Phosphodiesterase 4D Forms a cAMP Diffusion Barrier at the Apical Membrane of the Airway Epithelium*

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We demonstrated previously that Calu-3 airway epithelial cells sense adenosine on their luminal surface through adenosine A2B receptors coupled to adenyl cyclase. Occupancy of these receptors leads to activation of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel through protein kinase A (PKA) anchored at the apical membrane. Because luminal A2B receptor activation does not raise total cellular cAMP levels, we hypothesized that activation of phosphodiesterases (PDEs) confines cAMP generated by apical A2B receptors to a microdomain that includes the CFTR channel. Using reverse transcription-PCR, Western blotting, and activity measurements, PDE4D was identified as the major PDE species in airway epithelia. Consistent with these results, inhibitors of PDE4, but not PDE3, selectively abolished the lateral confinement of cAMP signaling in apical membrane patches during cell-attached recordings. Furthermore, stimulation of the CFTR in excised apical patches by rolipram and R825344 indicated that PDE4 is localized in close proximity to the CFTR channel. Indeed, immunohistochemistry of human airway sections revealed that PDE4D is localized in the apical domain of the cell. PDE4 was activated after luminal adenosine exposure in a PKA-dependent manner. Because PDE4 activity is positively regulated by PKA, our results support a model whereby the PDE diffusion barrier is proportional to the degree of receptor stimulation. These findings underscore the concept that subcellular localization of individual PDE isoforms is an important mechanism for confining cAMP signaling to functional domains within cells.

Numerous cellular processes are initiated, modulated, or terminated by cAMP (1). Acting on receptors and transducers, diverse extracellular stimuli elicit cAMP production by membrane-bound adenyl cyclases, but the spatial and temporal organization required to tailor cAMP signaling to individual cellular responses is not completely understood. In the case of the cystic fibrosis transmembrane conductance regulator (CFTR),1 we reported previously that luminal adenosine elevated juxtamembrane cAMP levels and stimulated the activity of protein kinase A (PKA) to increase CFTR open probability (2, 3). Furthermore, we showed that this signaling is dependent on the close proximity of each of these signaling components because cAMP-dependent stimulation of the CFTR initiated by adenosine A2B receptors requires locally anchored PKA (4). Interestingly, our patch clamp experiments also indicated that apical A2B receptors excluded from cell-attached patches did not stimulate the CFTR within cell-attached patches (2). This result suggests that diffusion of cAMP near the apical cell membrane is constrained in a manner similar to that observed in other model systems (5, 6).

The orchestration of signal transduction via the assembly of signaling protein complexes has been described for many cell systems (7). Signaling proteins are frequently organized into multi-protein complexes responsible for signal amplification or pathway specification as in the case of G protein-coupled receptors and calcium channels (8, 9). In epithelial cells, a complex composed of B2 adrenergic receptors, adenyl cyclase, PKA, and CFTR may be formed via interaction with A kinase-anchoring proteins (AKAPs) and the ezrin/moesin/radixin-binding protein, EBP50 (10). This complex promotes rapid cAMP-dependent phosphorylation and activation of the channel. In many instances, PKA also is associated with proteins involved in inactivation of effector proteins. For example, protein phosphatases can also directly bind AKAPs to promote rapid and specific dephosphorylation of specific substrates (11). Phosphodiesterases (PDEs), the enzymes that degrade and inactivate cAMP, have been found recently in complex with AKAPs and PKA (12, 13). This localization of PDEs in close proximity to PKA is thought to control the access of cAMP to binding sites on the regulatory subunit of the kinase (14, 15). PDEs are subdivided into 11 subgroups based on pharmacologic and structural properties, each varying in their regulation and cyclic nucleotide specificity (16). Although data exist concerning a role for phosphodiesterases in the compartmentation of cAMP in nonpolarized cells (17–19), little is known about the distribution and actions of cAMP in microdomains such as the apical regions of polarized epithelia. Several PDEs are ex-

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1 The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; PDE, phosphodiesterase; ADO, adenosine; AMPK, AMP-dependent protein kinase; AMPPCP, adenosine 5′-(β,γmethylene)diphosphate; WD-BHE, well-differentiated human bronchial epithelium; GPCR, G protein-coupled receptor; PKA, protein kinase A; AKAP, A kinase-anchoring protein.
pressed in both human airway and intestinal epithelia based on molecular (20, 21) and pharmacologic approaches (22, 23). At least one PDE, PDE3, can augment CFTR responses ex vivo (24), in some animal models (25), but not in humans (26). To define the role of PDE4 in signal compartmentalization in polarized cells, we extended our previous observations with regard to local cAMP signaling in human airway epithelium and assessed the impact of PDEs in limiting apical cAMP signaling and the regulation of CFTR. We find that PDE4D is a critical component of cAMP signaling at the apical membrane that controls the diffusion of cAMP from its site of generation and that this control is modulated through PKA activation.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals—All chemicals were purchased from either Sigma or Calbiochem unless otherwise noted. RS25344 was a generous gift of Roche Applied Science. These conditions resulted in a basal CFTR-mediated Ringer’s solution with gluconate. Bioelectric properties were digitally recorded from the output of voltage clamps (no. VCC600; Physiologic Instruments, San Diego, CA) using ACQUIRE software (Physiologic Instruments). Voltage was clamped to 0 mV except for 3-s pulses to ±10 mV every 60 s. Tissue conductance was calculated from the resulting current deflections. For each experiment, basal properties were recorded for 20–40 min. Test compounds (ADO, cilostamide, RS25344, and so forth) were added to the low-Cl bath 10 μM forskolin. Relative potency was determined by fitting curves to the fractional changes induced by sequentially increasing concentrations of test substances relative to the response to forskolin. These conditions resulted in a basal CFTR-mediated I Na of 30–40 μA/cm

Immunostaining—Human trachea sections were provided by the University of North Carolina Cystic Fibrosis Center Histology Core Facility, and immunostaining was performed as described previously (29) with modifications. Briefly, frozen tissue sections were fixed in 4% paraformaldehyde at room temperature (4 min), permeabilized in 100%
ethanol at 20 °C (4 min), and blocked in 20% goat serum in phosphate-buffered saline for 3 h. Sections were then incubated in primary antiserum (pan-4D8/H11001, PDE4; pan-4D3/H11002, PDE3; pan-4D5/H11002, PDE4B; and pan-4D1/H11001, PDE4D) monoclonal antibody (generously provided by ICOS, 1:5000), rabbit anti-4D3 antibody, or anti-4D5 polyclonal antibody as described previously (31). MAb and either cilostamine, RS25344, or neither. Cells were then stimulated for 5 min with 10 μM ADO at 37 °C. This was followed by cell lysis and cAMP enzyme immunoassay (Amersham Biosciences) according to the manufacturer’s recommendation.

**RESULTS AND DISCUSSION**

In preliminary patch clamp experiments, we used a pharmacological approach to determine which PDE gene families modulated adenosine-mediated activation of CFTR channel activity in cell-attached patches. We found that only inhibitors of PDE3 and PDE4 affected CFTR currents, with PDE4 inhibitors being washed extensively and analyzed by Western blot using the pan-PDE4D monoclonal antibody (generously provided by ICOS, 1:5000), rabbit anti-4D3 antibody, or anti-4D5 polyclonal antibody as described previously (31).

Reverse Transcription-PCR—RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) after RNA later treatment (Ambion, Austin, TX). Genomic DNA contamination was removed by DNase treatment. Sample RNA was quantified by spectrophotometry, and 200 ng was reverse-transcribed to cDNA in a total reaction volume of 22 μl. Briefly, a mixture of 200 ng of total RNA, 200 ng of random primers (Invitrogen), and 10 nmol of deoxynucleotide triphosphate in a total volume of 12 μl was denatured at 65 °C for 5 min and then incubated for 10 min at room temperature and 2 min at 42 °C with 4 μl of 5× First-Strand Buffer, 2 μl of 0.1 mM dithiothreitol (Invitrogen), and 40 units of RNase inhibitor (Promega). Two hundred units of Super-Script II reverse transcriptase (Promega) was added, and the mixture was incubated for 50 min at 42 °C. Finally, the mixture was incubated for 15 min at 70 °C to inactivate the enzyme. Primers and conditions used to amplify each of the phosphodiesterase isoforms can be supplied upon request.

cAMP Assay—Before ADO stimulation, Calu-3 cells were incubated for 1 h in serum-free Opti-MEM (Invitrogen) containing AMPCP and either cilostamine, RS25344, or neither. Cells were then stimulated for 5 min with 10 μM ADO at 37 °C. This was followed by cell lysis and cAMP enzyme immunoassay (Amersham Biosciences) according to the manufacturer’s recommendation.

**TABLE I**

Summary of reverse transcription-PCR of PDE3 and PDE4 expression profile in Calu-3 and WD-HBE cells

| Calu-3 | WD-HBE |
|--------|--------|
| PDE3A  | +      | +      |
| PDE3B  | +      | +      |
| PDE4A  | +      | +      |
| PDE4B  | +      | +      |
| PDE4C  | +      | -      |
| PDE4D  | +      | -      |
| 4D1/2  | +      | +      |
| 4D3    | +      | -      |
| 4D4    | +      | -      |
| 4D5    | +      | -      |
| 4D6    | -      | -      |
| 4D7    | -      | -      |
| 4D8    | -      | -      |

**FIG. 2.** Selective PDE4 inhibitor alters compartmentalized regulation of CFTR. A, effect of PDE inhibitors on regulation of CFTR-mediated Cl secretion by ADO. RS25344 (0.3 μM; ○), cilostamide (1.0 μM; ▲), or vehicle (H2O; ▼) was added to the luminal bath. ICER responses (CI secretion) to increasing concentrations of ADO were recorded and plotted as a percentage of the maximum forskolin-stimulated Cl secretion to increasing ADO. RS25344 (0.3 μM) and cilostamide (1.0 μM) were added, and the effect of 2 μM forskolin was plotted as a percentage of the maximum forskolin-stimulated Cl secretion. B, cAMP accumulation stimulated by 10 μM ADO in the presence or absence of phosphodiesterase inhibitors (1.0 μM cilostamide or 0.3 μM RS25344) and the effect of 2 μM forskolin. The intracellular cAMP concentrations are expressed in picomoles (n = 3); error bars indicate S.D. C, CFTR activity was recorded in cell-attached patches of Calu-3 cell apical membrane. Pipette voltage was clamped at +30 mV throughout. CFTR channels open as upward deflections. Cultured epithelia were pretreated with Me2SO/H2O (vehicle), 0.3 μM RS25344, or 1.0 μM cilostamide (data not shown). Activity was recorded for 240 s (basal period). ADO (10 μM) was introduced into the luminal bath, and channel activity was recorded for 300 s. Subsequently, forskolin (10 μM) was added to the luminal mixture to maximally stimulate CFTR. Inset shows a 10-s segment of activity at higher resolution. D, summary data from the experiments illustrated in C. For each experimental condition, the CFTR NPo (channel number × open probability) in the basal and ADO periods was calculated and normalized to the value observed during exposure to both ADO and forskolin. Error bars, S.E., * p < 0.001.
the most efficacious (data not shown). On the basis of this preliminary finding, we further characterized the expression of these two groups of PDEs in the Calu-3 cell line, a model system for exploring CFTR function in airway epithelia.

We monitored PDE enzyme activity in the presence of different inhibitors, and we found that PDE3 and PDE4 families of enzymes account for ~60% of the total activity (Fig. 1A). Because four distinct genes compose the PDE4 family, we further analyzed the pattern of PDE4 expression in Calu-3 cells using immunoprecipitation with isoform-specific antibodies. PDE4D accounted for the majority (60%) of PDE4 activity in Calu-3 cells and, by inference, up to 15% of total cAMP PDE activity (Fig. 1B). These results are consistent with previous reports of PDE expression in well-differentiated human bronchial epithelium (WD-HBE) (20, 21) and with in situ hybridization studies in mice (32). In a similar fashion, more than half of the total PDE activity in WD-HBE was due to PDE4, and ~50% of PDE4 activity in these cells was immunoprecipitated with PDE4D-selective antibodies.2

The presence of multiple PDE isoforms was confirmed by reverse transcription-PCR analysis of PDE3 and PDE4 mRNA expression in both Calu-3 cells and WD-HBE (Table I). Amplions corresponding to PDE3A, PDE3B, and the PDE4 families were readily detected, consistent with our previous observations using PDE activity with the exception of PDE4C; however, this may reflect a transcription/translation disparity. Of the nine known PDE4D gene-derived variants, transcripts corresponding to PDE4D3, PDE4D5, PDE4D7, and PDE4D9 were amplified from Calu-3 cell mRNA. Western blot analysis of immuno-enriched PDE4D from Calu-3 and WD-HBE cells indicated that the most abundant gene products migrated on SDS-PAGE as PDE4D5 and PDE4D7 (Fig. 1C). Western blots using a PDE4D5-specific antiserum confirmed that this immunoactivity is indeed due to PDE4D5 (data not shown).

We next tested whether inhibitors of PDE3 and PDE4 affect CFTR Cl− current in cultured Calu-3 monolayers after luminal application of ADO. RS25344 (33), an inhibitor of PDE4 isozymes, modestly shifted the dose-response curve for ADO stimulation of the CFTR to the left, whereas cilostamide, a selective PDE3 inhibitor, had no effect (Fig. 2A). We next asked whether PDE3 or PDE4 inhibitors affected total cellular cAMP while simultaneously increasing CFTR responses to A2B stimulation (Fig. 2B). In agreement with our previous observation (2), luminal ADO alone did not increase total cAMP levels in these cells. Interestingly, ADO in combination with PDE inhibitors also generated no detectable changes in total cAMP levels, even when we used concentrations of ADO and PDE inhibitors that maximally stimulated CFTR currents in patch clamp experiments (described below). These data suggest that A2B receptors stimulate cAMP production in a local compartment shared with AKAP-anchored PKA (4) and that the PKA in turn phosphorylates and activates CFTR. To further characterize the role of PDEs in local regulation of the CFTR, we next tested the effect of PDE inhibitors on the ability of apical A2B receptors to signal laterally to the CFTR contained within cell-attached patches. In this paradigm, ADO added to the luminal bath under control conditions did not effect the activation of the CFTR within the patch (Fig. 2C), consistent with our previous results (2). However, when Calu-3 cells were pretreated with 300 nM RS25344 (highly specific for PDE4 inhibition at this concentration), ADO applied to the bath readily stimulated the CFTR within the cell-attached patch. Indeed, when cultures were pretreated with RS25344, ADO stimulated CFTR almost as effectively as 10 μM forskolin (Fig. 2C). In contrast to the dramatic effect of RS25344, pretreatment with cilostamide, a selective inhibitor of PDE3 isozymes, had no significant effect on CFTR currents (Fig. 2D). Furthermore, similar results were observed when comparing rolipram (PDE4 inhibitor) and milrinone (PDE3 inhibitor; data not shown), suggesting that PDE4

**Fig. 3.** CFTR is regulated by apical PDE4 activity. Apical patches were generated from Calu-3 cells in the outside-out configuration. A, effect of rolipram on CFTR in excised outside-out patches. The horizontal dash at the left indicates channel closed. B, effect of rolipram on CFTR in excised outside-out patches with 10 μM PKI in the pipette. C, effect of milrinone on CFTR in excised outside-out patches. D, summary data for A (n = 11), B (n = 10), and C (n = 7). *, p < 0.02.

A. P. Barnes, G. Livera, M. Conti, and S. L. Milgram, unpublished observations.
Isozymes are critically involved in modulating the spread of cAMP signaling at the apical surface in Calu-3 cells. It is also important to note that the effect of RS25344 on ADO stimulation of CFTR in cell-attached patches appeared more dramatic than the modest shift induced in the adenosine CFTR $I_{sc}$ dose-response relation. The Calu-3 $I_{sc}$ is a composite of multiple electrochemical driving forces and does not directly measure apical conductance as described previously (34). Plausibly, stimulation of apical anion conductance due to PDE inhibition would not dominate $I_{sc}$ under these conditions.

Although these inhibitor studies strongly suggest that PDE4 isoforms limit the spread of subapical cAMP, we cannot rule out the possibility that the cell-permeant inhibitors we used acted indirectly through effectors located outside the subapical domain of the cell. Therefore, we generated excised apical membrane patches in the outside-out configuration from the Calu-3 cells and exposed them to PDE3 and PDE4 inhibitors. In this paradigm, traces indicate endogenous channel activity because the patches were not stimulated with ADO. Channel opening increased with increasing concentrations of the PDE4 inhibitor (Fig. 3A). To confirm that this observation results from cAMP signaling through PKA and not through nonspecific effects due to generation of the patch, we included PKI, a PKA-specific inhibitor, in the pipette solution (Fig. 3B). To test whether PDE3 isoforms were also associated with excised apical membrane patches, we added the PDE3 inhibitor milrinone to the bath, but we saw no significant effect on channel kinetics (Fig. 3, C and D). These data indicate that the membrane and submembranous complexes associated with the patch contain the components necessary for regulating CFTR signaling and that this regulation involves factors sensitive to inhibitors of PDE4. It is also important to note that in both cell-attached and outside-out patches, both RS25344 and forskolin are able to permeate the membrane, excluding the possibility that the effects observed represent a physical barrier created by the membrane geometry near the pipette tip.

The effect of PDE4 inhibitors on the CFTR in patches excised from the apical membrane suggested that PDE4 is recovered in the patch and, therefore, all the components controlling the opening of the channel are in close proximity to each other. To further assess this possibility, immunostaining was used to determine the subcellular location of PDE4D in human airway epithelium. The majority of PDE4D isoforms detected in human trachea, particularly PDE4D5 (Fig. 4), are localized at or near the apical membrane. This is consistent with the localization of other components of the CFTR complex (35–37). Interactions with RACK-1
Fig. 5. Protein kinase A activation of PDE4 after ADO stimulation. Calu-3 cells were incubated for 1 h in serum-free Opti-MEM containing 300 µM AMPCP and then stimulated for 5 min with 10 µM ADO at 37 °C. A, increased phosphodiesterase activity after apical ADO or forskolin stimulation. This increase is blocked by incubation with the PKA inhibitor Rp-cAMP (RP) (1 mM). B, cAMP metabolism at the apical membrane can be self-regulating, and its by-products also can regulate CFTR activity. Activation of the adenosine A2B receptor leads to stimulation of adenylyl cyclase by ADO. The resulting increase in cAMP triggers protein kinase A (Ir, regulatory; C, catalytic) that is anchored near CFTR by an AKAP. CFTR open probability is increased by PKA phosphorylation. PKA also increases PDE4 activity that leads to an attenuation of the cAMP signal. The by-product of cAMP degradation can activate adenosine monophosphate-activated kinase (AMPK), leading to CFTR down-regulation.

Our observations uncover a finely tuned orchestration of CFTR by cAMP and its metabolites. For example, CFTR current is reduced by AMP-dependent protein kinase (AMPK), a serine-threonine kinase activated by AMP and reported to directly bind the CFTR (43). Because of this direct interaction and because 5'-AMP is generated by the PDE-mediated metabolism of cAMP, we speculate that A2B receptor-driven stimulation of subapical PDE4 could initiate negative feedback on CFTR activity through AMPK. Preliminary data testing this possibility indicate that pretreating Calu-3 cell monolayers with the AMPK-activating compound 5-aminoimidazole-4-carboxamide riboside to fully activate AMPK reduces CFTR-mediated Cl⁻ secretion induced by luminal ADO (data not shown). Furthermore, recent work from Randak and Welsh (44) indicates that the CFTR harbors an intrinsic adenylyl kinase activity that may regulate channel gating by directly binding AMP. Taken together, these findings support a complex and interactive regulation of the CFTR through mechanisms that both regulate and respond to local levels of cAMP and its metabolites at the apical membrane.

Our data provide physical and functional evidence for a major role of PDE4 as a diffusion barrier to cAMP signaling in the apical membrane of airway epithelia. This spatial barrier of PDEs is sensitive and proportional to the levels of local cAMP (Fig. 5B). However, it should be noted that the effectiveness of this barrier would also be dependent on the intensity and duration of cellular stimulation because the excessive generation of cAMP in the microdomain may exceed the catalytic capacity of the available PDEs. This model also includes a potential downstream effector, AMPK. This kinase is known to localize to the apical membrane (43), can down-regulate CFTR currents in Calu-3 cells (45), and may be stimulated by the locally increased 5'-AMP generated by PDE activity near the activated receptor/channel complex. Additional experiments will be required to elucidate the exact relationship between the 5'-AMP generated by PDE4 and AMPK activity in Calu-3 cells.

Our observations uncover a finely tuned orchestration of signaling events at the apical membrane. Using cell-attached
important not only in understanding cellular physiology but also in controlling the apical signaling complex that regulates CFTR currents in the airway epithelium (2, 10). It also adds context to previous studies of the apical signaling complex that regulates CFTR currents otherwise identical cAMP signals. With the inclusion of phosphodiesterases as the cellular component required for limited ADO signaling, this work expands the earlier characterization of the apical signaling complex that regulates CFTR currents in the airway epithelium (2, 10). It also adds context to previous observations about PDE activity in airway and the role of cAMP and 5′-AMP in apical signaling. These themes are important not only in understanding cellular physiology but also in designing successful therapeutics for the treatment of cystic fibrosis.

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