RNA Silencing Identifies PDE4D5 as the Functionally Relevant cAMP Phosphodiesterase Interacting with β Arrestin to Control the Protein Kinase A/AKAP79-mediated Switching of the β2-Adrenergic Receptor to Activation of ERK in HEK293B2 Cells*

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PDE4B and PDE4D provide >90% of PDE4 cAMP phosphodiesterase activity in human embryonic kidney (HEK293B2) cells. Their selective small interference RNA (siRNA)-mediated knockdown potentiates isoprenaline-stimulated protein kinase A (PKA) activation. Whereas endogenous PDE4D co-immunoprecipitates with β arrestin, endogenous PDE4B does not, even upon PDE4D knockdown. Ectopic overexpression of PDE4B2 confers co-immunoprecipitation with β arrestin. Knockdown of PDE4D, but not PDE4B, amplifies isoprenaline-stimulated phosphorylation of the β2-adrenergic receptor (β2-AR) by PKA and activation of extracellular signal-regulated kinase (ERK) through Gi. Isoform-selective knockdown identifies PDE4D as the functionally important species regulating isoprenaline stimulation of both these processes. Ht31-mediated disruption of the tethering of PKA to AKAP scaffold proteins attenuates isoprenaline activation of ERK, even upon PDE4D knockdown. Selective siRNA-mediated knockdown identifies AKAP79, which is constitutively associated with the β2-AR, rather than isoprenaline-recruited gravi, as being the functionally relevant AKAP in this process. Isoprenaline-stimulated membrane recruitment of PDE4D is ablated upon β arrestin knockdown. A mutation that compromises interactions with β arrestin prevents catalytically inactive PDE4D5 from performing a dominant negative role in potentiating isoprenaline-stimulated ERK activation. β arrestin-recruited PDE4D5 desensitizes isoprenaline-stimulated PKA phosphorylation of the β2-AR and the consequential switching of its signaling to ERK. The ability to observe a cellular phenotype upon PDE4D5 knockdown demonstrates that other PDE4 isoforms, expressed at endogenous levels, are unable to afford rescue in HEK293B2 cells.

It is now well appreciated that cAMP signaling is compartmentalized in cells (1–4). This notion arose originally from elegant studies done on cardiac myocytes (2) and gained considerable credence because the discovery of anchor proteins (AKAPs) for protein kinase A (PKA)4 allowed gradients of cAMP to be sensed and acted upon accordingly (1, 4, 5). More recently, a number of CAMP sensors have been developed that have allowed gradients of cAMP to be identified and even visualized in cells (6–9). Paramount to the generation and shaping of intracellular gradients is the action of cAMP phosphodiesterases, which provide the sole means of degrading cAMP in cells (2, 10–14). Of these, the PDE4 family of enzymes has gained attention in view of the fact that chemical knock-out with selective inhibitors indicates that they perform an important role in regulating key processes such as inflammation and cognition (15–20). Four genes (4A, 4B, 4C, and 4D) encode a large family of PDE4 isoforms (15, 17). Little is known, however, about the functional significance of each PDE4 sub-family. Nevertheless, important insights into physiological function have come from gene knock-out studies on the PDE4B and PDE4D sub-families, which have implied distinct roles for these sub-families in macrophage functioning, in muscarinic regulation of airway contraction, and in depression (21–25).

β-adrenergic receptors (β2-AR) couple to the G-protein Gi in an agonist-dependent fashion so as to activate adenylyl cyclase and thereby increase intracellular cAMP concentrations (26–31). The rapid uncoupling of this response can be achieved by the action of the G-protein receptor-coupled kinase, GRK2. This kinase phosphorylates the plasma membrane-associated β2-AR and allows recruitment of β arrestins from the cytosol, which then sterically interdict coupling of the β2-AR with Gi. The importance of such β arrestin interaction to G-protein-coupled receptor function in vivo has been clearly established in knock-out mouse models (32, 33). Recently, a new feature of the GRK/β arrestin desensitization system associated with the β2-AR has been identified (34–37), namely that PDE4 isoforms from all four sub-families can interact with β arrestins, due to a common interaction site within their catalytic unit. This allows β arrestin to cause an agonist-dependent increase in cAMP degradation in the locality of the β2-AR by recruiting

4 The abbreviations used are: PKA, protein kinase A; PDE, cyclic 3’,5’-AMP phosphodiesterase; PDE4, rolipram-inhibited, cAMP-specific PDE; rolipram, 4-(3-cyclopentoxyl)-4-methoxyphenyl)-2-pyrrolidone; ERK, extracellular signal-regulated kinase; OA51, oligoadenylate synthase; gravi, also known as AKAP250 and by its gene reference AKAP12 (AKAP79, is the human homologue of rodent AKAP150) and is also known by its gene reference as AKAP5; β2-AR, β2-adrenergic receptor; siRNA, small interference RNA; GFP, green fluorescent protein; MEF, mouse embryo fibroblast; LF2000, Liptofectamine 2000; MIE, mitogen-activated protein kinase/extracellular signal-regulated kinase.
PDE4 isoforms to the $\beta_2$-AR. Such recruited PDE4 can regulate the activity of PKA at the plasma membrane, thereby influencing the PKA phosphorylation status of the $\beta_2$-AR itself (36). This has a functional correlate as the PKA-mediated phosphorylation of the $\beta_2$-AR allows it to switch its coupling from $G_s$ to $G_i$ (38). The consequence of this is that the activated $\beta_2$-AR can effect a transient activation of ERK, in a $G_s$ and PKA-dependent fashion (38). 

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Cell Culture, Membrane Isolation, and Transfection—HEK293B2 cells, a kind gift from Prof. Graeme Milligan (University of Glasgow, Glasgow, Scotland, UK), are a clone of HEK293 cells, which stably over-express a FLAG-tagged $\beta_2$-adrenoreceptor-GFP construct (43). These were cultured as described in Ref. 43. Transfection of these cells was done using Polyfect (Qiagen), following the manufacturer’s instructions. Pretreatments, for control experiments, were done 10 min prior to isoproterenol addition (10 $\mu$M) with the following compounds: rolipram (10 $\mu$M), H89 (1 $\mu$M), and U0126 (10 $\mu$M). Cells were pre-treated with pertussis toxin (25 mg/ml) for 16 h before challenge with isoproterenol.

$\beta$Arrestin1/2 (–/–; –/–) double knock-out mouse embryo fibroblasts (MEFs) (34), were a gift from Prof. R. J. Lefkowitz (Howard Hughes Medical Institute Laboratories, Duke University Medical Center), and grown in Dulbeccos modified Eagle’s medium supplemented with 10% fetal calf serum in 5% CO$_2$. Transfection of these cells with a $\beta$Arrestin 2 encoding vector (pcDNA3) was carried out using Lipofectamine 2000 (LF2000, Invitrogen) following the manufacturer’s instructions. Pre-treatments for control experiments were done 10 min prior to isoproterenol addition (10 $\mu$M). As indicated, cells were pre-treated with either pertussis toxin (25 mg/ml) or the PKA inhibitor KT5720 (1 $\mu$M) for 16 h and 20 min, respectively, before challenge with isoproterenol. A high speed pellet (P2) membrane fraction was isolated from HEK293B2 cells as described before by us (36).

Western Blotting—Immunoblotting was done as previously described (36) using 25 $\mu$g of cellular protein per lane. After treatment, HEK293B2 cells were washed twice with phosphate-buffered saline before being scraped into lysis buffer (25 mM HEPES, 150 mM NaCl, 50 mM Tris, pH 7.5) containing 1% Triton X-100, 10 mM EDTA, 100 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, pH 7.5 with added protease inhibitors (Complete protease inhibitor mixture, Roche Applied Science). Proteins were separated by PAGE and transferred to nitrocellulose for Western blotting. Protein concentrations of cell lysates were determined with bovine serum albumin as a standard using Bradford reagent as previously described (36).

Immunopurification—This was done for the four PDE4 sub-families as described before by us (41, 44). Briefly, detergent-soluble proteins were isolated from cells by lysis in buffer 1% (v/v) Triton X-100, 50 mM HEPES buffer, pH 7.2, 10 mM EDTA, 100 mM Na$_2$HPO$_4$, 2H$_2$O) containing Complete protease inhibitor mixture to 8% volume. Detergent-insoluble proteins were removed by centrifugation at 10,000 $\times g_{av}$ for 10 min, and the soluble fraction was retained. Note that in cells studied here this procedure extracted and solubilized efficiently the immunoreactive PDE4 species as assessed by immunoblotting (>94%). To quantify the proportion of total PDE4 activity accounted for by the activity of individual PDE4 sub-families, quantitative immunopurification was done using polyclonal anti-sera specific for the C-terminal regions of PDE4A, PDE4B, PDE4C, and PDE4D. Equal volumes of cell lysate containing 500 $\mu$g of protein were cleared by incubation with 30 $\mu$L of pre-immune serum and 30 $\mu$L of protein A slurry. The beads were then removed by centrifugation at 10,000 $\times g_{av}$ for 10 min at 4 °C, and cleared lysate was incubated at 4 °C for 2 h with constant agitation with a volume of antisera determined to immunoprecipitate all of the specific PDE4 sub-family. Immunoglobulins were then isolated by incubation with protein-A-coated Sepharose beads for 1 h before retrieval by refrigerated centrifugation at 10,000 $\times g_{av}$ for 5 min. PDE4 isofrom-immunoglobulin conjugates attached to the beads were then washed in phosphate-buffered saline twice before two further washes in KHEM (50 mM KCl, 10 mM EDTA, 50 mM HEPES, and 1.92 mM MgCl$_2$, pH 7.2, with added 0.001 mM dithiothreitol and Complete protease inhibitor mixture to 8% volume prior to use) and one wash in PDE4 assay buffer (20 mM Tris, pH 7.4) prior to use. In doing this we ensured that the complete and specific immunopurification of the entire pool of the indicated proteins was achieved.
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cated PDE4 sub-family was achieved by Western blotting the supernatant with antisera for the four PDE4 sub-families. Immunopurified material thus acquired was used for either Western blot analysis or PDE4 assay. The β2-AR was immunopurified from HEK293B2 cells as described before by us in detail (36).

**Immunoprecipitation Studies of Endogenous βArrestin and PDE4 Isoforms**—This was done as described before by us (34–36). Briefly, cells were washed in KHEM buffer, harvested, homogenized, and fractionated as described above. Immunoprecipitation of endogenous βarrestin was done by pre-clearing the S2 fraction (500 μg of protein) with 50 μl of KHEM-washed protein A beads (Amersham Biosciences) before the addition of either 5 μl of polyclonal antibody against βarrestin (A1CT) or 5 μl of pre-immune serum. The tubes were incubated at 4 °C with end-over-end mixing for 1 h before the addition of 50 μl of protein A beads. The tubes were mixed for a further 2 h at 4 °C before the beads were pelleted at 1300 × g for 3 min. The beads were washed three times in KHEM buffer before being boiled in Laemmli SDS sample buffer (45). The immunoprecipitates and lysate (1/20th total lysate) were run on SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with the PDE4D monoclonal antibody. Each experiment was carried out on three separate occasions, and the amount of PDE4D3 and PDE4D5 that co-immunoprecipitated with the PDE4D monoclonal antibody. The sequences described here result from the culmination of an extensive study to find siRNA duplexes that achieve efficient knockdown of the indicated species with no effect on expression of isoforms from the other PDE4 sub-families found in these cells.

**siRNA Knockdown**—To achieve the selective knockdown of the entire 4B or 4D sub-families, we designed double-stranded 21-mer RNA duplexes targeted at regions of sequence that are unique to each of these sub-families. The 21-mer duplexes were engineered to have a GC content between 30 and 50% and to begin with an adenosine dimer (AA). All oligonucleotide sequences were screened against the human expressed sequence tag library data base to ensure specificity of the siRNA. The PDE4B siRNA is located in the region of the PDE4B transcript that codes for residues 291–297 (PTQKDR, numbering as in the PDE4B isoform, GenBank™ accession number M97515) of the sequence, AAGCAUCUCAGCUUUGGAGU. The PDE4D siRNA is located in the region of the PDE4D transcript that codes for residues 456–461 (ELALMY, numbering as in the PDE4D5 isoform, GenBank™ accession number AF_012073) of the sequence, AAGAAGCUUGCUGAUUAGCUA. Each PDE4 sub-family siRNA was obtained from Dharmacon. The sequences described here result from the culmination of an extensive study to find siRNA duplexes that achieve efficient knockdown of the indicated species with no effect on expression of isoforms from the other PDE4 sub-families found in these cells.

A nonspecific siRNA control (duplex XII, Dharmacon) was obtained and used at the appropriate optimal concentration. Note that cells that had not been exposed to any form of siRNA gave data as per those treated with this non-targeted (control) siRNA (data not shown).

To deplete PDE4D3 and PDE4D5 selectively, a Dharmacon proprietary method using the SMARTpool (siDESIGN) algorithm held at Dharmacon’s website (www.Dharmacon.com) provided the necessary sequences of pools of duplexes required to deplete each of these PDE4D isoforms specifically and selectively. SMARTpools of 4 oligonucleotides were employed to knockdown the two human isoforms PDE4D3 and PDE4D5. The PDE4D3 (L20970) SMARTpool contained 5′-CAGCATA-GCTGTCTCAACA-3′, 5′-TACAGTGGAGAACAAGA-3′, 5′-TGTAGCAGTGGAATAATT-3′, and 5′-GAGGTGGAGAATTCTCATC-TGTA-3′. The PDE4D5 (AF012073) SMARTpool contained 5′-GACAAGAACCGGGAACCTT-3′, 5′-GACAAGCGGGAGACATT-3′, 5′-GAAAACGGTGCCCTGGAT-3′, and 5′-CAAGCTCTCTCCCAGTGC-3′.

βArrestin1/2-specific siRNA has been described previously (46). We generated this here by using oligonucleotide primer (19 nucleotides long) sequences, as per these investigators, using an in vitro transcription kit called the Silencer siRNA Construction Kit (Ambion, Inc.). This uses T7 RNA polymerase to generate individual strands of the siRNA consisting of ~21-bp double-stranded RNAs with 3′-dinucleotide overhangs. The resultant siRNA was transfected into HEK293B2 cells as described below.

AKAP79 and AKAP12 (gravin) siRNA were purchased commercially. Human AKAP79-specific siRNA (catalog no. sc-29660, NM_004857) was from Santa Cruz Biotechnology. AKAP12-siRNA (AKAP250/ gravin, NM_005100) was from Qiagen. Transcripts for OAS1 in control and siRNA transfected cells were determined by TaqMan analysis (47).

**Transfection of PDE4-specific siRNA**—Each siRNA oligonucleotide was delivered into target cells via the reagent LF2000 (Invitrogen). Specifically, an aliquot of LF2000 (1 mg/ml) was diluted 25-fold into the reduced serum medium Opti-MEM (plus Glutamax, Invitrogen), whereas separately, the siRNA samples were diluted in Opti-MEM to a final concentration of 100 nM. Both sample types were incubated at room temperature for 10 min after which time the LF2000 and siRNA were mixed and incubated for an additional 20 min. Concurrently, the spent medium from cells grown to 40% confluence in 6-well plates was removed and replaced by an 800-μl aliquot of Opti-MEM. The siRNA transfection complexes were added, and the plates were incubated for 6 h at 37 °C (5% CO2). These complexes were then removed, replaced with Dulbecco’s modified Eagle’s medium, and all samples were reincubated for the apposite time and temperature.

**Co-transfection of PDE4D-specific siRNA and Ectopically Overexpressed PDE4B2**—Aliquots of 1 μg of pEE7 harboring the full-length PDE4B2 (39) (GenBank™ number L20971), together with the PDE4D-specific siRNA, were incubated in Opti-MEM (at room temperature). The co-transfection mixture was mixed with diluted LF2000 and added to the target cells in 6-well plates (as above). Cell lysates were recovered after 72-h incubation (37 °C and 5% CO2).

**Treatment with Ht31 Species**—Stock solutions (25 mM) of Ht31 and Ht31P peptides were dissolved in Me2SO and stored at 4 °C. Cells were pretreated with the peptides at a concentration of 50 μM for 30 min prior to isoprenaline (10 μM) addition. Control cells were pre-treated with the equivalent concentration of Me2SO (0.2%). Expression of the Ht31-GFP fusion protein was achieved through transient transfection with 1 μg of DNA of the plasmid harboring this construct.

**Transfection with Dominant Negative PDE4D5 Species**—Transfection into HEK293B2 cells was done as described above using our previously described catalytically inactive, dominant negative form of PDE4D5 that is able to bind βarrestin normally (36). This has a single point mutation (Asp-556 → Ala) of a conserved aspartate group in the catalytic site, which disrupts interaction with the Zn2+ and Mg2+ ions essential for catalysis. Additionally we generated (QuikChange) a form of catalytically inactive PDE4D5 with a single point mutation (Arg-34 → Ala) in its unique N-terminal region of PDE4D5. Such a mutation has previously been shown (35) to severely compromise the ability of βarrestin to interact with PDE4D5 by destroying this additional βarrestin binding site within the unique N-terminal region of PDE4D5.

**PDE Assays**—PDE activity was assayed using a modification of the Thompson and Appleman two-step procedure (48) as described previously by us (49). Briefly, a 2 μM mixture of [3H]cAMP (Amersham Biosciences) and unlabeled cAMP in 20 mM Tris, pH 7.4, 10 mM MgCl2 assay buffer was mixed with PDE4 isoform immunoprecipitates or complete cell lysates to a final concentration of 1 μM cAMP. To determine the fraction of activity due to PDE3 and PDE4 families, PDE activity was measured when the selective inhibitors cilostamide (1 μM) or rolipram (10 μM), respectively, were included in the reaction mixture. Reactions were then incubated at 30 °C for 20 min with frequent agitation. cAMP hydrolysis was stopped by plunging the tubes into boiling water for 3
samples were frequently mixed by repeated inversion. Samples were each reaction, vortexed, and placed on ice for 15 min. During this period, Bradford (51) using bovine serum albumin as a standard.

Each substrate unbound to the discs was washed away with 1% v/v phosphoric acid. The amount of incorporated $\left[{^{32}P}\right]$ATP to the synthetic peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) in a substrate solution (50 mM potassium chloride, 10 mM ATP, 10 mM MgCl$_2$, 250 $\mu$g/ml bovine serum albumin, 50 mM Tris, pH 7.5) for 10 min at 30 °C. The reaction was stopped by spotting onto phosphocellulose discs P81 (Whatman), and any substrate unbound to the discs was washed away with 1% v/v phosphoric acid. The amount of incorporated $\left[{^{32}P}\right]$ATP was determined by scintillation counting using a Wallac 1419 liquid scintillation counter. To determine the percentage of total PKA that was activated in response to treatment, four conditions were set up for each sample. The first was the control sample, the second was sample in the presence of 1 mM of the PKA inhibitor PKI(6–22) to ascertain background activity, the third was sample to which 10 mM cAMP was added to ascertain the maximal PKA activity, and, finally, sample to which both the PKA inhibitor and camp activator were added simultaneously. The percentage of total PKA activity was determined using the differences between the first two conditions and the second two conditions.

Protein Assays—Protein was routinely measured by the method of Bradford (51) using bovine serum albumin as a standard.

RESULTS

Selective Ablation of PDE4B and PDE4D in HEK Cells—Using chemical knock-out with the selective PDE4 inhibitor rolipram, we see that, using 1 mM cAMP as substrate, PDE4 isoforms provide ~68% of the total cAMP PDE activity in HEK293B2 cells (Fig. 1a). Also shown are Western blots of lysates from cells treated with the indicated PDE4B- and PDE4D-specific siRNA. These data are representative of experiments performed at least 4 times. b, a densitometric quantification of the levels of expression of each of these three isoforms in either PDE4B siRNA- or PDE4D siRNA-treated cells relative to the levels of these isoforms in untreated cells (PDE4B2 (solid bars); PDE4D3 (hatched bars); and PDE4D5 (dotted)). Data are means ± S.D. for $n = 3$ different experiments. Using scrambled siRNA we observed no change in the levels of any of these isoforms (~5% change; data not shown). c, PKA activation ratio in unstimulated HEK293 cells (0 min) and those treated for 5 min with 10 mM isoproterenol (5 min). This was done for cells transfected with scrambled siRNA and then subjected to either no other treatment (control, hatched bars) or with 10 mM rolipram (plus/− rolipram, solid bars). Cells were also treated with siRNA to knock-down selectively either PDE4D (siD, vertical stripes) or PDE4B (siB, open bars) prior to challenge with isoproterenol. Shown are the PKA activation ratios as means ± S.D. for $n = 3$ separate experiments.

Selective Immunopurification of PDE4B and PDE4D—Using recombinant standards, with antisera specific for PDE4D5, and confirmation by transcript analysis using specific primers (data not shown and Refs. 35, 41, 44, and 52). Also shown are Western blots of lysates from cells treated with the indicated PDE4B- and PDE4D-specific siRNA. These data are representative of experiments performed at least 4 times. a, densitometric quantification of the levels of expression of each of these three isoforms in either PDE4B siRNA- or PDE4D siRNA-treated cells relative to the levels of these isoforms in untreated cells (PDE4B2 (solid bars); PDE4D3 (hatched bars); and PDE4D5 (dotted)). Data are means ± S.D. for $n = 3$ separate experiments.
allowed the selective immunopurification of the four PDE4 sub-families for activity analyses, as exemplified in studies done on monocytes and macrophages (41, 44). Applying such a strategy to HEK293B2 cells we see that ~92% of the PDE cAMP PDE activity in these cells is contributed by PDE4B and PDE4D together, with the remainder by PDE4A (Fig. 1b). There is no observable PDE4C activity (<2%).

Focusing on the major PDE4B and PDE4D activities in these cells, Western blotting identifies a single PDE4B immunoreactive species, namely the 68-kDa PDE4B2 short form together with two PDE4D immunoreactive species, the 98-kDa PDE4D3 and the 105-kDa PDE4D5 long isoforms (Fig. 2a). Transcripts confirming the identity of these isoforms are detected by RT-PCR (data not shown) using specific primers as specified previously by us (52).

Small interfering RNA (siRNA) strategies provide a powerful and novel means to achieve the selective knockdown of specific proteins in cells (53, 54). We employ this methodology here to deplete selectively the major PDE4B and PDE4D sub-families in these cells. This involves optimizing the design of oligonucleotides as well as the time and means of transfection so as to achieve the selective knockdown of these two families to high efficiency (see "Materials and Methods"). Under such optimized conditions we achieve ~95% selective knockdown of each of these PDE4 sub-families, as detected by Western blotting (Fig. 2, a and b). PDE4 sub-family-specific siRNA-mediated knockdown causes a comparable reduction in PDE4 activity to the level of activity we identify through immunopurification of the respective sub-family (Fig. 1b). This is consistent with specific knockdown being achieved by targeted siRNA.

We saw no compensating up-regulation, in terms of either PDE4 isoform expression or PDE activity, with knockdown of either PDE4D or PDE4B in this human cell line (Figs. 1 and 2). Neither did we see any up-regulation/induction of PDE4A and PDE4C isoforms with knockdown of PDE4B/D (data not shown). These observations strongly sup-

port observations made on various tissues from mice with targeted gene knock-out of PDE4B and PDE4D, which similarly did not show compensatory changes in PDE4 sub-family expression (21–23).

Knockdown of both PDE4B and PDE4D acts to potentiate the ability of 5 min treatment with isoprenaline to activate PKA in HEK293B2 cells (Fig. 2c). In neither case was the magnitude of this effect as great as using chemical knock-out with the PDE4 selective inhibitor rolipram, which inhibited all PDE4 enzymes (Fig. 2c). The effect of PDE4D knockdown was, however, greater than that achieved with PDE4B knockdown (Fig. 2c). This is consistent with PDE4D contributing a greater proportion of total PDE4 activity in HEK293B2 cells compared with PDE4B (Fig. 1).

Here we have used distinctly targeted sets of siRNA oligonucleotides to achieve the selective knockdown of either the PDE4B or the PDE4D subfamilies to compare the functional outcome on β2-AR switching and interaction with β-arrestin in a defined cell type. Clearly, these two sets of oligonucleotides act as an internal control for each other in identifying changes that are specific to the knockdown of a particular PDE4 subfamily. However, because it is possible that any common changes could be nonspecific, we also assess the action of non-targeted siRNA. Here we show that such non-targeted siRNA fails to affect total PDE activity, total rolipram-inhibited PDE4 activity, and total cilostamide-inhibited PDE3 activity in these cells (<6% change; n = 3 separate experiments).
Knockdown of PDE4D amplifies isoprenaline-stimulated PKA phosphorylation of the β2-AR. A time course of the PKA phosphorylation of the β2-AR is shown for cells challenged with isoprenaline and, as indicated, subject to knockdown of either PDE4D (a) or PDE4B (b). Detection is by Western blotting using a phospho-specific antisera. c, Western blotting data from cells having an amplified response to isoprenaline (5 min), through being treated with siRNA for PDE4D. These were then, as indicated, challenged with the PKA-selective inhibitors, either H89 (0.5 μM) or KT5720 (1 μM), as indicated. These data are representative of three separate experiments. d, relative levels of expression, as determined immunologically using the appropriate specific antisera, for the β2-AR, β-arrestin, and ERK. Data are means ± S.D. for n = 3 separate experiments. e, PKA phosphorylation of the β2-AR in HEK293B2 cells occurring upon challenge with isoprenaline in control cells (solid bars) and those treated with siRNA to either PDE4B (hatched bars) or PDE4D (shaded bars).

It also does not change either the pattern or the level of expression of the various PDE4 isoforms expressed in these cells (<5% change; n = 3 experiments; data not shown).

Although we use oligonucleotides in this study, it has been demonstrated that DNA vectors that express short hairpin RNAs from RNA polymerase III promoters can trigger an interferon response, as detected by induction of transcripts for OAS1 (47). However, we saw no increase (<3%) in transcripts for OAS1 in any of our transfections with any of the various siRNAs used in this study (data not shown).

β-arrestin Selectively Complexes PDE4D in HEK Cells—PDE4 enzymes from all four sub-families can interact with β-arrestin due to a common conserved binding site located within their catalytic unit (34). We have shown previously that β-arrestin binds to PDE4D isoforms in HEK293B2 cells and that β-arrestin-PDE4D complex is translocated to the membrane-bound β2-AR in an agonist-dependent fashion (34). Here we show that while immunoprecipitation of β-arrestin does allow pull-down of endogenous PDE4D3 and PDE4D5, it does not co-immunoprecipitate endogenous PDE4B2 (Fig. 3a).

There is an increasing realization that PDE4 isoforms are uniquely targeted to either membranes or scaffold proteins, which is thought to allow them to underpin compartmentalized signaling in many cell types (9, 15, 17, 55, 56). The inability of PDE4B2 to interact with β-arrestin implies that PDE4B2 may be tightly complexed with a competing protein in these cells such that there is no PDE4B2 available to bind β-arrestin. To evaluate such a proposal we attempted a “rescue” experiment through ectopic expression of recombinant PDE4B2. Here we see that overexpression of recombinant PDE4B2 in these cells neither affects endogenous levels of PDE4D3/5 nor the siRNA-mediated knockdown of PDE4D (Fig. 4, a and b). Neither does knockdown of either PDE4D or PDE4B, nor transfection with PDE4B2, affect levels of β-arrestin in these cells (Figs. 3b, 4c, and 4e). However, ectopic overexpression of recombinant PDE4B2 clearly allows this isoform now to co-immunoprecipitate with β-arrestin (Fig. 4d). Thus endogenous PDE4D, and not PDE4B, interacts with β-arrestin in these cells.

Knockdown of PDE4D, but Not PDE4B, Enhances the Isoprenaline-stimulated Phosphorylation of the β2-Adrenoreceptor by PKA—The β-agonist, isoprenaline, elicits the phosphorylation of the β2-AR both by GRK and by PKA (26, 27, 29, 31). The PKA phosphorylation of the β2-AR can be detected using specific phospho-antibodies. As shown before by us (36), isoprenaline challenge of these cells causes the transient phosphorylation of the β2-AR (Fig. 5). Although knockdown of PDE4B has no effect on this (Fig. 5b), knockdown of PDE4D profoundly potentiates both the speed and the magnitude of this response (Fig. 5, a

FIGURE 5. Knockdown of PDE4D amplifies isoprenaline-stimulated PKA phosphorylation of the β2-AR. A time course of the PKA phosphorylation of the β2-AR is shown for cells challenged with isoprenaline and, as indicated, subject to knockdown of either PDE4D (a) or PDE4B (b). Detection is by Western blotting using a phospho-specific antisera. c, Western blotting data from cells having an amplified response to isoprenaline (5 min), through being treated with siRNA for PDE4D. These were then, as indicated, challenged with the PKA-selective inhibitors, either H89 (0.5 μM) or KT5720 (1 μM), as indicated. These data are representative of three separate experiments. d, relative levels of expression, as determined immunologically using the appropriate specific antisera, for the β2-AR, β-arrestin, and ERK. Data are means ± S.D. for n = 3 separate experiments. e, PKA phosphorylation of the β2-AR in HEK293B2 cells occurring upon challenge with isoprenaline in control cells (solid bars) and those treated with siRNA to either PDE4B (hatched bars) or PDE4D (shaded bars).
Knockdown of PDE4D5, but Not PDE4D3, Enhances Both the Isoprenaline-stimulated ERK Activation. Knockdown of PDE4D5 potentiates the ability of isoprenaline to stimulate ERK phosphorylation. As shown before (36), we observed here that challenge of these cells with the β2-AR agonist, isoprenaline, triggers the transient activation of ERK (Fig. 6, a and c). This action is sustained and amplified upon siRNA-mediated knockdown of PDE4D (Fig. 6, a and c) but is unaffected by knockdown of PDE4B (Fig. 6, b and c). Note that knockdown of either PDE4B or PDE4D does not alter either ERK expression levels (Figs. 5d, 7a, and 7b) or the ability of PMA to activate ERK (data not shown).

We also see that the magnitude of amplification of isoprenaline-stimulated ERK activation achieved by PDE4D knockdown is identical to that achieved using chemical "knockdown" of the entire pool of PDE4 activity in these cells with the PDE4 selective inhibitor, rolipram (Fig. 7, a and c). The amplification of isoprenaline-stimulated ERK activation elicited upon PDE4D knockdown is clearly dependent upon both PKA and Gi-mediated ERK activation achieved by PDE4D knockdown is identical to that achieved using chemical "knockdown" of the entire pool of PDE4 activity in these cells with the PDE4 selective inhibitor, rolipram (Fig. 7, a and c). The amplification of isoprenaline-stimulated ERK activation elicited upon PDE4D knockdown is clearly dependent upon both PKA and Gi-mediated activation. This amplification is ablated upon PMA treatment of either the PKA inhibitors, H89 and KT5720 (Fig. 5c). Knockdown of either PDE4B or PDE4D does not alter the level of expression of the β2-AR (Fig. 5d).

Knockdown of PDE4D, but Not PDE4B, Enhances the Activation of ERK by Isoprenaline—Phosphorylation, by PKA, switches the coupling of the β2-AR to the Gi-mediated stimulation of ERK in these cells (36). As shown before (36), we observed here that challenge of these cells with the β2-AR agonist, isoprenaline, triggers the transient activation of ERK (Fig. 6, a and c). This action is sustained and amplified upon siRNA-mediated knockdown of PDE4D (Fig. 6, a and c) but is unaffected by knockdown of PDE4B (Fig. 6, b and c). Note that knockdown of either PDE4B or PDE4D does not alter either ERK expression levels (Figs. 5d, 7a, and 7b) or the ability of PMA to activate ERK (data not shown).

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of their unique N-terminal regions, which are encoded by distinct exons (15, 17, 57–59). Taking advantage of such differences, we utilized specific siRNA reagents targeted to these regions and successfully achieved their selective knockdown (Fig. 8a), without affecting expression of other PDE4 subfamilies in these cells (data not shown). Using these reagents we see that knockdown of PDE4D5, but not PDE4D3, enhances the ability of isoprenaline to elicit PKA phosphorylation of the $\beta_2$-AR (Fig. 8, b and d) and to activate ERK (Fig. 8, c and e).

Isoprenaline-stimulated Phosphorylation of $\beta_2$-Adrenoreceptor by PKA and Activation of ERK Is Dependent upon AKAP79 and Not Gravin—Gradients of cAMP in cells are sampled by anchored forms of PKA that bind to members of a family of proteins known collectively as AKAPs (1, 4). Although AKAPs are structurally unrelated, they all interact with PKA in the same manner, namely through a site formed from the dimerization interface of the regulatory, cAMP binding subunits (60, 61). The membrane-permeable, acylated form of the Ht31 peptide, as well as a plasmid that expresses a Ht31-GFP fusion protein, have been used to disrupt the tethering of PKA to AKAP in intact cells (42, 62, 63) and thereby to determine whether AKAP-bound PKA is involved in particular cellular processes (4). Here, we show that both such reagents attenuate the ability of isoprenaline to activate ERK in HEK293B2 cells (Fig. 9, a and b). Furthermore, these PKA-AKAP disruptors also attenuate the ability of siRNA-mediated PDE4D knockdown to enhance the isoprenaline-mediated activation of ERK (Fig. 9). Some cells are also treated, as indicated (Fig. 9b), with a control peptide (Ht31P), where two essential isoleucines are replaced by proline such that the helical nature of this peptide is destroyed, and so it becomes unable to disrupt AKAP-PKA interactions. No changes were seen using this control peptide (Fig. 9b).

It has been shown previously that the $\beta_2$-AR can be co-immunoprecipitated from rat brain with AKAP150, the rodent homologue of human AKAP79, indicating a constitutive interaction between these species (64). Additionally, these investigators demonstrated that co-transfection of HEK293B2 cells with both the $\beta_2$-AR and AKAP79 allowed such recombinant species to be co-immunoprecipitated (64).

Here we show that in HEK293B2 cells the $\beta_2$-AR can be co-immunoprecipitated with endogenous AKAP79 (Fig. 9c). Indeed, AKAP79 appears to be constitutively associated with the $\beta_2$-AR in HEK293B2 cells, and the level of its association with the $\beta_2$-AR is seemingly unaffected by challenge of cells with either isoprenaline ($I$) or isoprenaline plus rolipram ($I + R$) over a period (5 min). Such conditions suffice, however, to allow for the activation of PKA (Fig. 2c), PKA phosphorylation of the $\beta_2$-AR (Fig. 5e), and recruitment of farnesin-PDE4D complex to the $\beta_2$-AR/membrane fraction (34, 35). Similarly, over this time of challenge of HEK293B2 cells with either isoprenaline ($I$) or isoprenaline plus rolipram ($I + R$), there was no change in the amount of AKAP79 associated with the P2 membrane fraction (Fig. 9c). The siRNA-mediated knockdown of AKAP79, in HEK293B2 cells, prevented challenge either isoprenaline ($I$) or isoprenaline plus rolipram ($I + R$) from initiating both the PKA phosphorylation of the $\beta_2$-AR and activation of ERK (Fig. 9d), and also for $\beta_2$-AR immunoprecipitates ($IP$). In the right-hand panel immunoblots are shown for the membrane (P2) fraction (50 $\mu$g of protein). d, data for knockdown experiments done on AKAP149. These employ transfections done using either scrambled or AKAP179-specific siRNA, as indicated. Subsequent to knockdown assessed by immunoblotting (see "Materials and Methods"), cells were challenged for the indicated time (0 or 5 min) with either isoprenaline alone ($I$) or isoprenaline together with rolipram ($I + R$) before being harvested for analysis by Western blotting for P-Erk1/2, AKAP179, and the PKA-phosphorylated form of the $\beta_2$-AR. Experiments shown in a and b are as in c and d, respectively, except that in this case it is gravin that is subject to specific knock-down and detection, instead of AKAP179. Treatment and immunoblotting for P-Erk1/2 and the PKA phosphorylated form of the $\beta_2$-AR were done as in d and e. These various data are representative of three separate experiments.

FIGURE 9. Isoprenaline activation of ERK in HEK cells is dependent upon AKAP179. a, data for cells that either had or had not been treated with a plasmid expressing an Ht31-GFP fusion protein. This fusion protein was detected using an anti-Ht31 antiserum (lower panel). Cells were also transfected with non-targeted siRNA (control) as well as siRNAs targeted to either PDE4B (siB) or PDE4D (siD) sub-families as indicated. Specific antiserum was used to detect total ERK2 and P-ERK1/2, as indicated. Cells were challenged with isoprenaline and harvested either immediately (0 min) or after 5 min exposure (5 min), as indicated. b, similar experiments. However, instead of transfecting cells with a Ht31-GFP plasmid, cells either were or were not treated with a membrane permeable, acylated Ht31 peptide. Some were also treated, as indicated, with a control peptide (Ht31P). c, immunoblots are shown using an AKAP179-specific antibody for HEK cells challenged for the indicated times with either isoprenaline ($I$) alone or isoprenaline plus rolipram ($I + R$). In the left-hand panel immunoblots are shown for both whole cell lysates and also for $\beta_2$-AR immunoprecipitates ($IP$). In the right-hand panel immunoblots are shown for the membrane (P2) fraction (50 $\mu$g of protein). d, data for knockdown experiments done on AKAP149. These employ transfections done using either scrambled or AKAP179-specific siRNA, as indicated. Subsequent to knockdown assessed by immunoblotting (see "Materials and Methods"), cells were challenged for the indicated time (0 or 5 min) with either isoprenaline alone ($I$) or isoprenaline together with rolipram ($I + R$) before being harvested for analysis by Western blotting for P-Erk1/2, AKAP179, and the PKA-phosphorylated form of the $\beta_2$-AR. Experiments shown in a and b are as in c and d, respectively, except that in this case it is gravin that is subject to specific knock-down and detection, instead of AKAP179. Treatment and immunoblotting for P-Erk1/2 and the PKA phosphorylated form of the $\beta_2$-AR were done as in d and e. These various data are representative of three separate experiments.
RNA Silencing of PDE4 CAMP Phosphodiesterases

In A431 human epidermoid carcinoma cells, it has been shown (65, 66) that isoprenaline challenge elicits the PKA phosphorylation of gravin to the β2-AR, this was only really evident some 5 min after challenge (Fig. 9e). In contrast to knockdown of AKAP79, the siRNA-mediated knockdown of gravin did not alter the ability of either isoprenaline alone (I) or isoprenaline plus rolipram (I + R) to trigger the PKA phosphorylation of the β2-AR and subsequent activation of ERK (Fig. 9f). Challenge with siRNA for AKAP79 did not affect expression levels of gravin and vice versa (data not shown).

**β-arrestin Is Required for Isoprenaline-stimulated Translocation of PDE4D in HEK Cells**—It has previously been shown that isoprenaline fails to cause PDE4D translocation in mouse embryo fibroblasts (MEFs) from double knock-out β-arrestin1/2 (−/−) mice (34). However, isoprenaline-induced PDE4D translocation is reconstituted when such cells are transfected to express β-arrestin2 (34). Here we show that siRNA-mediated knockdown of endogenous β-arrestin1 and β-arrestin2 in HEK293B2 cells (Fig. 10a) ablates the isoprenaline-stimulated recruitment of PDE4D in these cells (Fig. 10b). Thus interaction with β-arrestins confers isoprenaline-stimulated membrane recruitment of PDE4D in HEK293B2 cells.

**Interaction with β-arrestin Is Required for PDE4D to Regulate Isoprenaline-stimulated Phosphorylation of ERK**—Here we see that isoprenaline caused a marked increase in the phosphorylation status of both ERK1 and ERK2 in MEFs from double knock-out β-arrestin1/2 (−/−) mice (Fig. 10c). The major fraction of this action was mediated through Gs as indicated by the inhibitory effect that pertussis toxin treatment exerted on this process (Fig. 10d). The ectopic expression of β-arrestin2 in these cells has been shown to reconstitute isoprenaline-mediated membrane translocation of PDE4D (34). Here we see that ectopic expression of β-arrestin2, in β-arrestin1/2 (−/−) double knock-out MEFs, attenuated the ability of isoprenaline to stimulate ERK phosphorylation (Fig. 10e). This is consistent with ectopic β-arrestin2 conferring isoprenaline-stimulated PDE4D membrane translocation, which attenuates switching in these cells.

Making a single point mutation in its catalytic site (36) can generate a catalytically inactive form of PDE4D. This species is able to bind β-arrestins and thereby exert a dominant negative role by displacing active endogenous PDE4D from interaction with β-arrestins as seen by its ability to enhance isoprenaline-stimulated P-ERK formation (36). We see here that expression of catalytically inactive PDE4D5 (DN-PDE4D5), in HEK293B2 cells, markedly increased the ability of isoprenaline to activate ERK1/2 (Fig. 10f, e and f). However, PDE4D5 preferentially interacts with β-arrestins through its isoform-specific N-terminal region and, as shown before by us (35), a single point mutation (Arg-34 → Ala) can ablate this unique property. We see here that the potentiating effect of dominant negative PDE4D5 on isoprenaline-stimulated ERK phosphorylation was ablated if a Arg-34 → Ala form of it is used (Fig. 10, e and f). This shows that the potentiating effect of dominant negative PDE4D5 on this response is dependent upon its ability to interact with β-arrestin.

**DISCUSSION**

The multigene PDE4 enzyme encodes around 20 different isoforms, all of which are capable of specifically hydrolyzing cAMP with similar kinetics (15, 17). The high conservation of PDE4 isoforms among species (15, 17) indicates a strong selective pressure to retain this diversity, implying that distinct isoforms have particular functional roles in cells. Indeed, PDE4 isoforms are selectively expressed in different cell types, presumably to tailor cAMP signaling on a cell type-specific basis (15, 17). Thus, for example, the long and short form groupings of PDE4 isoforms differ profoundly in their susceptibility and response to phosphorylation by PKA and ERK (67, 68). However, each PDE4 isoform is uniquely characterized by its N-terminal region, and there is a growing

**FIGURE 10. Interaction with β-arrestin is fundamental to the functional role of PDE4D in regulating β2-AR switching.** a, immunoblotting of cell lysates with antisera to α-tubulin, β-arrestin2, and β-arrestin1/2 from HEK293 transfected with either scrambled (Scr) siRNA or siRNA specific for β-arrestin1/2. The detected species are indicated in a typical experiment of one done at least three times. Equal protein loading with α-tubulin was used as additional loading control. b, the isoprenaline-mediated membrane translocation of PDE4D is shown for HEK293 cells transfected with either scrambled siRNA (solid bars) or siRNA targeted to knockdown β-arrestin1/2 (hatched bars). Shown is quantification for three separate experiments (means ± S.D.) of translocated PDE4D assessed by immunoblotting with an antibody specific to α-tubulin. c, isoform-specific ERK phosphorylation in mouse embryo fibroblasts (MEFs) from β-arrestin1/2 knock-out mice as detected with a P-ERK-specific antibody using an α-tubulin antibody as a loading control. Data are shown for mock transfected cells and for those transfected so as to express β-arrestin2 with shOWN data using mock-transfected β-arrestin1/2 (−/−) MEFs that had been pre-treated with pertussis toxin to inactivate Gs. Cells were, as indicated, challenged for 5 min with isoprenaline (iso) or not (ctr). These data represent a typical experiment of one done three times. e, HEK293 cells are transfected with isoprenaline for 0, 1, 3, 5, and 10 min before lysates were immunoblotted for P-ERK, total ERK, and the vsv-epitope tag. Cells were transfected, as indicated, using an empty vector (mock) and with vsv-epitope-tagged forms of either a dominant negative PDE4D5 (DN; solid bars) or an Arg-34 → Ala mutant form of dominant negative PDE4D5 (R34A; DN-4DS). The immunoblots shown are from a typical experiment with the quantification (f) for the level of P-ERK achieved in three separate experiments being shown (means ± S.D.).

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Making a single point mutation in its catalytic site (36) can generate a catalytically inactive form of PDE4D5. This species is able to bind β-arrestins and thereby exert a dominant negative role by displacing active endogenous PDE4D from interaction with β-arrestins as seen by its ability to enhance isoprenaline-stimulated P-ERK formation (36). We see here that expression of catalytically inactive PDE4D5 (DN-PDE4D5), in HEK293B2 cells, markedly increased the ability of isoprenaline to activate ERK1/2 (Fig. 10f, e and f). However, PDE4D5 preferentially interacts with β-arrestins through its isoform-specific N-terminal region and, as shown before by us (35), a single point mutation (Arg-34 → Ala) can ablate this unique property. We see here that the potentiating effect of dominant negative PDE4D5 on isoprenaline-stimulated ERK phosphorylation was ablated if a Arg-34 → Ala form of it is used (Fig. 10, e and f). This shows that the potentiating effect of dominant negative PDE4D5 on this response is dependent upon its ability to interact with β-arrestin.
appreciation that these regions play a key role in targeting of isoforms to either specific intracellular sites or to complexes with signaling scaffold proteins (15, 17). Indeed, recent evidence suggests that specific PDE4 isoforms underpin compartmentalized cAMP signaling in cardiac myocytes, as identified using genetically encoded cAMP sensors (9, 56), as well as in various other cells, as inferred from the selective action of PDE4 inhibitors (18–20, 69). It seems likely that this is underpinned by isoform-specific patterns of intracellular scaffolding/targeting. Consistent with there being specific functional roles for PDE4 isoforms, the targeted gene knock-out of the PDE4B and PDE4D sub-families has generated distinct phenotypes, as noted in altered functioning of macrophages, muscarinic receptor regulation of airway contraction, and models of depression (21–23).

β2-ARs provide an important role in regulating cellular function in many cell types. They act to elevate intracellular cAMP levels by stimulating adenyl cyclase through Gi (26–31). However, agonist-occupied β2-ARs become a target for phosphorylation by GRK2, which triggers recruitment of cytosolic βarrestins. Such βarrestin-bound β2-ARs are then unable to interact with Gi, causing rapid desensitization. Recently, an intriguing new facet of this process has been uncovered; namely that PDE4 isoforms from all four sub-families can interact with βarrestins (34–36). Thus, by binding PDE4, agonist-recruited βarrestin causes an increase in cAMP degradation in the locality of the β2-AR itself. This is likely to be of particular importance, because AKAP-tethered PKA at the plasma membrane plays a key role in phosphorylating the β2-AR and, thereby, switching its coupling from Gi-mediated activation of adenyl cyclase to Gi-mediated activation of ERK (31, 38, 64). Given that cells can be expected to express various tethering proteins for the different PDE4 isoforms they express, we set out to determine, in a defined cell type, whether particular PDE4s might associate with βarrestin and thereby regulate switching of β2-AR coupling to Gi-mediated activation of ERK.

HEK293B2 cells express PDE4A, PDE4B, and PDE4D isoforms, but not PDE4C. Selective immunopurification shows that the PDE4B and PDE4D sub-families provide the majority of PDE4 activity (Fig. 1). These sub-families generate the PDE4B2, PDE4D3, and PDE4D5 isoforms in these cells (Fig. 2). We designed siRNA reagents against regions that, although common to all isoforms in a particular sub-family, were also sub-family specific. These allowed us to achieve the highly effective and specific knockdown of the indicated PDE4 sub-family without ablated expression of isoforms from other sub-families (Fig. 1, 2). Consistent with this, selective knockdown of a sub-family caused a reduction in PDE4 activity that corresponded in magnitude to that achieved by selective immunopurification of the target PDE4 sub-family (Fig. 1b).

Interestingly, in achieving such selective knockdowns we observed no compensating up-regulation in either total PDE activity or the expression of PDE4 isoforms from other sub-families, as assessed immunologically. This lack of compensation strongly supports results from in vivo targeted gene knock-out studies done on PDE4B and PDE4D, which also appeared not to generate any up-regulation of other PDE4 isoforms in the various tissues studied (21, 22).

Despite the fact that PDE4B and PDE4D each provide major fractions of PDE4 activity in HEK293B2 cells, it is only endogenous PDE4D3 and PDE4D5 that co-immunoprecipitate with endogenous βarrestin and not any PDE4B2 (Fig. 3). In fact, even under conditions of PDE4D knockdown, endogenous PDE4B2 fails to co-immunoprecipitate with βarrestin (Fig. 3). This is not due to an inherent inability of PDE4B2 to interact with βarrestin, as we show here that the ectopic overexpression of PDE4B2 in HEK293B2 cells clearly allows PDE4B2 to co-immunoprecipitate with βarrestin (Fig. 4). Given the pre-disposition of PDE4 isoforms to interact with tethering proteins and, thereby, underpin compartmentalized responses in cells (15, 17), this suggests that endogenous PDE4B2 may be sequestered by a scaffold protein that precludes its interaction with βarrestin. Presumably such a putative scaffold has a higher affinity for binding PDE4B2 than has βarrestin and is expressed at such a level that it sequesters all endogenous PDE4B2 in HEK293B2 cells. Under such conditions there will be no pool of “free” PDE4B2 available for interaction with βarrestin. Clearly, identification of such a protein will provide a formidable analytical challenge, because PDEs have high specific activities with corresponding low levels of protein expression, making isolation and analysis of any binding partner extremely difficult and beyond the means of this present study. Nevertheless, a prediction of such a model would be that saturating the putative scaffold, by ectopic overexpression of PDE4B2, should provide a pool of free PDE4B2 that can interact with βarrestin, which is indeed what we observed here (Fig. 4). Such data indicate that PDE4D is compartmentalized distinctly from PDE4B2. The functional consequences of such compartmentalization of PDE4 isoforms are clearly demonstrated by our observations that, although PDE4B knockdown had no effect on isoprenaline stimulation of either the PKA phosphorylation of the β2-AR or activation of ERK, both of these processes were markedly amplified by PDE4D knockdown (Figs. 5 and 6). Furthermore, the pronounced amplification of the ERK response was clearly PKA- and Gi-dependent, being ablated by H89 and pertussis toxin, respectively (Fig. 7). That selective PDE4D knockdown amplifies β2-AR switching to ERK activation indicates the importance of the PDE4D subfamily in controlling the desensitization of this response in HEK293B2 cells.

These data indicate that βarrestin-recruited PDE4D controls the cAMP-mediated activation of PKA involved in a defined, compartmentalized process focused on the β2-AR. In this regard, there is a growing realization that, by tethering PKA to AKAPs, spatially distinct signaling modules can be formed whose activity is determined by the immediate gradient of cAMP (1, 4, 5). Key to forming such gradients is the activity of anchored cAMP phosphodiesterases, such as enzymes of the PDE4 family (15, 17). Here we demonstrate that by disrupting AKAP-PKA interactions, using either a membrane permeable Ht31 peptide or by overexpressing a Ht31-GFP chimera (42, 62, 63), we can markedly attenuate the ability of isoprenaline to activate ERK in HEK293B2 cells (Fig. 9). Not only that, but such reagents also attenuate the heightened ability of isoprenaline to activate ERK upon siRNA-mediated knockdown of PDE4D (Fig. 9). Thus the tethering of PKA to AKAPs is clearly fundamental to the regulation of PKA phosphorylation of the β2-AR controlled by βarrestin-recruited PDE4D. Interestingly, the β2-AR itself has been shown capable of interacting with specific AKAPs. In transfected cells, Lefkowitz, Scott, and colleagues have identified a constitutive association with AKAP79 (64), and Malbon and colleagues have identified a dynamic interaction with gravin, which occurs subsequent to the phosphorylation of gravin by PKA (65, 66). We show here that the β2-AR can interact with endogenously expressed forms of both of these AKAPs in HEK293B2 cells (Fig. 9). Furthermore, the interaction of the β2-AR with AKAP79 is indeed constitutive, whereas that with gravin is only seen subsequent to challenge of cells with isoprenaline (Fig. 9). However, employing for the first time an siRNA approach to achieve knockdown of AKAPs, we show here that the ability of isoprenaline to trigger the AKAP-mediated phosphorylation of the β2-AR, and consequential activation of ERK, is ablated only upon knockdown of AKAP79 and not upon knockdown of gravin (Fig. 9). Such data indicate that βarrestin-recruited PDE4D determines the activity of a discrete pool of AKAP79-tethered PKA that is responsible for causing the phosphorylation of the associated β2-AR. It is this fundamental, spatially and dynamically determined process that provides for the conse-
sequent switching of $\beta_2$-AR coupling to $G_i$ and thereby activation of ERK (Fig. 10).

The role of recruited gravin remains to be ascertained. However, we note here (Fig. 9) that gravin appears to be recruited more rapidly to the membrane fraction than it does to the $\beta_2$-AR. This might indicate that a major fraction of recruited gravin is functionally localized within a plasma membrane sub-domain distinct from that either sampled or occupied by $\beta_2$-AR-tethered AKAP79. In any event, selective siRNA-mediated knockdown of AKAP79 and gravin indicates that membrane-bound forms of these AKAPs serve distinct functional roles in HEK293B2 cells.

$\beta$Arrestin recruits and translocates both PDE4D3 and PDE4D5 isoforms to the $\beta_2$-AR in HEK293B2 cells (34). However, the ratio of PDE4D3 to PDE4D5 is greater in lysates compared with that bound to $\beta$Arrestin and that recruited to the $\beta_2$-AR, indicating that there is a preference for PDE4D5 to interact with $\beta$Arrestin (35). Such apparent preference for PDE4D5 to interact with $\beta$Arrestin may, as surmised for PDE4B2, be influenced by competing scaffolds but is also determined by the fact that PDE4D5, as well as having the common $\beta$Arrestin binding site in its conserved catalytic unit, has an additional site for interaction with $\beta$Arrestin located within its unique N-terminal region (35). The question then is whether PDE4D3 and PDE4D5 contribute equally or not in providing control of $\beta_2$-AR switching to ERK activation. To address this we again used a siRNA strategy, but this time with probes targeted at the unique 5′ region of PDE4D3 and PDE4D5 so as to elicit their selective knockdown (Fig. 8). Doing this we noted that selective knockdown of PDE4D3 had no observable effect on isoprenaline-stimulation of either the PKA phosphorylation of the $\beta_2$-AR or activation of ERK (Fig. 8). In contrast, selective knockdown of PDE4D5 elicited a profound amplification of both these isoprenaline-stimulated responses (Fig. 8). This indicates that PDE4D5 is the isoform of major import in regulating the ability of isoprenaline to stimulate the PKA phosphorylation of the $\beta_2$-AR and thus the switching of its signaling to the activation of ERK. The lack of effect seen with PDE4D3 knockdown may reflect the smaller size of the $\beta$Arrestin-PDE4D3 pool translocated, but equally it could reflect the fact that $\beta$Arrestin binds PDE4D5 distinctly due to its additional N-terminal interaction site (35), which may confer a functional advantage. Indeed, while over-expression of catalytically inactive PDE4D5 has been shown to provide a dominant negative role through displacing active endogenous PDE4D and enhancing isoprenaline-stimulated ERK phosphorylation (36), such an action is clearly ablated if the PDE4D5-specific site for interaction with $\beta$Arrestin is disrupted in the dominant negative PDE4D5 construct (Fig. 10, e and f). Consistent with this, using siRNA-mediated knockdown, we have been able to show in a defined cell type that, functionally, one particular PDE4 isoform, namely PDE4D5, is responsible for desensitizing the switching of the signaling of the $\beta_2$-AR to the activation of ERK.

Determination of the functional roles of particular PDE4 isoforms in cells has been hampered by the conservation of their catalytic units, which precludes the generation of useful, selective active site directed inhibitors. Here we have utilized small interfering RNA gene silencing to identify which PDE4 isoforms functionally interact with $\beta$Arrestin so as to influence the PKA phosphorylation status of the $\beta_2$-AR and hence its ability to switch its coupling to ERK activation. In doing this we have identified PDE4D5 as performing such a function in a defined cell type. Our studies provide a paradigm for the notion that, although cells invariably express a range of PDE4 isoforms, individual PDE4 isoforms are likely to be associated with specific functional roles. In this study we discover that loss of an individual PDE4 isoform generates a phenotype. From this it is apparent that, despite a panoply of PDE4 isoforms being expressed in HEK293B2 cells, there is clearly not simple redundancy, where another PDE4 isoform can simply take up the role of the depleted isoform. This points to the potential utility of developing PDE4 sub-family/isofrom-specific therapeutics and the use of siRNA reagents as targeted therapeutic agents for manipulating defined aspects of cAMP signaling through the knockdown of distinct PDE4 isoforms. Our study indicates that in cells where one signaling scaffold protein, such a $\beta$Arrestin, can potentially interact with a number of different PDE4 isoforms, then the functionally relevant species may well change in different cell types. A variety of factors will influence this, including not only differences in the affinity of a PDE4 isoform for the scaffold and the relative expression levels of PDE4 isoforms but also the presence of competing scaffolds whose concentration and affinity for binding the PDE4 isoform, relative to $\beta$Arrestin, will also contribute to the cell-type-specific profile of interacting partners.

The presence or absence of AKAP79 and $\beta$Arrestin-associated PDE4 can be expected to have a profound effect on the ability of the $\beta_2$-AR to become phosphorylated by PKA and switch its coupling to $G_i$ (Fig. 11). The AKAP79/PKA module constitutively associated with the $\beta_2$-AR ensures that once local cAMP levels have breached the threshold for activation of tethered PKA, then the $\beta_2$-AR will become PKA-phosphorylated and switch its coupling to $G_i$. Associated with such a potent system is a unique desensitization process that is provided by the $\beta$Arrestin-mediated delivery of PDE4D5. This increases the localized rate of degradation of cAMP, thereby de-activating PKA and uncoupling the $\beta_2$-AR from $G_i$. It is likely that cell-type-specific remodeling of the switching of $\beta_2$-AR signaling to ERK through this process will occur through alterations in the expression and availability of both AKAP79 and $\beta$Arrestin-bound PDE4 isoforms, such as PDE4D5.

**FIGURE 11. Schematic diagram of the central role for PDE4D5 in regulating the switching of signaling of the $\beta_2$-AR from adenylyl cyclase to ERK.** In the upper panel is shown the isoprenaline (iso)-mediated switching of signaling of the $\beta_2$-AR from the $G_s$-coupled activation of adenylyl cyclase (AC) to the $G_i$-coupled activation of ERK, upon phosphorylation of the $\beta_2$-AR by AKAP79-tethered PKA. In the lower left panel is shown the isoprenaline (iso)-mediated recruitment of GRK and the consequent phosphorylation of the $\beta_2$-AR by this kinase, which elicits (lower right panel) the recruitment of $\beta$Arrestin with bound PDE4D5. The recruited $\beta$Arrestin inhibits the coupling between the $\beta_2$-AR and $G_i$, whereas PDE4D5, in lowering local cAMP levels, attenuates the ability of AKAP79-bound PKA to phosphorylate the $\beta_2$-AR. Together this agonist-activated complex of $\beta$Arrestin and PDE4D5 achieves dual desensitization of $\beta_2$-AR signaling through both its $G_s$- and $G_i$-coupled pathways.
RNA Silencing Identifies PDE4D5 as the Functionally Relevant cAMP Phosphodiesterase Interacting with βArrestin to Control the Protein Kinase A/AKAP79-mediated Switching of the β2-Adrenergic Receptor to Activation of ERK in HEK293B2 Cells

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