least with reference to the β_2-AR and would highlight the power of the antisense RNA approach towards answering questions central to our understanding of desensitization.

Note Added in Proof. After we submitted this paper, Kong *et al.* (44) reported that overexpression of a βARK dominant negative mutant attenuated desensitization of β_2-AR *in vivo*.

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