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Differential inhibitor sensitivity between human recombinant and native photoreceptor cGMP-phosphodiesterases (PDE6s)

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Abstract

Human photoreceptor cGMP-phosphodiesterases (PDE6s) are important reagents in PDE inhibitor discovery. However, recombinant human PDE6s have not been expressed, and isolation of native human PDE6s is highly difficult. In this study, the catalytic subunit(s) of human rod and cone PDE6s (PDE6αβ and PDE6α′, respectively) were co-expressed or expressed separately as catalytically active enzymes. Sildenafil inhibited both the recombinant PDE6s in a dose-dependent manner with K_i values of 94 and 98 nM, respectively. These K_i values were four-fold higher than that (25 nM) of a human native PDE6 preparation. Similarly, 3-isobutyl-1-methylxanthine (IBMX)'s K_i values for the recombinant PDE6s were five- to eight-fold higher than that of the native enzyme. However, E4021 and zaprinast exhibited much (30–80-fold) lower potencies for the recombinant PDE6s than for the native enzyme. Additional PDE5 inhibitors representing other structural classes and possessing different selectivity against native PDE6 also showed different potencies against the recombinant and native PDE6s. In particular, one class of xanthine analogues exhibited significantly (5–15-fold) higher potencies for the recombinant PDE6s than for the native enzyme. Our data demonstrates that the recombinant and native PDE6s exhibit differential sensitivity to inhibitors, and cautions the use of recombinant catalytic subunits of PDE6 in drug discovery or in structural/functional studies.

Keywords: Phosphodiesterase; PDE6; Human; Recombinant; Inhibitor; Sildenafil

1. Introduction

Photoreceptor cGMP-phosphodiesterases (PDE6s) are key enzymes in the vertebrate visual transduction mediated by the rhodopsin-couple G protein, transducin [1–3]. The vertebrate retina is composed of rod and cone photoreceptor cells containing rod and cone PDE6s, respectively. Rod photoreceptors are much more sensitive to light stimulation than cones, but cones have a faster response/recovery time, being responsible mainly for adapting to a greater range of light intensities than rods [4–6]. The population of PDEs in the photoreceptors is almost exclusively restricted to the PDE6 family. The hydrolysis of cGMP by the PDE6s is the final step of signal amplification in the retinal phototransduction cascades.

Rod and cone PDE6s are biochemically highly related but distinct enzymes. The catalytic portion of rod PDE6 consists of an α subunit and a β subunit (PDE6α and PDE6β, respectively, both with a molecular mass of 99 kDa), that are tightly associated with two small identical γ subunits (11 kDa) acting as an internal enzyme inhibitor [7–9]. Cone PDE6 is composed of catalytic homodimeric α′ subunits (PDE6α′, also with a molecular mass of 99 kDa), and two or three small inhibitory subunits (13–15 kDa) [10,11]. The key role of these smaller subunits in both rod and cone is to inhibit cGMP hydrolysis by the catalytic subunits in the dark. Upon light stimulation of photoreceptors, PDE6s are stimulated through removal of these small inhibitory subunits by activated GTP-bound transducin [12].

PDE6α, PDE6β and PDE6α′ are encoded by different genes, and their cDNAs have been cloned in human and many other species [13–18]. Comparison of the deduced amino acid sequences reveals that the homology among PDE6α, PDE6β and PDE6α′ is as high as about 62–72%, and that the PDE6 sequences and subunit structure are well-conserved among different species [1,19]. The PDE6 family is most closely related to PDE5 family, as judged by

Abbreviations: PDE, phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine

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several criteria: (1) a high homology (45–48%) between their catalytic domains; (2) cGMP specificity; and (3) inhibitor sensitivity [20–23].

Several PDEs are important drug targets for the treatment of many diseases, such as PDE5 for erectile dysfunction [24] and PDE4 for inflammation [25]. PDE selectivity has been an issue in the development of PDE inhibitors as therapeutic agents. In particular, the PDE5 inhibitor sildenafil (VIAGRA™) also inhibits PDE6, resulting in the visual side effects [24,26]. Thus, for PDE selectivity evaluation in drug discovery, human PDE6 is an important reagent. However, large scale purification of native human PDE6 is highly difficult due to the limited availability of the retina. Moreover, in-depth structural/functional studies on PDE6 in any species requires the development of an effective system for expression of the recombinant proteins. There have been several studies on expression of catalytically active mouse or bovine recombinant PDE6 [27–29]. However, the comparative inhibitor sensitivity between the recombinant and native PDE6s has not been known, and there has been no report on functional expression of human PDE6.

In this study, we expressed catalytically active human rod PDE6αβ and cone PDE6α′ using the baculovirus/insect cell expression system. Unexpectedly, these recombinant enzymes exhibited inhibitor sensitivity that was significantly different from that of native human PDE6. Our data cautions the use of recombinant catalytic subunits of PDE6 in structural/functional studies or in drug discovery.

2. Materials and methods

2.1. Cloning of human rod PDE6 α and β subunits and cone PDE6α′

Both human rod PDE6 α and β cDNAs were amplified by PCR using human retina QUICK-Clone cDNA (Clontech) as template, based on the published α [17] and β [13] cDNA sequences. The forward and reverse primers for PDE6α were as follows: 5′-AGC AGA TAT CCA ATG GGC GAG GTG ACA GCA-3′ and 5′-GGA TCT AGA CTC TTA CTG GAT GCA GCA GGA CTT GGA-3′, respectively. The forward primer contained an *Eco*RV restriction enzyme site, while the reverse primer contained an *Xba*I site. For PDE6β, the forward primer containing a *Bam*HI site and the Kozak consensus sequence was 5′-TGC AGA TAT CCA ATG AGC CTC AGT-3′, and the reverse primer containing an *Xba*I site was 5′-GGA TCT AGA CTC TCA CAG GAT ACA GCA GGT TGA AGA CT-3′. The PCRs were carried out by the touchdown technique as described [30]. The PCR products were cloned into pcDNA3.1(+) vector (Invitrogen), followed by double-strand sequencing for confirmation. PCR-derived mutations were corrected using the QuickChange XL site-directed mutagenesis kit (Stratagene), and the correct sequence was further confirmed by double-strand sequencing.

Two parts of human cone PDE6α′ were amplified separately from human retina Marathon Ready cDNA (Clontech) by PCR based upon the published cDNA sequence [16], and then assembled together as a whole cDNA by DNA ligation. The first part, an 1154-bp fragment, between the nucleotide positions 131 and 1285 was amplified using a pair of primers (forward primer: 5′-GCC ACA CCA TGG GTG AGA TCA ACC-3′; and reverse primer: 5′-CCC AAC CAG TTT CGT CTA CAG-3′). The second part, an 1631-bp fragment, between the nucleotide positions 1248 and 2879 was amplified using another pair of primers (forward primer: 5′-CAC ATT TCC GAA AGG ACC TG-3′; and reverse primer: 5′-TTG CCC CTC GAG TTT TTG GGC TAG C-3′ containing an *Xho*I site). PCR conditions used were as follows: 94 °C for 1 min; 35 cycles of 94 °C for 30 s, 58 °C for 1 min, and 68 °C for 3 min; and finally 68 °C for 10 min, using Advantage cDNA PCR kit (Clontech). The PCR fragments were cloned into a pCR-Script SK vector using a PCR cloning kit (both from Stratagene), followed by double-strand sequencing for confirmation. PCR-derived mutations were corrected as above, and the correct sequence was further confirmed by double-strand sequencing. The two cDNA fragments were assembled together during subsequent construction of the recombinant baculovirus expression vector of cone PDE6α′.

2.2. Construction of recombinant pFASTBAC donor vectors and generation of recombinant baculoviruses

pFASTBAC1 vector (Invitrogen) was used to generate the human rod PDE6α and PDE6β as well as cone PDE6α′ recombinant donor vectors. The 2.6 kb cDNA encoding full-length rod PDE6α was released from the pcDNA3.1(+) vector by digestion with the restriction enzymes (*Bam*HI and *Xba*I), and then inserted into the multiple cloning site region of *Bam*HI/*Xba*I-digested pFASTBAC. This donor vector was used to generate the corresponding recombinant baculovirus. The 2.6 kb cDNA encoding full-length rod PDE6β was also cloned into a pFASTBAC vector as described above. To clone cone PDE6α′ cDNA into a pFASTBAC vector, the 1.1 kb cDNA fragment encoding the N-terminal portion of cone PDE6α′ was released from the pCR-Script SK vector by digestion with *Bam*HI and *Acc*I, while the 1.6 kb cDNA encoding the C-terminal portion of cone PDE6α′ was released from the vector by digestion with *Acc*I and *Not*I. These two cone PDE6α′ cDNA fragments were ligated together and inserted into *Bam*HI/*Not*I-digested pFASTBAC, thereby generating a baculovirus donor vector.

The recombinant rod PDE6α and PDE6β as well as cone PDE6α′ baculoviruses were generated using the BAC-TO-BAC baculovirus expression system (Invitrogen). The three recombinant baculoviruses were all confirmed by PCR using conditions described below.

2.3. Expression of recombinant human rod and cone PDE6 enzymes

The procedures for optimal expression of catalytically active rod PDE6αβ were as follows. 5×10^6 Sf9 cells in 10 ml of Sf-900 II serum-free medium (Invitrogen) were seeded in a 100-mm plate, and the cells were allowed to adhere to the plate for 1 h. After removing the medium, rod PDE6 α and β baculoviruses at 18 and 23 multiplicity of infection (MOI), respectively, were incubated with the adhered cells for 1 h at room temperature. The cells were incubated for 4 days following the addition of 17 ml of Sf-900 II medium. After removing the medium, the cells were harvested in 10 mM Tris–HCl buffer (pH 7.5) containing Protease Inhibitor Cocktail (Boehringer Mannheim) by scraping, followed by centrifugation at $350 \times g$ for 5 min. The cell pellet was resuspended in 0.5 ml of lysis buffer (10 mM Tris–HCl, pH7.5, containing Protease Inhibitor Cocktail), and then disrupted by sonicating twice (10 s each) on ice. The cell lysate was used directly for PDE6 assay.

For optimal expression of catalytically active cone PDE6α′, the conditions were essentially the same as described above except that the MOI used was 5.

Protein concentrations were determined using the Bio-Rad protein assay kit with BSA as the standard.

2.4. RNA isolation and RT-PCR analysis

Total RNA isolation and cDNA synthesis were performed as described [31]. For analysis of the expression of rod PDE6 α and β mRNAs, the following primer sets were used: PDE6α, forward primer: 5′-CCC AAC ACA GAG GAG GAT GA-3′, and reverse primer: 5′-GCA ACA TAA GCG GGG AGA C-3′ (covering a region in PDE6α between the nucleotide positions 460 and 1034, [17]); and PDE6β, forward primer: 5′-ATG AGC CTC AGT GAG GAG CAG-3′, and reverse primer 5′-TCC TGT TCG TCA AAG GGC TTC-3′ (covering a region in PDE6β between the nucleotide positions 1 and 1221, [13]). For analysis of the expression of cone PDE6α′ mRNA, the primer set used was that for cloning the first part of the PDE6α′ cDNA (described above). PCR conditions were as follows: 94 °C for 1 min; 35 cycles of 94 °C for 30 s, 58 °C for 1 min then 68 °C for 3 min; and finally 68 °C for 3 min.

2.5. SDS–PAGE and immunoblotting

SDS–PAGE was performed in 12% mini-gels. For immunoblotting, proteins were transferred to nitrocellulose membrane and analyzed using a rabbit anti-bovine PDE6 polyclonal antibody (CytoSignal). The antibody-antigen complexes were detected using horseradish peroxidase-conjugated anti-rabbit IgG and ELC reagent (both from Amersham Pharmacia Biotech).

2.6. Isolation of native human PDE5 and PDE6 proteins

Human native PDE5 from corpus cavernosum [32] and PDE6 from retina [33] were isolated by ion-exchange chromatography as described.

2.7. Assays for PDE5 and PDE6

The PDE5 assay was performed in duplicate using Amersham Pharmacia Biotech's [^{3}H]cGMP PDE SPA assay kit at a final concentration of unlabeled cGMP of 0.5 μM. The PDE6 assay was performed under the same conditions as those for PDE5, except that 0.2 mg/ml of histone was also present. Immediately before assay, the PDE sample was diluted appropriately with an enzyme diluent (10 mM Tris–HCl, pH 7.4, 1% BSA) so that less than 10% of the substrate was hydrolyzed. The assays were performed at 30 °C for 30 min. Inhibitors were dissolved in 100% DMSO at 1 or 10 mM, then diluted with water. The final concentration of DMSO in the assay mixture was 1%.

Sildenafil was extracted from VIAGRA™ tablets as described previously [32]. E4021 was synthesized in this Institute, while zaprinast and 3-isobutyl-1-methylxanthine (IBMX) were obtained from BIOMOL.

3. Results

3.1. Expression of recombinant human rod PDE6αβ and cone PDE6α′

In order to express human rod PDE6 α and β proteins, Sf9 cells were infected separately or co-infected with appropriate recombinant baculovirus(es). To demonstrate the expression of these proteins in the infected cells, rod PDE6 α and β mRNAs were first analyzed by RT-PCR using respective gene-specific primers. The 574-bp cDNA fragment of rod PDE6α was detected from cells infected with rod PDE6α baculovirus or co-infected with rod PDE6 α and β baculoviruses, but not with wild type baculovirus or rod PDE6β baculovirus (Fig. 1A). Similarly, the rod PDE6β cDNA fragment (1220-bp) was identified from cells infected with PDE6β baculovirus or co-infected with the two different viruses, but not with the PDE6α virus alone (Fig. 1A).

To demonstrate the expression of these rod PDE6 subunits at the protein level, the infected Sf9 cells were analyzed by immunoblotting using an anti-PDE6 polyclonal antibody. Proteins with the expected molecular mass of rod PDE6 α and β subunits were detected from cells infected with appropriate recombinant bacoluvirus(es), but not from wild type virus (Fig. 2A).

Similar experiments were performed to demonstrate the expression of cone PDE6α′. The cDNA fragment (1154-bp) as well as the protein with the expected

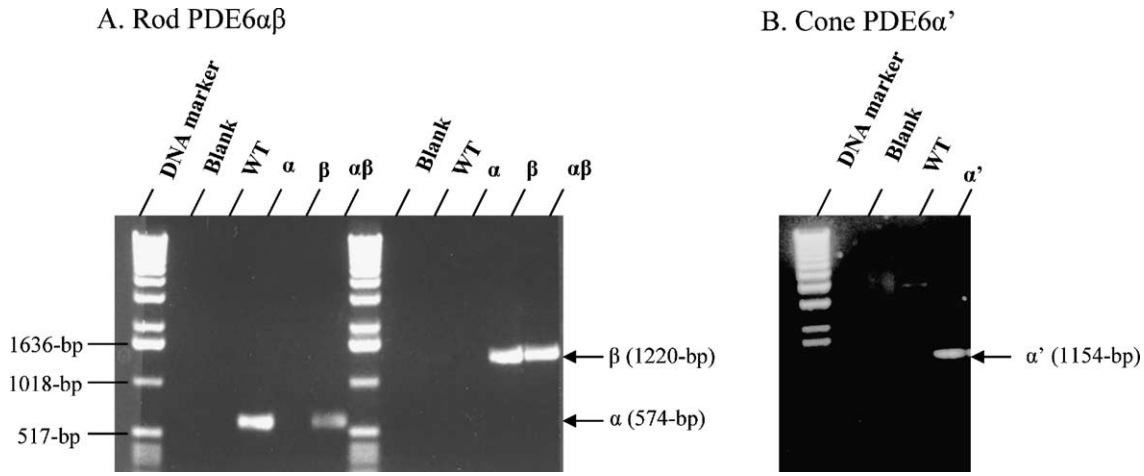

Fig. 1. RT-PCR analysis of human rod PDE6αβ (A) and cone PDE6α′ (B) expression in Sf9 cells. DNA marker: 1 kb DNA ladder obtained from Invitrogen. Blank was a reaction containing no cDNA template. WT, α, β, αβ, and α′ indicate analyzed cDNA samples from Sf9 cells infected with wild type, recombinant rod PDE6 α, β, both αβ, and cone PDE6α′ baculoviruses, respectively.

molecular mass of cone PDE6α′ were observed from cells infected with cone PDE6α′ baculovirus, but not with wild type virus (Figs. 1B and 2B).

3.2. Enzyme activity of recombinant human rod PDE6αβ and cone PDE6α′

PDE activity of the expressed proteins was assayed using cellular lysates of the infected Sf9 cells. A basal level of cGMP-hydrolyzing activity (about 25 pmol/min/mg protein) was observed from cells infected with wild type baculovirus (Fig. 3A). Only a minimal increase in cGMP-hydrolyzing activity over the basal level was detected from cells infected with either the rod PDE6 α or β recombinant baculovirus (data not shown). Co-infection with both the recombinant baculoviruses resulted in a four-fold increase in the enzyme activity (Fig. 3A). The cGMP-hydrolyzing activity of the recombinant cone PDE6α′ was evaluated similarly. As shown in Fig. 3B, the expression of cone PDE6α′ resulted in an about eight-fold increase in cGMP-hydrolyzing activity over the wild type control cells.

3.3. Inhibitor sensitivity of recombinant human rod PDE6αβ and cone PDE6α′ in comparison with that of native human PDE6

To compare the recombinant human rod PDE6αβ and cone PDE6α′ with native human PDE6, we first evaluated the sensitivity of these three different enzyme preparations to sildenafil. Our native PDE6 preparation presumably contained both rod and cone PDE6s [33], since we did

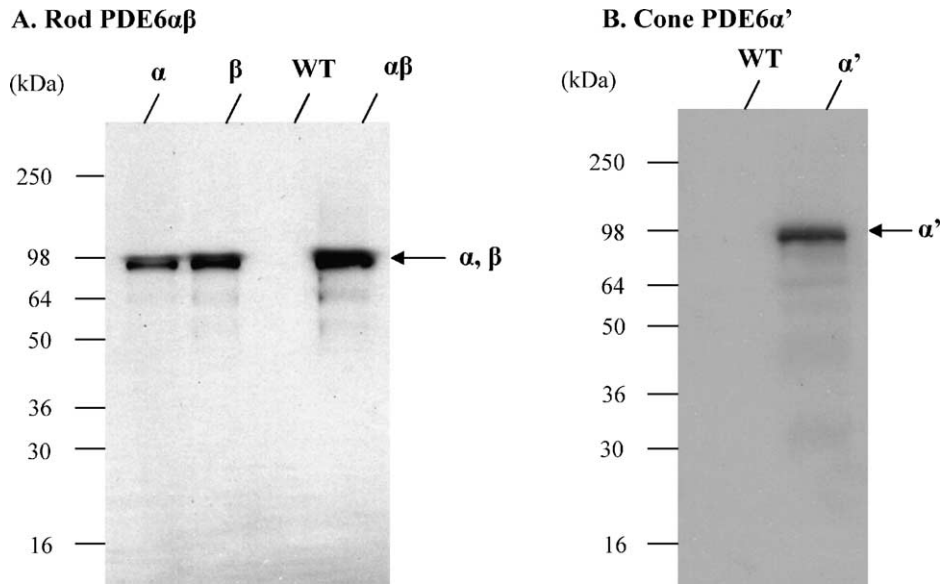

Fig. 2. Immunoblotting analysis of recombinant human rod PDE6αβ (A) and cone PDE6α′ (B) proteins expressed in Sf9 cells. WT, α, β, αβ, and α′ indicate analyzed cellular samples from Sf9 cells infected with wild type, recombinant rod PDE6 α, β, both αβ, and cone PDE6α′ baculoviruses, respectively.

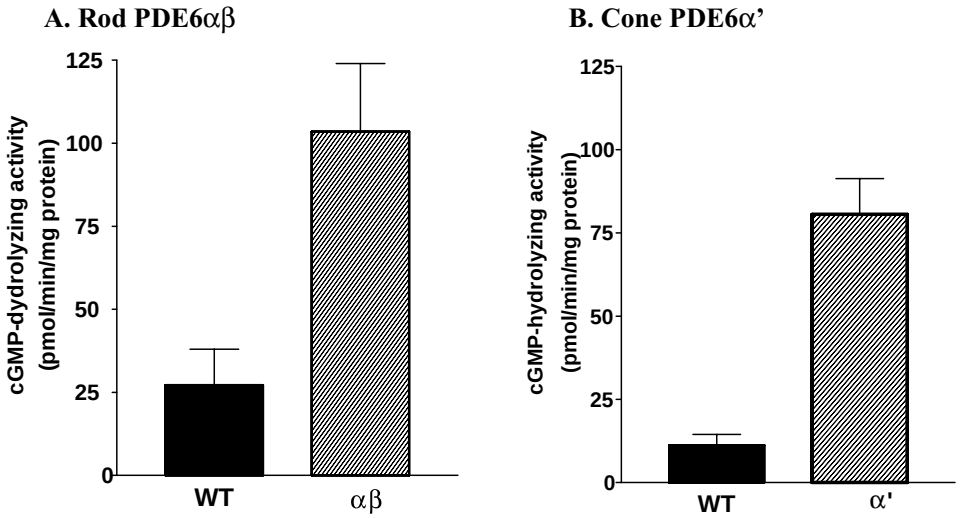

Fig. 3. cGMP-hydrolyzing activity of recombinant human rod PDE6αβ (A) and cone PDE6α′ (B). The results were from three typical experiments (mean ± S.E.M.). WT, αβ, and α′ indicate analyzed cellular samples from Sf9 cells infected with wild type, recombinant rod PDE6αβ (co-infection), and cone PDE6α′ baculoviruses, respectively.

not have sufficient amount of human retina for further separation between the rod and cone enzymes. Nevertheless, structurally distinct inhibitors have exhibited similar sensitivities between human native rod and cone PDE6s [26]. As shown in Fig. 4, sildenafil inhibited all the three enzyme preparations in a dose-dependent manner. The two recombinant enzymes exhibited similar K_i values for sildenafil. However, the K_i values of the recombinant enzymes were four-fold higher than that of the native PDE6 (Table 1). More surprisingly, E4021 (another PDE5 inhibitor known to have a potent inhibitory activity on native PDE6, [21]) inhibited the recombinant enzymes with K_i values that were 30 up to 50-fold higher than that for the native PDE6.

Two other benchmark PDE inhibitors, IBMX (a non-selective PDE inhibitor) and zaprinast (a dual PDE5/6 inhibitor) [34], were also used to compare inhibitor profiles of the human recombinant and native PDE6s. Like sildenafil, IBMX's K_i values for the recombinant PDE6s were

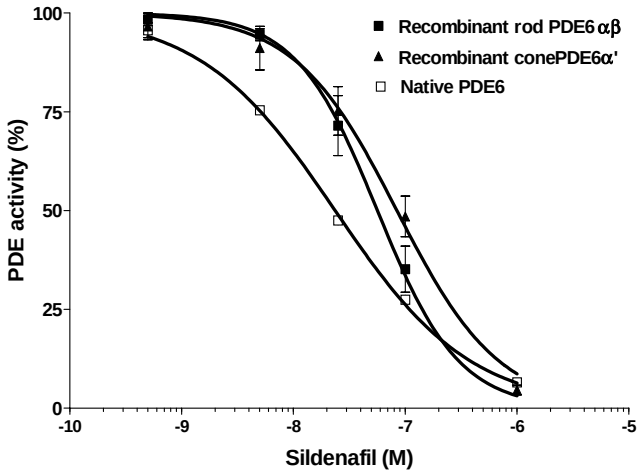

Fig. 4. Inhibition of human recombinant rod PDE6αβ and cone PDE6α′ and native PDE6 by sildenfil. The data were from three typical experiments (mean ± S.E.M.). The calculated K_i values are summarized in Table 1.

Table 1
Inhibitory effects of various PDE5/6 inhibitors on human native PDE6 and recombinant rod PDE6αβ and cone PDE6α′

Inhibitor	K_i ± S.D. (nM)			
	PDE5	Native PDE6	Recombinant rod PDE6αβ	Recombinant cone PDE6α′
Sildenafil	2.2 ± 0.1	24.5 ± 1.5	93.6 ± 11.7	98.4 ± 2.9
E4021	13.2 ± 0.2	3.5 ± 0.5	101.4 ± 20.5	173.5 ± 7.8
Zaprinast	204.3 ± 12.3	177.8 ± 15.8	14376.2 ± 1228.1	11452.2 ± 1023.4
IBMX	3547.7 ± 91.1	12118.5 ± 1066.7	60838.2 ± 1257.3	94853.8 ± 1247.6
Compound A	4.3 ± 0.3	176.3 ± 23.2	361.6 ± 7.8	660.8 ± 81.9
Compound B	5.4 ± 1.8	17.3 ± 0.5	1205.7 ± 131.6	1605.3 ± 206.6
Compound C	10.2 ± 2.4	18.3 ± 0.5	2.6 ± 0.3	3.6 ± 0.6
Compound D	8.9 ± 1.1	721.5 ± 166.4	48.7 ± 2.9	54.6 ± 3.4

The data were from at least two experiments. K_i values were calculated using the Cheng–Prusoff equation [41]. Chemical names of compounds A–D: A, 2-bromo-5-ethyl-7,8-dihydro-1-[(4-hydroxyphenyl)methyl]-7(*R*)-(phenylmethyl)-1*H*-imidazo[2,1-*b*]purin-4(5*H*)-one; B, 5-ethyl-7,8-dihydro-2,7(*R*)-bis(phenylmethyl)-1*H*-imidazo[2,1-b]purin-4(5*H*)-one; C, 8-bromo-1-ethyl-3,7-dihydro-7-[(4-methoxyphenyl)methyl]-3-(2-methylpropyl)-1*H*-purine-2,6-dione; and D, 3,7-dihydro-8-[(1-hydroxymethyl)-3-cyclopenten-1-yl]amino-7-[(4-methoxyphenyl)methyl]-1,3-dimethyl-1*H*-purine-2,6-dione.

five- to eight-fold higher than that of the native enzyme. On the other hand, zaprinast was similar to E4021 in that it exhibited much (30–80-fold) lower potencies for the recombinant enzymes than for the native PDE6 (Table 1).

To further confirm the differential sensitivity of inhibitors to the recombinant and native PDE6s, the sensitivity to four additional PDE5 inhibitors (compounds A–D in Table 1) with different structures and selectivity against native human PDE6 was also evaluated. Compounds A and B belong to the structural class of cyclic guanine [35], while compounds C and D are from a particular xanthine-containing structural class [36]. All these compounds distinguished between the recombinant and native enzymes to various degrees, from 2- or 4-fold (compound A) up to about 90-fold (compound B) differences. Interestingly, the different structural classes exhibited differential sensitivities between the recombinant and native enzymes. Thus, the cyclic guanine compounds were more potent against the native PDE6 than against the recombinant enzymes, whereas the xanthine-containing inhibitors were relatively more potent against the recombinant PDE6s.

4. Discussion

Expression of recombinant mouse rod PDE6αβ [29], mouse/bovine rod PDE6αβ [27] and bovine cone PDE6α′ [28] has been reported. Although the recombinant PDE6 proteins were catalytically active, they were not characterized. For instance, it is not known whether recombinant and native PDE6s have similar enzymatic properties including inhibitor sensitivity. In this study, catalytically active recombinant human rod PDE6αβ and cone PDE6α′ were expressed for the first time. Efficient expression of recombinant human PDE6 may be particularly important, since large scale isolation of native human PDE6 is highly difficult due to the limited availability of the retina. However, our study reveals that the inhibitor sensitivity of the recombinant human rod PDE6αβ and cone PDE6α′ is significantly different from that of native human PDE6. Our data, hence, questions the appropriateness of using recombinant rod PDE6αβ or cone PDE6α′ for in-depth structural/functional studies or for drug discovery.

The inhibitors tested, sildenafil [24], E4021 [35], IBMX, zaprinast [20], compounds A and B (cyclic guanine compounds [35]), and compounds C and D (from a particular xanthine-containing class [36]) represent six different structural classes. The differential sensitivities of recombinant and native enzymes to structurally distinct inhibitors suggest that the recombinant and native proteins may assume different conformations. The change of the recombinant PDE6s in inhibitor sensitivity might be due to that the small PDE6 subunit (γ or γ′ for rod or cone PDE6, respectively) was not co-expressed. Qin and Baehr [29] reported that co-expression of murine PDE6αβγ in the insect cells gave rise to a higher enzyme activity than that of PDE6αβ, despite the expression of the γ subunit being very low compared to α and β subunits. Their study suggests that the presence of the γ subunit may affect the conformation and/or processing of the PDE6αβ, and that in the insect cells there may not be a mammalian PDE6γ-like endogenous protein. It would be interesting to see, in future studies, whether recombinant PDE6αβγ and PDE6α′β′ have similar enzymatic properties to those of the respective native PDE6s.

Another possible reason for the changed inhibitor sensitivity was expression of the enzymes in the insect cells. PDE6s are known to undergo multiple biologically important post-translational modifications [29]. It is conceivable that PDE6s expressed in insect and human cells may undergo differential post-translational modifications. Although bovine PDE6α/murine PDE6β [27] and bovine PDE6α′ [28] were expressed in human cells (HEK293), no evaluation of their inhibitor profile has been reported. Whether human recombinant PDE6s expressed in human cells have inhibitor profiles similar to that of native PDE6 remains to be determined.

Inhibition of PDE6 results in the troublesome visual side effects exhibited by VIAGRA™ [24,26]. Moreover, it has been reported that PDE6 inhibition results in rod and cone death [37], and that certain PDE6 mutations lead to blindness in the *rd* mouse [38] or night blindness in humans [39]. The use of PDE5 inhibitors for other indications such as pulmonary hypertension [40] and the development of PDE5 inhibitors with long plasma half-lives such as tadalafil (CIALIS™, [26]) further necessitate clinical use of PDE5 inhibitors with high selectivity against human PDE6. For selectivity evaluation in PDE5 inhibitor discovery, the present study strongly supports the use of native human PDE6.

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