Small-molecule allosteric activators of PDE4 long form cyclic AMP phosphodiesterases

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Cyclic AMP (cAMP) phosphodiesterase-4 (PDE4) enzymes degrade cAMP and underpin the compartmentalization of cAMP signaling through their targeting to particular protein complexes and intracellular locales. We describe the discovery and characterization of a small-molecule compound that allosterically activates PDE4 long isoforms. This PDE4-specific activator displays reversible, noncompetitive kinetics of activation (increased Vmax with unchanged Km), phenocopies the ability of protein kinase A (PKA) to activate PDE4 long isoforms endogenously, and requires a dimeric enzyme assembly, as adopted by long, but not by short (monomeric), PDE4 isoforms. Abnormally elevated levels of cAMP provide a critical driver of the underpinning molecular pathology of autosomal dominant polycystic kidney disease (ADPKD) by promoting cyst formation that, ultimately, culminates in renal failure. Using both animal and human cell models of ADPKD, including ADPKD patient-derived primary cell cultures, we demonstrate that treatment with the prototypical PDE4 activator compound lowers intracellular cAMP levels, restrains cAMP-mediated signaling events, and profoundly inhibits cyst formation. PDE4 activator compounds thus have potential as therapeutics for treating disease driven by elevated cAMP signaling as well as providing a tool for evaluating the action of long PDE4 isoforms in regulating cAMP-mediated cellular processes.

PDE4 | PDE4 activator | cyclic AMP | ADPKD | phosphodiesterase

Cyclic AMP (cAMP) is a universal second messenger found in cells of all biological systems. In mammalian cells, it controls critical actions as diverse as cardiac function, cell proliferation, learning, and memory, for example. Even within a cell, it can differentially control distinct processes by virtue of compartmentalization, where targeted degradation by cAMP phosphodiesterases (PDEs) forms and shapes gradients that underpin this spatiotemporal organization. Here, we describe a small-molecule allosteric activator of PDE4 long isoforms. This discovery is poised to have considerable impact for both developing therapeutics and as a probe for gaining insight into the impact of PDE4 long isoforms upon the fundamental cellular processes that cAMP controls in health and disease.


Significance

Cyclic AMP (cAMP) is a universal second messenger found in cells of all biological systems. In mammalian cells, it controls critical actions as diverse as cardiac function, cell proliferation, learning, and memory, for example. Even within a cell, it can differentially control distinct processes by virtue of compartmentalization, where targeted degradation by cAMP phosphodiesterases (PDEs) forms and shapes gradients that underpin this spatiotemporal organization. Here, we describe a small-molecule allosteric activator of PDE4 long isoforms. This discovery is poised to have considerable impact for both developing therapeutics and as a probe for gaining insight into the impact of PDE4 long isoforms upon the fundamental cellular processes that cAMP controls in health and disease.

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inactive (9, 12). These various UCR1/2 combinations confer distinct differences in the ability of PDE4 isoforms to be regulated by phosphorylation through the action of a variety of protein kinases, allowing PDE4 isoforms to serve as key nodes for integrating responses between different signaling pathways (8, 16).

In particular, UCR1-targeted phosphorylation, with concomitant activation, of PDE4 long isoforms by PKA provides a pivotal component of the cellular cAMP signaling desensitization system (17). Such functionality requires only a relatively small increase (~30–100% range) in the cAMP degrading activity of PDE4 long isoforms to exert a profound effect on diminishing cellular cAMP levels (17–19). Furthermore, MK2 [MAP kinase-activated protein kinase 2 (MAPKAPK2)], the key downstream kinase of the p38 MAP kinase inflammatory pathway, also phosphorylates PDE4 long isoforms, thereby achieving subtle inhibitory manipulation of the level of activation of PDE4 long isoforms conferred by PKA phosphorylation (18).

Interestingly, PDE4 long isoforms, but not short isoforms, show similar levels of activation in response to the binding of phosphatic acid to UCR1 (20) and the binding of a short “UCR1-related” peptide (UCR1C), which has been suggested to function by disrupting the interaction between the UCR1 and UCR2 (21). Thus, long, but not short, isoforms from all four PDE4 subfamilies have an inherent ability to be activated endogenously through a variety of routes. Such activation appears to involve the UCR1 regulatory domain and requires the enzyme to adopt a dimeric conformation (22). This particular quaternary structural status is uniquely associated with long PDE4 isoforms because two key dimerization interfaces are involved, one located within the catalytic domain and the other involving the long-specific UCR1 domain (23).

Here, we describe a drug-like compound that allosterically activates long, but not short, PDE4 isoforms. This PDE4-selective long-isoform activator demonstrably lowers intracellular cAMP levels leading to reduced PKA activity. Compounds exerting such functional effects may have utility in treating diseases where chronically elevated cAMP levels drive the molecular pathology. Indeed, using in vitro kidney cell models of one such disease, namely autosomal dominant polycystic kidney disease (ADPKD), where chronically elevated cAMP levels drive cyst formation (24–28), we show that the PDE4 activator compound MR-L2 inhibits cyst formation.

**Results**

**Small-Molecule Activators of Long-Form PDE4 Isoforms.** During the course of a chemistry program to develop PDE4 structure–function probes and PDE4-selective inhibitors, we uncovered a substituted triazole compound able to activate PDE4 long isoforms in a concentration-dependent manner. Taking this compound as a starting point, we then embarked upon a campaign to develop additional PDE4 activators as tool compounds with which to further investigate their biological effects in vitro and assess their therapeutic potential. This resulted in the generation of a series of small-molecule PDE4 activators, exemplified by MR-L2 in Fig. 1 (additional examples in SI Appendix, Fig. S1).

Four distinct genes (PDE4A4, PDE4B1, PDE4C3, and PDE4D5) encode the PDE4 family of enzymes (8–10, 12, 29). As well as being separated into four distinct gene families, catalytically active PDE4 isoforms can be segregated into long, short, and supershort isoform variants based upon the presence (or absence) of so-called UCR1 and UCR2 sequences (Fig. 2A) (9, 29). MR-L2 (Fig. 1A), similarly activates representative PDE4 long-isoform variants (PDE4A4, PDE4B1, PDE4C3, PDE4D5) from each of the four PDE4 genes (Fig. 1B and SI Appendix, Fig. S2A), albeit with a slightly enhanced magnitude of activation observed for PDE4A4. Thus, for example, the commonly expressed PDE4D5 isoform exhibited a ~60% increase in activity over basal levels at maximally effective concentrations of MR-L2. This is of a similar magnitude to the level of endogenous activation of PDE4D5 by PKA that elicits profound downregulation of cAMP (17, 29). As might be predicted, the stimulatory effects are specific to the PDE4 family, as demonstrated by the failure of MR-L2 to enhance the activity of exemplars from any of the other 10 families within the PDE superfamily (SI Appendix, Fig. S2B).

**The Activation of PDE4 Long Isoforms Mirrors Activation by PKA Phosphorylation.** Interestingly, the magnitude of PDE4 long-form activation by MR-L2 (Fig. 1B) is comparable to the degree of activation observed when PDE4 long isoforms are activated by the PKA-mediated phosphorylation of a conserved serine located within the UCR1 domain that is unique to long PDE4 isoforms and absent from short/supershort isoforms (17, 19, 30, 31) (Fig. 2A and SI Appendix, Fig. S2C). This suggested to us that the activator

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Compounds may phenocopy the PKA activation process. Indeed, such levels of activation are physiologically relevant, providing a major regulatory mechanism in terms of PKA-mediated feedback allowing desensitization to cAMP signaling in PDE4 long-isoform-expressing cells (17, 18). This feedback exerts a major impact on the magnitude and duration of elevation of cAMP levels in cells subsequent to the activation of adenylyl cyclase (16–18, 32, 33). In contrast to its ability to activate PDE4 long isoforms, MR-L2 failed to activate short PDE4 isoforms, which lack the regulatory UCR1 domain and so are insensitive to activation by PKA (Fig. 1C). Moreover, the compound failed to activate engineered PDE4 species that comprise solely a core, functional catalytic unit deprived of both the regulatory UCR1 and UCR2 domains and the C-terminal domain (Fig. 1D), again highlighting the requirement for protein domains distal to the catalytic site in pharmacological activation of PDE4 species. Furthermore, if the stimulatory effects of the activator compound phenocopy the activation process engendered by PKA phosphorylation of long PDE4 isoforms, then such actions should not be additive. It is possible to mimic the PKA phosphorylated state of PDE4 long isoforms by engineering a Ser:Asp swap in the "Arg–Arg–Glu–Ser–Phe" PKA consensus motif conserved in the UCR1 region of all PDE4 long isoforms (Fig. 2A) (19, 30). The advantage of using such a mutant species is that it ensures that the entire PDE4 long-form population under evaluation is in an activated state reflecting that achieved by PKA phosphorylation. Indeed, we observed (Fig. 2B) that the S126D-PDE4D5 mutant, which reflects a population of constitutively activated enzyme (19, 30), exhibits a near-complete ablation to pharmacological activation by challenge with MR-L2 (additional in SI Appendix, Fig. S2D).

MAPKAPK2 (MK2) also phosphorylates PDE4 long forms in UCR1, but at a different site (Ser133 of PDE4D5; Fig. 2C; ref. 32).
18) from that at which PKA acts. Instead of activation, MK2 phosphorylation serves to attenuate the level of activation elicited by PKA phosphorylation. We therefore investigated whether the activity of MR-L2 could be influenced by MK2 phosphorylation using an engineered MK2 phosphomimetic mutant (S133D-PDE4D5) that is known to mimic effectively the MK2-phosphorylated protein state (18). We noted that the S133D-PDE4D5 mutant, when examined in isolation, was activated by MR-L2 to a similar extent to wild-type PDE4D5 (Fig. 2C). Interestingly, while the PKA-phosphomimicking S126D-PDE4D5 mutant was insensitive to pharmacological activation by MR-L2 (Fig. 2B), the addition of a MK2 phosphomimetic mutation alongside the phosphomimetic PKA mutation (S126D:S133D-PDE4D5) acted to partially restore sensitivity of the PKA phosphomimetic mutant of PDE4D5 to pharmacological activation by MR-L2 (Fig. 2C). This demonstrates that additional posttranslational modification of long PDE4 isoforms can influence the pharmacological activation elicited by MR-L2, including where prior PKA mediated phosphorylation and activation of long-form PDE4 enzymes is present.

In this instance, it seems likely that MR-L2 acts to overcome the attenuating effect that MK2 phosphorylation exerts on PKA activation of PDE4 long isoforms.

The growth-promoting kinase Erk is able to phosphorylate a serine at the C-terminal end of the conserved PDE4 catalytic domain, thereby leading to diminished intracellular Ca^2+ levels and, thereby, the chronic activation of Ca^2+-inhibited adenylyl cyclase-5/6. This results in abnormally elevated cAMP levels that drive proliferative signaling and enhanced fluid transport, resulting in the formation, expansion, and swelling of multiple cystic structures within the kidney (25, 26, 41). Madin–Darby canine kidney (MDCK) cells maintain the normal integrity of epithelial cell polarity and adherens junctions, and can be triggered to form cysts (cystogenesis) upon chronic stimulation of adenylyl cyclase, providing a cellular model for cAMP-dependent cyst formation in ADPKD.

To confirm that the suppression of cAMP was due to the pharmacologically enhanced activity of long-form PDE4 enzymes, we then conducted a series of assays in the presence of the PDE4-specific inhibitor, roflumilast (100 nM). We hypothesized that inhibition of PDE4 activity, through occupancy of the catalytic site by the PDE4 inhibitor, would ablate the suppression of cAMP accumulation by inactivating the catalytic activity of the PDE4 long-form target of the allosteric activator. Indeed, we found that, in MDCK cells, the inhibition of PDE4 activity, by a saturating concentration of roflumilast (100 nM), ablated the suppressive effect of MR-L2 (3 μM), thereby confirming that the catalytic activity of PDE4 is required for the action of MR-L2 (Fig. 3C).

Interestingly, cAMP is known to be excreted from the kidney epithelia (see, e.g., ref. 43). For that reason, we investigated whether the MR-L2–mediated reduction in intracellular cAMP could be due to enhanced excretion from the MDCK cells. Thus, we assessed the extracellular cAMP levels after forskolin (3 μM) treatment of cells, with and without MR-L2 (3 μM) pretreatment. We found that, in parallel to the MR-L2–elicited suppression of intracellular cAMP levels, the extracellular level of cAMP was also significantly reduced by MR-L2 treatment (Fig. 3D). This demonstrates that cAMP excretion is not enhanced by MR-L2 treatment and that reducing intracellular cAMP levels, consequentially, leads to decreased cAMP export.
Fig. 3. MR-L2 reduces intracellular cAMP in MDCK cells without affecting PDE4 expression or cell viability. (A) MDCK cells were grown in 2D culture and treated with increasing concentrations of MR-L2. Western blot analysis of protein extracts shows the expression of short-form PDE4A and PDE4B and PDE4D, as well as long forms of PDE4C and PDE4D. Based upon their observed gel migration and predicted molecular weights, these bands are likely to represent PDE4A1, PDE4B2, PDE4C1/3, PDE4D1/2, PDE4D5/7, and PDE4D3/8/9 (in some cases, multiple isoform variants comigrate due to similar molecular weights). The expression of these isoform variants was not affected by 1-h incubation with MR-L2. β-Actin was used as a protein loading control. Quantitation of multiple Western blot analysis is presented in SI Appendix, Fig. S2E. (B) A 1-h pretreatment with MR-L2 suppresses cAMP accumulation in response to acute treatment with 3 μM forskolin (15 min). Mean of n = 7 independent experiments ±SD. Data points represent independent experimental results. (C) The suppressive effects of 1-h pretreatment with 3 μM MR-L2 on forskolin (3 μM, 15 min)-stimulated cAMP accumulation was ablated by cotreatment with the PDE4 inhibitor roliflumast (100 nM). Results show the mean of three independent experiments, and error bars represent SD. (D) Extracellular cAMP was assessed in response to MR-L2 (3 μM) incubation for 1 h before forskolin (3 μM, 15 min) challenge. Extracellular cAMP accumulation was reduced in the MR-L2 samples, indicating that the intracellular reduction in global cAMP was not due to enhanced excretion from the cell. Data from three independent experiments are shown. (E) MR-L2 treatment exhibited no deleterious effects on the proliferation of MDCK cells in 2D culture using the xCELLigence impedance-based assay system. MR-L2 was added to the culture 24 h after inception of the assay.

We confirmed that the activator compounds were not acting as cytotoxic agents in these 2D culture assays using the xCELLigence electrical-impedance–based assay system (Fig. 3E). The expected exponential growth rate was observed for untreated cells, and there was no effect of MR-L2 on cell number, demonstrating a lack of overt cytotoxicity.

The Pharmacological Activation of Long PDE4 Isoforms Suppresses MDCK Cyst Formation. Using the MDCK cell line as a model for kidney cyst formation in 3D culture (24, 42), we then interrogated whether PDE4 activity can modulate cAMP-mediated cyst expansion using the archetypal PDE4 inhibitor rolipram. We found that the inhibition of PDE4 exacerbates agonist-driven cyst formation in a concentration-dependent manner (52). In this regard, it is possible that pan-PDE4 inhibitors used clinically (7, 13, 14) may adversely exacerbate cyst formation in individuals either suffering from ADPKD or harboring mutations that may lead to the clinical manifestations of ADPKD.

Having established that modulation of PDE4 activity regulates the cAMP-mediated cystic response, we then tested whether amplifying PDE4-mediated cAMP degradation, using MR-L2, could suppress the cystic phenotype. We evaluated this in cells challenged with a fixed concentration of PGE2 (300 nM), observing a concentration-dependent suppression of cyst formation by MR-L2 (Fig. 4B and C), with an EC50 of 1 μM. Interestingly, in the cellular context, we found that the potency of MR-L2 was higher than in the biochemical PDE4D3 enzyme activation assay. Such enhanced cellular potency was seen in both the cAMP assay and the cyst formation assay. This suggests that the functional long-form PDE4 target in these cells has an enhanced sensitivity to MR-L2 compared with the ectopically expressed PDE4 long isoforms in HEK293 cells, which may be due to cell type–conferring differences in the conformation and/or posttranslational modification status of the PDE4 long isoform. Indeed, there is a large literature showing that PDE4 isoforms can show dramatically different sensitivities to selective inhibitors dependent upon cellular context (see, e.g., refs. 44–49). Such dramatic differences are thought to be due to altered conformational changes in PDE4 that may be triggered when PDE4 isoforms either interact with certain partner proteins or are subject to specific types of posttranslational modification. Examples of this are provided by, for example, the PKA-mediated phosphorylation of PDE4D3 and the interaction of human PDE4A species with various SH3 domain–containing proteins (9, 13, 22, 30, 50, 51).

To establish that the elevated catalytic activity PDE4 long isoforms elicited by MR-L2 is required to suppress cyst formation, we investigated whether cotreatment with the potent PDE4 inhibitor roliflumast (100 nM) was sufficient to ablate the functionality of MR-L2 by inhibiting the catalytic activity of its PDE4 target. We observed that treatment with roliflumast did, indeed, interdict the suppression of cystogenesis elicited by the pharmacological PDE4 activator MR-L2 (3 μM, Fig. 4D).

ATP levels have previously been connected with cyst formation and expansion in the MDCK cell model (52). We therefore set out to determine whether downstream modulation of ATP signaling, in response to long-form PDE4 activation, may contribute to the suppression of cyst formation. We assessed this by measuring the abundance of DNA-normalized ATP levels in whole-cell cystic cultures, where we observed a slight reduction (15.6 ± 30% SD) in the ATP/DNA ratio when cells were grown in the presence of MR-L2 (3 μM; Fig. 4E).

The differential expression and activity of adenylyl cyclase isoforms has been shown to occur in ADPKD (53, 54), and the AC3, AC5, and AC6 isoforms are expressed in MDCK cells (53, 54). To investigate whether the suppression of cAMP-driven cystogenesis was independent of the mode of activation of adenylyl cyclase, we evaluated a single, nonmaximally effective, concentration of MR-L2 (3 μM) against either (i) incremental increases in forskolin concentration (Fig. 5A), to drive cystogenesis by direct activation of adenylyl cyclase, or (ii) PGE2 stimulation (Fig. 5B), to evaluate cystogenesis driven by the Gi-coupled receptor–promoted activation of adenylyl cyclase. The activator, MR-L2, clearly suppressed the maximal cyst response elicited by both such direct and indirect activation of adenylyl cyclase.
The cystic fibrosis MDCK cyst formation is regulated by PDE4 and Omar et al. We further studied the action of MR-L2 in human of a selective CFTR inhibitor (CFTR

channel in cyst expansion in this model is well recognized (24, 25, 42, 58, 59). PDE4D long isoforms localize with the CFTR signaling complex and regulate channel activity by lowering local cAMP levels and, thereby, associated PKA activity (56, 57). In ADPKD, activated CFTR facilitates fluid transport into the cystic lumen (55).

MDCK cells retain expression of CFTR, and the role of this ion channel in cyst expansion in this model is well recognized (24, 25, 42, 58, 59). We investigated whether increased cAMP hydrolysis, induced by MR-L2, could suppress CFTR activation in MDCK cells using a membrane depolarization (60). We found that PGE2 treatment depolarized MDCK cell membrane potential in a concentration-dependent manner (Fig. 5C) and this was reversed by the addition of a selective CFTR inhibitor (CFTRinh172, 100 μM) (SI Appendix, Fig. S3B). Interestingly, PGE2-elicited CFTR activity was clearly suppressed in a concentration-dependent manner upon MR-L2 pretreatment (Fig. 5D). Interestingly, the dose dependency of MR-L2, for both inhibition of MDCK cystogenesis and CFTR depolarization, was near identical when employing PGE2 stimulation, at either 300 or 100 nM (Fig. 5 E and F, respectively).

PDE4 Activation Suppresses Cyst Formation in Human Cell Models of ADPKD. We further studied the action of MR-L2 in human models of ADPKD by first profiling the P4E4 isoform composition in four normal (CL5, CL8, CL11, and RFH), and four ADPKD patient-derived cell lines (SKI-001, SKI-002, OX938, and OX161) (42, 61) (SI Appendix, Fig. S3C). We found that long PDE4B1, PDE4B3, PDE4C3, PDE4D5, PDE4D7, and PDE4D9 isoform as well as short PDE4B2 and PDE4D1/ PDE4D2 isoform transcripts were expressed in these cells. Of these transcripts, we found that those for long PDE4D5 and for short PDE4D1/2 were up-regulated in ADPKD cells (SI Appendix, Fig. S3C). This is consistent with the elevated levels of cAMP driving increased gene expression by virtue of functional CRE motifs known to be located within the promoter elements of their genes (62, 63). Interestingly, we observed the expression of long PDE4C species in the MDCK cell model (Fig. 3A) and detected an elevated mRNA expression in two of the human ADPKD mRNA samples. PDE4C exhibits a very restricted expression pattern compared with other PDE4 subfamilies, but it has been connected with the regulation of a PC-2/AKAP150/ PKA localized signalosome in the primary cilia and, furthermore, the regulation of PDE4C isoforms by HNF-1 has been linked to ADPKD (64).

We selected one of these immortalized ADPKD kidney cell lines to examine the action of MR-L2 in a cellular context. The immortalized OX161 line was first isolated from a patient with ADPKD who expressed a truncated, nonfunctional form of polycystin-1 (PC1) (42). As with MDCK cells, OX161 cells are responsive to agonists of CAMP signaling, such as forskolin and PGE2, which drive the formation of small cystic structures when cells are grown in a 3D matrix (42). As with MDCK cell model, we found that, in OX161 cells, cystogenesis is enhanced upon challenge with the PDE4 inhibitor, rolipram (Fig. 6A), highlighting the fact that PDE4 activity serves to regulate cystic

Fig. 4. MDCK cyst formation is regulated by PDE4 activity and is suppressed by pharmacological activation of PDE4 long isoforms. (A) MDCK cells were grown in a 3D collagen matrix and treated with increasing concentrations of the PDE4-selective inhibitor, rolipram. Rolipram exacerbates the PGE2-induced formation of large cystic structures, demonstrating that PDE4 enzymes are localized within compartments that control cyst expansion. (B) PGE2-induced MDCK cell cyst formation was suppressed by MR-L2 (EC50 1.2 μM). Data are displayed as a percentage of PGE2 stimulation alone, and error bars represent the SEM of all cysts measured in each condition. (C) Representative images of MDCK cysts treated with increasing concentrations of MR-L2. The images illustrate a concentration-dependent suppression of cyst size. (D) Cotreatment of PGE2 (300 nM)-stimulated MDCK cystic cultures with rolipram (100 nM) and/or MR-L2 (3 μM) shows that inhibition of PDE4 catalytic activity ablates the suppressive effects of MR-L2 on cyst growth. Error bars represent the SEM. Representative images of MR-L2, and rolipram/MR-L2-treated cultures are shown. (E) DNA normalized ATP concentrations within the cystic cultures were used as a measurement ATP in the cystic cultures. The balance of ATP signaling in the cystic cultures was not perturbed by MR-L2 treatment.

Activation of PDE4 Long Isoforms Suppresses cAMP-Dependent Cystic Fibrosis Transmembrane Receptor Activation. The cystic fibrosis transmembrane receptor (CFTR) chloride channel is expressed in many tissues including kidney epithelia (55). In response to elevated cAMP, PKA phosphorylates the CFTR at multiple intracellular residues, resulting in channel activation and chloride transport (55). PDE4D long isoforms localize with the CFTR signaling complex and regulate channel activity by lowering local cAMP levels and, thereby, associated PKA activity (56, 57). In ADPKD, activated CFTR facilitates fluid transport into the cystic lumen (55).
potential of human ADPKD cells. Similar to what we observed with MDCK cells, we found that MR-L2 attenuated the PGE2-stimulated cystogenesis of OX161 cells (Fig. 6B).

**PDE4 Activation Suppresses Cyst Formation in Primary Cultures of ADPKD Patient-Derived Kidney Epithelial Cells.** We then went on to assess the functionality of MR-L2 in primary human cell cultures that were collected from the cysts of an ADPKD patient’s kidney. Such cells form fluid-filled cysts in 3D culture and are responsive to stimuli that increase cAMP, such as forskolin (SI Appendix, Fig. S4A) and PGE2 (SI Appendix, Fig. S4B). Interestingly, while immortalized culture models such as OX161 cells fail to respond to vasopressin, an important accentuator of cyst formation, these primary cells were found to be vasopressin-sensitive (SI Appendix, Fig. S4C), thus mirroring the pathological situation in vivo. It must be noted, however, that at the concentrations of AVP tested, this Gs-coupled receptor agonist elicited a lower level of stimulation than did the Gs-coupled receptor agonist PGE2. This may reflect cAMP compartmentalization such that these receptors activate distinct pools of adenylyl cyclases, showing not only different degrees of activation but also different spatial localization within the plasma membrane conferring distinct cellular functionalities and potential for being differentially regulated by distinct long-form PDE4 populations. Consistent with their origins in diseased kidney tissue, these primary cell cultures spontaneously form large cystic structures in vitro (Fig. 6C). Furthermore, MR-L2 clearly suppressed the number of cysts formed in 3D culture in both unstimulated (Fig. 6C) and vasopressin-treated (10 nM) cell cultures (Fig. 6D) without adversely affecting cell viability (Fig. 6E–G).

**Discussion**

PDE4 family isoforms are ubiquitously expressed and play a fundamental role in determining the spatial compartmentalization of cAMP signaling in cells. They also act as nodes for conferring cross talk via inputs from various other signaling pathways through multisite phosphorylation (8, 10, 12, 13, 29). Furthermore, long PDE4 isoforms play a key role in determining cellular desensitization to cAMP signaling through a feedback loop involving their PKA-mediated phosphorylation and activation (17, 18, 65). This process is restricted to long PDE4 isoforms, as PKA phosphorylates a serine residue located within UCR1 (a regulatory module conserved across long isoforms from all PDE4 subfamilies) but that is absent from PDE4 short and super-short forms and is not found in any other PDE family (19, 30, 31).

The adjacent UCR2 region, although found in all PDE4 isoforms (whether long or short), exerts an autoinhibitory action that is specific to the long forms and serves to diminish, but does not fully abolish, their catalytic activity (37). The mechanistic basis for this arises from the interaction of UCR2 with UCR1. Thus, together, UCR2 and UCR1 interact to form a combined regulatory module (66) that docks over the catalytic pocket (38). This UCR1/2 module imposes a constraint on long-form PDE4 catalytic activity that is ablated by PKA phosphorylation of UCR1, leading to an increase in activity of, typically, some 40–80% (19, 22, 29, 37, 38). Given the high catalytic turnover of PDE4, this degree of activation has the ability to generate a profound decrease in cAMP levels in cells, thereby regulating cAMP-responsive processes (17–19, 65).

Here, we describe the discovery of a long-form PDE4-activating ligand, namely, the N-substituted-2-(3-aryl-1H-1,2,4-triazol-1-yl)acetamide chemotype of MR-L2 (Fig. 1A). It acts to...
phenoctype the stimulatory effect exerted by PKA phosphorylation on dimeric PDE4 long isoforms (19, 31, 35, 37). Thus, we have shown that the stimulatory effect of MR-L2 (i) is restricted to PDE4 family enzymes and specific to long-form variants; (ii) that it is not additive with the stimulatory effect of PKA on long isoforms; (iii) that it requires the dimeric state that is adopted by long, but not short, PDE4 isoforms; (iv) that it is operative on PDE4 long isoforms showing attenuated activity due to phosphorylation by Erk MAP kinase; and (v) that it elicits comparable levels of activation to those caused by PKA-mediated phosphorylation of PDE4 long isoforms.

The action of MR-L2 is reversible and exhibits kinetics of non-competitive activation. These data are consistent with the compounds binding to an allosteric site on the enzyme. We propose that, as has been suggested for PKA phosphorylation-mediated activation, this drives an equilibrium shift away from a UCR1/UCR2-capped autoinhibited conformational state toward dis-inhibited conformational state(s) (29, 37, 38). It is known that UCR1 engages with UCR2 directly to form a tandem regulatory module (66). Insight into a mechanism whereby the UCR1/UCR2 module might regulate catalytic activity has been gauged from crystal structures showing that a helix within UCR2 can fold across the entrance to the catalytic pocket to gate substrate entry (67). Although complete structural characterization of full-length PDE4 species remains elusive, further advances with crystallographic studies (40) have now unambiguously confirmed hypotheses (40, 68) that UCR2 gating of the catalytic pocket involves cross-capping within the dimeric long-form PDE4 structures. Thus, regulatory control of long-form PDE4 activity requires the enzyme to be in a dimeric configuration with alterations in activity likely mediated by modulation of the equilibrium position between the UCR2-capped and -uncapped states, thereby exerting $V_{\text{max}}$ kinetic control over cAMP hydrolysis. Presumably, such a transition leading to uncapping and increased catalytic activity can be triggered either natively, when PKA phosphorylates UCR1, or pharmacologically, when a small-molecule activator binds to its allosteric site.

As with PKA phosphorylation (17–19, 30), we show that the prototypical allosteric activator, MR-L2, can decrease intracellular cAMP levels in cells where long PDE4 isoforms are expressed. Moreover, it is expected that the observed reduction in global cAMP concentration will not be homogenously distributed throughout the cell, and that effects may be more pronounced at key nanodomain signaling complexes. At such sites in intact cells, the protein complexation and/or posttranslational modification status of target PDE4 species may give rise to altered sensitivity to activator compounds relative to that seen in our biochemical enzyme assay based on cell lysates. Such compounds might have therapeutic potential in diseases where chronic cAMP elevation provides the underpinning molecular pathology. One such disease is ADPKD (21, 26, 41, 69). This is a genetically defined disease that is underpinned by mutations in PKD1 or PKD2, leading to loss or diminution of their function. The consequences of this are lowered intracellular Ca$^{2+}$ levels that lead to inhibition of adenyl cyclases-5/6 and thence to elevation of cAMP. Such chronically elevated cAMP levels drive the formation and swelling of kidney cysts that, eventually, lead to kidney failure. The variety of PDE4 long isoforms that kidney epithelial cells express (70, 71) (Fig. 3D and SI Appendix, Fig. S3C) provide targets for activator compounds such as MR-L2, suggesting a potential therapeutic strategy. Indeed, we show here that not only does MR-L2 decrease intracellular cAMP but that it also attenuates CFTR activity, which is stimulated by cAMP-driven activation of PKA, and cyst formation in the model MDCK cell system. MR-L2 was also successful in suppressing cyst expansion in an immortalized kidney cell line, OX161, derived from an ADPKD patient, and we further show that the compound suppresses the formation of cysts in 3D cultures of primary human kidney epithelial cells isolated from an ADPKD patient.

**Fig. 6.** MR-L2 inhibits cyst formation in human models of ADPKD. (A) Treatment with the PDE4-selective inhibitor, rolipram, exacerbates the expansion of cysts formed by the OX161 cell line when grown in 3D culture. Data are expressed as a percentage of vehicle [0.1% (vol/vol) DMSO] control treatment and displayed as mean ± SEM of all cysts measured in each condition. (B) MR-L2 suppresses the formation of PGE2-stimulated OX161 cell cysts in a concentration-dependent manner. Mean cyst size is shown as a percentage of the PGE2-alone treatment group, where MR-L2 reduces mean cyst size to below unstimulated [0.1% (vol/vol) DMSO] levels. (C) Primary human cells from a patient with ADPKD were cultured from single kidney cysts. MR-L2 suppresses the spontaneous cyst formation of diseased primary human kidney epithelial cells. Cyst number decreases in response to MR-L2 treatment. Error bars represent the SD of four treatments. (D) MR-L2 suppresses the vasopressin-exacerbated, ADPKD-driven, cyst formation of diseased primary human kidney epithelial cells. Cyst number decreases in response to MR-L2 treatment. Error bars represent the SD of four treatments. (E and F) Primary human ADPKD cell viability was not adversely affected by MR-L2 treatment, as assessed using a luminescence-based detection of ATP as a function of living cells. Data are displayed in relative luminescence units, with error bars representing the SD of four treatments. (G) Composite confocal microscopy of primary human cystic cultures under treatment with 10 nM vasopressin and concentrations of MR-L2. Cystic structures are seen as spherical, lumen-forming, cellular outgrowths. Epithelial tubules and supporting cellular matrices are also present within the cultures but are not overtly affected by the presence of MR-L2 (additional imaging available in SI Appendix, Fig. S4).
In summary, MR-L2 pharmacologically activates dimeric PDE4 long-form variants through a mechanism that phenocopies both the magnitude and mechanism of PKA-mediated PDE4 long-isofrom activation. This provides a route to evaluate the consequences of specifically activating this group of PDE4 isoforms. Moreover, the discovery and initial characterization of this drug-like PDE4 activator, together with the demonstration that it can inhibit the in vitro formation of kidney cysts that characterize ADPKD, suggests that direct pharmacological activation of PDE4 long forms may have therapeutic utility in diseases driven by chronically up-regulated cAMP levels.

Materials and Methods

Chemicals and Reagents. Unless otherwise specified, reagents were sourced from Sigma-Aldrich. MR-L2 was synthesized as described in patent WO2016151300.

Cell Culture. HEK293 and MDCK (ATCC) cells were cultured in DMEM (Lonza) supplemented with 10% (vol/vol) FBS (Invitrogen). Human primary epithelial cells were sourced from DiscoveryBioMed and cultured in proprietary media supplied by DBM. Transfection into mammalian cells was conducted using the Lipofectamine 3000 reagent (Invitrogen) as per the manufacturer’s instruction.

Molecular Biology. pcDNA3.1 (Invitrogen) was used as the standard vector for mammalian expression, and all site-directed mutagenesis was conducted using the QuickChange mutagenesis kit (Agilent Technologies).

cAMP Phosphodiesterase Assay. Cells were collected in KHEM buffer [50 mM KCl, 10 mM EGTA, 50 mM Hepes (pH 7.2), 1.92 mM MgCl₂, pH 7.4 with KOH] and lysed by mechanical disruption. Lysate was preclarified by centrifugation at 2,000 × g for 10 min before centrifugation at 100,000 × g for 30 min. The protein concentration of the supernatant was determined by BCA assay (Sigma). The cAMP PDE assays were then conducted as previously described, using protein concentrations (typically 200 ng to 1 μg per reaction) and incubation times (10 min) that yielded linear rates of reaction as described before us by (72).

Western Blot. Cells were lysed for 20 min in a whole-cell lysis buffer [1% (vol/vol) Triton X-100, 25 mM Hepes, 2.5 mM EDTA, 150 mM NaCl, 50 mM NaF, and 30 mM NaPi] containing a protease inhibitor mixture (Roche). Insoluble material was removed by centrifugation at 14,000 × g for 10 min. Concentration was measured by standard BCA assay (Sigma) before the addition of SDS sample buffer (Invitrogen). The antisera raised against PDE4 have previously been described.

cAMP ELISA. Assays were conducted using a cAMP ELISA kit (Enzo) following the manufacturer’s instructions and using pre-prepared reagents supplied by the manufacturer.


42. E. Parker et al., Inhibitors of phosphodiesterase IV (PDE IV) allosteric modulators for the regulation of CFTR function and inhibition. J. Biol. Chem. 289, 3531–3537 (2014).


