Title:

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

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Running title:  CFTR activation through PDE3 and PDE4D inhibition in T84 cells

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31 pages,  
1 table,  
9 figures,  
40 references  
246 words (Abstract) 750 words (Introduction), 942 words (Discussion).

Abbreviations:

AKAP, A-kinase anchoring protein; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; COPD, chronic obstructive pulmonary disease; Isc, short circuit current; cAMP, 3′,5′-cyclic adenosine monophosphate; Cpd-A (L-826,141), the 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; cGMP, 3′,5′-cyclic guanidine monophosphate; Cilomilast (Ariflo, SB-207499), cis-4-[cyano-4-(3-cyclopentyloxy-4-methoxyphenyl)-(R)-1-cyclohexane carboxylic acid; H89, N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide; PDE, 3′,5′-cyclic nucleotide phosphodiesterase; PDE3, type 3 3′,5′-cyclic nucleotide phosphodiesterase; PDE4, type 4 3′,5′-cyclic nucleotide phosphodiesterase; Roflumilast, 3-(cyclopropyl-methoxy)-N-(3,5-dichloropyridin-4-yl)-4-difluoromethoxy benzamide; Rolipram, 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyridinone.
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 CFTR activation may represent new therapeutics in their treatment. Herein we report the activation of CFTR-mediated chloride secretion by PDE4 inhibitors in T84 monolayer using $^{125}$I anion as tracer. In the absence of forskolin, the iodide secretion was insensitive to PDE4 inhibitor L-826,141, Roflumilast or to PDE3 inhibitor Trequinsin. However, these inhibitors potently augmented iodide secretion following 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. Paralleled with an increased phosphor-CREB formation, the CFTR activation dissociated from a global cAMP elevation and was blocked by H89. 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 AC-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.
Introduction

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 which leads to chronic 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). LTB₄ is elevated in the epithelial lining fluid of CF patients (Konstan et al., 1993). CF-tissues have abnormally high levels of pro-inflammatory arachidonic acid with values of the heterozygous parents halfway 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 classical CF, approximately 3.3% of the US 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 PP2C cluster at the apical membrane of epithelial cells to ensure the specificity of the cAMP-mediated CFTR activation (Zhu et al., 1999; Huang et al., 2001a; Sun et al., 2000). 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, TNFα, 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 (Mehats et al., 2003). A number of 2nd generation PDE4 inhibitors, exemplified by Roflumilast, 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 respiratory disorders among CF-
carriers and improve the lung function of COPD patients. This partly stems from the observation that a) levels of the residual CFTR activity appear to be predictive of the CF-disease severity; and b) cAMP-elevating agents, either through receptor stimulation, AC-activation, nonselective PDE or PDE3 inhibition, activated the wild type-CFTR and partially restored the defective chloride conductance of many CFTR mutants including ∆F508-CFTR from CF-mice and patients (Kelley et al., 1997; Haws et al., 1996; Al Nakkash and Hwang, 1999). Effects of PDE4 inhibitors on CFTR activity had been controversial. Rolipram and RP-73401 were either inactive or weakly modulated the chloride conductance of Calu3 and T84 cells under conditions where PDE3 inhibition potently increased the short-circuit current measurement (Kelley et al., 1995; Cobb et al., 2003; O'Grady et al., 2002), whereas RS25344 significantly augmented the adenosine-induced apical anion conductance in Calu3 cells (Barnes et al., 2005). By directly monitoring the chloride secretion using $^{125}$I as tracer in T84 monolayer, we report here that PDE4 inhibitors, particularly the PDE4D-selective inhibitors, potently augmented CFTR-mediated iodide secretion with their efficacy coupled to the AC activation state.
Methods

Chemicals  Buffer chemicals were from Sigma/Aldrich. [3H]-cyclic adenosine 3’, 5’-monophosphate was from Amersham. Complete\textsuperscript{TM} protease inhibitor tablet was from Boehringer Mannheim. PDE4 inhibitors, L-826,141 (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 (Claveau et al., 2004; Reid, 2002; Marfat et al., 2001). Forskolin, H89 and Trequinsin were from Calbiochem. Sodium iodide (\textsuperscript{125}I) with a typical specific activity of 2000 Ci/mmole was from Draximage Inc.

Cell culture  T84 cells (ATCC, Rockville, MD) were grown as a monolayer in a 1:1 mixture of DMEM/ F12 media supplemented with 5% FBS, 100U/ml penicillin, 100 µg/mL streptomycin. The epithelial cells (1 x 10^6 cells /well) were plated onto the 9.5 cm^2 culture dishes and incubated at 37°C in a humidified atmosphere of 5% CO\textsubscript{2} 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.

\textsuperscript{125}I efflux assay  Confluent T84 monolayer in 6-well dishes with a density of 4 x10^6 cells/well was labeled with 5 µCi/mL Na\textsuperscript{125}I in 1 mL of HPBR buffer (135 mM NaCl, 5mM KCl, 3.33 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.83 mM Na\textsubscript{2}HPO\textsubscript{4}, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM glucose and 5 mM HEPES, pH 7.2) for 30 min at 37 °C. Labeled cells were washed with 3 x 5 mL HPBR buffer within 30 s to completely remove extracellular radioactivity. Efflux of the intracellular \textsuperscript{125}I 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 3\textsuperscript{rd} buffer replacement at 1.5 min. Temperature was maintained at 37°C. NaOH (1 mL, 0.1N) was added after 9 min to lyse the cells by sonication (2 min, Branson bath sonicator, Model 2510). The radioactivity in
each time point and the final cell lysate were 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 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) per manufacture’s directions.

**PDE activity assay**

Confluent T84 cells (~ 100x10⁶) were harvested by trypsinization and washed twice using cold PBS by centrifugation. Cells were suspended in 1 mL of a buffer containing 50 mM HEPES (pH 7.5), 5 mM EDTA, 0.05 mM DTT, 1 mM BAEE, 1 mM Benzamide, 10 µg/mL Pepstatin A and “Complete” EDTA-free proteases inhibitors tablet. After sonication (5 x 10 s pulses, power setting: 3, 4 ºC), the lysate was collected after a 10,000g spin (30 min at 4 ºC). 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 Bioscience (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, 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 Kᵢ. PDE1 was purified from dog heart and other PDEs were human recombinant enzymes.

**Phosphor-CREB induction and Western Blot analysis**

Confluent T84 monolayer in 6-well plate at 37 ºC was incubated with compounds or DMSO in efflux buffer for specified time. After
the removal of incubation buffer, 300 µl of cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na₄P₂O₇, 0.1 mM vanadate, 250 µM H₂O₂ 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-PAGE under denaturing condition. Following electrophoresis, proteins were transferred to PVDF 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-phosphor-CREB (Ser-133, polyclonal, Cell Signaling Technology) in 5% BSA/TTBS overnight at 4°C. After 3 x 15 min washes with TTBS, the membranes were incubated with 1: 10000 anti-rabbit IgG horseradish peroxidase (Santa Cruz Biotechnology) in 5% skim milk/TTBS for 1 h at RT. After 3 x 15 min washes with TBS containing 0.3% Tween-20, it was developed with the ECL kit (Amersham) and digitized with LAS-1000 plus Image Reader (Fuji Photo Film Co. Ltd). For detecting CREB, the membrane was stripped using Restore™ stripping buffer (Pierce) at RT for 15 min and washed 3 x 15 min with TTBS. After blocking with 5% skim milk/TTBS for 1 h, the membrane was incubated with 1:1000 polyclonal anti-CREB (Cell Signaling Technology) and detected using the same secondary antibody as described above.

**Data analysis**

\[ \frac{125}{I} \text{efflux rate was calculated following } \left[ \ln(R_2) - \ln(R_1) \right] / (t_2-t_1), \]

where \( R_x \) is the percent radioactivity remaining in the monolayer at time \( t_x \) as previously described (Rogers et al., 1990). Data were expressed as mean ± s.e. of \( \geq 3 \) independent experiments unless otherwise specified. Dose-response curves and IC_{50} values were analyzed by a non-linear iterative regression routine using Grafit™ (Erithacus Software).
Results

*PDE3 and PDE4 are the Major cAMP-PDE 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). Earlier studies also established that its cAMP-mediated chloride secretion, sensitive to the over-expression of a mutant PKA, occurred primarily through CFTR channel (Bell and Quinton, 1992; Rogers, et al., 1990). 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 non-recombinant 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 Cpd-A (L-826,141), Roflumilast, and the PDE3 inhibitor Trequinsin. As summarized by their intrinsic potencies against the multiple recombinant cAMP-PDEs in Table 1, Cpd-A and Roflumilast are potent PDE4 inhibitors with IC₅₀ 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 Roflumilast are over 400-fold and 10,000-fold more potent than their weaker PDE3 inhibition respectively (Claveau et al., 2004). Trequinsin, with IC₅₀ value of ~ 0.05 nM against PDE3A and 3B, is over 6000-fold more potent compared to its weaker PDE4 inhibition. The inhibition of the cAMP-PDE activity of T84 lysate by Cpd-A and Roflumilast were biphasic (Fig. 1A). Each inhibited ~ 55% of the total activity 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 3,000 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 with the corresponding inflection points at ~ 0.1 nM and ~ 400 nM respectively. Thus, PDE4 and PDE3 accounted for approximately 55% and 30% of the cAMP-PDE activity, respectively, in T84 lysate under the assay conditions. Most of the remaining activity (10 to 15% of the total) was inhibited by 10 µM IBXM, a nonselective PDE inhibitor. This was thought to be contributed by PDE1 from its sensitivity toward 8-MM-IBMX (O'Grady, et al., 2002). The abundant PDE4 and PDE3 expression in T84 cells compared well with that in airway epithelial cells.

Forskolin-stimulated dynamic \(^{125}\text{I}\) efflux in T84 cells. To monitor chloride secretion in T84 cells, we selected the well-established \(^{125}\text{I}\) efflux assay for its sensitivity and robustness. Earlier head to head studies comparing \(^{125}\text{I}\) and \(^{36}\text{Cl}\) have established that \(^{125}\text{I}\) is an ideal alternative for the highly penetrating \(^{36}\text{Cl}\) in monitoring chloride secretion from T84 cells (Venglarik et al., 1990). After removing extracellular \(^{125}\text{I}\) through extensive buffer washing, the efflux of intracellular \(^{125}\text{I}\) into the incubation media was continuously monitored every 30 s by replacing only 80% of the incubation buffer with fresh one over a 9-min period. The partial buffer replacement minimized physical perturbation to the monolayer from repeated buffer exchange, thus significantly improved data reproducibility over earlier procedures. Activators were added via the efflux buffer at 1.5 min with their concentrations maintained throughout the remaining time course. Over 85% of the intracellular \(^{125}\text{I}\) was released into the culture media over the 9-min duration in response to a saturating concentration (10 µM) of forskolin stimulation (O, Fig. 2A). Over 90% of the released radioactivity co-migrated with authentic \(^{125}\text{I}\) by HPLC analysis,
indicating a negligible biotransformation of the tracer. Compared with the spontaneous efflux (▼, Fig 2B), significantly elevated $^{125}$I efflux slowly emerged after > 5.5 min incubation with 0.1 µM forskolin (Fsk, O, Fig. 2B). Higher forskolin concentrations elicited a faster and transient increase in $^{125}$I efflux in a dose-dependent manner that remained elevated at approximately 2 ~ 3 times 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 ~ 0.55 µM. 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 µM of forskolin stimulation, illustrating the rapid responsiveness of the system. The efflux rate peaked and then fell rapidly above 1 µM 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 over-expressing 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 µM of Cpd-A or 1 µM of Trequinsin, with their efflux curves overlapped with the spontaneous efflux of the cell (□, Fig. 3). Since these concentrations were over 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 µM Cpd-A plus 1 µM 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 µM of 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 Adenylyl Cyclase Activation. In contrast to the lack of efficacy under the basal AC state, PDE3 or PDE4 inhibition each potently augmented iodide secretion following forskolin stimulation. The presence of 0.1 µM forskolin, which only slightly increased the efflux after > 5.5 min incubation (O, Fig. 2B), significantly increased the efficacy of 1 µM Cpd-A by elevating the efflux rate to 0.055 min⁻¹ over the vehicle control, with the enhanced efflux emerged after ~ 3 min instead. Under the same AC state, 0.1 and 1 µM of Trequinsin augmented the peak efflux to 0.09 and 0.16 min⁻¹ respectively. Further AC activation by increasing forskolin to 0.3 µM amplified the responsiveness of the iodide secretion toward PDE4 and PDE3 inhibition further, as represented by the efflux curves of Cpd-A (Fig. 4A) and Trequinsin (Fig. 4B). 3 nM Cpd-A (▼, Fig. 4A) or 1 nM Trequinsin (▼, Fig. 4B) each significantly prolonged the iodide efflux induced by 0.3 µM of forskolin (O. Fig. 4A & 4B), 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 ~ 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 to 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.4A and 4B. At the submaximally activated AC state by 0.3 $\mu$M forskolin, the ablation of PDE4 and PDE3 activity by Cpd-A (1 $\mu$M) plus Trequinsin (1 $\mu$M) elicited a peak efflux response (O, Fig. 5) which was comparable to that from a full AC activation by 10 $\mu$M forskolin (Δ, 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. 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.**

Phosphor-CREB (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 Cpd-A (1 $\mu$M) 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 μM forskolin non-significantly elevated the pCREB level over the DMSO-control after 10 min (1.6-fold, p < 0.2, Fig. 6). Further cAMP elevation by combining 0.3 μM forskolin and 1 μM 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 μM) for 10 min prior to Cpd-A and forskolin stimulation, with H89 concentration maintained throughout the remaining duration. Approximately 55% of the efflux induced by Cpd-A (1 μM) plus forskolin (0.3 μM) 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 ~ 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 μM forskolin. This pair of enantiomers was discovered by scientists at Pfizer (Marfat et al., 2001). The (S)-isomer stereoselectively inhibited PDE4D with an IC50 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 a ~ 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 to that elicited by the nonselective 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.

**CFTR activation dissociated from global intracellular cAMP elevation.** The cAMP content of T84 cells in response to forskolin stimulation and PDE4 or PDE3 inhibition was analyzed as detailed in methods. Significantly elevated cAMP was only detected after 10 min treatment with > 1 µM forskolin (Fig. 9). In the absence of forskolin, treatment with 1 µM of Cpd-A, Roflumilast and Trequinsin separately or their combination elicited a negligible global cAMP elevation. In the presence of 0.3 µM forskolin, elevated cAMP was only detected after treatment with above 5 µM of Trequinsin (FSK*/Treq(5)) or Roflumilast (FSK*/Rof (5)), which are > 1000-fold higher than the minimal dose capable of prolonging the efflux duration. Therefore, the global cAMP elevation from either forskolin stimulation or in combination with PDE3 or PDE4 inhibition all dissociated from their more potent activation of iodide secretion. It is also less sensitive than the pCREB induction index.
Discussion

The present results demonstrate that PDE3, PDE4 and PDE4D inhibitors each dynamically augment the CFTR-mediated iodide secretion in T84 cells following 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 a) there is a highly compartmentalized cAMP-mediated CFTR activation through PKA activation in T84 cells, and b) 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. Earlier 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 AKAPs (protein kinase A anchoring protein) has been identified following receptor stimulation (Sun et al., 2000; Naren et al., 2003). Augmentation of
the adenosine-induced apical anion conductance by RS25344 supports the PDE4 proximity to the signaling complex (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 AKAPs and/or activated by PKA-mediated phosphorylations which provide additional controls to ensure a localized cAMP signaling through PDE4 regulation (Conti et al., 2003; Laliberte et al., 2002). Despite its lower abundance in T84 cells, PDE3 inhibition by Trequinsin appears to be a more effective activator of iodide efflux with a quicker response, compared to 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 potentially closer proximity or from the approximately 10-fold enhanced cAMP affinity of PDE3. Earlier sub-fractionation 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 (Claveau et al., 2004; Hatzelmann and Schudt, 2001). 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 \( C_{\text{max}} \) 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 secretary response signified a possible CFTR activation in the gastrointestinal track 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 undergoing 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 \( \beta \)-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 CFTR regulation there. In view of
the increased cardiotonic 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.

Acknowledgements.

We thank Merv Turner, Joseph Mancini, Brian Kennedy, Gary O’Neill, Dwight Macdonald,
Yves Girard, Don Nicholson and Robert Young at Merck Frosst and Professor John Hanrahan at
McGill University for many helpful discussions.
References


**Legends for Figures**

Fig. 1A. Inhibition of T84 lysate-catalyzed cAMP hydrolysis by Roflumilast (O) and Cpd-A (△). The biphasic curves have the 1st 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 2nd inflection point at ~ 3,000 nM. Roflumilast starts to inhibit the remaining activity above 5000 nM. Mean (± SE, n=3).

Fig. 1B. 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 2nd response has an inflection point near 400 nM. Mean (± SE, n=3).

Fig. 2A. Time course of forskolin-induced 125I efflux (% of total) from T84 monolayer at 37 °C (mean ± SE, n=6). Forskolin was added at 1.5 min as marked by the arrow and its concentration was maintained throughout the remaining duration. 10 µM forskolin (O), 0.3 µM forskolin (△), vehicle (DMSO) (□) overlapped with that in the absence of DMSO.

Fig. 2B. Time course of increased 125I efflux in response to increased forskolin stimulation. The efflux rate (min⁻¹) was calculated from the efflux curves in Fig. 2A as detailed in methods. The latency to reach the peak efflux rate after forskolin stimulation is defined as the response time as illustrated.

Fig. 3: PDE3, PDE4 and dual PDE3 and PDE4 inhibition elicited 125I 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 Cpd-A (10 µM) or Trequinsin (1 µM) overlapped with the spontaneous efflux of DMSO control (□, DMSO). 10 µM Cpd-A plus 1 µM Trequinsin (▲) elicited an efflux response with a comparable peak efflux.
rate but a prolonged efflux duration in comparison with that induced by 0.3 µM of forskolin (▼). (mean ± SE, n = 2 to 4).

Fig. 4A. Time course of Cpd-A elicited 125I efflux in the presence of 0.3 µM forskolin. Increased concentrations of Cpd-A and 0.3 µM of forskolin were added simultaneously at 1.5 min as marked by the arrow with their concentrations maintained throughout the remaining duration. DMSO-control (*); 0.3 µM forskolin (O); 0.3 µM forskolin plus 3 nM Cpd-A (▼); 0.3 µM forskolin plus 100 nM Cpd-A (□); 0.3 µM forskolin plus 1000 nM Cpd-A (△) (mean ± SE, n=2 to 4).

Fig. 4B. Time course of Trequinsin elicited 125I efflux in the presence of 0.3 µM forskolin. Increased concentrations of Trequinsin and 0.3 µM forskolin were added simultaneously at 1.5 min as marked by the arrow with their concentrations maintained throughout the remaining duration. DMSO control (*); 0.3 µM forskolin (O); 0.3 µM forskolin plus 1 nM Trequinsin (▼); 0.3 µM forskolin plus 10 nM Trequinsin (□); 0.3 µM forskolin plus 1000 nM Trequinsin (△). (mean ± SE, n=2 to 4).

Fig. 4C: Net peak efflux rate from PDE3 and PDE4 inhibition in the presence of 0.3 µM forskolin. The average net peak efflux rates over the DMSO-control in response to increased PDE3 and PDE4 inhibition were summarized. Cpd-A (O), Roflumilast (▼) and Trequinsin (△). Data were from cells with similar passage numbers (mean ± SE, n= 4 to 6).

Fig. 5. Chloride efflux from maximal PDE3, PDE4, and PDE3 plus PDE4 inhibition in the presence of 0.3 µM of forskolin compared with that from 10 µM of forskolin. All compounds were added at 1.5 min as marked by the arrow with their concentrations maintained throughout the remaining duration. DMSO control (□); 0.3 µM forskolin plus 1 µM Cpd-A (▼, peak efflux
~ 0.3 min\(^{-1}\), response time ~ 180 s); 0.3 µM forskolin plus 1 µM Trequinsin (*, peak efflux ~ 0.4 min\(^{-1}\), response time 150 to 180 s); 0.3 µM forskolin plus 1 µM Trequinsin and 1 µM Cpd-A (O, peak efflux ~ 0.53 min\(^{-1}\), response time ~ 120 s); 10 µM forskolin (∆, peak efflux ~ 0.52 min\(^{-1}\), response time ~ 60 s). Same batch of cells was used (mean ± SE, n=2 to 4).

Fig. 6. Phosphor-CREB induction after Cpd-A and forskolin stimulation. pCREB levels after 5 and 10 min treatment with forskolin (0.3 µM), Cpd A (1 µM) and forskolin (0.3 µM) plus Cpd-A (1 µM) were analyzed by Western blot analysis. Top panels: representing pCREB and CREB blot images after drug treatment for 5 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 ± SE, n= 4). DMSO-treatment had no effect on pCREB level. Its induction by 0.3 µM forskolin (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.

Fig. 7A. H89 inhibition of \(^{125}\)I efflux induced by Cpd-A plus forskolin. H89 (10 µM) was added 10 min prior to the efflux initiation and its concentration was maintained throughout the remaining time course. Cpd-A (1 µM) plus forskolin (0.3 µM) was added at 1.5 min simultaneously as marked by the arrow to initiate the active \(^{125}\)I secretion. 1 µM Cpd-A plus 0.3 µM forskolin (∆), 10 µM H89 plus 1 µM Cpd-A plus 0.3 µM forskolin (▽), vehicle DMSO (□). H89 suppressed the \(^{125}\)I efflux by ~ 55 % from the reduced area under the curve over the DMSO control.
Fig. 7B. The corresponding pCREB levels after 10 min drug treatment as in Fig. 7A. Forkolin (Fsk, 0.3 μM) plus Cpd-A (1 μM) increased pCREB level by 2.2 (± 0.4)-fold (p < 0.04) over the DMSO-control. Pretreatment with 10 μM H89 reduced the pCREB induction by ~ 66%. (mean ± SE, n=2).

Fig. 8. Stereoselective augmentation of the peak efflux rate in response to increased PDE4 inhibition by Cpd-B and Cpd-C. The net peak efflux rates (over DMSO-control) were determined in the presence of 0.3 μM forskolin. The PDE4D-selective (S)-isomer (Δ, Cpd-B) stereoselectively augmented the chloride efflux over the less potent and less selective (R)-isomer (O, Cpd-C) in T84 cells. (mean ± SE, n=3 to 4).

Fig. 9. Intracellular cAMP content of T84 cells after drug treatment for 10 min. The cAMP levels after forskolin treatment for 3 and 6 min were similar to those shown after 10 min treatment. FSK (x), Rof (x) and Treq (x) represent after treatment with x μM of forskolin, Roflumilast and Trequinsin respectively. FSK*/Rof (x) and FSK*/Treq (x) represent after treatment with 0.3 μM forskolin plus x μM Roflumilast or Trequinsin respectively; * P < 0.05; ** P < 0.001; (mean ± SE, n=6)
### Table 1  
**Potency and selectivity of inhibitors against cAMP-PDEs (IC$_{50}$, nM).**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Roflumilast$^a$ (L-826,141)</th>
<th>Cpd-A$^a$ (S)-isomer</th>
<th>Cpd-B (R)-isomer</th>
<th>Trequinsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td><img src="image1.png" alt="Structure" /></td>
<td><img src="image2.png" alt="Structure" /></td>
<td><img src="image3.png" alt="Structure" /></td>
<td><img src="image4.png" alt="Structure" /></td>
</tr>
<tr>
<td>PDE1</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>7,800 (1500)</td>
<td>4,200 (700)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5,300 (1000)</td>
</tr>
<tr>
<td>PDE3A</td>
<td>&gt;10,000</td>
<td>2,100 (400)</td>
<td>0.04 (0.02)</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>PDE3B</td>
<td>~10,000</td>
<td>1,100 (100)</td>
<td>0.05 (0.01)</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>PDE4A</td>
<td>0.2 (0.05)</td>
<td>1.3 (0.2)</td>
<td>360 (240)</td>
<td>315 (40)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>156 (48)</td>
</tr>
<tr>
<td>PDE4B</td>
<td>0.1 (0.02)</td>
<td>0.4 (0.3)</td>
<td>437 (490)</td>
<td>200 (50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>76 (26)</td>
</tr>
<tr>
<td>PDE4C</td>
<td>0.6 (0.1)</td>
<td>2.4 (1.8)</td>
<td>790 (630)</td>
<td>970 (140)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1160 (450)</td>
</tr>
<tr>
<td>PDE4D</td>
<td>0.1 (0.03)</td>
<td>0.3 (0.2)</td>
<td>230 (180)</td>
<td>1.4 (0.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39 (10)</td>
</tr>
<tr>
<td>PDE7A</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>9,000 (1500)</td>
<td>&gt;10,000</td>
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<td>&gt;10,000</td>
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<tr>
<td>PDE8B</td>
<td>&gt;10,000</td>
<td>7,200 (400)</td>
<td>2,100 (500)</td>
<td>&gt;10,000</td>
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<td></td>
<td></td>
<td></td>
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<td>&gt;10,000</td>
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</tbody>
</table>

IC$_{50}$ values represent mean (± SD) with n ≥ 3.  
$^a$The potency of Roflumilast and Cpd-A had been recently disclosed (Claveau et al., 2004).
Fig. 1A.

Graph showing cAMP hydrolysis (%) against Inhibitor (nM) with data points for Cpd A and Roflumilast.
Fig. 1B

Inhibitor (nM) vs. cAMP hydrolysis (%)

- Trequinsin
Fig. 2A:

![Graph showing 125I efflux (%) over time in response to different concentrations of Fsk and DMSO.]
Fig. 2B

![Efflux rate vs Time graph](image)

- **Response time**

![Graph legend](image)

- 30 uM (n=2)
- 10 uM (n=6)
- 1 uM (n=2)
- 0.3 uM (n=6)
- 0.1 uM (n=4)
- DMSO (n=6)

**Time (min)**

- 0
- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9

**Efflux rate (min⁻¹)**

- 0
- 0.1
- 0.2
- 0.3
- 0.4
- 0.5
- 0.6

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Fig. 3.
Fig. 4A

![Graph showing efflux rate over time for different concentrations of 0.3 µM Fsk + [Cpd A]. The graph compares efflux rates for 1000 nM, 100 nM, 3 nM, and 0 nM of the compound. Each concentration is represented by different symbols on the graph.]
Fig. 4B.

![Graph showing efflux rate (min⁻¹) over time (min) for different concentrations of DMSO or Treq (1000 nM). The graph includes data for 0.3 µM Fsk + [Treq] at concentrations of 1000 nM, 10 nM, 1 nM, and 0 nM.](image-url)
Fig. 4C.

![Graph showing net peak efflux rate (min⁻¹) vs. PDE inhibitor (nM)]

- △ - Trequinsin
- ▽ - Roflumilast
- ○ - Cpd A
Fig. 5.

![Graph showing efflux rate over time.](image-url)
Fig. 6

The diagram shows a Western blot analysis of pCREB and CREB levels after treatment with different compounds.

- **DMSO**: Baseline control.
- **Fsk**: Positive control with moderate fold induction.
- **Cpd A**: Shows slight fold induction compared to controls.
- **Cpd A + Fsk**: Significant fold induction compared to controls.

Significant differences in fold induction are indicated by p-values:

- **p=0.005** between **Cpd A + Fsk** and **DMSO**.
- **p=0.02** between **Fsk** and **DMSO**.
- **p=0.06** between **Cpd A** and **DMSO**.

The p-values suggest that **Cpd A + Fsk** and **Fsk** have a statistically significant effect on fold induction compared to **DMSO**, while **Cpd A** shows a trend but not as significant.
Fig. 7A.
Fig. 7B

![Bar graph showing fold induction of pCREB and CREB](image)

- **DMSO**
- **Cpd A + Fsk**
- **Cpd A + Fsk + H89**

Fold induction:
- DMSO: 1
- Cpd A + Fsk: 2
- Cpd A + Fsk + H89: 1
Fig. 8

![Graph showing net peak efflux rate (min⁻¹) against inhibitor concentration (nM). The graph compares Cpd B and Cpd C.](image-url)
Fig. 9.

![Bar graph showing cAMP (pmole/well) for different treatments.](chart.png)