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Characterization of the Cellular Activity of PDE 4 Inhibitors Using Two Novel PDE 4 Reporter Cell Lines

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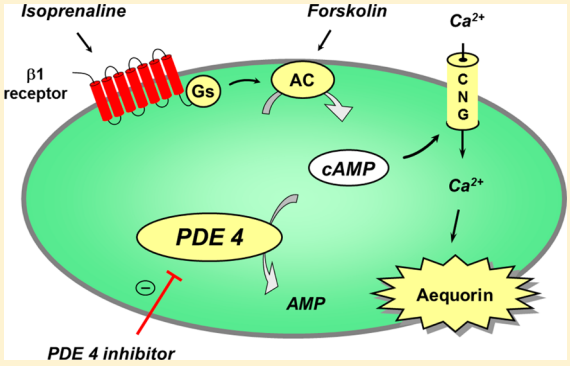
ABSTRACT: We report here the generation and pharmacological characterization of two novel PDE 4B1 and PDE 4D3 reporter cell lines. Intracellular cAMP levels are monitored in these cells by a cAMP-sensitive biosensor. We used the recombinant PDE 4B1 and PDE 4D3 reporter cell lines to characterize the cellular effects of various competitive and allosteric PDE 4 inhibitors. In addition, we compared the cellular activity of these PDE 4 inhibitors with the *in vitro* inhibition of full-length PDE 4D3 and a truncated enzyme comprising the PDE 4D3 catalytic domain. Two different groups of PDE 4 inhibitors could be identified. The first group, including competitive inhibitors like roflumilast, cilomilast and piclamilast, shows similar *in vitro* activity on full-length and truncated PDE 4D3 and comparably low cellular activity. The second group, including the allosteric inhibitors PMNPQ, D159153, and D159404, shows much better inhibition of full-length versus truncated PDE 4D3. In addition, these compounds show high cellular activity. Our data obtained with the prototype PDE 4 inhibitor rolipram show that rolipram has properties intermediate between the two groups. The results imply that these novel PDE 4 reporter cell lines are well-suited for the characterization of the cellular activity of PDE 4 inhibitors and may also support a better understanding of the complex PDE 4 pharmacology.



KEYWORDS: *PDE 4, cAMP, CNG channel, reporter assay*

INTRODUCTION

Cyclic nucleotide-specific phosphodiesterases (PDEs) play an essential role in cellular signal transduction by regulating the intracellular levels of 3′,5′-cyclic adenosine monophosphate (cAMP) and 3′,5′-cyclic guanosine monophosphate (cGMP) and, therefore, are important pharmacological targets. To date, 21 mammalian PDE genes have been identified and are subgrouped into 11 isozyme families based on their substrate specificity, regulation, and pharmacology.[1]

Among the most targeted PDEs are the members of the cAMP-specific PDE 4 isozyme family (PDE 4A-D). A role for PDE 4 isozymes in the pathophysiology of asthma, COPD, as well as additional inflammatory and neurological diseases has been proposed.[2,3] Therefore, members of the PDE 4 isozyme family have gained considerable attention as targets for new drug development. As the result of continuous medicinal chemistry efforts, a growing number of potent and selective PDE 4 inhibitors have been identified in recent years. Following the characterization of the prototype PDE 4-selective inhibitor rolipram, additional PDE 4 inhibitors with higher potency were identified. These include second and third generation inhibitors like roflumilast, cilomilast, oglemilast, and piclamilast.[4−6] In addition, novel PDE 4 inhibitors which are claimed to inhibit PDE 4 isozymes allosterically, have also been described.[7,8] However, the development of novel PDE 4 inhibitors is largely hampered by dose-limiting side effects, and the only marketed PDE 4 inhibitor is roflumilast.[3,9−11]

Classically, biochemical PDE assays were used for the characterization of PDE pharmacology and for drug discovery. Only few reports on cell-based assays for the characterization of the cellular activity of PDE inhibitors, which are suitable for high-throughput screening (HTS), are available. Recently, reporter gene assays and fluorescence-based assays using biosensors to detect intracellular cAMP levels and PDE activity have been introduced.[12−16]

We have shown previously that the olfactory CNGA2 channel is well-suited as a biosensor for the detection of intracellular cGMP levels. In addition, we described two cellular PDE reporter assays which can be used for the characterization of the cellular activity of PDE 2A and PDE 9A inhibitors.[17,18]

We report here the generation and pharmacological characterization of two novel PDE 4 reporter cell lines. In these PDE 4B1 and PDE 4D3 reporter cell lines, cAMP levels can be monitored in real-time using a heteromultimeric CNG channel as a cAMP biosensor. The CNG channel shows high sensitivity for cAMP ($k_{1/2}$ < 3 μM) and reduced cGMP sensitivity ($k_{1/2}$ ∼ 50 μM).[19,33,34] We used the recombinant PDE 4B1 and PDE 4D3 reporter cell lines to characterize the cellular effects of various competitive and allosteric PDE 4

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Table 1. Primers and Fluorescent Probes[a]

PDE 1A:	forward primer: 5′-TGTCAGCTGTGGACCTGAAG-3′
	probe: 5′-(FAM)TTTCAAAAACAACCTGGTGGACATCA(TAMRA)-3′
	reverse primer: 5′-CCACCTCTCTTTGTTCTGCTG-3′
PDE 1B:	forward primer: 5′-GAATGTGCCATCCTGTACAAC-3′
	probe: 5′-(FAM)ATCGCTCGGTGCTGGAGAATCAC(TAMRA)-3′
	reverse primer: 5′-CCTGCATCATTCGAAAAACA-3′
PDE 1C:	forward primer: 5′-CAAAGCCAAGAAGGAAGCTG-3′
	probe: 5′-(FAM)CCTGGCTGCTGAGGAAAAGCAAAAG(TAMRA)-3′
	reverse primer: 5′-GGTCTTGGGCTTCCATTTC-3′
PDE 2A:	forward primer: 5′-ACAGGCCAGATCCTGAACAT-3′
	probe: 5′-(FAM)CATACGCCCATCCGCTTTTCTATCG(TAMRA)-3′
	reverse primer: 5′-GAAGCCTGTGCTGTCGTCTA-3′
PDE 3A:	forward primer: 5′-CTCCTGCACAGCACTCCTC-3′
	probe: 5′-(FAM)CAAAGAGGAAGAGGAAGAGAAGGGGA(TAMRA)-3′
	reverse primer: 5′-GGGTCTCCTCTGCTCTTGG-3′
PDE 3B:	forward primer: 5′-GGCGGATATTTTGTCAGCTA-3′
	probe: 5′-(FAM)TGCACCATCTCACTGAAAACCACAA(TAMRA)-3′
	reverse primer: 5′-TCTTCTTCTATGATTTCCTTCCA-3′
PDE 4A:	forward primer: 5′-AGCAAATGGGGCCTGAATA-3′
	probe: 5′-(FAM)TTTGTGTGTCGGAGTACGCTGGAGG(TAMRA)-3′
	reverse primer: 5′-ATGATGCAGCTGAGTGAACG-3′
PDE 4B:	forward primer: 5′-TCCATCCACTGTGGGAGAC-3′
	probe: 5′-(FAM)CCTGGTTCAGCCTGATGCTCAAGAC(TAMRA)-3′
	reverse primer: 5′-CTGGGGTATCATGCTCTGGT-3′
PDE 4C:	forward primer: 5′-CAGGAGCTGTTGGACACCTT-3′
	probe: 5′-(FAM)TCAGAGTAGGATACCCTGCAGCCCC(TAMRA)-3′
	reverse primer: 5′-GTGTTGACCCATGGTGTGG-3′
PDE 4D:	forward primer: 5′-TGTGGACAATGGTACATCAGC-3′
	probe: 5′-(FAM)ACGGAGTCCCTTGGATCCCATGAC(TAMRA)-3′
	reverse primer: 5′-TGTGGACAAAGTTTGCTTGG-3′
PDE 5A:	forward primer: 5′-CCTGACCCATGTTTCAGAGG-3′
	probe: 5′-(FAM)CCTTGCTGGATGGCTGCAAGAAGA(TAMRA)-3′
	reverse primer: 5′-GGCTTGCCATTTCTGTCTGT-3′
PDE 6A:	forward primer: 5′-GCTGGATGGGATAACCAACA-3′
	probe: 5′-(FAM)AAGGAATGGAAGGCACTGGCTGATG(TAMRA)-3′
	reverse primer: 5′-GCCTTCATCTTGGCCTCATA-3′
PDE 6B:	forward primer: 5′-AGATCCTGCCCATGTTTGAC-3′
	probe: 5′-(FAM)CAGAAAGGAGTGGAAAGCGCTAGCTG(TAMRA)-3′
	reverse primer: 5′-GCCTTGACTTTGGCCTCATA-3′
PDE 6C:	forward primer: 5′-AGAGAAATCACCCCCATGCT-3′
	probe: 5′-(FAM)CGGCCTTCAGAATAACAGAGTGCAA(TAMRA)-3′
	reverse primer: 5′-TCACCTTCGCCTCATACTCA-3′
PDE 7A:	forward primer: 5′-GACAATGCTTGGACATGTGG-3′
	probe: 5′-(FAM)ATAAAGCGAGCTGGAAGGGACTGCA(TAMRA)-3′
	reverse primer: 5′-CTCACTGCTGGGCTGTTGT-3′
PDE 7B:	forward primer: 5′-ACTGATTCCCATGCCAAAAT-3′
	probe: 5′-(FAM)TGGGATTGCTACCTGTATAAACAATGGC(TAMRA)-3′
	reverse primer: 5′-TTAAAGTTAAGGCCTTTATCTGC-3′
PDE 8A:	forward primer: 5′-CCTGCATTTGAAACAACGAT-3′
	probe: 5′-(FAM)TAGGGAAGGAGTTAGCACAAGTGCCC(TAMRA)-3′
	reverse primer: 5′-GCAAGTCCCCTTTCTTTTCA-3′
PDE 8B:	forward primer: 5′-AGGGGCTACCTGTTGTGATG-3′
	probe: 5′-(FAM)TTCGACCGTAATACCTGCAGCATCC(TAMRA)-3′
	reverse primer: 5′-GAAGGAGATCTGGGACTTGG-3′
PDE 9A:	forward primer: 5′-TTGACATCTGGGGGCACT-3′
	probe: 5′-(FAM)TGCCACAGAGAAGAACAGAGATGTGAA(TAMRA)-3′
	reverse primer: 5′-GAGGAGAATGGCCTTCACTG-3′
PDE 10A:	forward primer: 5′-TCTCAACCAGTGGGAGAAGG-3′
	probe: 5′-(FAM)ATTCGCGGGGAGGAGACTGCG(TAMRA)-3′
	reverse primer: 5′-CCTTTGCCAGAAATCCACTG-3′
PDE 11A:	forward primer: 5′-GTGAAGGTCAACGCCAAACT-3′
	probe: 5′-(FAM)CCCATGCTAGATTTAGTGGCTGCCA(TAMRA)-3′

Table 1. continued

	reverse primer: 5′-AACTCCTCCCACTTCCTTCG-3′
hPDE 4B1:	forward primer: 5′-TGATACCTCAAAGTCCCTCACC-3′
	probe: 5′-(FAM)CCACTGGACGAGCAGAACAGGG(TAMRA)-3′
	reverse primer: 5′-CCATCAGACCCTGGCAGT-3′
hPDE 4D3:	forward primer: 5′-TGCAGAACATGGTGCACTG-3′
	probe: 5′-(FAM)CTGACCTGTCCAACCCCACCAAGC(TAMRA)-3′
	reverse primer: 5′-GGTCCACTGACGGTACAGC-3′
hADRB1:	forward primer: 5′-TTCTGCGAGCTGTGGACCT-3′
	probe: 5′-(FAM)TGGACGTGCTGTGCGTGACGG(TAMRA)-3′
	reverse primer: 5′-ACACACAGGGTCTCGATGCTG-3′
L32:	forward primer: 5′-CTGGCGGAAACCCAGAG-3′
	probe: 5′-(FAM)AGATTCAAGGGCCAGATCCTGATGC(TAMRA)-3′
	reverse primer: 5′-TGCTTGGTTTTCTTGTTGCTC-3′

[a]FAM, 5-carboxyfluorescein; TAMRA, 5-carboxytetramethylrhodamine.

inhibitors. In addition, we compared the cellular activity of these PDE 4 inhibitors with the *in vitro* inhibition of PDE 4D3 full-length enzyme and its catalytic domain.

MATERIALS AND METHODS

Quantitative Real-Time RT-PCR Analysis. Quantitative TaqMan analysis was performed using the Applied Biosystems PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA). CHO cell mRNAs were digested with DNase I and were reverse transcribed using random hexamers. Selective probes were carefully designed and comparable probe efficiencies were assured by titration of genomic hamster DNA. Normalization was performed using the housekeeping gene *Rpl32* (ribosomal protein L32) as a control. The resulting expression is given in arbitrary units as mean values ± SEM (standard error of the mean, n = 3). The primers and fluorescent probes used are shown in Table 1.

Generation of Stable PDE 4B1 and PDE 4D3 Reporter Cell Lines. A stable cAMP reporter cell line was generated as described previously. Briefly, a CHO cell line, stably expressing cytosolic apoaequorin, and a cAMP biosensor CNG channel, was generated by zeocin (0.25 mg/mL) and hygromycin (0.6 mg/mL) selection.[19] The cAMP reporter cell line was stably cotransfected with plasmid constructs encoding human PDE 4B1 (acc. no. NM_002600) or PDE 4D3 (acc. no. NM_006203) and a β1-adrenoceptor (acc. no. NM_000684) plasmid construct. Stable PDE 4 clones were identified by 100 nM isoprenaline stimulation in the absence or presence of 100 nM roflumilast (data not shown). In addition, a β1-adrenoceptor reporter cell line was generated by transfection with the β1-adrenoceptor plasmid construct only. Stable clones were identified by isoprenaline stimulation (data not shown). Positive clones were purified once by the limited dilution technique.

Cell Culture Conditions and Aequorin Luminescence Measurements. Cells were cultured at 37 °C and 5% CO_2 in D-MEM/F12, supplemented with 10% (v/v) inactivated fetal calf serum, 4 mM GlutaMAX, 20 mM HEPES, 1.4 mM sodium pyruvate, 2 mM sodium bicarbonate, 50 units/mL penicillin, and 50 μg/mL streptomycin. In addition, 1 mg/mL geneticin was added to the cell culture medium used for the PDE 4 and the β1-adrenoceptor reporter cell lines.

Luminescence measurements were performed on opaque 384-well microtiter plates (MTPs). Either 2000 cells/well or 1500 cells/well were plated and were cultured for 24 or 48 h, respectively. After removal of the cell culture medium, cells were loaded for 3 h with 2.5 μg/mL coelenterazine in Ca^{2+}-free Tyrode (130 mM NaCl, 5 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$, 4.8 mM $NaHCO_3$ at pH 7.4) at 37 °C and 5% CO_2. Isoprenaline and PDE inhibitors were added for 10 min in Ca^{2+}-free Tyrode containing 0.1% BSA. Immediately before adding calcium ions (final concentration 3 mM), the measurement of the aequorin luminescence was started by using a charge-coupled device (CCD) camera (Hamamatsu Corp., Shizuoka, Japan) in a light tight box. Luminescence was monitored continuously for 60 s.

Biochemical Inhibitor Studies. Full length human PDE 4D3 (acc. no. NP_006194.2) and PDE 4D3 catalytic domain (amino acids 223−613) were recombinantly expressed in HEK293-6E or Sf9 cells. Cell extracts were prepared by sonication and removal of cell debris by centrifugation. Enzyme inhibition studies were carried out using the commercially available ^{3}H-cAMP Scintillation Proximity Assay (SPA, Perkin-Elmer) system as described previously.[17]

Compounds. Roflumilast, roflumilast *N*-oxide, piclamilast, oglemilast, D159153, D159404, PMNPQ, and lirimilast were synthesized by the medicinal chemistry department of Bayer Pharma AG. The PDE 2A inhibitor BAY 60-7550 was purchased from Axxora Life Sciences Inc. (San Diego, CA). Cilomilast and zardaverine were purchased from AxonMedChem (Groningen, Netherlands). YM976 and BRL 50481 were obtained from Tocris (Bristol, UK). Tadalafil was purified from commercially available tablets. Dipyridamole, etazolate, milrinone, rolipram, vinpocetine, 8-methoxymethyl-IBMX, and the PDE 9A inhibitor BAY 73-6991 were obtained from Sigma-Aldrich (Taufkirchen, Germany). Compounds were diluted from 10 mM DMSO stock solutions.

Statistics. The data are presented as mean values with standard deviation errors. The GraphPad Prism software (Version 5.03, GraphPad Software Inc., San Diego, CA) was used for curve-fitting and calculation of the half-maximal effective and inhibitory concentrations (EC_{50} and IC_{50}, respectively). pEC_{50} and pIC_{50} values are given as means ± SEM (n = 3−10).

RESULTS

Generation of the PDE 4B1, PDE 4D3, and β1-Adrenoceptor Reporter Cell Lines. A cAMP reporter cell line, recombinantly expressing apoaequorin and a cAMP-sensitive CNG channel, was generated as described previously.[19] The cAMP reporter cell line was stably cotransfected with plasmid constructs encoding human PDE 4B1 or PDE

4D3 and a β1-adrenoceptor encoding plasmid construct. Stable PDE 4B1 and PDE 4D3 clones were identified by 100 nM isoprenaline stimulation in the absence or presence of 100 nM roflumilast. Cell clones which showed increased luminescence signals in the presence of the PDE 4 inhibitor were expanded and further analyzed. In addition, a β1-adrenoceptor reporter cell line was generated by transfection with the β1-adrenoceptor construct only. Stable clones were identified by isoprenaline stimulation. Positive clones were purified once by the limited dilution technique, and three clonal cell lines, referred to here as the PDE 4B1, PDE 4D3, and β1-adrenoceptor reporter cell lines, were used for further experiments. In our PDE 4 reporter cell lines, activation of the β1-adrenoceptor results in cAMP synthesis and concomitant cAMP hydrolysis by PDE 4. PDE 4 inhibitors prevent cAMP degradation, which results in the opening of the CNG channel and enhanced aequorin luminescence in the presence of extracellular calcium ions.

Analysis of PDE Expression in CHO Cells. To characterize the expression of endogenous and recombinant PDEs in our CHO reporter cell lines, we analyzed the expression levels by quantitative RT-PCR. As shown in Figure 1A, we were able to

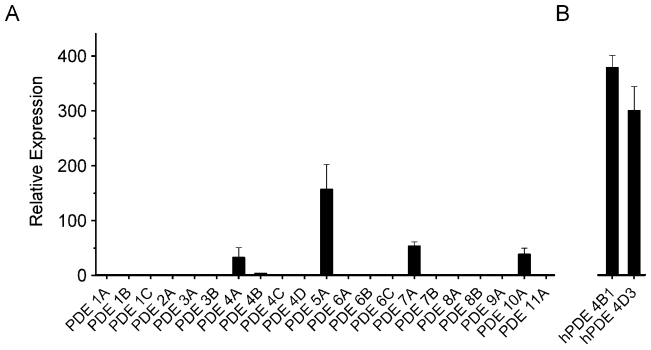

Figure 1. Characterization of PDE expression levels. Quantitative real-time RT-PCR analysis was performed to measure (A) endogenous PDE or (B) recombinant hPDE 4B1 and 4D3 expression levels using specific oligonucleotide probes. Expression levels were normalized to ribosomal protein L32. Relative expression is given in arbitrary units as means with SEM (n = 3).

detect very low expression levels of endogenous PDE 1A-C, 2A, 3A, 3B, 4B-D, 6A-C, 7B, 8A, 8B, 9A, and 11A in the parental CHO, the PDE 4B1, and PDE 4D3 cell lines. Low expression was detected for PDE 4A, 7A and 10A, and significant expression was found for the cGMP-specific PDE 5A. As expected, we could verify very high expression of human PDE 4B1 and PDE 4D3 in our reporter cell lines (Figure 1B). The β1-adrenoceptor expression levels were found to be similar to PDE 4B1 levels in the PDE 4B1 reporter cell line and ~14-fold higher in the PDE 4D3 reporter cell line (data not shown).

Characterization of the β1-Adrenoceptor Reporter Cell Line. As shown in Figure 2, isoprenaline stimulated concentration-dependent luminescence signals on the β1-adrenoceptor reporter cell line with an EC_{50} value of 0.23 ± 0.01 nM. Isoprenaline-mediated luminescence signals were not significantly affected by the presence of increasing concentrations of roflumilast (10−1000 nM, Figure 2A). In the absence of isoprenaline, roflumilast did not stimulate significant luminescence signals (Figure 2B). In addition, application of 1−100 nM isoprenaline stimulated maximal luminescence signals, which were not affected by increasing roflumilast

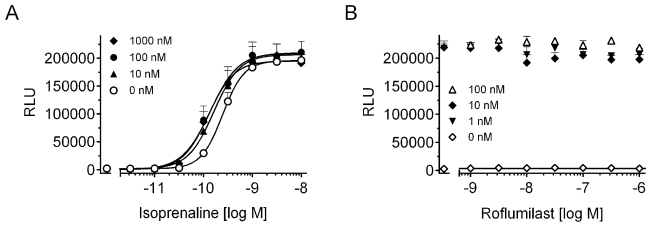

Figure 2. Effects of isoprenaline and roflumilast on the β1-adrenoceptor reporter cell line. (A) Stimulation with isoprenaline in the absence (○) or presence of 10 nM (▲), 100 nM (●), and 1000 nM (◆) roflumilast. (B) Treatment of the reporter cell line with roflumilast in the absence (◇) or presence of 1 nM (▼), 10 nM (◆), and 100 nM (△) isoprenaline. Isoprenaline and roflumilast were added for 10 min in Ca^{2+}-free Tyrode, and luminescence measurements were started immediately before the addition of Ca^{2+} (final concentration 3 mM). Results are expressed as relative light units (RLU), and data are presented as means with SD.

concentrations. Similar results were obtained with other PDE 4 inhibitors (data not shown).

Characterization of the PDE 4B1 and PDE 4D3 Reporter Cell Lines. We characterized our newly established PDE 4B1 and PDE 4D3 reporter cell lines by isoprenaline stimulation in the absence or presence of the selective PDE 4 inhibitor roflumilast. In the absence of roflumilast, isoprenaline was not able to stimulate significant luminescence signals (Figure 3). In contrast, in the presence of the PDE 4 inhibitor,

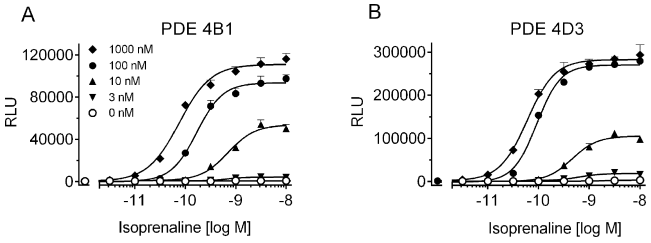

Figure 3. Effects of the PDE 4 inhibitor roflumilast on isoprenaline-mediated luminescence signals. Stimulation of (A) the PDE 4B1 and (B) the PDE 4D3 reporter cell lines with isoprenaline. Luminescence signals mediated by isoprenaline in the absence (○) or presence of 3 nM (▼), 10 nM (▲), 100 nM (●), and 1000 nM (◆) roflumilast. Isoprenaline and roflumilast were added for 10 min in Ca^{2+}-free Tyrode, and luminescence measurements were started immediately before the addition of Ca^{2+} (final concentration 3 mM). Data are presented as means with SD.

isoprenaline-mediated luminescence signals became visible. With increasing concentrations, roflumilast significantly enhanced the luminescence signals mediated by isoprenaline stimulation and induced leftward shifts of the corresponding concentration−response curves. The PDE 4 inhibitor effects were characterized up to a concentration fairly above (>1000-fold) the biochemical IC_{50} value. In the presence of the highest concentration of 1 μM roflumilast, isoprenaline stimulated luminescence signals on the PDE 4B1 and PDE 4D3 reporter cell lines with EC_{50} values of 0.14 ± 0.03 nM and 0.06 ± 0.005 nM, respectively (Figure 3).

Next, we characterized the effect of isoprenaline on roflumilast concentration−response curves. When applied to the PDE 4B1 and PDE 4D3 reporter cell lines under basal conditions in the absence of isoprenaline, roflumilast did not stimulate any significant luminescence signals (Figure 4). In

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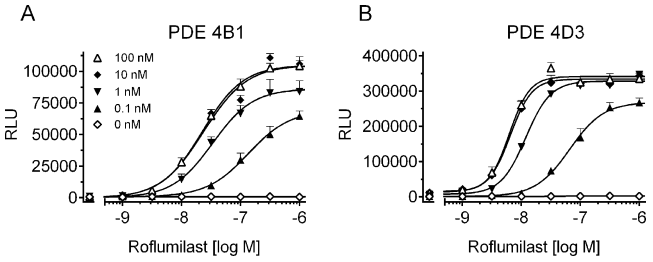

Figure 4. Effects of isoprenaline on roflumilast-mediated luminescence signals. Treatment of (A) the PDE 4B1 and (B) the PDE 4D3 reporter cell lines with roflumilast. Luminescence signals mediated by roflumilast in the absence (◇) or presence of 0.1 nM (▲), 1 nM (▼), 10 nM (◆), and 100 nM (△) isoprenaline. Isoprenaline and roflumilast were added for 10 min in Ca^{2+}-free Tyrode, and luminescence measurements were started immediately before the addition of Ca^{2+} (final concentration 3 mM). Data are presented as means with SD.

contrast, with increasing isoprenaline concentrations (0.1−100 nM), luminescence signals became visible, and leftward shifts of the corresponding roflumilast concentration−response curves were observed. At a concentration of 10 nM, isoprenaline-mediated stimulation of the luminescence signals became saturated. A higher isoprenaline concentration of 100 nM was also used; however, no further leftward shift of the roflumilast concentration−response curves was observed (Figure 4). Under these conditions, roflumilast stimulated luminescence signals with EC_{50} values of 17.8 ± 1.5 nM and 7.0 ± 0.4 nM on the PDE4B1 and the PDE4D3 reporter cells, respectively. Similar results were obtained by stimulation with the direct adenylate activator forskolin (1−30 μM) instead of isoprenaline (data not shown). We also characterized the roflumilast-mediated luminescence signals obtained after 1, 10, and 60 min isoprenaline stimulation. Slightly higher luminescence signals were obtained after 10 min stimulation (data not shown). Therefore, cells were incubated for 10 min with isoprenaline and PDE inhibitors in all experiments.

Characterization of the Cellular Activity of PDE 4 Inhibitors. We used our newly established PDE 4B1 and PDE 4D3 reporter cell lines for the characterization of the cellular activity of various PDE 4 inhibitors. In particular, competitive inhibitors like roflumilast, cilomilast, and piclamilast and allosteric inhibitors, including PMNPQ, D159153, and D159404, were characterized.[7] All PDE 4 inhibitors analyzed stimulated potent luminescence signals on both reporter cell lines in the presence of 100 nM isoprenaline. Representative concentration−response curves are shown in Figure 5. The results are summarized in Table 2. As expected, most of the PDE 4 inhibitors tested showed similar activities on both reporter cell lines.

Table 2. Characterization of the Cellular Activities of PDE 4 Inhibitors on PDE 4B1 and PDE 4D3 Reporter Cell Lines[a]

PDE 4 inhibitor	PDE 4B1 cellular EC_{50} (nM)	PDE 4D3 cellular EC_{50} (nM)	factor EC_{50} (PDE 4B1)/ EC_{50} (PDE 4D3)
roflumilast	10.0 ± 1.5	6.7 ± 1.0	1.5
roflumilast *N*-oxide	33.4 ± 7.6	12.8 ± 2.1	2.6
piclamilast	21.2 ± 3.6	8.0 ± 1.0	2.6
oglemilast	11.4 ± 1.1	4.4 ± 0.7	2.6
cilomilast	7112 ± 1044	823 ± 80	8.6
YM976	139 ± 17	36.2 ± 4.6	3.8
zardaverine	7181 ± 1074	1801 ± 199	4.0
etazolate	>30 000	15 553 ± 2614	
rolipram	44.6 ± 5.2	600 ± 56	0.1
PMNPQ	0.47 ± 0.06	0.29 ± 0.05	1.6
D159153	28.5 ± 3.0	6.6 ± 0.8	4.3
D159404	1415 ± 227	157 ± 9	9.0
lirimilast	6.9 ± 0.8	23.4 ± 5.9	0.3

[a]EC50 values are given in nM as means ± SEM.

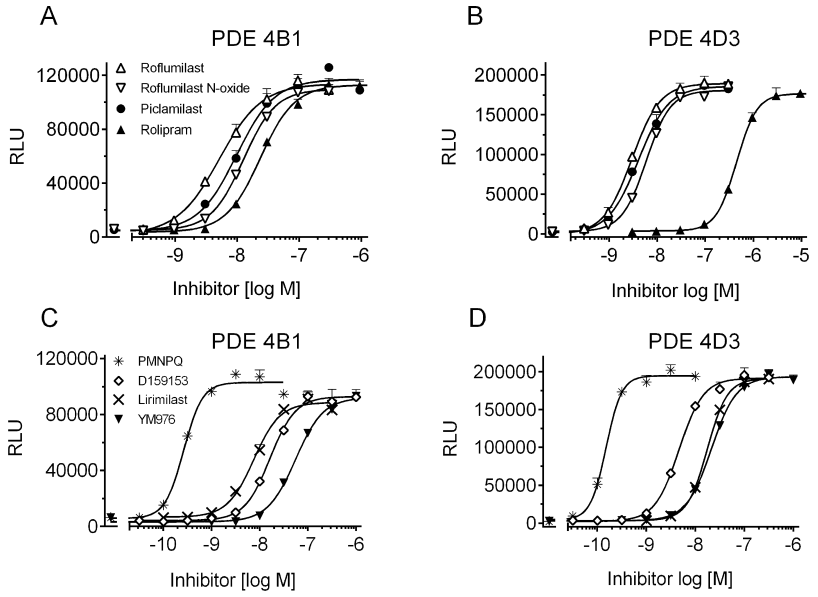

Figure 5. Characterization of the cellular activity of PDE 4 inhibitors. Stimulation of (A, C) the PDE 4B1 and (B, D) the PDE 4D3 reporter cell line with 100 nM isoprenaline in the presence of (A, B) roflumilast (△), roflumilast *N*-oxide (▽), piclamilast (●), and rolipram (▲) or (C, D) PMNPQ (∗), D159153 (◇), lirimilast (×), and YM976 (▼). Isoprenaline and PDE inhibitors were added for 10 min in Ca^{2+}-free Tyrode, and luminescence measurements were started immediately before the addition of Ca^{2+} (final concentration 3 mM). Data are presented as means with SD.

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Characterization of the Cellular Activity of Non-PDE 4 Inhibitors. We next sought to determine whether non-PDE 4 inhibitors were able to enhance isoprenaline-stimulated luminescence signals. Therefore, we tested the PDE inhibitors 8-methoxymethyl-IBMX (PDE 1), BAY 60-7550 (PDE 2), milrinone (PDE 3), tadalafil (PDE 5), BRL 50481 (PDE 7), BAY 73-6691 (PDE 9), vinpocetine (PDE 1), and dipyridamole (nonselective PDE inhibitor) up to a concentration of 30 μM. However, even in the presence of 100 nM isoprenaline, these PDE inhibitors did not stimulate significant luminescence signals on our PDE 4B1 and PDE 4D3 reporter cell lines at relevant concentrations (Figure 6). The PDE 2 inhibitor BAY 60-7550 and the PDE 9 inhibitor BAY 73-6691 stimulated weak luminescence signals on the PDE 4D3 reporter cell line at concentrations above 3 μM, which is most likely due to unspecific inhibition of PDE 4D3 at high concentrations.[17,20]

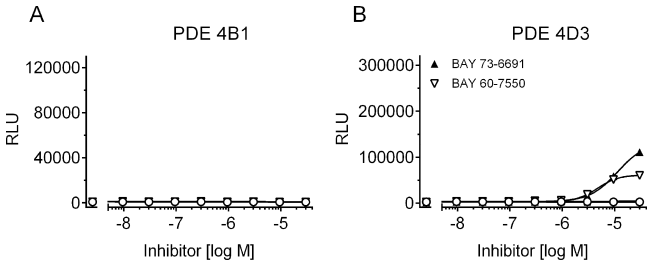

Figure 6. Effects of non-PDE 4 inhibitors on PDE 4B1 and PDE 4D3 reporter cells. Stimulation of (A) the PDE 4B1 and (B) the PDE 4D3 reporter cell lines with 100 nM isoprenaline in the presence of 8-methoxymethyl-IBMX (●), BAY 60-7550 (▽), milrinone (+), tadalafil (◆), BRL 50481 (○), BAY 73-6691 (▲), vinpocetine (×), and dipyridamole (◇). Isoprenaline and PDE inhibitors were added for 10 min in Ca^{2+}-free Tyrode, and luminescence measurements were started immediately before the addition of Ca^{2+} (final concentration 3 mM). Data are presented as means with SD.

Characterization of the *in Vitro* Activity of PDE 4 Inhibitors. The competitive and allosteric PDE 4 inhibitors which were previously characterized on the PDE 4 reporter cell lines were also tested for their biochemical *in vitro* activity. The inhibitory activity was tested on full length PDE 4D3 and a truncated enzyme comprising the PDE 4D3 catalytic domain only (amino acids 223−613). All PDE 4 inhibitors used showed potent inhibition of full length PDE 4D3. In contrast, when tested on the PDE 4D3 catalytic domain, several inhibitors showed clearly lower potencies. The results are summarized in Table 3.

In addition, we compared the biochemical *in vitro* activity (IC_{50} values) on full length and truncated PDE 4D3 with the cellular EC_{50} values obtained with our newly established PDE 4D3 reporter cell line. The calculated factors are summarized in Table 3. Two different groups of PDE 4 inhibitors could be identified. The first group, including competitive inhibitors like roflumilast, cilomilast, and piclamilast, show similar *in vitro* activity on full-length and truncated PDE 4D3 and comparably low cellular activity. The second group, including the allosteric inhibitors PMNPQ, D159153, and D159404, shows high cellular activity and much better inhibition of full-length versus truncated PDE 4D3.

DISCUSSION

In recent years, selective PDE 4 inhibitors were identified and have been evaluated as potential therapeutics. However, due to frequently observed side effects, the only marketed PDE 4 inhibitor so far is roflumilast.[3,11,21] Several ways to circumvent these side effects and to increase the therapeutic window have been proposed, namely, PDE 4 subtype-selective inhibitors, the use of PDE 4 inhibitors which do not pass the blood−brain barrier, selectivity for inhibition of low versus high affinity rolipram binding states of the enzyme ("LARBS" and "HARBS"), and allosteric PDE 4 inhibition.[2,3,7,22]

For the development of novel PDE inhibitors, the characterization of their biochemical *in vitro* as well as their cellular activity is mandatory. This is important, since clear differences between the cellular activity and the *in vitro* inhibition of isolated PDE 4 have been reported for several PDE 4 inhibitors.[23−25] Therefore, the development of a sensitive, recombinant assay for the characterization of the cellular activity of PDE 4 inhibitors is highly desirable. In recent years, cellular assays for the characterization of PDE 4 inhibitors have been published. However, these assays require the use of radioactivity or long incubation times and do not allow real-time detection of intracellular cAMP generation and turnover in living cells.[13,23,26]

Table 3. Characterization of PDE 4 Inhibitors[a]

PDE 4 inhibitor	PDE 4D3 cellular EC_{50}	PDE 4D3 *in vitro* $IC_{50(full\text{-}length)}$	PDE 4D3 *in vitro* $IC_{50(cat.domain)}$	factor $IC_{50(full\text{-}length)}/IC_{50(cat.domain)}$	factor $EC_{50}/IC_{50(full\text{-}length)}$	factor $EC_{50}/IC_{50(cat.domain)}$
roflumilast	6.7 ± 1.0	0.076 ± 0.015	0.039 ± 0.007	2.0	89	174
roflumilast *N*-oxide	12.8 ± 2.1	0.26 ± 0.02	0.079 ± 0.003	3.3	48	162
piclamilast	8.0 ± 1.0	0.12 ± 0.01	0.064 ± 0.008	1.9	67	126
oglemilast	4.4 ± 0.7	1.1 ± 0.1	0.50 ± 0.04	2.1	4	9
cilomilast	823 ± 80	10.1 ± 1.0	20.5 ± 1.4	0.5	81	40
YM976	36.2 ± 4.6	0.73 ± 0.06	0.34 ± 0.03	2.1	50	106
zardaverine	1801 ± 199	65.3 ± 4.7	163 ± 30	0.4	28	11
etazolate	15,553 ± 2614	240 ± 20	143 ± 25	1.7	65	109
rolipram	600 ± 56	72.8 ± 8.3	510 ± 25	0.14	8	1
PMNPQ	0.29 ± 0.05	0.43 ± 0.07	115 ± 10	0.0037	0.67	0.0025
D159153	6.6 ± 0.8	68.3 ± 13.2	20,500 ± 957	0.0033	0.10	0.0003
D159404	157 ± 9	380 ± 129	20,000 ± 408	0.019	0.41	0.0079
Lirimilast	23.4 ± 5.9	54.3 ± 12.2	3725 ± 542	0.015	0.43	0.0063

[a]Cellular activity on the PDE 4D3 reporter cell line and *in vitro* inhibition of full-length and truncated (catalytic domain) PDE 4D3 from cell lysates. Cellular EC50 values and *in vitro* IC50 values are given in nM as means ± SEM.

In this report we describe a novel approach using recombinant PDE 4B1 and PDE 4D3 reporter cell lines. In our PDE 4 reporter cells, intracellular cAMP levels can be indirectly monitored by activation of a cAMP-sensitive CNG channel.[19] Activation of the β1-adrenoceptor by isoprenaline (or direct activation of adenylate cyclase by forskolin) stimulates intracellular cAMP synthesis and concomitant cAMP hydrolysis by PDE 4B1 or PDE 4D3, respectively. PDE 4 inhibitors prevent cAMP degradation, which results in increased intracellular cAMP levels, opening of the CNG channel, calcium influx, and enhanced luminescence signals.

We characterized our reporter cell lines by measuring the expression levels of endogenous PDEs and of the heterologously expressed human PDE 4 variants. Similar high levels of PDE 4B1 and PDE 4D3 expression were detected. In addition, the PDE 4D3 cells express higher levels of the β1-adrenoceptor. Presumably, this is the reason for the higher RLU values obtained with the PDE 4D3 reporter cells.

A similar cell-based PDE 4 reporter assay was described recently. However, in this reporter cell line PDE 4 was not recombinantly expressed, and the endogenous PDE 4 subtypes and splice isoforms are not known. In addition, very low signal-to-background (S/B) ratios were reported.[16] In contrast, in our PDE 4B1 and PDE 4D3 reporter cells the identity of the PDE 4 splice isoform is well-defined, and much higher S/B ratios are observed.

We further characterized our newly generated PDE 4B1 and PDE 4D3 reporter cell lines by using selective and nonselective PDE 4 inhibitors which stimulate concentration-dependent luminescence signals on the reporter cells in the presence of isoprenaline. According to literature data, most of the PDE 4 inhibitors used have no preference for PDE 4B or PDE 4D isozyme inhibition *in vitro* and, accordingly, show similar effects on both reporter cell lines. Only cilomilast and D159404 stimulate luminescence signals with slightly higher potencies on the PDE 4D3 cell line, whereas rolipram is more potent on the PDE 4B1 reporter cells. These results correspond well with published data for inhibition of isolated PDE 4B and PDE 4D isozymes *in vitro*.[7,27,28]

As expected, non-PDE 4 inhibitors are not able to stimulate significant effects on our PDE 4 reporter cells. In addition, roflumilast and other PDE 4 inhibitors have no effect on isoprenaline-stimulated luminescence signals in our β1-adrenoceptor control cell line, where no PDE 4 is recombinantly expressed. The results indicate that our PDE 4 reporter cells specifically monitor cellular PDE 4B1 and PDE 4D3 activity and that endogenous PDEs are not involved in luminescence signal generation.

We also compared the cellular activity of the PDE 4 inhibitors with the *in vitro* inhibition of full-length PDE 4D3 and a truncated enzyme comprising the PDE 4D3 catalytic domain. Thereby, we identified two different groups of PDE 4 inhibitors.

The first group, including the competitive inhibitors roflumilast, cilomilast, oglemilast, and piclamilast, shows similar *in vitro* activity on full-length and truncated PDE 4D3 and comparably low cellular activity. Within this inhibitor group, cellular EC_{50} values on the PDE 4D3 reporter cells are 5- to 90-fold higher compared to the biochemical IC_{50} values for inhibition of full-length PDE 4D3. In addition, within this group we observe very similar *in vitro* inhibitory activity on PDE 4D3 full-length enzyme and catalytic domain. The results imply that the presence of the PDE 4D3 N-terminal domain has no impact on inhibitor activity and is not necessary for potent PDE 4 inhibition by these compounds.

The second PDE 4 inhibitor group, including PMNPQ, D159153, D159404, and lirimilast, shows very high cellular activity and much better inhibition of full-length versus truncated PDE 4D3. The observed cellular EC_{50} values are even lower compared to the IC_{50} values for *in vitro* inhibition of full-length PDE 4D3. PMNPQ, D159153, and D159404 are so-called allosteric inhibitors since their *in vitro* activity is known to be massively influenced by the presence of the N-terminal regulatory upstream conserved regions UCR1 and UCR2.[7] Lirimilast also belongs to this inhibitor group, which is somehow unexpected with respect to its distinct structure.

Our results obtained with rolipram imply that rolipram has properties intermediate between the two groups. The *in vitro* activity of rolipram is increased by ~7-fold in the presence of the N-terminal regulatory domains, which points to a modulation of the rolipram inhibitory activity by the UCR domains. However, the effect is much smaller as compared to the other allosteric PDE 4 inhibitors which show 50- to 300-fold increased *in vitro* activity on full-length PDE 4D3. In addition, the cellular activity of rolipram is lower compared to the *in vitro* activity on the full-length enzyme, a behavior shared with the classical competitive inhibitors of the first group.

The PDE 4 pharmacology is known to be quite complex, due to the existence of four different isozymes and a large number of long, short, and supershort splice isoforms. The activity of PDE 4 inhibitors can be influenced by the presence of and interaction with N-terminal UCR domains, dimerization, the phosphorylation status of the enzyme, its subcellular localization, and interaction with other proteins in the cell. In addition, a molecular understanding of the high and low affinity rolipram binding states of PDE 4 is only beginning to emerge.[8,21,29]

Therefore, we can only speculate about the reason for the comparably high cellular activity of the second allosteric PDE 4 inhibitor group. These inhibitors are believed to interact with both the catalytic domain and the N-terminal UCR regulatory domains. UCR domains control the activity of the catalytic center and confer regulatory functions on PDE 4 by regulating the effect of phosphorylation by PKA and ERK.[9,29]

PKA-mediated phosphorylation of PDE 4D3 at Ser-54 was previously shown to result in an increased sensitivity to inhibition by PDE 4 inhibitors. The reported effects vary between a relatively small 8-fold increase for rolipram and huge 100- to 330-fold increases for RS-25344 and RS-33793, respectively.[30−32] Interestingly, RS-25344 and RS-33793 are structurally very similar to PMNPQ and the other allosteric inhibitors used in this study. These results provide a rationale for our observation that allosteric PDE 4 inhibitors show very high cellular activity and fit very well to our data obtained with rolipram and the other allosteric inhibitors.

Therefore, it is tempting to speculate that isoprenaline stimulation in the presence of a PDE 4 inhibitor results in intracellular cAMP accumulation in our reporter cells and subsequent PKA-mediated PDE 4D3 phosphorylation at Ser-54. This phosphorylation in turn induces a conformational change and an increased sensitivity to inhibition by allosteric PDE 4 inhibitors. In contrast, the phosphorylation status of PDE 4D3 at Ser-54 in cellular lysates from nonstimulated HEK 293 cells is likely to be low. Therefore, a reduced *in vitro* activity of these allosteric inhibitors can be anticipated.

In summary, the results imply that our novel PDE 4B1 and PDE 4D3 reporter cell lines are valuable tools for the characterization of the cellular activity of PDE 4 inhibitors and for novel drug development, and will also support a better understanding of the complex PDE 4 pharmacology.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CNG, cyclic nucleotide-gated; MTP, microtiter plate; PDE, phosphodiesterase; RLU, relative light unit; HTS, high-throughput screening

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