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A Novel PDE2A Reporter Cell Line: Characterization of the Cellular Activity of PDE Inhibitors

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Abstract: We report here the generation and pharmacological characterization of a phosphodiesterase 2A (PDE2A) reporter cell line. Human PDE2A was stably transfected in a parental cell line expressing the atrial natriuretic peptide (ANP) receptor and the cyclic nucleotide-gated (CNG) cation channel CNGA2, acting as the biosensor for intracellular cGMP. In this reporter cell line, cGMP levels can be monitored in real-time via aequorin luminescence stimulated by calcium influx through the CNG channel. By using different PDE inhibitors, we could show that our PDE2A reporter assay specifically monitors PDE2A inhibition with high sensitivity. In the absence of ANP stimulation, the PDE2A selective inhibitors EHNA, BAY 60-7550 and PDP did not increase basal luminescence levels in this experimental setting. However, in combination with ANP, these inhibitors stimulated luminescence signals and induced leftward shifts of ANP concentration−response curves. Similar results were obtained when using IBMX, trequinsin and dipyridamole, which inhibit PDE2A nonselectively with lower potency. PDP, the most potent PDE2A inhibitor known to date, was found to exhibit much lower cellular activity as anticipated from its biochemical PDE2A inhibitory activity. By cellular uptake and transport studies we could show that PDP's cell permeability is low and that the compound is a substrate for an efflux transporter. Other PDE inhibitors including vinpocetine, milrinone, rolipram, sildenafil, zaprinast, BRL 50481 and BAY 73-6691 did not stimulate luminescence signals on our PDE2A reporter cell line. The results imply that this novel PDE2A reporter assay provides an efficient, high throughput means for the identification and characterization of PDE2A inhibitors.

Keywords: ANP receptor; cGMP; CNG channel; PDE2A; reporter assay

Introduction

Cyclic nucleotide-specific phosphodiesterases (PDEs) represent an important target class for drug discovery and development. PDEs regulate cellular signaling pathways by modulating the intracellular levels of the second messenger molecules 3′,5′-cyclic adenosine monophosphate (cAMP) and 3′,5′-cyclic guanosine monophosphate (cGMP). To date, 21 mammalian PDE genes have been identified and subgrouped into 11 isoenzyme families based on their substrate specificity, regulation and pharmacology.[1,2]

PDE2A, a member of this isoenzyme family, was originally purified from bovine tissues and was characterized as a cGMP-stimulated PDE.[3] Subsequently, PDE2A cDNAs were cloned from bovine adrenal cortex and brain, and later on human and rat orthologues were also identified. Three N-terminal splice variants with different subcellular localization have been described. PDE2A1 is found in the cytosol,

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whereas PDE2A2 and PDE2A3 are localized at the cell membrane.[4−6,37] Biochemical characterization showed that PDE2A is a dual substrate enzyme that has a high V_{max} and low K_m for hydrolysis of both cAMP and cGMP. Recently, crystal structures of the PDE2A catalytic and regulatory domains have been published.[7,8]

Potent and selective PDE2A inhibitors have been identified in recent years. Following the characterization of the first PDE2A-selective inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA),[9] additional selective inhibitors with higher potency were identified. These PDE2A inhibitors include 2-(3,4-dimethoxybenzyl)-7-{(1*R*)-1-[(1*R*)-1-hydroxyethyl]-4-phenylbutyl}-5-methylimidazo[5,1-*f*][1,2,4]triazin-4(3*H*)-one (BAY 60-7550), 9-(6-phenyl-2-oxohex-3-yl)-2-(3,4-dimethoxybenzyl)-purin-6-one (PDP), IC933 and oxindole.[10−13]

PDE2A expression has been detected in a wide variety of tissues, however, highest PDE2A mRNA levels are found in heart, brain and adrenal glomerulosa cells. Accordingly, PDE2A is involved in a variety of physiological processes.[1,2] In the adrenal gland, PDE2A has been shown to mediate the inhibitory effects of atrial natriuretic peptide (ANP) on aldosterone secretion from adrenal glomerulosa cells.[14,15] In the central nervous system, PDE2A inhibition was shown to increase synaptic plasticity and improve memory performance.[10] In cardiac myocytes PDE2A, in contrast to PDE5A, was shown to functionally couple to ANP receptors and to control the subsarcolemmal concentrations of cAMP and cGMP.[16,17] Thereby, PDE2A is able to blunt cardiac β-adrenergic receptor mediated cAMP-signaling in a negative feedback control loop.[18] Recently, TNF-α-dependent PDE2A upregulation in endothelial cells was observed and a role for PDE2A in the regulation of endothelial barrier function was proposed. Accordingly, PDE2A inhibition might become a novel therapeutic option in sepsis.[11,19]

In recent years, PDEs have gained considerable attention as targets for new drug development.[1,2,20] 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 CNG channels as biosensors to detect intracellular cAMP levels and PDE activity have been introduced.[21−24] In addition, a FRET-based method has also been described.[25]

We have shown previously that the olfactory CNGA2 channel is well suited for the detection of intracellular cGMP

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synthesis mediated by soluble guanylyl cyclase (sGC) in an uHTS assay format.[26] In addition, we could show that this reporter assay can be used for the characterization of the cellular activity of PDE9A inhibitors.[27] We describe in this report the development of a novel, luminescence- and cell-based PDE2A reporter assay. To achieve optimal sensitivity for PDE2A inhibition, PDE2A was stably coexpressed with the ANP receptor in an aequorin cGMP reporter cell line. We used our newly established PDE2A reporter cell line for the pharmacological and kinetic characterization of the cellular activity of PDE2A inhibitors.

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 efficencies were assured by titration of genomic hamster DNA. Normalization was performed using GAPDH as control, and relative expression was calculated using the formula: relative expression $= 2^{(18-(Ct(probe)-Ct(GAPDH)))}$. The parameter Ct is defined as the threshold cycle number at which the amplification plot passed a fixed threshold above baseline. The resulting expression is given in arbitrary units as mean values ± SEM ($n = 3$). The primers and fluorescent probes used are shown in Table 1.

Generation of the ANP Receptor and PDE2A Reporter Cell Lines. A recombinant CHO cell line expressing cytosolic apoaequorin was stably cotransfected with a plasmid construct encoding the bovine CNGA2 channel (acc. no. X55010) and a plasmid providing zeocin resistance, as has been described previously.[26] This parental cGMP reporter cell line was cotransfected with a pCMV3 plasmid construct encoding the rat ANP receptor (kindly provided by D. L. Garbers, University of Texas; acc. no. X14773) and pcDNA3 (G-418 resistance). Stably transfected clones were obtained by G-418 (Geneticin) selection and were characterized by ANP stimulation (data not shown). Positive clones were purified once by the limited dilution technique, and one clonal cell line was selected for further experiments (referred to here as the ANP receptor cell line).

Thereafter, the ANP receptor cell line was cotransfected with a pcDNA1.1 Amp plasmid construct encoding human PDE2A3 (acc. no. NM_002599) and a plasmid providing hygromycin resistance. PDE2A3 expressing clones were identified by ANP stimulation in the absence or presence of 1 μM BAY 60-7550 (data not shown). Positive clones were again purified once by the limited dilution technique. One clonal cell line, referred to here as the PDE2A reporter cell line, was selected for further characterization. All plasmid vectors, with the exception of pCMV3, were purchased from Invitrogen (Carlsbad, CA).

Cell Culture Conditions and Aequorin Luminescence Measurements. Cells were cultured at 37 °C and 5% CO_2 in Eagle's MEM alpha medium with ribonucleosides and deoxyribonucleosides, supplemented with 10% (v/v) inactivated fetal calf serum, 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, 1 mg/mL G-418 and 0.25 mg/mL zeocin. In addition, 0.6 mg/mL hygromycin B was added to the cell culture medium used for the PDE2A reporter cell line. Cells were passaged using enzyme-free/Hanks'-based cell dissociation buffer. All cell culture reagents were obtained from Invitrogen (Carlsbad, CA).

Luminescence measurements were performed on opaque 384-well microtiter plates (MTPs). Either 2500 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 0.3 μg/mL coelenterazine in Ca^{2+}-free Tyrode solution (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. ANP and PDE inhibitors were added for 6 min in Ca^{2+}-free Tyrode containing 0.1% bovine serum albumin. Immediately before adding Ca^{2+} ions (final concentration 3 mM), measurement of the aequorin luminescence was started by using a charge-coupled device (CCD) camera (Hamamatsu Corporation, Shizuoka, Japan) in a light tight box. Alternatively, a conventional luminometer may also be used.[26] Luminescence was monitored continuously for 60 s. For the real-time detection of cGMP generation, coelenterazine loading and agonist stimulation were performed in Tyrode solution containing 2 mM calcium ions.[26] For the luminescence measurements performed on 1536-well MTPs, 400 cells/well were plated in medium containing 5 μg/mL coelenterazine and were cultured for 24 h at 30 °C and 5% CO_2.

Transport Studies in Cells. Caco-2 cells were purchased from the Deutsche Sammlung für Mikroorganismen; Braunschweig. Caco-2 cells were seeded at a density of 40,000 cells/well and grown for 15 days on 24-well filter insert plates

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Table 1. Primers and Fluorescent Probes[a]

PDE1B	forward primer: 5′-CAGATCCATGCAGCTGATGTG-3′
	probe: 5′-(FAM)TCCATTGCTTTCTGCTCCGCACAG(TAMRA)-3′
	reverse primer: 5′-TCTGACAGGCAGTGCACCAT-3′
PDE1C	forward primer: 5′-GCCAAGAAGGAAGCTGAAGA-3′
	probe: 5′-(FAM)AGCAAAAGGAAATGGAAGCCAAAAGCCA(TAMRA)-3′
	reverse primer: 5′-TGTCTTTTTCTCAGCTTTGC-3′
PDE2A	forward primer: 5′-CGGAGTAGACGACAGCACAG-3′
	probe: 5′-(FAM)CGCGCAACATCCTCTGCTTCCCTAT(TAMRA)-3′
	reverse primer: 5′-CCTCCTGGTTCTCGTTTTTG-3′
PDE3A	forward primer: 5′-TGTCCCGGCCAGAGTATAAC-3′
	probe: 5′-(FAM)AACCTGGACCATGTGGAATTTAAGCA(TAMRA)-3′
	reverse primer: 5′-GCCAAAATTGCTTCAATGACT-3′
PDE3B	forward primer: 5′-TTGTGGCCTTTCTTGAGTGA-3′
	probe: 5′-(FAM)TTCTGCTCATGGCAGTACACTGTTCA(TAMRA)-3′
	reverse primer: 5′-CAAAGCTGGGAGAACCACAT-3′
PDE4A	forward primer: 5′-GGGCCTGAACATCTTTTGTG-3′
	probe: 5′-(FAM)TCGGAGTACGCTGGAGGCCG(TAMRA)-3′
	reverse primer: 5′-TCGTGTACATGATACAGCTGAGTG-3′
PDE4B	forward primer: 5′-TCCTGGTGTCAGTTTCAGTTTG-3′
	probe: 5′-(FAM)TGTCATGCTTCAGTTCAGCATTATGACA(TAMRA)-3′
	reverse primer: 5′-TGCACATTTGTGCCATTTTC-3′
PDE5A	forward primer: 5′-ATTCTCAGTGGCCTCTCCAT-3′
	probe: 5′-(FAM)CCACACTGAAAATAATCAAGCAAGCA(TAMRA)-3′
	reverse primer: 5′-ACAGTGCTAGGTCTGTAGCTAAAAT-3′
PDE7A	forward primer: 5′-TCCTTGGGATATCTTGCTGAG-3′
	probe: 5′-(FAM)TTAATTGCAGCCGCCACTCATGATC(TAMRA)-3′
	reverse primer: 5′-TGATTAACACCTGGGTGATCC-3′
PDE8A	forward primer: 5′-TATCAATGCTGCCCAGGAA-3′
	probe: 5′-(FAM)CAGTCCTGTGCCTGTGACAGAAGCC(TAMRA)-3′
	reverse primer: 5′-ATATCCAGCACACGGTTCAA-3′
PDE9A	forward primer: 5′-TGCATGACAACTACAGGAACAAC-3′
	probe: 5′-(FAM)CCACAACTTCCGGCACTGCTTCTG(TAMRA)-3′
	reverse primer: 5′-CAGACCATGCTGTACATCATCTG-3′
PDE10A	forward primer: 5′-ATCCGCAAAGCCATCATC-3′
	probe: 5′-(FAM)TTTGGGAACAGGAAGCAGTTGGAG(TAMRA)-3′
	reverse primer: 5′-GACCCGGTCTGGTACATCTC-3′
PDE11A	forward primer: 5′-TGACTTTTCTCTTGATGTTGATGC-3′
	probe: 5′-(FAM)CACAGCTGCTCTCCGGATGTTCATG(TAMRA)-3′
	reverse primer: 5′-TTCTGTACCATCCCCAGCTC-3′
GAPDH	forward primer: 5′-GGCTACACCGAGGACCAGG-3′
	probe: 5′-(FAM)TGTCTCCTGCGACTTCAACAGTGAC(TAMRA)-3′
	reverse primer: 5′-CATCAAAGGTGGAAGAGTGGG-3′
hPDE2A	forward primer: 5′-TGAACATCCCTGACGCATATG-3′
	probe: 5′-(FAM)CTACCGCGGCGTGGACGACAG(TAMRA)-3′
	reverse primer: 5′-CGTGCGGAAGCCGGT-3′

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

(Costar, Corning, NY) in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 5 mL/500 mL nonessential amino acids, 1 mM sodium pyruvate, 0.1 mg/mL streptomycin and 100 U/mL penicillin (Invitrogen, Carlsbad, CA) at 8% CO_2. P_{app} values for both directions and efflux ratios were determined at a compound concentration of 2 μM after 2 h incubation at 37 °C. Concentrations of the test compounds were determined by LC−MS/MS using an Agilent 1100 liquid chromatography system (Cohesive Technologies, Aschaffenburg, Germany) equipped with a Synergy Fusion RP column (20 × 2 mm, 4 μm particle size; Merck, Darmstadt, Germany) coupled to an ABI 3000 mass spectrometer (Applied Biosystems, Aschaffenburg, Germany) equipped with a turbo ion spray source for electrospray ionization. For the determination of intracellular concentrations of BAY 60-7550 and PDP, PDE2A reporter cells were plated on 12-well MTPs (400,000 cells/well) and were cultured for 24 h. Cells were treated with BAY 60-7550 or PDP (500 nM each) in HBSS supplemented with 5 mM HEPES and 20 mM glucose for 5 min at 37 °C ($n = 3$). Cells were washed three times with ice-cold HBSS and lysed with acetonitril. Compound concentrations within the cell

lysate were determined by LC−MS/MS. P_{app} values and efflux ratios were calculated as described before.[28]

L-MDR1 cells were purchased from The Netherlands Cancer Institute in Amsterdam.[29] Cells were cultured and transport studies were performed as described earlier.[28] Efflux ratios across LLC-PK1 and L-MDR1 cells were determined at a concentration of 2 μM. After 2 h incubation at 37 °C, samples were taken from both compartments and analyzed by LC−MS/MS.

Compounds. The PDE2A inhibitor 2-(3,4-dimethoxybenzyl)-7-{(1*R*)-1-[(1*R*)-1-hydroxyethyl]-4-phenylbutyl}-5-methylimidazo[5,1-*f*][1,2,4]triazin-4(3*H*)-one (BAY 60-7550) was purchased from Axxora Life Sciences Inc. (San Diego, CA). The PDE2A inhibitor 9-(6-phenyl-2-oxohex-3-yl)-2-(3,4-dimethoxybenzyl)-purin-6-one (PDP) was synthesized by the chemistry department of Bayer HealthCare AG. We could confirm that PDP is a highly selective PDE2A inhibitor (IC_{50} = 0.6 nM; data not shown). Sildenafil was purified from commercially available tablets. The PDE9A inhibitor 1-(2-chlorophenyl)-6-[(2*R*)-3,3,3-trifluoro-2-methylpropyl]-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidine-4-one (BAY 73−6991) and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma-Aldrich (Taufkirchen, Germany). All other PDE inhibitors were obtained from Calbiochem (San Diego, CA).

Statistics. The data are presented as mean values with standard deviation errors. The GraphPad Prism software (Version 4.02, 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). For the determination of pEC_{50} and pIC_{50} values, three to six independent experiments were performed in quadruplicate. pEC_{50} and pIC_{50} values are given as means ± SEM.

Results

Generation of the Recombinant ANP Receptor and PDE2A Reporter Cell Lines. A cGMP reporter cell line, recombinantly expressing apoaequorin and the CNGA2 channel, was generated as has been described previously.[26] This cell line was cotransfected with plasmid constructs encoding the rat ANP receptor and a plasmid providing Geneticin resistance. Active clones were characterized by ANP stimulation and were purified by the limited dilution technique. One clonal cell line was used for further characterization (referred to as the ANP receptor cell line). Thereafter, this ANP receptor cell line was cotransfected with a plasmid construct encoding human PDE2A3 and a plasmid providing hygromycin resistance. Positive clones were identified by ANP stimulation in the absence or presence of the PDE2A inhibitor BAY 60-7550.[10] PDE2A expressing clones were purified by the limited dilution technique and one clonal cell line, referred to here as the PDE2A cell line, was used for further experiments.

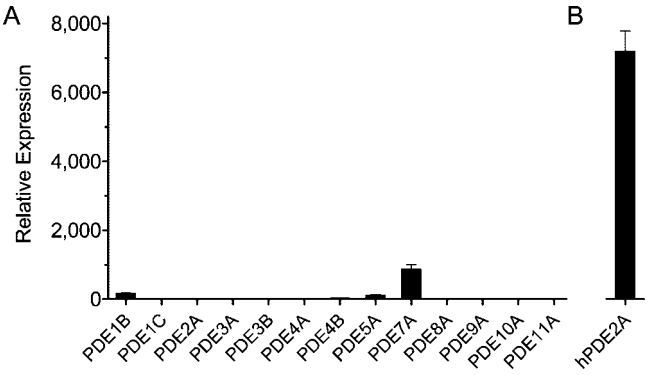

Figure 1. Characterization of PDE expression levels. Quantitative real-time RT-PCR analysis was performed on the ANP receptor (A) or the PDE2A (B) reporter cell line using specific oligonucleotide probes. Expression levels were normalized to GAPDH. Relative expression is given as means with SEM.

Analysis of PDE Expression in CHO Cells. To characterize the expression of endogenous 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 detect very low expression levels of endogenous PDE1C, PDE2A, PDE3A, PDE3B, PDE4A, PDE4B, PDE8A, PDE9A, PDE10A and PDE11A in the parental ANP receptor cell line. Low expression was detected for PDE1B and PDE5A, moderate expression was found for PDE7A. Similar results were obtained with the PDE2A cell line (data not shown). As expected, we could verify high expression of human PDE2A in our PDE2A reporter cell line (Figure 1B).

Characterization of the ANP Receptor and PDE2A Reporter Cell Lines. We characterized our newly established ANP receptor and PDE2A cell lines by ANP stimulation in the absence or presence of the PDE2A selective inhibitors EHNA, BAY 60-7550 and PDP. As shown in Figure 2A−C, ANP stimulated concentration-dependent luminescence signals on the ANP receptor cell line with a pEC_{50} value of 10.40 ± 0.08. ANP-mediated luminescence signals were not affected by the presence of increasing concentrations of EHNA (0.1−10 μM), BAY 60-7550 (0.01−1 μM) or PDP (0.01−1 μM).

Completely different results were obtained with the PDE2A reporter cell line. In the absence of a PDE2A inhibitor, ANP was not able to stimulate significant luminescence signals (Figure 2D−F). In contrast, in the presence of the respective PDE2A inhibitor, ANP-mediated luminescence signals became visible. With increasing concentrations, all three PDE2A inhibitors significantly enhanced the

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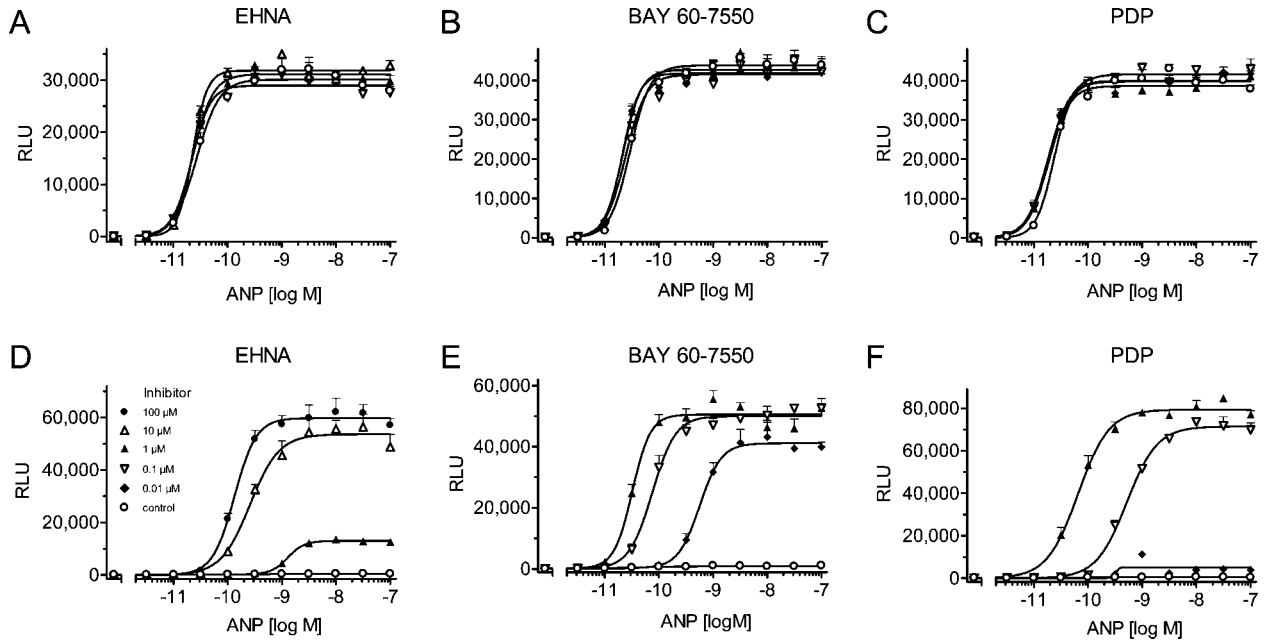

Figure 2. Effects of the PDE2A inhibitors EHNA, BAY 60-7550 and PDP on ANP mediated luminescence signals. Stimulation of the parental ANP receptor (A−C) or the PDE2A reporter cell line (D−F) with ANP was performed in the absence (○) or presence of 0.01 μM (◆), 0.1 μM (▽), 1 μM (▲), 10 μM (△), and 100 μM (●) of the PDE2A inhibitors EHNA, BAY 60-7550 and PDP. ANP and inhibitors were added for 6 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.

luminescence signals mediated by ANP stimulation and induced leftward shifts of the corresponding concentration−response curves. PDE2A inhibitor effects were characterized up to concentrations fairly above (100−1600-fold) their biochemical IC_{50} values. In the presence of the highest concentration of EHNA (100 μM), BAY 60-7550 (1 μM) or PDP (1 μM), ANP stimulated luminescence signals with pEC50 values of 9.97 ± 0.05, 10.54 ± 0.03 and 10.06 ± 0.06, respectively.

We also studied the effects of ANP on PDE2A inhibitor concentration−response curves. When applied to both, the parental ANP receptor or the PDE2A reporter cell lines under basal conditions without ANP stimulation, none of the three PDE2A inhibitors were able to induce significant luminescence signals (Figure 3). However, with increasing ANP concentrations (0.1−100 nM), luminescence signals became visible. On the parental ANP receptor cell line, ANP-mediated luminescence signals were unaffected by increasing PDE2A inhibitor concentrations (Figure 3A−C). In contrast, the PDE2A inhibitors EHNA, BAY 60-7550 and PDP stimulated concentration-dependent luminescence signals on the PDE2A cell line in combination with ANP (Figure 3D−F). In the presence of 10 nM ANP, pEC_{50} values of 5.76 ± 0.14, 8.24 ± 0.02 and 7.40 ± 0.03 were observed, respectively. A higher ANP concentration (100 nM) was also used, but was not able to further shift the PDE2A inhibitor concentration−response curves leftward (pEC_{50} = 5.78 ± 0.12, 8.22 ± 0.05 and 7.40 ± 0.06; data not shown). With respect to EHNA and BAY 60-7550, the observed pEC50 values corresponded well to the biochemical IC_{50} values for PDE2A inhibition of 800 nM and 4.7 nM, respectively.[9,10] In contrast, the cellular activity of PDP was clearly lower (~60-fold) as anticipated from the reported PDE2A IC_{50} value of 0.6 nM.[11]

Activities of Other PDE Inhibitors on the PDE2A Reporter Cell Line. We next sought to determine whether additional PDE inhibitors were able to enhance ANP-stimulated luminescence signals. Therefore, we tested the PDE inhibitors (highest concentration used is given) vinpocetine (30 μM), milrinone (100 μM), rolipram (100 μM), sildenafil (10 μM), zaprinast (30 μM), BRL 50481 (100 μM) and BAY 73-6691 (30 μM). However, even in the presence of 10 nM ANP, these PDE inhibitors did not stimulate significant luminescence signals on our ANP receptor or PDE2A reporter cell lines (data not shown). Subsequently, we tested additional PDE inhibitors which are known to inhibit PDE2A nonselectively at higher concentrations in the micromolar range. As expected, the nonselective PDE inhibitor IBMX, the PDE3A inhibitor trequinsin, and the PDE5A inhibitor dipyridamole stimulated concentration-dependent luminescence signals in the presence of 0.1−10 nM ANP (Figure 4). In combination with 10 nM ANP, pEC_{50} values of 4.13 ± 0.05, 5.32 ± 0.12 and 5.19 ± 0.02 were obtained, respectively. Similar results were observed when using 100 nM ANP (pEC_{50} = 4.14 ± 0.01, 5.50 ± 0.03 and 5.15 ± 0.01; data not shown). The observed pEC_{50} values for IBMX, trequinsin and dipyridamole correlate well to their reported biochemical IC_{50} values for PDE2A inhibition of 20−50 μM, 0.5−2 μM and 3.6 μM, respectively.[30,31]

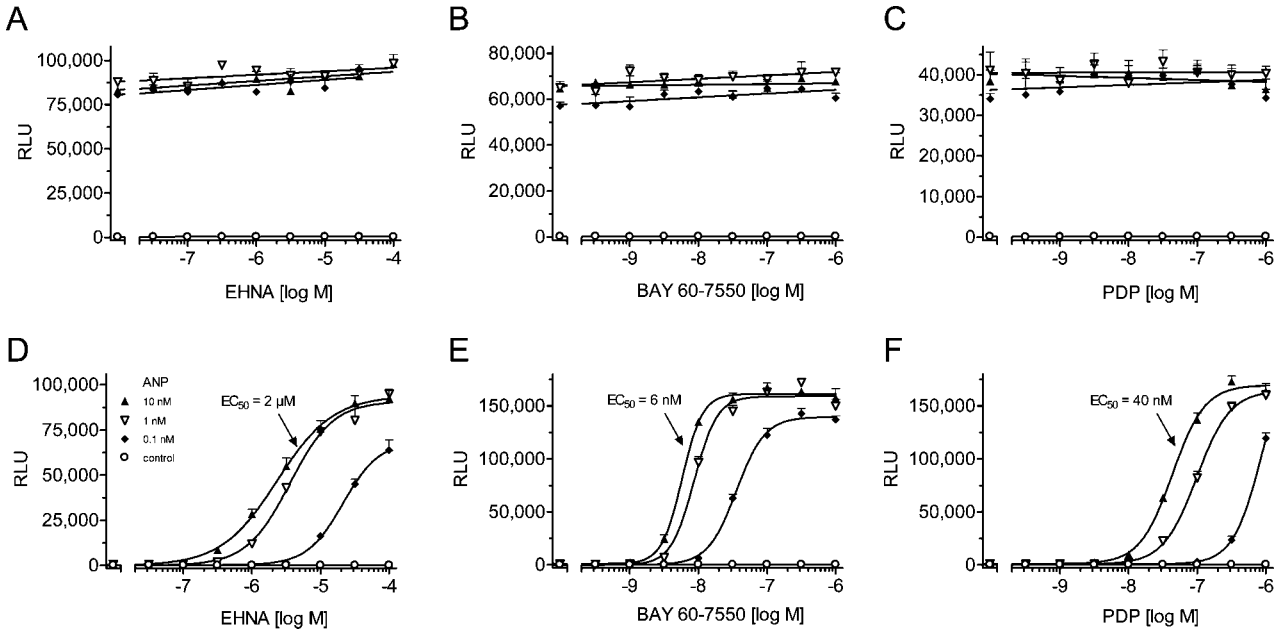

Figure 3. Characterization of the cellular activity of PDE2A inhibitors on the parental ANP receptor (A−C) or the PDE2A reporter cell line (D−F). Luminescence signals generated in the absence (○) or presence of 0.1 nM (◆), 1 nM (∇), and 10 nM (▲) ANP. Stimulation by 100 nM ANP did not further increase luminescence responses (data not shown). PDE2A inhibitors and ANP were added for 6 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.

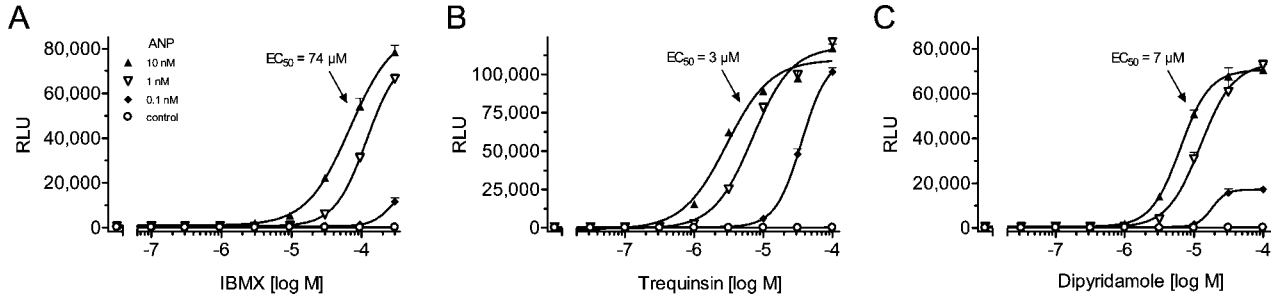

Figure 4. Characterization of additional PDE inhibitors on the PDE2A reporter cell line. Luminescence signals generated by the nonselective PDE inhibitors (A) IBMX, (B) trequinsin and (C) dipyridamole in the absence (○) or presence of 0.1 nM (◆), 1 nM (∇), and 10 nM (▲) ANP. Biochemical IC_{50} values for PDE2A inhibition of IBMX, trequinsin and dipyridamole are 20−50 μM, 0.5−2 μM, and 6 μM, respectively. PDE inhibitors and ANP were added for 6 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.

Real-Time Detection of cGMP Synthesis. To monitor intracellular cGMP accumulation in real-time, increasing concentrations of ANP (1−100 nM) were added to the ANP receptor and PDE2A reporter cell lines in the presence of 2 mM calcium ions ("kinetic mode").[26] Luminescence measurements were started immediately before agonist addition. As shown in Figure 5A, ANP concentration-dependently stimulated luminescence signals on the ANP receptor cell line with transient signal kinetics. Lag times in the range of 9 s (at 0.01 nM) to 2 s (at 100 nM) were observed. At the highest ANP concentration of 100 nM, maximum luminescence signals were reached after 8 s. In the presence of the PDE2A inhibitor BAY 60-7550 (1 μM), ANP stimulated very similar luminescence signals and lag times ranging from 10 s (0.01 nM) to 2 s (100 nM) were obtained (Figure 5B). Maximum luminescence signals were reached after 8 s (at 100 nM ANP).

Next, we measured ANP-mediated signal kinetics on the PDE2A cell line. In the absence of a PDE2A inhibitor, luminescence signals stimulated by ANP were greatly

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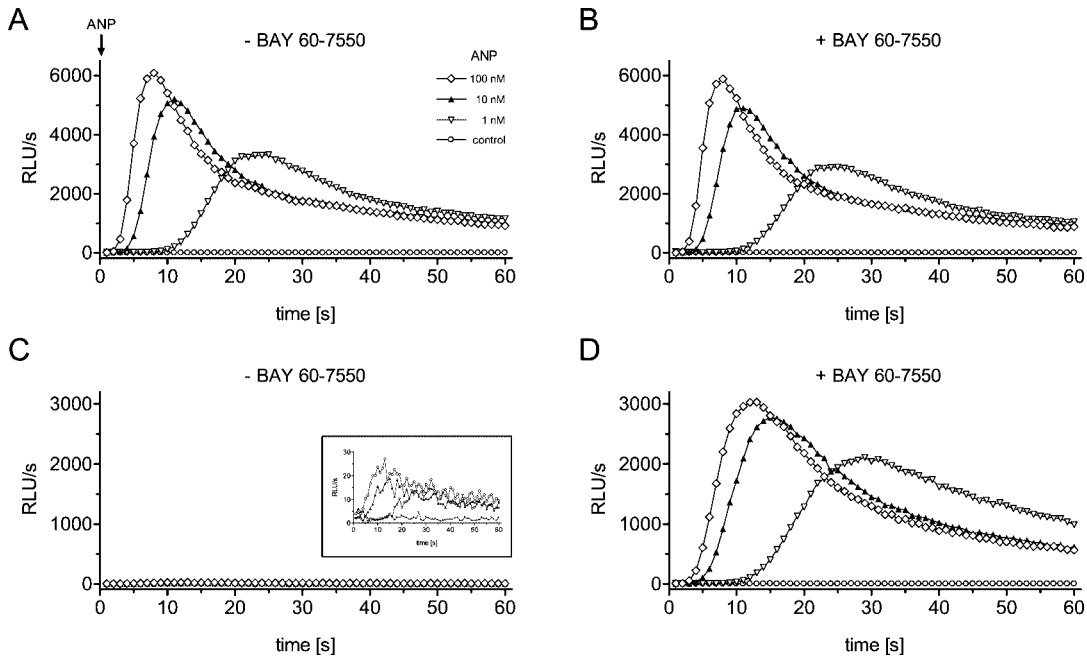

Figure 5. Real-time detection of intracellular cGMP accumulation. Kinetics of the luminescence signals generated by ANP on the parental ANP receptor (A, B) or the PDE2A reporter cell line (C, D) in the presence of 2 mM calcium ions. Cells were stimulated with 0 nM (○), 1 nM (▽), 10 nM (▲), and 100 nM (◇) ANP in the absence (A, C) or the presence (B, D) of 1 μM BAY 60-7550. Inset in (C) shows luminescence signals at higher magnification.

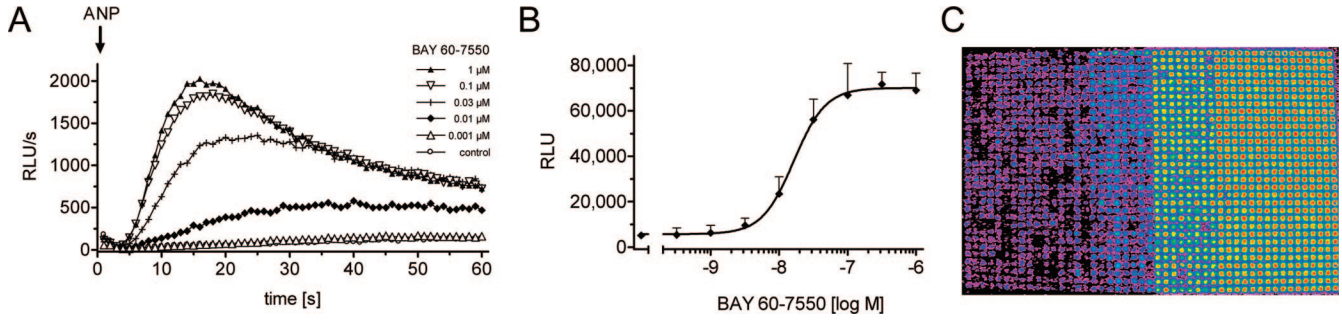

Figure 6. Luminescence measurements on 1536-well MTPs. PDE2A reporter cells were stimulated with 10 nM ANP in the absence (○) or presence of 0.001 μM (△), 0.01 μM (◆), 0.03 μM (+), 0.1 μM (▽) and 1 μM (▲) BAY 60-7550. (B) Corresponding concentration−response curve. (C) Image of a 1536-well MTP after stimulation of the PDE2A reporter cell line with 10 nM ANP in the absence or presence of 0.001−1 μM BAY 60-7550. Data in (B) are presented as means with SD.

diminished (Figure 5C). Only very small responses to ANP with lag times of 14 s (at 0.01 nM) to 3 s (at 100 nM) could be detected (inset in Figure 5C). In contrast, addition of the PDE2A inhibitor BAY 60-7550 (1 μM) greatly enhanced ANP-mediated luminescence signals. As shown in Figure 5D, lag times in the range of 10 s (0.01 nM ANP) to 2 s (100 nM ANP) were observed. At 100 nM ANP concentration, maximum luminescence signals were reached after 12 s.

Luminescence Measurements on 1536-Well MTPs. To evaluate if our PDE2A reporter cell line is suitable for the identification of novel PDE2A inhibitors by uHTS, we also performed luminescence measurements on 1536-well MTPs. Cells were cultured for 24 h at 30 °C in the presence of the aequorin cofactor coelenterazine. The PDE2A inhibitor BAY 60-7550 (0.001−1 μM) was added for 2 min and luminescence measurements were started immediately before the addition of 10 nM ANP. As shown in Figure 6A, ANP stimulated luminescence signals that were concentration-dependently increased by the PDE2A inhibitor. Total luminescence was calculated by integrating the area under the curve from which the corresponding concentration−response curve was derived (Figure 6B). A picture of a 1536-well MTP after stimulation with ANP in the absence or presence of 0.001−1 μM BAY 60-7550 is shown in Figure 6C.

Results from Cellular Transport Studies. In additional experiments, we tried to find a reason for the unexpectedly low cellular activity of PDP. Therefore, we compared the apparent permeabilities (P_{app}) for BAY 60-7550 and PDP across Caco-2 cell monolayers. P_{app} values and calculated efflux ratios for both compounds are shown in Figure 7A. A high apparent permeability value (P_{app}) of 1.30×10^{-5} cm/s and low P_{app} value of 2.69×10^{-7} cm/s for the apical (A) to basal (B) direction was determined for BAY 60-7550 and PDP, respectively. For the B to A orientation, BAY 60-

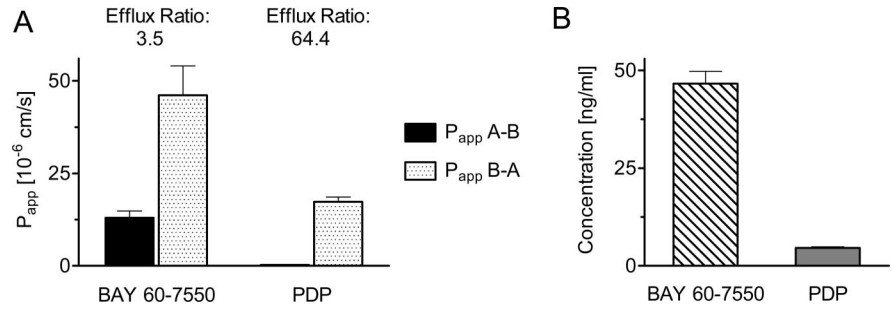

Figure 7. Results from cellular transport studies. (A) P_{app} values and calculated efflux ratios for BAY 60-7550 and PDP from Caco-2 cells. (B) Concentration of BAY 60-7550 and PDP in cell lysates after 5 min incubation (500 nM each) on the PDE2A reporter cell line at 37 °C. Data are presented as means with SD.

7550 and PDP showed P_{app} values of 4.61×10^{-5} cm/s and 1.73×10^{-5} cm/s, respectively. Thus, for PDP a high efflux ratio of 64.4 is calculated, showing that this compound is a substrate for an efflux transporter. This finding was further corroborated by measuring the intracellular concentrations of both inhibitors in the PDE2A reporter cell line. As shown in Figure 7B, treatment of the PDE2A cell line with identical concentrations of BAY 60-7550 and PDP for 5 min (500 nM each) resulted in a 10-fold higher intracellular concentration of BAY 60-7550, compared to PDP (46.6 ± 3.2 vs 4.6 ± 0.3 μg/L). Using LLC-PK1 cells overexpressing the transporter protein P-gp (L-MDR1 cells), we found that PDP and BAY 60-7550 are both P-gp substrates (data not shown). However, due to its higher permeability, BAY 60-7550 can overcome the active efflux and permeate into the cell.

Discussion

It has now become widely accepted that cyclic nucleotide-specific PDEs, by regulating the intracellular levels of cAMP and cGMP, play important roles in many (patho-)physiological processes. Therefore, targeting specific PDEs might offer novel treatment opportunities for different disease states. In recent years, selective inhibitors could be identified for several of the PDE isoenzymes and have been evaluated as potential therapeutics.[1,2,20] Following the identification of EHNA, the first selective, albeit low potency PDE2A inhibitor, Bayer has developed several selective PDE2A inhibitors with higher potency and selectivity. These inhibitors have been reported to inhibit edema formation and to enhance neuronal plasticity and memory performance in animal models.[10,11,19] Therefore, although studies in humans are lacking, selective PDE2A inhibition might become a novel treatment option for sepsis and memory disorders such as Alzheimer's disease.

For some PDE inhibitors a discrepancy between the cellular activity and the *in vitro* activity on the isolated enzyme has been reported.[32,33] Therefore, development of a simple and sensitive assay for the characterization of the cellular activity of PDE inhibitors is highly desirable. In this report we have described a novel approach using our cell-based cGMP reporter assay[26] for the characterization of the cellular activity of PDE2A inhibitors.

In cardiomyocytes, PDE2A was shown to functionally couple to ANP receptors and to exclusively control the subsarcolemmal concentrations of cAMP and cGMP. In addition, PDE5A, in contrast to PDE2A, was shown to control the cytoplasmic cGMP pool synthesized by sGC.[16−18] In accordance to their colocalization and functional interplay in cardiac myocytes, we coexpressed PDE2A with the ANP receptor in our PDE2A reporter cell line, to achieve maximal sensitivity for PDE2A activity and inhibition. For the detection of intracellular cGMP accumulation mediated by ANP receptor stimulation, we used the cGMP sensitive, olfactory CNG channel CNGA2. Homooligomeric CNGA2 channels enable the real-time monitoring of intracellular cGMP production via calcium influx measurements.[26]

We have previously used this assay principle for the identification of sGC activators by automated uHTS, and for the characterization of the cellular activity of PDE9A inhibitors.[26,27] In our PDE9A reporter cell line sGC and PDE9A, two enzymes which are colocalized in the cytosol, were stably coexpressed. As described in our previous report, PDE9A expression in the sGC reporter cell line reduced the ability of sGC activating compounds to stimulate luminescence signals. The addition of PDE9A inhibitors significantly enhanced these luminescence signals and induced leftward shifts of the corresponding concentration−response curves.[27]

The results obtained when using our PDE2A reporter cell line show that the presence of PDE2A had even stronger effects on the luminescence signals induced by ANP receptor activation, which were nearly completely inhibited. In the absence of a PDE2A inhibitor, only very small residual ANP-mediated signals could be detected in our kinetic measurements. Addition of the PDE2A inhibitor BAY 60-7550 enhanced these luminescence signals dramatically (~130-fold). These strong effects might be related to the direct colocalization of PDE2A with the ANP receptor at the

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plasma membrane and to the high levels of PDE2A expression within our reporter cell line. In addition, PDE2A is an enzyme with high V_{max} and low K_m for cGMP hydrolysis and its enzymatic activity is allosterically stimulated by cGMP.[1] As has been shown in cardiac myocytes, this might lead to the observed cyclic nucleotide compartimentalization mediated by PDE2A and may hinder the spreading of cGMP from the microdomains where it is synthesized by the ANP receptor. This in turn might also compromise cGMP detection by the CNG channel, although CNG channels are well suited to detect local changes in cyclic nucleotide levels near the cell membrane.[34]

As shown in this report, our PDE2A reporter cell line enables the characterization of the cellular activity of PDE2A inhibitors. So far, only few selective PDE2A inhibitors have been described. The first published PDE2A inhibitor, albeit with low potency, was EHNA.[9] Later on, BAY 60-7550, PDP, IC933 and oxindole were described as selective PDE2A inhibitors.[10−13] Unfortunately, IC933 and oxindole are not commercially available and, therefore, could not be used in this study.

Using our reporter cell lines we compared the cellular activities of the available PDE2A inhibitors EHNA, BAY 60-7550 and PDP. In control experiments using the parental ANP receptor cell line we could show that all three inhibitors were without effect on the luminescence signals mediated by ANP receptor stimulation. In contrast, when using the PDE2A cell line ANP-mediated luminescence signals were significantly enhanced by the inhibitors, as discussed above. We found that the cellular activities of EHNA and BAY 60-7550 closely mirror their biochemical activities for PDE2A inhibition. We determined slightly higher cellular EC_{50} values compared to the biochemical IC_{50} values. This implies that both inhibitors are highly cell permeable and inhibit cellular PDE2A activity with high efficiency.

In contrast, PDP showed much less cellular activity as anticipated from its biochemical PDE2A inhibitory potency. PDP is the most potent, selective PDE2A inhibitor known so far, with a published IC_{50} value of 0.6 nM for PDE2A inhibition *in vitro*.[11] We have confirmed this IC_{50} value by in-house experiments (A. Tersteegen, personal communication). Despite its higher (~8-fold) *in vitro* PDE2A inhibitory potency compared to BAY 60-7550, the cellular activity of PDP was found to be significantly lower. By cellular transport studies using Caco-2 cells we could show that BAY 60-7550 is highly cell-permeable, whereas the cell-permeability of PDP was found to be low. Comparing the apparent permeabilities in the absorptive and secretory directions, PDP was classified as a strong substrate for an efflux transporter, whereas BAY 60-7550 only shows a weak efflux. These findings were further corroborated by the determination of clearly higher intracellular concentrations of BAY 60-7550 compared to PDP in our PDE2A reporter cell line. These findings are particularly intriguing, since both compounds are very similar in structure. As a result of the low permeability of PDP, the rank order of the cellular activity of the PDE2A inhibitors is BAY 60-7550 > PDP > EHNA, which is different from the rank order of PDE2A inhibitory activity *in vitro* (PDP > BAY 60-7550 > EHNA).

In additional experiments, we could show that the nonselective PDE inhibitor IBMX, the PDE3A inhibitor trequinsin and the PDE5A inhibitor dipyridamole, which inhibit PDE2A nonselectively at micromolar concentrations, were also able to stimulate luminescence signals in the presence of ANP. The observed EC_{50} values using our PDE2A cell line are in the same range, being about 2−3-fold higher, compared to the respective IC_{50} values for PDE2A inhibition *in vitro*. Therefore, it is highly likely that these PDE inhibitors stimulate luminescence signals due to their PDE2A inhibitory activity and not due to inhibition of other PDEs.

We also tested the effects of additional PDE inhibitors, including vinpocetine, milrinone, rolipram, sildenafil, zaprinast, BRL 50481 and BAY 73-6691. These PDE inhibitors were not able to stimulate significant luminescence signals on our two reporter cell lines in the presence of ANP. Therefore, the inhibition of endogenous PDEs by these inhibitors is not sufficient to significantly amplify ANP-mediated signals. This might be related to the very low endogenous expression of most PDEs, with the exception of PDE7A, for which moderate expression levels could be detected. In addition, our reporter cell lines, which are specifically designed for the detection of intracellular cGMP, are not well suited for monitoring the inhibition of cAMP-specific PDEs like PDE7A, which would result in cAMP accumulation.

Taken together, the results of the pharmacological characterization of our PDE2A reporter cell line using different selective as well as nonselective PDE inhibitors imply that the cell line specifically monitors PDE2A inhibition and is a useful tool for the characterization of the cellular activity of PDE2A inhibitors.

In addition, we have shown that the PDE2A reporter assay can be successfully transferred to the 1536-well MTP format with minor variations. Therefore, this reporter cell line provides a further example for the implementation of the CNG channel assay technology in an uHTS format.[26,27,35,36] In summary, the results presented in this report show that our novel reporter assay is well suited for the characterization

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of PDE2A pharmacology, but can also be used for drug discovery by uHTS.

Abbreviations Used

ANP, atrial natriuretic peptide; CNG, cyclic nucleotide-gated; MTP, microtiter plate; PDE, phosphodiesterase; RLU, relative light unit; uHTS, ultrahigh-throughput screening.

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