Mechanisms of Signal Transduction: Dynamic Regulation, Desensitization, and Cross-talk in Discrete Subcellular Microdomains during β2-Adrenoceptor and Prostanoid Receptor cAMP Signaling

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Dynamic Regulation, Desensitization, and Cross-talk in Discrete Subcellular Microdomains during β₂-Adrenoceptor and Prostanoid Receptor cAMP Signaling

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The β₂-adrenergic receptor (β₂AR)² and prostanoid receptors (EP2R and EP4R) are Gα-linked receptors that, upon agonist occupancy, activate adenylyl cyclases (ACs) to produce a diverse number of cAMP-dependent biological actions. Many tissues types, including airway epithelium and cardiac muscle, endogenously express both classes of G-protein-coupled receptor to generate cellular increases in cAMP following appropriate stimuli (1, 2). Differential agonist actions are thus conjectured to be permitted via compartmentalization of cAMP signals to regions close to individual G-protein-coupled receptor subtypes (3, 4). These local cAMP signals can then mediate specific downstream events through the regulation of neighboring effector molecules such as protein kinase A (PKA), exchange protein directly activated by cAMP, and cyclic nucleotide-gated channels (CNGCs). Temporally distinct, localized cAMP signals can be produced by two main factors: firstly, rapid cAMP hydrolysis by spatially organized cAMP phosphodiesterases (PDEs) (3–9) and, secondly, rapid receptor uncoupling or “desensitization” causing reduced Gα-linked receptor responsiveness to subsequent or prolonged agonist application (10, 11).

The study of separate pools of cAMP deriving from different receptors in the same cell is most directly examined at the single cell level. Recent real-time, single cell approaches have provided some insight into the composition of signaling complexes whose tethered PDEs serve to shape a localized cAMP signal (9, 12, 13). However, the characteristics of the other key regulators comprising rapid attenuation of cAMP signal and receptor desensitization have not been addressed at this essential level. In addition, comparison of the regulation of local cAMP dynamics mediated by different receptor subtypes in the same cell, and their dependence upon a number of known signaling molecules, remains to be addressed. One key question of such compartmentalized events is whether activation of a specific Gα-linked receptor type is able to influence cAMP signals generated by other Gα-linked receptors in neighboring compartments of the cell.

In the current study we have used a single cell approach to investigate the dynamics of specific cAMP signaling events in real-time. Using an adenovirus expressing a mutant CNGC as a high sensitivity probe for direct read-out of sub-plasmalemmal cAMP, we have assessed the role of specific signaling components in β₂AR-mediated and EP-R-mediated signaling in HEK293 cells. In particular, we have examined the role of known signaling molecules in the local degradation of cAMP and subsequent loss of signaling efficiency. The functional role...
of individual proteins, including the β-arrestins, PKA, and PDE4 isoforms, was systematically assessed using specific small interfering RNAs (siRNA). In complementary experiments we overexpressed catalytically inactive, “dominant negative” versions of PDE4D isoforms, to assess the role of these proteins during both isoproterenol- and prostaglandin-evoked receptor activation. By displacing such cognate, anchored endogenous PDE4 isoforms from their functionally relevant sites, these constructs provide a unique insight into signaling functions of spatially constrained PDE4 isoforms (14, 15).

Our results reveal that distinct signaling components are associated with the β2-AR and EP-R receptors. A dynamic framework of specific isoforms of β-arrestin, PKA regulatory subunit, and PDE4D subtypes governs the local cAMP dynamics and desensitization of β2-AR-mediated signals; in contrast, dynamic regulation of EP-R signals was independent of β-arrestin recruitment and largely dependent upon PKA-R1I and PDE4D5 activity. Despite highly efficient compartmentalization of cAMP signaling, we provide evidence for heterologous unidirectional desensitization of β2-AR signaling as a consequence of EP-R activation, which suggests organized communication between discrete signaling pathways.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK293 cells were grown in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum and maintained at 37 °C (5% CO2). For all experiments requiring real-time assessment of cAMP changes using mutant CNGCs, cells were plated onto 25-mm poly-l-lysine-coated coverslips 24 h prior to transfection.

Preparation and Transfection of Specific siRNAs—The design and preparation of PDE4 and β-arrestin1 and -2 siRNA oligonucleotides was as described previously (14) with the PKA-R1I and -RII-specific siRNA prepared as in a previous study (16). Each siRNA was transfected into 40–60% confluent HEK293 cells using Lipofectamine 2000 reagent (Lf2000, Invitrogen). siRNA samples were first diluted in Opti-MEM to a final concentration of 100 nM, and 4 μl of Lf2000 (1 mg/ml) was diluted in 96 μl of Opti-MEM. After 10-min incubation the diluted Lf2000 reagent (100 μl) was added to siRNA sample (100 μl), and after 20 min the mixture was added to cells plated onto poly-l-lysine-coated coverslips in 6-well plates. 800 μl of Opti-MEM was also added to each well giving a total volume of 1 ml. After incubation at 37 °C (5% CO2) for ~6 h the solution was replaced with 2 ml of serum-containing MEM per well. All effects of selected knockdowns were assessed ~72 h after transfection.

Transfection with Dominant Negative Forms of PDE4D3 and PDE4D5—Rolipram, a simple competitive inhibitor, acts on both anchored and free PDE4 isoforms. To address the specific functioning of anchored forms of PDE4 a dominant negative approach has previously been developed. This takes advantage of the known three-dimensional structure of the PDE4 catalytic domain (17) to generate forms of PDE4D that, although retaining their ability to bind β-arrestin, are catalytically inactive due to a single point mutation made deep within the catalytic pocket (15, 16). PDE4 dominant negative proteins stem from wild-type cDNA of PDE4D3 and PDE4D5 cloned into the vector pcDNA3.1 and mutated via site-directed mutagenesis. The generation of a single point mutation that prevents binding of essential divalent cation in the cAMP binding site of either PDE4D3 (D484A) (16) or PDE4D5 (D556A) ablated catalytic activity of the enzymes without altering β-arrestin-binding properties (15). HEK293 cells were transfected with such dominant negative constructs using Lf2000 reagent in a manner similar to that described for siRNA transfections using 0.1–0.2 μg of cDNA per well. Again cells were plated onto Poly-l-lysine-coated coverslips ~24 h prior to transfection.

Cell Lysate Preparation and Western Blotting—HEK293 cells were harvested using phosphate-buffered saline containing 2 mM EDTA and washed twice in phosphate-buffered saline. Detergent-soluble proteins were isolated from cells by disruption in lysis buffer (1% (v/v) Triton X-100, 50 mM HEPES buffer, pH 7.2, 10 mM EDTA, 100 mM NaHPO4, 2H2O) containing Sigma protease inhibitor mixture, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-phosphoglycerol, and 1 mM Na3VO4. After mixing on ice for 30 min detergent-insoluble proteins were removed by centrifugation at 12,000 gav for 15 min at 4 °C, and supernatant containing soluble proteins was retained for Western blot analysis. Protein concentration of each sample was determined using the Bradford method with bovine serum albumin as standard, with ~50 μg of protein run per well.

CNGC Adenovirus Expression—One day prior to making single cell cAMP measurements HEK293 cells that had been plated onto 25-mm coverslips and transfected with specific siRNA oligonucleotides or dominant negative cDNA were infected with adenovirus encoding the α subunit of the rat olfactory CNGC with mutations C460W and E583M. The mutant CNGC adenovirus was constructed using the QuikChange site-directed mutagenesis kit (Strategene) and purified as described previously (8). A multiplicity of infection of 10 plaque forming units per well was used, and after 2 h hydroxyurea (2 mM) was added to each coverslip to inhibit adenovirus vector replication.

cAMP Measurements Monitored by Ca2+ Influx—CNGC-infected HEK293 cells were loaded with 4 μM fura-2/AM and 0.02% pluronic F-127 (Molecular Probes) for 40 min in extracellular buffer containing (mM): 140 NaCl, 4 KCl, 1 CaCl2, 1 MgCl2, 11 glucose, 10 Hepes, pH 7.4. After loading, cells were washed and then imaged using a CoolSnap charge-coupled device camera (Photometrics) and monochromator system (Cairn Research) attached to a Nikon TMD microscope (~40 objective). Emission images (D510/80M) at 340 nm and 380 nm excitation were collected at 1 Hz using MetaFluor software (Universal Imaging). Data were plotted as 340/380 nm ratio changes relative to the pre-stimulus fluorescence ratio. Only cells that gave >20% increase in fura-2 ratio over baseline in response to the first application of agonist were analyzed. Solutions were applied directly to the bath to enable rapid solution change.

RT-PCR Experiments—Reverse transcription PCR reactions were performed by using total cellular RNA isolated with the SV Total RNA Isolation System (Promega). The PCR primer sequences were designed and synthesized on the basis of published human cDNA sequences and are as follows: EP2-R (for-
ward, 5′-GCTGCTGCTCTTATTGTCTCG-3′ and reverse, 5′-TCCGACAACAGAGGACTGAACG-3′ (392 bp)), EP3-R (forward, 5′-GAGTACTCTTGGATACTACTAGAC-3′ and reverse, 5′-GCAGTGCTCAACTGATGTCTAGC-3′ (293 bp)), and EP4-R (forward, 5′-ATCTTACTCTAGGCCACCGG-3′ and reverse, 5′-CTCATGGCTTGTAGCAGGAC-3′ (212 bp)). A control for genomic DNA contamination of the RNA preparations, parallel samples were not reverse-transcribed. PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide.

RESULTS

The Role of Specific PDE4 Isoforms in β2-AR cAMP Signal Compartmentalization and Desensitization—Previous studies have highlighted the important role of cAMP-dependent phosphodiesterase-4 (PDE4) activity in the compartmentalization of cAMP signals during cellular AC activity (3, 5–9). To assess the specific role of PDE4 in shaping sub-plasmalemmal cAMP dynamics and in down-regulating signaling efficiency during β2-AR activation, cells were treated with an adenoviral vector expressing a mutant α-subunit of the rat olfactory CNGC (C460W/E583M CNGC). Homologous expression of the C460W/E583M CNGC subunit produces a functional cation-conducting channel with ~10-fold greater sensitivity to cAMP over cGMP (K1/2 values of 1.4 μM versus 11 μM for cAMP and cGMP, respectively) (8). Activity of the CNGCs in individual HEK293 cells was assessed by monitoring Ca2+ influx as a function of cAMP-mediated channel activation. In parallel experiments using uninfected HEK293 cells Gs-coupled agonists did not produce any changes in fura-2 signal, demonstrating that any Ca2+ influx observed was due to activation of the mutant CNGCs expressed.

Under control conditions application of 1 μM isoproterenol for 1 min generated a rise in cAMP that, upon agonist removal, rapidly returned to basal (Fig. 1A). This behavior is thought to be due primarily to the rapid hydrolysis of sub-plasmalemmal cAMP (5, 9, 18), although cAMP diffusion though the cell cytosol may also play a role (cAMP diffusion rate is estimated to be high, ~0.8 × 10−5 cm2 s−1 (19), but is thought to be restricted to ~5 × 10−9 cm2 s−1 for movements between sub-plasmalemmal microdomains and the bulk cytosol (20)). Subsequent 1-min applications of 1 μM isoproterenol given at 5-min intervals elicited no detectable rise in [cAMP] (Fig. 1A) indicating “desensitization” of AC-coupled signaling. At least 90% loss of signaling efficiency was detected in all cells tested (>400 cells) under control conditions following a 1-min application of 1 μM isoproterenol. This desensitization was quantified by “ratioint” the peak amplitude of cAMP increase during the first and second agonist application, with 100% representing complete desensitization. A similar degree of desensitization was seen when the interval between 1 μM isoproterenol applications was reduced to just 3 min (see supplemental Fig. S1). However, when the interval was increased to 10 min clear signs of recovery of β2AR signaling were seen, and at 15 min after the initial isoproterenol challenge up to 80% of the initial signaling efficiency was recovered in some cells (supplemental Fig. S1). For the purpose of this study we examined the effects of specific signaling components on the loss of cAMP signal seen at 5 min after the initial receptor stimulus as this generated a robust, reproducible loss of signaling efficiency.

Using this protocol we found that co-application of 1 μM isoproterenol with the PDE4-selective inhibitor, rolipram (10 μM), eliminated the apparent desensitization to yield a robust cAMP rise at a time point that showed complete loss of isoproterenol responsiveness under control conditions (Fig. 1B). In previous single cell studies we found that, although the immediate recovery of sub-plasmalemmal cAMP signals is predominantly driven by PDE4 activity, inhibition of this enzyme does not directly influence the amplitude of cAMP rise (and subsequent CNGC activation) (9). Thus, the significant increase in cAMP signal seen in Fig. 1B upon co-application of isoproterenol and rolipram following a 1-min pre-treatment with isoproterenol implicates an essential role for PDE4 in isoproterenol-mediated desensitization. The PDE3-selective inhibitor, cilostamide (10 μM), had no significant effect on the rate of cAMP hydrolysis or degree of desensitization (data not shown).

PDE4 represents the major fraction (~70%) of total cAMP PDE activity in HEK293 cells (14). Hence, it is not surprising that this enzyme plays such an important role in the rapid hydrolysis of sub-plasmalemmal cAMP signals. However, the PDE4 group of enzymes comprises 4 families and around 20 isoforms, of which members are selectively expressed in different cell types (21). It is not known which PDE4 isoform is predominantly responsible for compartmentalizing near membrane β2AR-mediated cAMP signals. >90% of total PDE4 activity in HEK293 cells is provided by the PDE4B and PDE4D subfamilies. Of this, PDE4D3 and PDE4D5 contribute ~60% of total PDE4 activity, with PDE4B2 generating a further ~30% (14). Using siRNA to selectively knock down the subfamilies of PDE4 in HEK293 cells we were able to examine the relative actions of each PDE4 class with respect to the dynamics of isoproterenol-induced cAMP signaling via β2ARs. Transfection of HEK293 cells with siRNA oligonucleotides directed against PDE4B and PDE4D markedly reduced endogenous levels of PDE4B2 (Fig. 1C), or PDE4D3 and PDE4D5, respectively (Fig. 1D). Cell lysates were collected 3 days post-transfection and subjected to Western blot analyses to confirm knockdown of endogenous protein levels. Although modest amounts of PDE4A activity (~8% of total PDE4 activity) have been reported previously for HEK293 cells (14) no PDE4A immunoreactivity was found in the present study (data not shown). Previous investigation has shown that the PDE4B- and PDE4D-selective siRNA oligonucleotides are highly specific for their chosen isoform and do not lead to compensatory up-regulation of other PDE isoforms (14).

Knockdown of PDE4B had little effect on the transient cAMP rise evoked by isoproterenol application (Fig. 1F), with rates and percentage of cAMP recoveries comparable to those seen under resting (basal) conditions (Fig. 1H). In contrast, following knockdown of PDE4D, recovery from isoproterenol-evoked increases in [cAMP] was greatly attenuated (Fig. 1G and H). Such large reductions in the degree of cAMP recovery (just 9 ± 8% in PDE4D siRNA-treated cells versus 89 ± 11% in controls) are consistent with the requirement for local PDE4D activity to rapidly hydrolyze cAMP following β2-AR occupancy. Although PDE4D activity was clearly involved in shaping isoproterenol-

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\text{Essential Components of Dynamic cAMP Signaling}
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two AR desensitization. A, sub-plasmalemmal cAMP changes in response to repeated 1 μM isoproterenol (Iso) application in a single CNGC-expressing cell with complete loss of responsiveness after the first agonist application. B, representative trace of single cell data in which receptor desensitization is reversed by 10 μM rolipram. Mean ± S.E. data reveal a significant effect of PDE4 inhibition (*, p < 0.001, n values in parentheses). C, Western blot analysis to confirm knockdown of endogenous PDE4B, and D, PDE4D levels in HEK293 cell lysate 72-h post-transfection with selective siRNA. Each blot is typical of findings from three separate experiments. Representative single cell cAMP measurements in control (E), PDE4B siRNA (F), and PDE4D siRNA-treated (G) cells. H, charts show quantification of recovery rates and % recoveries of cAMP signal in control cells (n = 34), PDE4B siRNA-treated cells (n = 27), and PDE4D siRNA-treated cells (n = 28); *, p < 0.01.

Selective Knockdown of β-Arrestin Isoforms and PKA Regulatory Subunits—Previous studies in a HEK293 cell line that constitutively overexpressed β2ARs revealed that stimulation with 10 μM isoproterenol transiently recruited endogenous β-arrestin-associated PDE4D isoforms to the plasma membrane (15, 22, 23) and increased local cAMP hydrolysis (14, 23). This PDE4 recruitment, and degradation of local cAMP, was shown to influence other factors intimately associated with receptor desensitization, such as PKA phosphorylation of β2ARs, receptor switching from Gs to Gi, and subsequent activation of ERK in those cells (14, 15, 22, 24).

In Fig. 1 we showed that PDE4D plays a critical role in shaping dynamic changes in cAMP following modest activation of endogenous β2ARs. However, it is unclear if the critical PDE4D activity involved in shaping the β2AR agonist response is pre-associated in the immediate vicinity of the β2AR and plays a direct role in the loss of its signaling in response to subsequent isoproterenol applications, or if the actions of PDE4D are dependent on other signaling components that are reportedly involved in β2AR desensitization, particularly β-arrestin, which sequesters PDE4 isoforms and allows for their recruitment to agonist-stimulated β2ARs (15, 22, 23). In a first step to unravel this issue we investigated the effects of selective knockdown of specific signaling components on real-time cAMP production during β2AR stimulation using CNGC activity as a functional gauge of signaling efficiency.

The traces in Fig. 2 (A–E) are representative of typical sub-plasmalemmal cAMP changes evoked in single HEK293 cells during two 1-min exposures to 1 μM isoproterenol, with a 5-min interval between agonist applications. Again in control studies a second isoproterenol challenge was unable to elicit any rise in sub-plasmalemmal cAMP (Fig. 2A). Knockdown of either endogenous β-arrestin1 (Fig. 2B) or endogenous PKA-RI levels (Fig. 2D) had little influence on the profile of the cAMP changes. In contrast, knockdown of either β-arrestin2 (Fig. 2C) or PKA-RII subunits (Fig. 2E) dramatically attenuated cAMP recovery compared with controls (Fig. 2F, p < 0.01). The effectiveness of PKA-RII knockdown on
The cAMP recovery profile was maximal immediately following the first agonist addition. Knockdown of β-arrestin2 was equally effective at decreasing the extent of cAMP recovery following β₂AR stimulation; however, the peak knockdown effect was delayed, occurring from ~200 s onward. The presence of marked cAMP hydrolysis at the onset of the 5-min period of wash was consistent with recruitment of β-arrestin2, and its associated proteins, prior to influencing the sub-plasmalemmal cAMP signal. These data show a defined specificity for a regulatory module, involving PDE4D, β-arrestin2, and PKA-RII, that shapes signaling through the β₂AR.

Significant knockdown of endogenous β-arrestins and PKA-RII levels by specific siRNA oligonucleotides were confirmed by Western blot analyses (Fig. 2, G and H, respectively). Using an antibody that recognizes both β-arrestin isoforms we detected bands corresponding to endogenous β-arrestin1 and β-arrestin2 in control cell lysates. In the upper panel of Fig. 2G the top band is nonspecific with the smaller middle band representing β-arrestin1 and the lower band β-arrestin2. A clear decrease in expression of β-arrestin2 is confirmed in lane 3. However, the intensity and broadness of the band for β-arrestin2 made it difficult to confirm down-regulation of the β-arrestin1 isoform (upper blot, lane 2). To overcome the restricted separation of the two β-arrestin isoforms we used an antibody specific to β-arrestin1 to confirm significant knockdown of this protein in cells expressing β-arrestin1 specific siRNA (Fig. 2G, lane 1).
middle panel, lane 2). Fig. 2H confirms almost complete knockdown of endogenous PKA-RII levels at 72 h post-transfection with PKA-RII-specific siRNA oligonucleotides.

**Gi Does Not Influence cAMP Dynamics and Desensitization of \( \beta_2 \text{AR Signaling} \)**—One potential component of \( \beta_2 \text{AR} \) desensitization is the PKA-dependent switching of receptor coupling from \( \text{Gs} \) to \( \text{Gi} \), with a subsequent decrease in AC activity and activation of the ERK signaling pathway (15, 25). This mode of regulation occurs in various, but not all, cell types, whereby signaling from cAMP to ERK pathway is complex with a variety of possible modes of coupling (26). To establish the significance of \( \text{Gi} \) activation under our experimental conditions, we pretreated HEK293 cells with 100 ng/ml pertussis toxin (PTX) at 37 °C for 16 h in serum-free media; this ADP-ribosylates \( \text{Gi} \) and prevents it coupling to inhibitory G-protein-coupled receptors. We confirmed the effective abolition by PTX of \( \text{Gi} \)-mediated AC inhibition in forskolin (FSK)-stimulated cells following the activation of endogenous \( \text{Gi} \)-linked somatostatin receptors. Application of 10 \( \mu \text{M} \) FSK produced a sustained rise in sub-plasmalemmal cAMP that decreased upon addition of 2 \( \mu \text{M} \) somatostatin (Fig. 3A, upper panel), which activates \( \text{Gi} \)-coupled receptors in HEK293 cells (27, 28). This \( \text{Gi} \)-mediated effect was ablated when cells were pre-treated overnight with PTX (Fig. 3A, middle panel and bar chart). PTX treatment, however, did not affect the recovery profile of isoproterenol-mediated cAMP production or minimize the degree of \( \beta_2 \text{AR} \) desensitization (Fig. 3B). These data suggest that \( \text{Gi} \) activation does not contribute to the rapid desensitization observed under our experimental conditions as detected by the CNGCs. Furthermore, these data indicate that any switching of \( \beta_2 \text{AR} \) coupling to \( \text{Gi} \) is not sufficient to account for the rapid return of cAMP levels to baseline between agonist applications.

**Use of PDE4D Isoform-specific siRNA to Compare Roles for PDE4D3 Versus PDE4D5**—siRNA oligonucleotides have been developed that selectively knockdown either PDE4D3 or PDE4D5, the two major isoforms of PDE4D present in HEK293 cells (14). Both PDE4D isoforms can associate with \( \text{beta}-\text{arrestins} \), allowing their recruitment to the plasma membrane as a consequence of \( \beta_2 \text{AR} \) stimulation (15, 23). However the unique N-terminal sequence of PDE4D5 accommodates an extra \( \beta-\text{arrestin} \) association site promoting the selective sequestration of this isoform to \( \beta-\text{arrestin} \) and its subsequent, agonist-dependent recruitment to the \( \beta_2 \text{AR} \) (22, 29, 30). In cardiac myocytes and in HEK293 cells stably transfected to overexpress \( \beta_2 \text{ARs} \), PDE4D5 can regulate receptor phosphorylation by PKA, conferring \( \text{Gi} \)-dependent ERK activation (14, 29, 30). We have used the same siRNAs to characterize the direct effects of selective PDE4D3 and PDE4D5 knockdown on the real-time sub-plasmalemmal cAMP changes that accompany isoproterenol-induced desensitization of endogenous \( \beta_2 \text{ARs} \) in HEK293 cells.

Western blotting suggested approximately equivalent levels of endogenous PDE4D3 and PDE4D5 in HEK293 cells used in the present study, with selective siRNA expression producing...
significant knockdown of each isoform at 72 h post-transfection (Fig. 4A). Interestingly, when the consequences of the knockdown assays were assessed by investigating isoproterenol-mediated activation of CNGCs, both PDE4D3 (Fig. 4C) and PDE4D5 (Fig. 4D) had significant effects, compared with controls (Fig. 4B), on the initial rate of cAMP recovery and the degree of desensitization following a 1-min application of isoproterenol (Fig. 4, E and F, p < 0.01). If we assume, as indicated by our experiments, comparable knockdown efficiencies for PDE4D3 and PDE4D5 siRNAs, it would appear that both isoforms contribute approximately equally to β2AR signal desensitization. By silencing individual PDE4D isoforms, rather than eliminating all PDE4D activity (Fig. 1G), we can more readily determine a clear role for PDE4D in the desensitization of β2AR signaling. Furthermore, we can conclude that the activities of PDE4D3 and PDE4D5 most likely generate additive effects on the spatiotemporal dynamics of cAMP signal and desensitization of β2AR signaling in the intact cell.

**Overexpression of Catalytically Inactive PDE4D Isoforms Confirms a Role for PDE4D3 and PDE4D5 in Desensitization**—A complementary method to examine the role of the PDE4D isoforms in receptor desensitization is to overexpress catalytically inactive versions of PDE4D3 and PDE4D5 in cells. The overexpression of such species displaces the cognate endogenous active isoform from its sequestering proteins. Thus such species can be expected to give specific information on spatially constrained PDE4 isoforms, if they provide a dominant negative function (15, 16). In contrast to this approach, siRNA-mediated knockdown will interrogate the entire target population, in both free and tethered populations. These constructs contain a single point mutation (D484A (PDE4D3) or D556A (PDE4D5)) that renders the enzymes catalytically inactive, but still capable of binding to and being recruited by the β-arrestins (14, 15, 23, 29). Overexpression of such catalytically inactive versions of the two PDE4D isoforms specifically expressed in HEK293 cells was confirmed by blotting cell lysates with PDE4D-specific antibody 72 h post-transfection (Fig. 5A). In a similar fashion to that seen in experiments using siRNA-mediated knockdown of PDE4D isoforms, expression of the catalytically dead constructs attenuated the degree of β2AR desensitization evoked by a 1-min application of isoproterenol (Fig. 5, C and D). This identified clear transient cAMP signals in response to a second agonist application that were significantly greater (Fig. 5F, p < 0.01) than the very modest cAMP increase seen under control conditions (Fig. 5B). However,
in contrast to the siRNA knockdown approach presented in Fig. 4, expression of PDE4D3 or PDE4D5 dominant negative did not slow the initial rate of cAMP recovery following the first agonist stimulus (Fig. 5E). This difference indicates that free as well as tethered forms of PDE4D3 and PDE4D5 can contribute to the dynamics of desensitization, because all populations are knocked down using siRNA, whereas overexpression of catalytically inactive isoforms only probes the function of tethered PDE4D subpopulations.

FIGURE 5. Expression of dominant negatives forms of PDE4D3 and PDE4D5 confirm a role for both isoforms in β₂AR desensitization. A, overexpression of catalytically inactive PDE4D3 and PDE4D5 confirmed by Western blot analysis of cell lysates 72 h post-transfection. B–D, single cell analyses of the effects of dominant negative expression on sub-plasmalemmal cAMP changes during repeated 1-min applications of 1 μM isoproterenol. E, mean ± S.E. data for all cells analyzed shows no significant effects of the dominant negatives on rates of cAMP return to baseline. F, mean ± S.E. data for all cells reveals marked increases in the amplitude of cAMP response to a second application of 1 μM isoproterenol following expression of catalytically inactive PDE4D3 or PDE4D5 (*, p < 0.01).

components of cAMP signal termination and desensitization following Prostanoid Receptor Activation—The foregoing data support significant roles for PDE4D3, PDE4D5, PKA-RII, and β-arrestin2 in the compartmentalization and desensitization of signals arising from β₂AR activation. However, HEK293 cells also express endogenously prostanoid receptors (EP-R). We thus set out to investigate whether similar mechanisms of compartmentalization and desensitization apply to the EP-Rs. In addition we aimed to determine whether there is any communication, regarding the desensitization of cAMP signaling, between Gₛ-linked EP-Rs and the β₂ARs, which are known to co-reside in a number of tissue types (1, 2).

Fig. 6B shows a typical recording of sub-plasmalemmal cAMP signals evoked by two 1-min applications of 1 μM PGE₁ under control conditions with a 5-min interval between each application. Analogous to β₂AR activation, a transient rise in local cAMP is evoked by an initial 1-min PGE₁ application. However, unlike the case with β₂ARs, the subsequent EP-R response is only partially attenuated, with an estimated 45 ± 7% desensitization of EP-R signaling, compared with 100% desensitization of β₂AR signaling in parallel isoproterenol experiments. The degree of PGE₁-evoked signal desensitization was not enhanced further by additional applications of the agonist (see Fig. 8D). RT-PCR analyses to identify the endogenous EP-R subtypes in these HEK293 cells suggested that the source of the PGE₁-evoked cAMP signals could be EP2- and/or EP4-Rs (Fig. 6A), because both are linked to Gₛ activation and AC activity (11). The Gi-linked EP3-R subtype was also present in these HEK293 cells.

To examine the role of specific signaling components in the compartmentalization and desensitization of the local EP2/4-R generated signals, siRNA were used to knock down selectively endogenous levels of PDE4B, PDE4D, β-arrestin1, β-arrestin2, PKA-RI, and PKA-RII (Fig. 6B). However, we observed that only consequent to knockdown of either PDE4D or PKA-RII was there any significant effect on the cAMP-recovery profile compared with control (Fig. 6, C (p < 0.001) and B). In contrast...
FIGURE 6. Components that shape dynamic cAMP events mediated via prostanoid receptor activation. A, RT-PCR analysis reveals positive bands corresponding to EP2-, EP3-, and EP4-receptor subtypes in HEK293 cells. B, representative examples of single cell cAMP measurements to examine the effects of expressing selective siRNA for PDE4B, PDE4D, β-arrestin1, β-arrestin, PKA-RI, or PKA-RII on PGE1-evoked cAMP changes. The effects of overnight pre-treatment with 100 ng/ml PTX were also examined. All applications of 1 μM PGE1 (see bars) were for 1 min with a 5-min interval. C, mean ± S.E. data illustrating significant effects of PDE4D and PKA-RII knockdown on cAMP recovery; **, p < 0.001. n values for each experimental condition are shown in parentheses.
to our observations concerning the desensitization of β_{2}-AR-mediated cAMP dynamics, the effects of PDE4D on local EP2/4-R-generated cAMP signals were clearly independent of β-arrestin recruitment, with β-arrestin1 and 2 knockdown assays having no effect on the cAMP-recovery profile or the degree of desensitization of the EP-R-mediated cAMP signal (Fig. 6, B and C). Overnight treatment of cells with PTX (100 ng/ml) was also without effect, which excluded any significant role for G_{i}-linked EP3-R activation in shaping the local cAMP changes during repeated applications of PGE_{1}.

Selective Roles of PDE4D Subtype in the Regulation of PGE_{1}-evoked cAMP Signals—Using both siRNA and dominant negative approaches we were able to characterize distinct roles for both PDE4D3 and PDE4D5 in the compartmentalization of PGE_{1}-evoked cAMP signaling via endogenous EP2/4-Rs in HEK293 cells (Fig. 7). Interestingly, the effects of both PDE4D5 siRNA (Fig. 7A) and overexpression of catalytically inactive PDE4D5 (Fig. 7C) were significant from the onset of the recovery phase following the first agonist application. This identified a marked slowing of the initial rate of cAMP recovery upon agonist removal, implicating a role for PDE4D5 in the earliest stages of signal termination (Fig. 7, B and D, upper bar charts). In contrast, both PDE4D3 siRNA (Fig. 7A) and overexpression of catalytically inactive PDE4D3 (Fig. 7C) had no significant effect on the early component of cAMP recovery but generated a delayed rise in local cAMP levels ~2 min into the period of washing, suggesting a role for sequestered or tethered PDE4D3 in maintaining basal cAMP levels within the vicinity of the plasma membrane under normal conditions (Fig. 7, A and C).

In both PDE4D3 siRNA- and PDE4D5 siRNA-expressing cells, or in cells overexpressing catalytically inactive PDE4D3 and PDE4D5, the amplitude of cAMP rise evoked by a second application of 1 μM PGE_{1} was significantly reduced compared with control conditions (Fig. 7, B and D, lower bar charts, p < 0.01). This result was somewhat surprising, because we had anticipated larger responses as a consequence of the reduced level of cAMP hydrolysis, but it may be due to elevated levels of local cAMP minimizing the effects of a second PGE_{1} application in these cells. The reduced responsiveness of cells expressing catalytically inactive PDE4D3 and PDE4D5 isoforms suggests that a spatially constrained pool of PDE4D is involved in local cAMP signaling. Any potential effects of PDE4D isoform on EP-R desensitization may also be masked by the possible activation of the G_{i}-linked EP3-R subtype when PDE4D3 or PDE4D5 activity is reduced. In ~20% of PDE4D3 siRNA-expressing cells, the second application of PGE_{1} evoked an apparent G_{i}-mediated event producing a decrease in local cAMP signal (Fig. 7A, bottom right panel). Although our data point to distinct roles for PDE4D3 and PDE4D5 in shaping EP-R-mediated cAMP signals, a lack of reliable isoform-selective inhibitors for the prostanoid receptors limits isolation of specific signaling events associated with any particular EP-R subtype.

Cross-talk between cAMP Microdomains Gives Rise to Heterologous Receptor Desensitization—Having established that there are distinct differences in the organization of the signaling complexes responsible for the rapid cAMP dynamics associated with the β_{2}-AR and EP-R in HEK293 cells, which indicates a non-overlap of shared elements and therefore distinct compartmentalization, we next examined the possibility that the two receptor pools could communicate with each other (Fig. 8). Indeed we found significant interplay between PGE_{1}- and isoproterenol-mediated cAMP signals despite rapid hydrolysis of these modest, locally compartmentalized cAMP signals. A 1-min pre-treatment of HEK293 cells with 1 μM PGE_{1} was sufficient to decrease β_{2}-AR signaling efficiency by ~75% (Fig. 8, B and F) suggesting that the rapidly attenuated cAMP signal emanating from EP2/4-Rs can propagate sufficiently to influence the state of neighboring β_{2}-ARs. It has previously been suggested that prolonged pre-treatment of cell populations with PGE_{1}, or the AC-activator FSK can activate PKA sufficiently to phosphorylate β_{2}-ARs and inhibit coupling of the receptor to G_{s} (31) giving rise to heterologous receptor desensitization (32–34). Strikingly, here pre-treatment with 10 μM FSK failed to attenuate isoproterenol-mediated cAMP increases, which suggests that the effects of PGE_{1} were due to a much more localized stimulation of AC activity.

These results are the first evidence to suggest that very rapid changes in cAMP can dramatically influence signaling in neighboring receptor domains. Stimulation of EP2/4-Rs generates a transient rise in local cAMP that can significantly attenuate subsequent β_{2}-AR-mediated cAMP signaling. Bidirectional interplay between the β_{2}-ARs and EP2/4-Rs cAMP signaling microdomains was not seen, because isoproterenol pre-treatment failed to influence the amplitude or dynamics of subsequent PGE_{1}-mediated cAMP signals (Fig. 8, E and F). This unidirectional interplay between the prostanoid- and β_{2}-ARs provides compelling evidence of complex signaling interplay within discrete cellular compartments.

DISCUSSION
In the present study we examined compartmentalization of β_{2}-AR- and EP-R-mediated cAMP signals in real-time. Using mutant CNGCs to monitor changes in sub-plasmalemmal cAMP with high temporal resolution and sensitivity provides a unique and powerful methodology to accurately measure both the dynamics and the efficiency of β_{2}-AR- or EP-R G_{s}-coupled signaling at the single cell level. Our data provide the first detailed information on local changes in cAMP during agonist-induced β_{2}-AR and EP-R desensitization. In addition, we observe evidence of unidirectional cross-talk between the neighboring cAMP signaling pathways. Stimulation of AC activity in naïve cells, using either 1 μM isoproterenol or 1 μM PGE_{1} to activate endogenous G_{s}-coupled receptors, was sufficient to induce transient local rises in cAMP. Surprisingly, these seemingly modest and tightly regulated cAMP signals were sufficient to mediate either complete, or partial, loss of cAMP signaling via β_{2}-AR, or EP-R, respectively. As a consequence of this homologous receptor desensitization subsequent applications of agonist were incapable of generating a full cAMP response.

The initial rapid termination of sub-plasmalemmal cAMP signals generated in response to either β_{2}-AR or EP-R stimulation was largely dependent upon PDE4D and PKA-RII activity, with siRNA-mediated knockdown of either signaling component producing sustained local cAMP increases following agonist stimulation. Our data suggest that PDE4D and PKA-RII...
also play an essential role in desensitization, resulting in reduced amplitude of cAMP signals upon subsequent agonist applications. This may be attributed to either a direct effect of PDE4D activity on the local cAMP signal or to a PDE4D effect mediated via mechanism(s) downstream of the regulated cAMP signal. Because basal PDE4 activity is low in HEK293 cells (9), the former can only be true if we assume a relatively prolonged enhancement or recruitment of PDE4D activity last-
Essential Components of Dynamic cAMP Signaling

FIGURE 8. Heterologous desensitization of β2-ARs. A, repeated 1-min applications of 1 μM isoproterenol at 5-min intervals shows complete homologous desensitization of cAMP signaling. B, pre-treatment of CNGC expressing HEK293 cells with 1 μM PGE1 significantly reduces sensitivity of β2-ARs to subsequent 1 μM isoproterenol applications. C, pre-treatment with 10 μM FSK has little effect on β2-AR responsiveness. D, partial, homologous desensitization of prostanoid receptors in response to 1 μM PGE1 application. E, pre-treatment of cells with 1 μM isoproterenol does not reduce EP-R-mediated cAMP signaling. F, mean ± S.E. data quantifying the effects of various means of adenylyl cyclase pre-stimulation β2-AR and EP-R responsiveness; **, p < 0.001.

β-arrestin1 and β-arrestin2 isoforms can associate with PDE4D (22, 23), there is little evidence for a role of β-arrestin1 under our experimental conditions. Such selectivity for β-arrestin2 over β-arrestin1 in regulating sub-plasmalemmal cAMP dynamics is consistent with cell population studies showing increased cAMP accumulation following siRNA-mediated knockdown of β-arrestin2 (38) and provides further evidence for β2-ARs preferential recruitment of β-arrestin2 (39). Here we reveal that the involvement of β-arrestin2 is maximal from 3 min after the initial β2-AR stimulation, allowing time for recruitment of the scaffold protein in response to receptor occupancy and G-protein-coupled receptor kinase-mediated receptor phosphorylation, which is thought to trigger the recruitment of β-arrestin and its associated proteins to β2-ARs (40, 41). However, the specific involvement of G-protein-coupled receptor kinases in the attenuation of prostanoid signaling in the present study was not addressed directly. The ability of PDE4D activity to influence cAMP dynamics prior to this time point is likely to reflect an additional role for pre-associated or free local PDE4D molecules.

PDE4D3 and PDE4D5 isoforms are present in the cytosol and at the plasma membrane of HEK293 cells, where PDE4D5 is preferentially co-recruited to the cell membrane in complex with β-arrestin2 during β2-AR occupancy (14, 22, 23, 29). However, while β-arrestin2-sequestered PDE4D5 has been shown to regulate the phosphorylation of the β2-AR by AKAP79-tethered PKA (14), nothing is known about whether the PDE4D isoforms can affect dynamics of the local cAMP signal and desensitization of the β2-AR. Using siRNA and dominant negative approaches we reveal that both PDE4D3 and PDE4D5 significantly contribute to the rapid hydrolysis of β2-AR-mediated cAMP signals and to receptor desensitization. The peak effect of each PDE4D isoform on the degree of signal desensitization occurred around 5 min following initial stimulation of β2-ARs. However, PDE4D5 played a significant role from as early as 3 min (data not shown), which may reflect the preferential interaction of PDE4D5 with β-arrestin (22, 29). It is likely that effects of PDE4D3 and PDE4D5 knockdown on the rate of cAMP hydrolysis immediately following AC stimulation are at least partly due to the additional loss of non-recruited PDE4D3/5 activity that already resides within cellular microdomains adjacent to the β2-ARs. Thus, PDE4D3 and PDE4D5, which are free
or pre-associated at the plasma membrane, as well as recruited forms of PDE4D3 and PDE4D5, can contribute to β2AR signaling.

Previous studies in cardiac and in HEK293 cells have provided evidence for PDE4-dependent, dynamic changes in sub-plasmalemmal cAMP in response to the EP-R agonist, prostaglandin E1 (PGE1) (4, 8, 9). In HEK293 cells, rapid PDE4-dependent hydrolysis of the local PGE1-evoked cAMP signal depends on a membrane-associated PDE4/PKA/AKAP250 (gravin) signaling complex (9). This AKAP-based complex was also shown to play a lesser, delayed role in shaping cAMP transients following β2AR activation (9). However, the role of PDE4 isoforms associated with other scaffolding proteins, such as the β-arrestins, in attenuating the local cAMP signal as a consequence of prostanoid receptor activation has not been established. By comparing the regulation of local β2AR-mediated signals with EP-R-mediated signals at the sub-plasmalemmal level in real-time we can begin to unravel the complexities and specificities of cAMP compartmentalization available to cells expressing several types of Gs-linked receptor. In addition, we can examine the possibilities for cross-talk between such localized, dynamic cAMP events.

In sharp contrast to β2AR signaling, the involvement of PDE4D isoforms in PGE1-mediated signal termination and desensitization of EP2/4-R signaling was completely independent of β-arrestin recruitment, which suggests a predominant role for free or pre-associated PDE4D isoforms. Consistent with this, previous work in our laboratory has identified a PDE4D/PKA/AKAP250 (gravin) signaling complex that plays an essential role in shaping PGE1-evoked sub-plasmalemmal cAMP signals in HEK293 cells (9). Interestingly, the use of PDE4D3- and PDE4D5-selective siRNA, or overexpression of catalytically inactive PDE4D3 and PDE4D5, in the present study indicates that it is a sequestered pool of PDE4D5 that provides a dominant role in attenuating the initial rate of cAMP recovery following application of PGE1. By comparison, PDE4D3 siRNA or dominant negative generates a substantial and delayed increase in local cAMP following EP-R stimulation. One interpretation of these data could be that pre-associated PDE4D5 isoforms are preferentially activated in close proximity to the EP2/4-Rs, whereas PDE4D3 activity may play a more significant role within the signaling microdomain over a slower time period (Fig. 9A). The efficiency of PDE4D isoforms in shaping the sub-plasmalemmal cAMP signals is in contrast to a recent report suggesting that PDE4B regulates cAMP in this cellular domain following PGE1 activation of HEK293 cells (13).

Unexpectedly, the use of selective siRNA and catalytically inactive versions of either PDE4D3 or PDE4D5 appeared to...
reduce rather than enhance the amplitude of cAMP transients evoked by subsequent PGE$_1$ applications, disputing a role for the enzymes in desensitization at EP2/4-Rs. However, it is feasible that such effects may be obscured by the elevated local [cAMP] that is established in response to the initial agonist application in cells in which PDE4D3/5 activity is reduced. This is likely to minimize any potential rises in local cAMP, and is further prejudiced (at least in the case of PDE4D3 knockdown) by the unrolling of a G$_i$-linked response to PGE$_1$.

RT-PCR analyses identified three distinct prostanoid receptors in HEK293 cells; EP2-, EP3-, and EP4-R. Both EP2- and EP4-isomers are known to associate with G$_i$ and may be responsible for the cAMP events mediated by PGE$_1$ in this study. Previous reports suggest that the EP4-R, but not the EP2-R, can display rapid agonist-induced desensitization due to multiple phosphorylation sites within its C-terminal sequence (42, 43). Furthermore, it is suggested that this effect is attenuated in β-arrestin antisense-expressing cells (44). It is conceivable that the desensitization of EP2/4-Rs seen here is also due to PKA-mediated receptor phosphorylation. However, the β-arrestin independence of cAMP events may implicate either a dominance of EP2-R-mediated signaling and desensitization under our experimental conditions, or a mode of EP4-R desensitization that is independent of β-arrestin. The G$_i$-coupled EP3-R appears to have little activity under control conditions, as assessed by PTX experiments, but it may become more active as a consequence of PDE4D silencing.

In conclusion, the local cAMP signals produced in response to G$_i$-coupled AC activity during isoproterenol or PGE$_1$ stimulation appear upon first observation quite similar in terms of their dynamics. However, by probing the role of specific signaling molecules in shaping the spatiotemporal characteristics of the cAMP signals, it is clear that the signaling microenvironments associated with β$_2$ARs and EP2/4-Rs are distinct. The rapid hydrolysis of cAMP by two major isoforms of PDE4D is critical for both pathways although the association of the enzymes with specific scaffolding proteins and/or the time course of their recruitment are quite different. Similarly, the role of β-arrestins in β$_2$AR desensitization is not mirrored in EP2/4-R desensitization.

Consequently, it seems remarkable that such compartmentalized signaling pathways can display significant interplay; with modest stimulation of EP-Rs by 1 μM PGE$_1$ resulting in significant desensitization of β$_2$ARs, but not the converse. Additionally, general stimulation of AC activity throughout the cell using FSK was ineffective, which suggests that the localized cAMP signal generated by EP2/4-R stimulation was highly specific in its ability to down-regulate neighboring β$_2$ARs. The precise mechanism for this heterologous desensitization of β$_2$ARs by PGE$_1$ is not clear. Previous studies in cell populations suggested that a sustained rise in cAMP resulting from both PGE$_1$ and FSK stimulation could produce up to a 30% loss of signaling efficiency at β$_2$ARs (34), possibly due to enhanced PKA activity, which can phosphorylate the β$_2$AR and inhibit its coupling to G$_i$ (31). Here we report around 75% loss of β$_2$AR signaling efficiency following a 1-min stimulation of AC activity with PGE$_1$, with little effect of FSK pre-treatment. In contrast, isoproterenol pre-treatment did not affect signaling via the EP2/4-R in response to subsequent PGE$_1$ application (Fig. 9B).

Thus, even in these simple model cells three effective compartments of membrane-localized pools of cAMP can be considered (Fig. 9).

The use of mutant CNGCs in this study to quantify local cAMP production in individual cells has provided several surprising insights into the generation of dynamic cAMP signals in the vicinity of specific G$_i$-coupled receptors. Our findings highlight the cell’s ability to utilize different combinations of signaling molecules to propagate unique compartmentalized signaling events. In addition, there is scope for restricted interactions between these compartments to yield unsuspected modulated events. The application of these single cell approaches promises further revelations in the sophistication of cell signaling.

REFERENCES