Dynamic Activation of Cystic Fibrosis Transmembrane Conductance Regulator by Type 3 and Type 4D Phosphodiesterase Inhibitors

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ABSTRACT

The diseases of cystic fibrosis, chronic obstructive pulmonary disease (COPD), and chronic bronchitis are characterized by mucus-congested and inflamed airways. Anti-inflammatory agents that can simultaneously restore or enhance mucociliary clearance through cystic fibrosis transmembrane conductance regulator (CFTR) activation may represent new therapeutics in their treatment. Herein, we report the activation of CFTR-mediated chloride secretion by phosphodiesterase (PDE) 4 inhibitors in T84 monolayer using 125I anion as tracer. In the absence of forskolin, the iodide secretion was insensitive to PDE4 inhibitor L-826,141 [4-[2-(3,4-bis-difluoromethoxyphenyl)-2-[4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenyl]-ethyl]-3-methylpyridine-1-oxide], roflumilast, or to PDE3 inhibitor trequinsin. However, these inhibitors potently augmented iodide secretion after forskolin stimulation, with efficacy coupled to the activation states of adenylyl cyclase. The iodide secretion from PDE3 or PDE4 inhibition was characterized at first by a prolonged efflux duration, followed by progressively elevated peak efflux rates at higher inhibitor concentrations. Compared with an increased phosphor-cAMP response element-binding protein formation, the CFTR activation dissociated from a global cAMP elevation and was blocked by H89 [N-[2-[(p-bromocinnamyl)amino]ethyl]-5-isouquinolinesulfonamide]. 2-(4-Fluorophenoxy)-N-[(1S)-1-(4-methoxyphenyl)ethyl]nicotinamide, a stereoselective PDE4D inhibitor, augmented iodide efflux more efficiently than its less potent (R)-isomer. The peak efflux from maximal PDE4 and PDE3 inhibition matched that from full adenylyl cyclase activation. These data suggest that PDE3 and PDE4 (mainly PDE4D) form the major cAMP diffusion barrier in T84 cells to ensure a compartmentalized CFTR signaling. Together with their potent anti-inflammatory properties, the potentially enhanced airway mucociliary clearance from CFTR activation may have contributed to the efficacy of PDE4 inhibitors in COPD and asthmatic patients. PDE4 inhibitors may represent new opportunities to combat cystic fibrosis and other respiratory diseases in future.

Respiratory epithelia continuously remove inhaled particles, microbes, and metabolites of resident and migratory cells through a mucociliary clearance process that requires the secretion of fluid and electrolytes. Cystic fibrosis transmembrane conductance regulator (CFTR) is the primary cAMP-activated chloride channel on the apical membrane of airway epithelia, thereby playing an integral role in controlling the electrolyte/fluid balance and mucociliary clearance process (Pilewski and Frizzell, 1999). Most CFTR mutations lead to either a reduced expression or proteins with a compromised chloride conductance in response to physiological stimuli. The major disease mutation ΔF508-CFTR with a decreased epithelial expression in CF patients (Kalin et al., 1999) remains partially functional with a reduced open probability and sensitivity to stimulation by cAMP agonists (Wang et al., 2000a). The functional defects of CFTR mutants result in multiple organ dysfunctions, including a severely impaired airway mucociliary clearance that leads to chronic

ABBREVIATIONS: CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; PKA, protein kinase A; AC, adenylyl cyclase; PDE, 3’,5’-cyclic nucleotide phosphodiesterase; COPD, chronic obstructive pulmonary disease; RP-73401, 3-(cyclopentyl oxy)N-(3,5-dichloropyridin-4-yl)-4-methoxybenzamide; Cpd-A (L-826,141), active enantiomer of 4-[2-(3,4-bis-difluoromethoxyphenyl)-2-[4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenyl]-ethyl]-3-methylpyridine-1-oxide; Cpd-B, 2-(4-fluorophenoxy)-N-[(1S)-1-(4-methoxyphenyl)ethyl]nicotinamide; Cpd-C, 2-(4-fluorophenoxy)-N-[(1R)-1-(4-methoxyphenyl)ethyl]nicotinamide; H89, N-[2-[(p-bromocinnamyl)amino]ethyl]-5-isouquinolinesulfonamide; DMSO, dimethyl sulfoxide; RS25344, 8-aza-1-(3-nitrophenyl)-3-(4-pyridylmethyl)-2,4-quinazolinedione; RT, room temperature; TTBS, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 0.1% (v/v) Tween 20; pCREB, phosphor-cAMP response element-binding protein; CREB, cAMP response element-binding protein.
secondary bacteria infections and respiratory failure in CF patients.

It is being increasingly recognized that there is an elevated inflammation in CF patients, which plays a major role in the pathogenesis of CF lung disease (Chmiel et al., 2002). Early pulmonary inflammation with neutrophilia can be detected in CF infants before the onset of bacterial colonization (Khan et al., 1995). Leukotriene B4 is elevated in the epithelial lining fluid of CF patients (Konstan et al., 1993). CF tissues have abnormally high levels of proinflammatory arachidonic acid with values of the heterozygous parents half-way between CF patients and healthy controls (Freedman et al., 2004). These data suggest the possible presence of an excessive eicosanoid-based inflammatory response in CF patients and CF carriers. Diseases associated with CFTR mutations continue to widen. In addition to the classic CF, approximately 3.3% of the U.S. population are CF carriers who are predisposed to a number of related airway diseases, including chronic bronchitis and rhinosinusitis (Kostuch et al., 2000; Wang et al., 2000b).

The key regulatory event for CFTR activation is the PKA-mediated serine phosphorylations of its R-domain, which facilitates its ATP binding, with the sequential ATP hydrolysis triggering the channel opening and closing. CFTR is dynamically regulated by the activities of adenylyl cyclase (AC) and cAMP-phosphodiesterase (PDE) through the activation of PKA. Multiple signaling elements, including receptors, G proteins, AC, PKA, CFTR, and protein phosphatase 2C cluster at the apical membrane of epithelial cells to ensure the specificity of the cAMP-mediated CFTR activation (Zhu et al., 1999; Sun et al., 2000; Huang et al., 2001a). cAMP hydrolysis in airway epithelial cells is mainly regulated through PDE4s and PDE3s (Wright et al., 1998). There are four PDE4 (4A to 4D) and two PDE3 (3A and 3B) genes, each producing multiple spliced variants with specific tissue distribution and subcellular localization. PDE4s are abundantly expressed in proinflammatory cells and airway epithelial cells. PDE4 inhibition attenuates the overproduction of many proinflammatory mediators and cytokines, including arachidonic acid, leukotrienes, reactive oxygen species, and tumor necrosis factor-α, and suppresses the infiltration of neutrophils and eosinophils in inflamed airways (Torphy, 1998). PDE4D, the major cAMP-PDE species in bronchial epithelia, plays a dominant role in controlling airway smooth muscle contraction (Mehtats et al., 2003). A number of second-generation PDE4 inhibitors, exemplified by roflumilast [3-(cyclopropyl-methoxy)-N-(3,5-dichloropryridin-4-yl)-4-di-fluoromethoxy benzamide], are presently in advanced clinical development and may emerge as new therapeutics for asthma and COPD (Huang et al., 2001b).

Restoring CFTR activity and simultaneously reducing the excessive airway inflammatory response may represent a promising strategy in CF treatment. In addition, enhancing the mucociliary clearance via CFTR activation may reduce the lung function of COPD patients. This partly stems from the observation that 1) levels of the residual CFTR activity seem to be predictive of the CF disease severity; and 2) cAMP-elevating agents, either through receptor stimulation, AC activation, or nonselective PDE or PDE3 inhibition, activated the wild type-CFTR and partially restored the defective chloride conductance of many CFTR mutants, including ΔF508-

### Materials and Methods

**Chemicals.** Buffer chemicals were from Sigma-Aldrich (St. Louis, MO). [3H](Cyclic adenosine 3′,5′-monophosphate was from Amersham Biosciences, Inc. (Piscataway, NJ). Complete protease inhibitor tablet was from Roche Diagnostics (Indianapolis, IN). PDE4 inhibitors L-882,141 (Cpd-A), the comparator roflumilast, 2-(4-fluorophenoxy)-N-(1S)-1-(4-methoxyphenyl)ethyl|nicotinamide (Cpd-B) and its (R)-enantiomer (Cpd-C) were prepared according to literature (Marfat and Chambers, 1998; Reid, 2002; Claveau et al., 2004). Forskolin, H89, and trequinsin [9,10-dimethoxy-2-mesitylimino-3-methyl-2,3,6,7-tetrahydro-(1,2)-isquinolin-4-one, HCl] were from Calbiochem (San Diego, CA). Sodium iodide (125I) with a typical specific activity of 2000 Ci/mmol was from Draximage Inc. (Kirkland, QC, Canada).

**Cell Culture.** T84 cells (American Type Culture Collection, Manassas, VA) were grown as a monolayer in a 1:1 mixture of Dulbecco’s modified Eagle’s medium/F-12 supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. The epithelial cells (1 × 105 cells/well) were plated onto the 9.5-cm2 culture dishes and incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Culture medium was changed every 48 h. It becomes confluent in 4 to 5 days. Only cell viability exceeded 90% by trypan blue exclusion was used for further experiments.

**125I Efflux Assay.** Confluent T84 monolayer in six-well dishes with a density of 4 × 105 cells/well was labeled with 5 µCi/ml Na125I in 1 ml of HBPR buffer (135 mM NaCl, 5 mM KCl, 3.33 mM NaH2PO4, 0.83 mM Na2HPO4, 1 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 5 mM HEPES, pH 7.2) for 30 min at 37°C. Labeled cells were washed with 3 × 5 ml of HBPR buffer within 30 s to completely remove extracellular radioactivity. Efflux of the intracellular 125I was sampled by replacing 80% (0.8 ml) of the incubation buffer with fresh buffer every 30 s up to 9 min. Activators were added via the assay buffer immediately after the third buffer replacement at 1.5 min. Temperature was maintained at 37°C. NaOH (1 ml, 0.1 N) was added after 9 min to lyse the cells by sonication (2 min, Branson model 2510 bath sonicator). The radioactivity in each time point was determined on a Microbeta liquid scintillation counter in 24-well format. The maximal forskolin-induced efflux rate over the DMSO control typically ranged from 0.35 to 0.45 min−1 for cells up to 10 passages. Studies investigating multiple conditions were paired using cells of same passage.

**cAMP Measurement.** Confluent T84 cells in 96-well plate were incubated with drug in 200 µl of efflux assay buffer at RT. After quenched with 20 µl of 1 N HCl, cells were lysed by sonication for 2 min. The acidic lysate (20 µl) was neutralized with 80 µl of 250 mM Tris-HCl, pH 7.5, and its cAMP levels were quantified using the cAMP-Biotrak SPA screening assay kit (Amersham Biosciences, Inc.) per manufacturer’s directions.
PDE Activity Assay. Confluent T84 cells (~100 × 10^6) were harvested by trypsinization and washed twice using ice-cold phosphate-buffered saline by centrifugation. Cells were suspended in 1 ml of a buffer containing 50 mM HEPES, pH 7.5, 5 mM EDTA, 0.05 mM diithiothreitol, 1 mM N-a-benzoyl-L-arginine ethyl ester, 1 mM benzamide, 10 μg/ml pepstatin A, and Complete EDTA-free proteases inhibitors tablet. After sonication (5 × 10-s pulses; power setting 3; 4°C), the lysate was collected after a 10,000g spin (30 min at 4°C). The lysate cAMP-PDE activity was determined by monitoring the hydrolysis of 0.1 μM [3H]cAMP in 10 mM MgCl2 and 50 mM HEPES, pH 7.2, using the SPA-PDE assay kit from Amersham Biosciences, Inc. (Laliberte et al., 2000). The potencies of inhibitors against multiple PDEs in Table 1 were determined using 0.1 μM [3H]cAMP for PDE1, PDE4s, PDE7A, and PDE8B and 0.01 μM [3H]cAMP for PDE3A and PDE3B under the same assay conditions as detailed previously (Claveau et al., 2004). Under the conditions, they were close to the apparent K_i, PDE1 was purified from dog heart, and other PDEs were human recombinant enzymes.

Phospho-CREB Induction and Western Blot Analysis. Confluent T84 monolayer in six-well plate at 37°C was incubated with compounds or DMSO in efflux buffer for specified time. After the removal of incubation buffer, 300 μl of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2P_2O_7, 0.1 mM vanadate, 250 μM H_2O_2, plus protease inhibitor (EDTA-free) cocktail tablet) was added. Cells were lysed in the bath sonicator (2 min/4°C). Supernatant was collected after a 10,000g centrifugation (10 min/4°C) and concentrated by Speed-Vac. Approximately 100 μg of protein was loaded per lane on SDS-polyacrylamide gel electrophoresis under denaturing condition. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes, and blocked with 5% skim milk in TTBS [20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 0.1% (v/v) Tween 20] for >60 min. After briefly washing in TTBS, it was incubated with 1:1000 anti-phospho-CREB (Cell Signaling Technology Inc.) and detected using the same secondary antibody as described above.

Data Analysis. 125I efflux rate was calculated following \([ln(R_2)-ln(R_1)]/(t_2-t_1)\), where \(R_i\) is the percentage of radioactivity remaining in the monolayer at time \(t_i\) as described previously (Rogers et al., 1990). Data are expressed as mean ± S.E. of three or more independent experiments unless otherwise specified. Dose-response curves and IC_{50} values were analyzed by a nonlinear iterative regression routine with Grafit (Erichthus Software, Horley, Surrey, UK).

Results

PDE3 and PDE4 Are the Major cAMP-PDEs in T84 Cells. Human colonic T84 cells express abundant CFTR, AC, PKA, and multiple cAMP-PDEs as in human airway epithelial cells (Cohn et al., 1992; Sun et al., 2000; O’Grady et al., 2002). Previous studies also established that its cAMP-mediated chloride secretion, sensitive to the overexpression of a mutant PKA, occurred primarily through CFTR channel (Rogers et al., 1990; Bell and Quinton, 1992). In addition, T84 monolayer possesses several key characteristics that are similar to native secretory epithelial cells, including the formation of tight junctions and the maintenance of a vectorial chloride transport in the confluent stage. Thus, it provides the advantage of a nonrecombinant system to study CFTR regulation in response to AC activation and PDE inhibition (Halm et al., 1988).

To dissect the expression of cAMP-PDEs in T84 cells, the hydrolysis of cAMP by T84 lysate was titrated with the PDE4 inhibitor L-826,141 (Cpd-A), rolflumilast, and the PDE3 inhibitor trequinsin. As summarized by their intrinsic potencies against the multiple recombinant PDEs-PDEs in Table 1, Cpd-A and rolflumilast are potent PDE4 inhibitors, with IC_{50} values ranging from 0.2 to 2.4 nM and from 0.1 to 0.6 nM for the inhibition of PDE4A, 4B, 4C, and 4D, respectively. Cpd-A and rolflumilast are >400- and 10,000-fold more potent than their weaker PDE3 inhibition, respectively (Claveau et al., 2004). Trequinsin, with IC_{50} value of ~0.05 nM against PDE3A and 3B, is >6000-fold more potent compared with its weaker PDE4 inhibition. The inhibition of the cAMP-PDE activity of T84 lysate by Cpd-A and rolflumilast were biphasic (Fig. 1A). Each inhibited ~55% of the total activity.

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cpd-A (L-826,141)</th>
<th>Cpd-B (S)-isomer</th>
<th>Cpd-C (R)-isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE1</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>PDE3A</td>
<td>&gt;10,000</td>
<td>2100 (400)</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>PDE3B</td>
<td>~10,000</td>
<td>1100 (100)</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>PDE4A</td>
<td>0.2 (0.05)</td>
<td>1.3 (0.2)</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>PDE4B</td>
<td>0.1 (0.02)</td>
<td>0.4 (0.3)</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>PDE4C</td>
<td>0.6 (0.1)</td>
<td>2.4 (1.8)</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>PDE4D</td>
<td>0.1 (0.03)</td>
<td>0.3 (0.2)</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>PDE7A</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>PDE7B</td>
<td>&gt;10,000</td>
<td>7200 (400)</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

IC_{50} values represent mean ± S.D. (in nanomolar), with n ≥ 3. The potency of rolflumilast and Cpd-A have recently been disclosed (Claveau et al., 2004).
during the first titration phase, with inflection points at ~0.4 and ~0.1 nM, respectively. These values were consistent with their intrinsic potencies against the recombinant PDE4s listed in Table 1. Approximately 30% of the total activity was further inhibited by higher concentrations of Cpd-A with a second inflection point near 3000 nM, which is likely the consequence of its weaker PDE3 inhibition. This is supported by the biphasic titration of trequinsin from its potent PDE3 inhibition and weaker PDE4 inhibition in Fig. 1B. Its first and second titration phases each eliminated ~30 and ~55% of the total activity. The second response has an inflection point near 400 nM. Mean ± S.E. (n = 3).

Fig. 1. A, inhibition of T84 lysate-catalyzed cAMP hydrolysis by roflumilast (○) and Cpd-A (△). The biphasic curves have the first inflection points at ~0.1 nM for roflumilast and ~0.4 nM for Cpd-A, with an overlapping plateau at 40 to 45% of the total activity. Cpd-A has a second inflection point at ~3000 nM. Roflumilast starts to inhibit the remaining activity above 5000 nM. Mean ± S.E. (n = 3). B, inhibition of T84 lysate-catalyzed cAMP hydrolysis by trequinsin. The biphasic curve has its first inflection point at ~0.1 nM with a plateau near 65% of the total activity. The second response has an inflection point near 400 nM. Mean ± S.E. (n = 3).

Fig. 2. A, time course of forskolin-induced 125I efflux (percentage of total) from T84 monolayer at 37°C. Mean ± S.E. (n = 6). Forskolin was added at 1.5 min as marked by the arrow, and its concentration was maintained throughout the remaining duration. Forskolin (10 μM) (○), 0.3 μM forskolin (△), and vehicle (DMSO) (□) overlapped with that in the absence of DMSO. B, time course of increased 125I efflux in response to increased forskolin stimulation. The efflux rate (minute^-1) was calculated from the efflux curves in A as detailed under Materials and Methods. The latency to reach the peak efflux rate after forskolin stimulation is defined as the response time as illustrated.
vated $^{125}\text{I}$ efflux slowly emerged after $>5.5$ min incubation with 0.1 $\mu$M forskolin (Fig. 2B, Fsk, $\bigcirc$). Higher forskolin concentrations elicited a faster and transient increase in $^{125}\text{I}$ efflux in a dose-dependent manner that remained elevated at approximately 2 to 3 times that of the spontaneous level at the end of the 9-min period. The peak efflux rate extrapolated from the efflux curves saturated in response to increasing forskolin concentrations with an $EC_{50}$ of $\sim 0.55$ $\mu$M. In addition, the increased forskolin concentration progressively reduced the latency to reach the maximum efflux response (response time) as marked in Fig. 2B. These results are consistent with the presence of a tightly coupled AC-cAMP-CFTR signaling in T84 cells, reflecting a progressively activated CFTR from increased AC activation by forskolin. The iodide secretion peaked within 60 s after 30 $\mu$M forskolin stimulation, illustrating the rapid responsiveness of the system. The efflux rate peaked and then fell rapidly above 1 $\mu$M forskolin. The later phase of the biphasic response that causes the efflux rate to decline could be contributed either by tracer depletion, local ATP depletion, or desensitization. The forskolin-stimulated iodide efflux and its responsiveness in T84 cells echoed that in CFTR-overexpressing cells (Haws et al., 1996).

Synergistic CFTR Activation from Dual PDE3 and PDE4 Blockade under the Basal AC State. In the absence of forskolin, the iodide efflux was insensitive to the presence of up to 10 $\mu$M Cpd-A or 1 $\mu$M trequinsin, with their efflux curves overlapped with the spontaneous efflux of the cell (Fig. 3, □). Since these concentrations were $>1000$-fold above their intrinsic PDE4 and PDE3 potencies, respectively, a complete suppression of PDE4 and PDE3 activity would be expected under the conditions, even after considering the potentially reduced potency from increased protein binding in the whole cell environment. Yet, the ablation of PDE3 or PDE4 activity alone, under the basal AC state, was insufficient to activate the iodide secretion. On the other hand, dual PDE4 and PDE3 blockade (10 $\mu$M Cpd-A plus 1 $\mu$M trequinsin; Treq) induced a significantly elevated iodide secretion (Fig. 3, ▲), yielding an efflux curve that has a comparable peak efflux rate but with a prolonged duration to that elicited by 0.3 $\mu$M forskolin alone (Fig. 3, ▼). These data suggest that dual suppression of PDE3 and PDE4 activity is synergistic, instead of additive, in stimulating the cAMP-mediated CFTR activation under the basal AC state with a limited cAMP turnover.

**Increased Efficacy of PDE3 and PDE4 Inhibitors upon Adenyllyl Cyclase Activation.** In contrast to the lack of efficacy under the basal AC state, PDE3 or PDE4 inhibition each potently augmented iodide secretion after forskolin stimulation. The presence of 0.1 $\mu$M forskolin, which only slightly increased the efflux after $>5.5$ min incubation (Fig. 2B, $\bigcirc$), significantly increased the efficacy of 1 $\mu$M Cpd-A by elevating the efflux rate to 0.055 min$^{-1}$ over the vehicle control, with the enhanced efflux emerged after $\sim 3$ min instead. Under the same AC state, 0.1 and 1 $\mu$M trequinsin augmented the peak efflux to 0.09 and 0.16 min$^{-1}$, respectively. Further AC activation by increasing forskolin to 0.3 $\mu$M amplified the responsiveness of the iodide secretion toward PDE4 and PDE3 inhibition, as represented by the efflux curves of Cpd-A (Fig. 4A) and trequinsin (Fig. 4B). Cpd-A (3 $nM$) (Fig. 4A, ▼) or 1 $nM$ trequinsin (Fig. 4B, ▼) each significantly prolonged the iodide efflux induced by 0.3 $\mu$M forskolin (Fig. 4, A and B, $\bigcirc$), and 3 $nM$ roflumilast elicited a similar response to that of Cpd-A (data not shown). Higher inhibitor concentrations augmented the peak efflux rate in a dose-dependent and biphasic manner, with the response of Cpd-A nearly superimposed onto that of roflumilast (Fig. 4C). The first phases of Cpd-A and roflumilast's biphasic responses elevated the peak efflux rate by $\sim 0.06$ min$^{-1}$ over the forskolin response, with inflection points between 2 to 5 nM. Their second response phases started above 1000 nM, increasing the peak efflux rate by another $\sim 0.09$ min$^{-1}$. In comparison, trequinsin augmented the peak efflux rate by $\sim 0.09$ min$^{-1}$ over the forskolin response with an initial inflection point between 1 to 3 nM. Its second response phase occurred above 100 nM, elevating the peak efflux rate by another $\sim 0.05$ min$^{-1}$, which is comparable with that derived from PDE4 inhibition by Cpd-A or roflumilast. The potentiating effects through PDE3 or PDE4 inhibition start to diminish at higher AC activation states, with a minimal augmentation detected at or above 10 $\mu$M forskolin (data not shown).

The increased PDE3 or PDE4 inhibition also progressively reduced the response time. This became more apparent near their saturating doses, particularly judging from the faster rising phase of their efflux curves in Fig. 4, A and B. At the submaximally activated AC state by 0.3 $\mu$M forskolin, the ablation of PDE4 and PDE3 activity by 1 $\mu$M Cpd-A plus 1 $\mu$M trequinsin elicited a peak efflux response (Fig. 5, $\bigcirc$) which was comparable with that from a full AC activation by 10 $\mu$M forskolin (Fig. 5, ▲). The combination of higher PDE3 and PDE4 inhibitor concentrations did not augment the peak efflux rate nor shorten the response time further, supporting that PDE3 and PDE4 are mainly responsible for degrading the cAMP pool involved in CFTR activation in T84 cells.

![Fig. 3. PDE3, PDE4, and dual PDE3 and PDE4 inhibition elicited $^{125}$I efflux in the absence of forskolin stimulation. Compounds were added at 1.5 min as marked, with their concentrations maintained throughout the remaining duration. The efflux curves of 10 $\mu$M Cpd-A or 1 $\mu$M trequinsin overlapped with the spontaneous efflux of DMSO control (□, DMSO). Cpd-A (10 $\mu$M) plus 1 $\mu$M trequinsin (▲) elicited an efflux response with a comparable peak efflux rate but a prolonged efflux duration in comparison with that induced by 0.3 $\mu$M forskolin (▼). Mean ± S.E. (n = 2–4).](image-url)
The average net peak efflux rates over the DMSO control in response to increased PDE3 and PDE4 inhibition were summarized. Cpd-A (H11005), 0.3 mM forskolin plus 1 mM Cpd-A (\(\bullet\)), peak efflux \(-0.3 \text{ min}^{-1}\), response time \(-180 \text{ s}\), 0.3 mM forskolin plus 3 nM trequinsin (\(\triangledown\), peak efflux \(-0.4 \text{ min}^{-1}\), response time 150–180 s), 0.3 mM forskolin plus 1 mM trequinsin (\(\ast\)), peak efflux \(-0.53 \text{ min}^{-1}\), response time \(-120 \text{ s}\), and 10 mM forskolin (\(\ast\), peak efflux \(-0.52 \text{ min}^{-1}\), response time \(-60 \text{ s}\)). The same batch of cells was used. Mean \(\pm\) S.E. (n = 2–4).

Compared with forskolin stimulation, there remains a delayed response in activating the chloride secretion from blocking cAMP degradation through PDE3 plus PDE4 inhibition. The exact timing difference remains to be quantified with a faster sampling protocol.

**CFTR Activation, Paralleled with Phosphor-CREB Formation, Is Blocked by H89.** pCREB induction is a surrogate for PKA activation from cAMP elevation. The CFTR activation through PDE4 inhibition was compared with increased pCREB formation under the same condition. Treatment of T84 cells with 1 mM Cpd-A alone caused no pCREB induction, echoing its negligible stimulation of iodide secretion. In contrast to the robust activation of iodide secretion after 2 min, 0.3 mM forskolin nonsignificantly elevated the pCREB level over the DMSO control after 10 min (1.6-fold; Fig. 6). Further cAMP elevation by combining 0.3 mM forskolin and 1 mM Cpd-A resulted in a statistically significant pCREB induction over the DMSO control (1.8-fold at 5 min, \(p < 0.02\); 3-fold at 10 min, \(p < 0.001\); Fig. 6). Thus, pCREB induction is a less sensitive marker for PKA activation in comparison with the active iodide secretion.

To confirm the increased iodide secretion from PDE4 inhibition was through PKA activation, cells were pretreated with the PKA inhibitor H89 (10 \(\mu\)M) for 10 min before Cpd-A and forskolin stimulation, with H89 concentration maintained throughout the remaining duration. Approximately 55% of the efflux induced by 1 mM Cpd-A plus 0.3 mM forskolin was blocked by H89 treatment, judging from the reduced area under the curves over the DMSO control (Fig. 7A). The H89-suppressed efflux was associated with \(\sim\)66% reduced pCREB formation (Fig. 7B).

**Stereoselective Activation of the Iodide Efflux by a PDE4D-Selective Inhibitor.** To delineate the PDE4 subtype involved, we compared the efficacy of 2-(4-fluorophenoxy)-N-[(1S)-1-(4-methoxyphenyl)ethyl]nicotinamide (Cpd-B) with its \((R)\)-isomer (Cpd-C) in augmenting the iodide efflux in the presence of 0.3 \(\mu\)M forskolin. This pair of enan-
pCREB level. Its induction by 0.3 μM forskolin was 3-fold after 10 min; the CREB levels were unchanged among all samples. Image intensities of pCREB levels over the DMSO control were plotted in the bar graph. Mean ± S.E. (n = 4). DMSO treatment had no effect on pCREB level. Its induction by 0.3 μM forskolin was 1.6-fold after 10 min; p < 0.2) and by Cpd-A were statistically insignificant. The combination of Cpd-A plus forskolin significantly elevated pCREB formation by 1.8-fold after 5 min (p < 0.02), which increased to 3-fold after 10 min (p < 0.001). These values were also statistically higher than the pCREB levels after forskolin or Cpd-A treatment alone as indicated by the p values on the graph.

tomers was discovered by scientists at Pfizer, Inc. (Marfat and Chambers, 1998). The (S)-isomer stereoselectively inhibited PDE4D with an IC_{50} of 1.4 nM under our assay conditions (Table 1). It is >140-fold more potent against PDE4D in comparison with its inhibition of PDE4A, 4B, and 4C. In addition, the (S)-isomer is an ~28-fold more potent PDE4D inhibitor than the less potent and less selective (R)-isomer. As shown in Fig. 8, significantly augmented iodide secretion over the forskolin-control was detected at 1 nM (p < 0.05; n = 4) and 5 nM (p < 0.001; n = 4) of the (S)-isomer, which are comparable with that elicited by the nonspecific Cpd-A or roflumilast within a similar concentration range. The efficacy of the (R)-isomer decreased in parallel by approximately 10- to 20-fold at augmenting the peak efflux rate. Thus, the efficacy difference of the two stereoisomers closely matched their PDE4D potency difference, supporting that PDE4D is the predominant PDE4 component in regulating the active iodide secretion in T84 cells.

**Discussion**

The present results demonstrate that PDE3, PDE4, and PDE4D inhibitors each dynamically augment the CFTR-mediated iodide secretion in T84 cells after forskolin stimulation, with their efficacy coupled to the forskolin concentration. The enhanced iodide secretion in response to increased PDE4 or PDE3 inhibition was characterized initially by a prolonged efflux duration, followed by a progressively increased efflux rate and reduced response time at higher inhibitor concentrations. The peak iodide efflux from PDE4 plus PDE3 blockade matched the maximal response from a full AC-activation, with approximately 33% of the total efflux rate attained by PDE4 inhibition and the remaining by PDE3 inhibition. The increased iodide secretion from forskolin stimulation and PDE4 inhibition was associated with an increased pCREB formation at higher activation states and dissociated from a global cAMP elevation. H89 antagonized
the forskolin-stimulated and PDE4 inhibitor-potentiated iodide secretion and pCREB formation in parallel. A possible explanation for these data is 1) the presence of a highly compartmentalized cAMP-mediated CFTR activation through PKA activation in T84 cells; and 2) PDE3 and PDE4, mainly through PDE4D, are responsible for ensuring the compartmentalized signaling by restricting cAMP diffusion through degradation.

The localized cAMP-CFTR signaling relies on the formation of a supermolecular assembly for specificity and efficiency. Previous studies have elegantly demonstrated the presence of a cAMP microdomain near its production site, with restricted diffusion access of cAMP to the bulky cytosol. PDE inhibitors reduced the compartmentalized response to various degrees (Rich et al., 2001; Jurevicius et al., 2003). A macromolecular complex including β2-adrenoceptor, PKA, and CFTR through the interaction of protein kinase A anchoring proteins has been identified after receptor stimulation (Sun et al., 2000; Naren et al., 2003). Augmentation of the adenosine-induced apical anion conductance by RS25344 and CFTR through the interaction of protein kinase A anchoring proteins and/or activated by PKA-mediated phosphorylations, which provide additional controls to ensure a localized cAMP signaling through PDE4 regulation (Barnes et al., 2005). Stimulation of the β2-adrenoceptor also rapidly recruits β-arrestins with bound PDE4s to the plasma membrane within minutes as part of the desensitization machinery to limit the spread of the cAMP pool (Baillie et al., 2003). Some PDE4s are associated with PKA via protein kinase A anchoring proteins and/or activated by PKA-mediated phosphorylations, which provide additional controls to ensure a localized cAMP signaling through PDE4 regulation (Laliberte et al., 2002; Conti et al., 2003). Despite its lower abundance in T84 cells, PDE3 inhibition by trequinsin seems to be a more effective activator of iodide efflux with a quicker response, compared with that from PDE4 inhibition by Cpd-A under an identical AC state. It is unclear whether the enhanced PDE3/CFTR coupling is due to their potential proximity or from the approximately 10-fold enhanced cAMP affinity of PDE3. Previous subfractionation study indicated a similarly abundant PDE4 over PDE3 distribution on the T84 membrane (O’Grady et al., 2002). Whether PDE3, PDE4, or PDE4D is physically associated with the CFTR regulatory complex remains to be clarified.

Airway disease from mucus accumulation, recurring bacterial colonization, and chronic inflammation is the major cause of morbidity and mortality in CF. Asthma, COPD, and chronic bronchitis are also characterized by chronic airway inflammation, mucus-congested airways, and hyperplasia of goblet cells. The shifted balance from water secretion to mucus secretion around the periciliary layer of the disease airways may compromise the mucociliary clearance process, with the resulting accumulation of mucus, bacterial, and host-response products from infiltrating neutrophils and eosinophils contributing significantly to the pathogenesis of disease states (O’Byrne and Postma, 1999). In contrast to CF, most asthma, COPD, and chronic bronchitis patients have functional CFTR channel. Agents such as PDE4 and PDE4D inhibitors that can reduce the exuberant inflammation response and simultaneously enhance mucociliary clearance through CFTR activation in airway may provide additional benefits over antiinflammatory treatment alone in their management. Cpd-A and roflumilast belong to the second generation nonselective PDE4 inhibitors, effectively suppressing the overproduction of leukotrienes and a variety of proinflammatory cytokines in vitro and in animal models (Hatzelmann and Schudt, 2001; Claveau et al., 2004). Treatment with roflumilast at 0.5 mg once daily has significantly improved airway function in asthmatic and COPD patients, with its plasma concentration reached a Cmax of 3.8 ng/ml (~9.5 nM) and the active N-oxide metabolite being several-fold higher (Reid, 2002). Since the combined exposure is higher above the minimal dose required for CFTR activation in T84 cells, it is possible that an enhanced airway mucociliary clearance from CFTR activation may have contributed to its improved airway function in clinic. Slightly increased diarrhea incidence has been noted as a treatment-related adverse event in roflumilast trials with its mechanism(s) unresolved. Whether the enhanced secretory response signi-
fied a possible CFTR activation in the gastrointestinal tract remains to be clarified. The present data clearly demonstrated that the cellular efficacy of PDE inhibitors is coupled to the AC activation state. If the flux-mediated sensitivity phenomenon is transferred in vivo, it would support the notion that PDE4 inhibitors are more effective at targeting disorders and potentiating pathways underlying higher CAMP turnover. In view of the high flux sensitivity of PDE4 inhibitors in activating CFTR identified here, one might expect that the combination of an oral PDE4 inhibitor with an inhaled β-agonist may augment their efficacy and improve the therapeutic index further from the airway-specific activation of CAMP signaling.

In summary, the CAMP-mediated CFTR activation in T84 cells is almost exclusively under PDE3 and PDE4 (mainly PDE4D) regulation. The similarly abundant PDE4 and PDE3 expression in human airway epithelia supports an analogous CAMP regulation there. In view of the increased cardiotoxic risk from the chronic administration of PDE3 inhibitors, restoring or enhancing the airway mucociliary clearance and risk from the chronic administration of PDE3 inhibitors, restoring or enhancing the airway mucociliary clearance and suppressing the inflammatory responses with the emerging PDE4 inhibitors may provide exciting new opportunities to combat cystic fibrosis and other airway diseases, including asthma, COPD, and chronic bronchitis in near future.

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