Turunen, Teemu; Koskelainen, Ari

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Electrophysiological determination of phosphodiesterase-6 inhibitor inhibition constants in intact mouse retina

Teemu T. Turunen, Ari Koskelainen

Department of Neuroscience and Biomedical Engineering, Aalto University School of Science, P.O. Box 12200, FI-00076 Aalto, Finland

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A B S T R A C T

Cyclic nucleotide phosphodiesterases (PDEs) hydrolyze the second messengers cAMP and cGMP. PDEs control numerous cellular processes making them promising targets for the development of therapeutic agents. Unfortunately, many PDE inhibitor molecules are non-selective among PDE classes and efficient methods for quantitative studies on the isoform-specificity of PDE inhibitors in the natural environments of PDEs are unavailable. The PDE in photoreceptors, PDE6, mediates the conversion of photon information into electrical signals making the retina an exceptional model system for examinations of the pharmacological effects of PDE inhibitors on PDE6. Here we introduce electroretinography-based methods for determining the inhibition constants of PDE inhibitors towards the naturally occurring light-activated and spontaneously activated forms of PDE6. We compare our results to earlier biochemical determinations with trypsin-activated PDE6 with disintegrated PDE6 γ-subunit. The potencies of PDE inhibitors were determined by stimulating the photoreceptors of isolated mouse retinas with light and tracking the inhibitor-induced changes in their electrical responses. The methods were tested with three PDE inhibitors, 3-isobutyl-1-methylxanthine (IBMX), sildenafil, and zaprinast. The inhibition constants towards light-activated, spontaneously activated, and trypsin-activated PDE6 differed significantly from each other for sildenafil and zaprinast but were closely similar for IBMX. We hypothesize that this is due to the ability of the PDE6 γ-subunit to compete with sildenafil and zaprinast from the same binding sites near the catalytic domain of PDE6. The introduced methods are beneficial both for selecting potent molecules for PDE6 inhibition and for testing the drugs targeted at other PDE isoforms against adverse effects on visual function.

1. Introduction

Vertebrate cyclic nucleotide phosphodiesterases (PDEs) form a superfamily of enzymes that contains 11 related gene families (PDE classes 1–11) coding a vast number (~100) of protein isoforms (Ahmad et al., 2015). PDEs regulate the level of cyclic nucleotide second messengers in cells by catalyzing their hydrolysis. The high therapeutic value of PDE inhibitors has long been recognized because PDEs take part in almost every regulatory system in the body (Lugnier, 2006), and PDE inhibitors are used for pharmacological treatments of various disorders such as erectile dysfunction, congestive heart failure, and inflammatory airway disease (Boswell-Smith et al., 2006; Essayan, 1999; Tenor et al., 2011). However, the structural similarity of the catalytic domains among the PDE classes results in poor specificity of these PDE inhibitor drugs and they have been reported to cause several side effects including hearing impairment and increased sensitivity to light (Boswell-Smith et al., 2006; Kerr and Danesh-Meyer, 2009; Khan et al., 2011). The pharmaceutical interest in PDE inhibitors has recently revived due to advances in understanding the structural and functional properties of the PDEs. The development of truly isoform-specific PDE targeting drugs could help treat a broad range of diseases (Ahmad et al., 2015; Maurice et al., 2014).

Photoreceptor PDE (PDE6) expressed in the retina has been shown to be non-discriminant towards many so-called “specific” PDE inhibitor drugs that effectively inhibit PDE6 in addition to their target PDE isoform (Zhang et al., 2005). The potency of PDE inhibitors towards PDE6 is typically determined biochemically in vitro from purified or...
recombinant PDE6. These approaches, while intrinsically very specific, have several drawbacks. Typically, the experiments are conducted in conditions that lack the native cellular environment of PDE6. In addition, the sensitivity to inhibitors may differ between the native and the recombinant PDE (Zhang et al., 2004). Further, the inhibition constant of a PDE6 inhibiting molecule is commonly determined using trypsin-activated PDE6 from which the inhibitory γ-subunits are irreversibly dissociated with trypsin.

In their native environment, PDE6 molecules are activated in two ways, spontaneously due to thermal energy or in a light-induced process called phototransduction. Phototransduction begins when a photon is absorbed by a G protein-coupled receptor, rhodopsin, in the photoreceptor outer segment. Activated rhodopsin activates G proteins, transducins, the α-subunits (Gt,α) of which bind to and displace the inhibitory PDE6 γ-subunits, and thereby reveal the catalytic sites of PDE6. This activation of PDE6 allows the enzyme to catalyze the hydrolysis of cGMP nearly at diffusion limited rate (Reingruber et al., 2013). Deactivation of the enzyme takes place when the Gt,α dissociates from PDE6. This dissociation is catalyzed by the GTPase-accelerating protein complex (for a review, see e.g. Fu and Yau, 2007). In contrast to physiological activation of PDE6, the trypsin-activation causes permanent change in the PDE6 structure and full activation of the enzyme. At present, there are no methods for quantitative investigations of the inhibition potency of drugs towards the light-activated PDE6 in functional mammalian photoreceptors. An ex vivo or in vivo method, where structurally intact PDE6 molecules are in their native environment and the PDE6 activity can be controlled in a physiologically relevant manner, is needed to quantify the physiological effect of PDE inhibitors on the photoreceptors and vision.

In this study, we introduce two methods for determining the inhibition efficiency of drugs towards rod PDE6 in intact photoreceptor cells. In the methods, we stimulate an isolated retina with light and quantify the PDE inhibitor-induced effect from the resulting ex vivo electroretinogram (ERG) signal. The first method is applied for light-activated PDE6 and it is based on the ability of PDE inhibitors to decrease the molecular gain of phototransduction. In the second method, the PDE inhibitor efficiency is determined towards spontaneously activated PDE6 by defining the increase in the ERG signal amplitudes caused by the inhibitor-induced growth in intracellular cGMP concentration. The methods were tested with three known PDE inhibitors: the non-selective 3-isobutyl-1-methylxanthine (IBMX), the selective PDE5 inhibitor sildenafil, and the less selective PDE5 inhibitor zaprinast. These model drugs were assumed to function as competitive PDE6 inhibitors that block the entry of cGMP to the catalytic pocket of PDE6 (Cobbs, 1991; Gillespie and Beavo, 1989; Simon et al., 2006). We found significant differences between the inhibition constants towards light-activated and spontaneously activated PDE6 but also between our values and the values published previously using in vitro methods (D’Amours et al., 1999; Zhang et al., 2004; Zhang et al., 2005). We hypothesize that these differences are caused by the interaction of the drug molecules with the PDE6 γ-subunit, which is relocated during the light-activation of PDE6 but completely dismantled in vitro studies. The introduced methods enable quantitative examination of the effectiveness of PDE inhibitor molecules and the possible inhibitory effects of other drugs on PDE6 in its natural environment, inside the photoreceptor cells in the living retina.

2. Methods

2.1. Experiments

2.1.1. Ethical approval

The use and handling of the animals were in accordance with the Finland Animal Welfare Act 2006, guidelines of the Animal Experiment Board in Finland, and with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

2.1.2. Electroretinography

Electroretinography (ERG) registers light-induced extracellular voltage changes originating in the retina. ERG can be recorded in vivo from humans/living animals or ex vivo from eyecups or from isolated retinas. In this study, we conducted ex vivo ERG recordings both transretinally (TERG) and locally across the photoreceptor outer segments (LERG-OS) from isolated mouse retinas. When recorded from dark-adapted isolated mouse retinas, the ex vivo ERG signal originates mainly from rod photoreceptors because the cone density is < 1/30 of the rod density (Ortin-Martinez et al., 2014) and the rods are over 1000-fold more sensitive to light compared to cone photoreceptors when studied with ex vivo and in vivo ERG b-wave recordings (Vinberg et al., 2014). In addition, the recovery kinetics of cone responses are substantially faster compared to rod responses (Heikkinen et al., 2008). Hereby, the dark-adapted ERG responses are expected to be practically “cone-free” excluding the small contribution from cones superimposed to the fast transient peak in the beginning of the response to strong flash for cone contribution, see e.g. (Turunen and Koskelainen, 2017; their Fig. 2) or Heikkinen et al. (2008); their Fig. 1.

2.1.3. Transretinal ERG experiments

Female and male wild type mice (C57BL/6) between the age of two and six months were used in the experiments. The mice were housed in cages in 24 °C and kept in 12/12 h dark/light cycle. Before experiments, the mice were allowed to dark adapt overnight and they were sacrificed with CO₂ inhalation and cervical dislocation. The eyes were enucleated and cut open along the equator of the eye. The retinas were detached in cooled nutrition medium under dim red light. The isolated retinas were flat-mounted photoreceptors upwards in a specimen holder on a filter paper (Donner et al., 1988). Retinas were perfused with a constant flow (3–4 ml/min) of nutrition medium and the experiments were conducted either at 25 °C or 37 °C. The temperature was controlled with a heat exchanger in contact with the specimen holder and monitored constantly with a thermistor (30K6A309); BetaTHERM; Measurement Specialties, Inc., Hampton, VA, USA) located in the bath close to the retina. The nutrition medium consisted of (in mM) Na⁺, 133.4; K⁺, 3.3; Mg²⁺, 2.0; Ca²⁺, 1.0; Cl⁻, 142.7; glucose, 10.0; EDTA, 0.01; HEPES, 12.0. The pH was adjusted to 7.5 (at room temperature) with NaOH. Sodium aspartate (2 mM) was used to block synaptic transmission to the second-order neurons. 50 μM BaCl₂ was used in the nutrition medium to block the K⁺ currents of Müller cells generating the glial component (Bolnick et al., 1979; Nymark et al., 2005). Leibowitz culture medium L-15, 0.72 g/l, was added to improve the viability of the retina in all experiments. PDE inhibitors 3-isobutyl-1-methylxanthine (IBMX), sildenafil and zaprinast were used at concentrations of 10–150 μM, 1–2000 nM and 50–2000 nM, respectively. The retinas were always allowed to reach a steady state after each medium change. This was verified by monitoring the inhibitor-induced changes in the ERG signals.

The light stimuli composed of 2 ms flashes with homogeneous full-field illumination to the distal side of the retina. The stimulus lights were generated with a 532 nm laser diode module (IQSC (532-100)L74; Power Technology Inc., Little Rock, AR, USA) and for longer light steps with a 633 nm HeNe laser (25 LHR 151; Melles Griot, Carlsbad, CA, USA). The light strength at the retina were controlled with computer driven neutral density filters and wedges and an electronic shutter (Oriel 76,992; Irvine, CA, USA). The uniformity of the beam at the level of the retina was confirmed with a camera-based beam profiler (Model SP503U; Spiricon Laser Beam Diagnostics, Ophir-Spiricon Inc., Logan, UT, USA) and the absolute intensity of the unattenuated laser beam (photons mm⁻² s⁻¹) incident on the retina was measured regularly with a calibrated photodiode (HUV-1000B; EG&G, URS Corporation, Gaithersburg, MD, USA; calibration by the National Standards Laboratory of Finland). The number of photoisomerizations (R*rad⁻¹)
produced by the stimulating flash light in a rod was calculated using the pigment template by Govardovskii et al. (2000) as described in Heikininen et al. (2008).

The transretinal potential was recorded with two silver/silver chloride microelectrode electrodes (EP2; World Precision Instruments [WPI], Hitchin, UK). One electrode was inserted into an electrode space connected to the proximal side of the retina through a 1.2 mm hole covered with the filter paper. This set the active recording area to be 1.1 mm². The other electrode was connected through a porous plug to the nutrition medium perfusing the distal side of the retina. Both electrode spaces were filled with a chloride solution containing 115 mM Na⁺, 122.3 mM Cl⁻, 3.3 mM K⁺, and 2.0 mM Mg²⁺.

2.1.4. Local ERG experiments

In local ERG, the signal was recorded with two microelectrodes, composed of glass capillaries filled with chloride solution and connected to silver/silver chloride microelectrode electrodes (EP2; WPI). The reference microelectrode, with a tip diameter of ca. 30 μm, was positioned on the retinal surface and the recording electrode, with a tip diameter of 2–5 μm, was advanced to the level of rod ciliun. The recording electrode was positioned with a hydraulic micromanipulator (MC-35A, 0.2-μm resolution; Narishige International Ltd., London, UK) and the reference electrode was moved with an electronic micro-micromanipulator (MR 471843; Carl Zeiss AG, Oberkochen, Germany). With the used electrode configuration, we could record voltage changes across the rod outer segment layer, i.e. voltage changes directly proportional to the changes in the circulating outer segment current (Penn and Hagnis, 1969; Turunen and Koskelainen, 2017). The surface of the retina was identified by voltage shifts that occur when the recording electrode penetrates the surface of the retina. The specimen holder was similar to the one used in TERG experiments except a 2 mm diameter electrode penetrates the surface of the retina. The specimen holder was connected to the proximal side of the retina through a 1.2 mm hole (Ø 1.2 mm2). The other electrode was connected through a porous plug to the nutrition medium perfusing the distal side of the retina. Both electrode spaces were filled with a chloride solution containing 115 mM Na⁺, 122.3 mM Cl⁻, 3.3 mM K⁺, and 2.0 mM Mg²⁺. The transretinal potential was recorded with two silver/silver chloride electrolyte solutions (EP2; WPI). The reference microelectrode, with a tip diameter of ca. 30 μm, was advanced to the level of rod ciliun. The recording electrode was positioned with a hydraulic micromanipulator (MC-35A, 0.2-μm resolution; Narishige International Ltd., London, UK) and the reference electrode was moved with an electronic micro-micromanipulator (MR 471843; Carl Zeiss AG, Oberkochen, Germany). With the used electrode configuration, we could record voltage changes across the rod outer segment layer, i.e. voltage changes directly proportional to the changes in the circulating outer segment current (Penn and Hagnis, 1969; Turunen and Koskelainen, 2017). The surface of the retina was identified by voltage shifts that occur when the recording electrode penetrates the surface of the retina. The specimen holder was similar to the one used in TERG experiments except a 2 mm diameter electrode penetrates the surface of the retina. The specimen holder was connected to the proximal side of the retina through a 1.2 mm hole (Ø 1.2 mm2). The other electrode was connected through a porous plug to the nutrition medium perfusing the distal side of the retina. Both electrode spaces were filled with a chloride solution containing 115 mM Na⁺, 122.3 mM Cl⁻, 3.3 mM K⁺, and 2.0 mM Mg²⁺.

2.1.5. Data and statistical analysis

In transretinal ERG experiments, the data acquisition and laser controls where handled with a data acquisition card (PCI-6024E; National Instruments, Austin, TX, USA) and a custom-made LabWindows software. In Local ERG experiments, the data acquisition and LED controls were handled with a data acquisition card (PCIe-6351; National Instruments) and a custom-made LabVIEW software. In both recording modalities the recorded DC signal was sampled at 1000 Hz and low-pass filtered with f = 500 Hz (8-pole Bessel filter). The collected data was pre-analyzed with a custom-made program to provide linear baseline correction, digital low-pass filtering with f = 100 Hz, and averaging of flash response traces recorded with same stimulus light strength in similar experimental conditions. One response was used or up to five responses were averaged for each stimulus strength. Further data analysis was conducted with OriginPro 2017 including plotting graphics, and fitting of linear, exponential and Gaussian (Eq. (6)) models to the data. The statistical analysis was conducted with Microsoft Excel 2016. Unpaired, two-tailed two-sample Student’s t-test with unequal variance was used as the statistical test and level of probability below 5% (p < 0.05) was considered statistically significant. The data averaged from several experiments are expressed as mean ± SEM values. Number of experiments (n) is given in the parentheses.

2.1.6. Materials

All chemicals were purchased from Sigma-Aldrich (Espoo, Finland).

2.2. Theoretical background and calculations

In this study, we introduce two methods for the determination of the inhibition constant for PDE6 inhibitor drugs towards naturally occurring activation states of PDE6: spontaneously activated PDE6 and light-activated PDE6. The methods are based on the premise that we can detect changes in the cytoplasmic cGMP concentration by recording the changes in the extracellular voltage of the retina and photoreceptors with ex vivo ERG. The next section presents the theoretical background behind the methods and describes the mathematical models connecting the inhibitor action towards PDE6 to the biochemical reactions in photoreceptor outer segments and to ERG recordings.

2.2.1. Determination of inhibition constant towards light-activated PDE6

Activated PDE6 is known to hydrolyze cGMP nearly at diffusion limited speed in vertebrate photoreceptor cells (Reingruber et al., 2013), resulting in a rapid drop in the cytoplasmic cGMP content in photoreceptor outer segments and in a closure of cyclic nucleotide-gated (CNG) channels in the photoreceptor outer segment cell membrane. Reduction of the outer segment conductance results in a decrease in the circulating dark current. This causes the radial extracellular potential change that is recorded with ERG. PDE inhibitors weaken the catalytic activity of PDE6, which leads to a decrease in the molecular amplification of the phototransduction cascade. In our approach to determine the inhibition constant towards light-activated PDE6, we used the Lamb and Pugh (1992) activation model to quantify the changes in the PDE6 catalytic activity. The following theoretical analysis adopts similar logic used in their original work (Lamb and Pugh, 1992). A more thorough analysis of the phototransduction cascade is presented in Pugh and Lamb (2000).

The hydrolysis of cGMP in the presence of a competitive PDE inhibitor can be described as:

\[ \text{PDE}^* + [\text{cGMP}] \rightleftharpoons k_{-1} \text{PDE}^* \text{cGMP} + [\text{I}] \]

where \([\text{PDE}^*]\) is the concentration of light-activated PDE, \([\text{I}]\) is the concentration of inhibitor, \([\text{cGMP}]\) is the concentration of cGMP, and \(k_{-1}\) is the rate constant of the reaction examined. When the reaction has reached equilibrium, the rate of cGMP hydrolysis can be stated as

\[ \frac{d[\text{cGMP}]}{dt} = \frac{k_{-1} [\text{PDE}^*][\text{cGMP}]}{[\text{cGMP} + K_M (1 + \frac{[\text{I}]}{K_{I_{\text{light}}}})]] \]  

(2)

where \(K_M\) is the Michaelis constant of the hydrolysis reaction and \(K_{I_{\text{light}}}\) is the inhibition constant of the competitive inhibitor towards PDE6 in light-activated state. The Michaelis constant \(K_M\), and thus the term \(K_{M}(1 + \frac{[\text{I}]}{K_{I_{\text{light}}}})\), can be assumed substantially larger than the cGMP level at any moment (Pugh and Lamb, 2000), and the Eq. (2) reduces to

\[ \frac{d[\text{cGMP}]}{dt} = \frac{k_{-1} [\text{PDE}^*][\text{cGMP}]}{K_M (1 + \frac{[\text{I}]}{K_{I_{\text{light}}}}) \text{cGMP}} = \beta_{\text{sub}} \text{I} \text{PDE}^* [\text{cGMP}] \]  

(3)

where the rate constant for cGMP hydrolysis, \(\beta_{\text{sub}}\), \(\text{I}\), represents the
maximum speed by which one active PDE6 subunit can hydrolyze cGMP and it is described by the equation

\[ \beta_{\text{sub},i} = \frac{k_3}{K_M (1 + \frac{[I]}{K_{I,\text{light}}})} \]  

(4)

Effectively, the competitive PDE6 inhibitor increases the apparent Michaelis constant \( K_M \) and thus the rate constant for cGMP hydrolysis decreases by a factor of \( 1 + \frac{[I]}{K_{I,\text{light}}} \), giving

\[ \beta_{\text{sub},i} = \frac{\beta_{\text{sub}}}{1 + \frac{[I]}{K_{I,\text{light}}}} \]  

(5)

Lamb and Pugh (1992) developed an activation model based on the known molecular interactions in rod disc membranes to estimate the phototransduction amplification. According to the model, the very beginning of the flash response leading edge can be described with a delayed Gaussian equation

\[ F(t) = e^{-\frac{1}{2}(t-t_0)^2} \]  

(6)

where the activation coefficient \( A \) describes the molecular amplification of phototransduction

\[ A = \gamma_{\text{GCAP}} c_{\text{GR}} \beta_{\text{sub}} c_{\text{GMP}} \]  

(7)

\( \gamma_{\text{GR}} \) is the rate by which an activated rhodopsin can activate transducins, \( c_{\text{GR}} \) is the coefficient defining the coupling efficiency of \( \gamma_{\text{GR}} \) to PDE6 and \( c_{\text{GMP}} \) is the Hill coefficient for the CNG channel activation (Pugh and Lamb, 2000). The activation coefficient in the presence of a competitive PDE inhibitor is

\[ A_f = \gamma_{\text{GCAP}} c_{\text{GR}} \beta_{\text{sub},i} c_{\text{GMP}} \]  

(8)

Combining Eqs. (5), (7) and (8) results in a linear relation between the inhibitor concentration and the ratio of activation coefficient without and in the presence of the inhibitor

\[ \frac{A_{\text{control}}}{A_i} = \frac{[I]}{K_{I,\text{light}}} + 1 \]  

(9)

2.2.2. Determination of inhibition constant towards spontaneously active PDE6

Inhibitor-induced changes in cGMP hydrolysis activity can be utilized in determination of the inhibition constant towards spontaneously active PDE6. This requires that no light-activated PDE6 is present, i.e. the experiments are conducted in darkness, and that the rate of cGMP synthesis stays constant. The latter is achieved by conducting the experiments on guanylate cyclase activating protein knockout (GCAPs−/−) mice, in which the calcium mediated regulation of cGMP synthesis by guanylate cyclase is not functional.

In darkness, the synthesis (\( \alpha_{\text{dark}} \)) and hydrolysis (\( \beta_{\text{dark}}[\text{cGMP}]_{\text{dark}} \)) rates of cGMP are equal

\[ \frac{d[\text{cGMP}]}{dt} = \alpha_{\text{dark}} - \beta_{\text{dark}}[\text{cGMP}]_{\text{dark}} = 0 \]  

(10)

Introduction of a PDE6 inhibitor decreases the spontaneous PDE6 activity to a new value \( \beta_{\text{dark},i} \) and reduces the rate of cGMP hydrolysis leading to an increase in the intracellular cGMP concentration. With GCAPs−/− mouse rod photoreceptors \( \alpha_{\text{dark}} \) is constant and after reaching a steady state in the presence of the PDE6 inhibitor holds

\[ \alpha_{\text{dark}} - \beta_{\text{dark},i}[\text{cGMP}]_{\text{dark},i} = 0 \]  

(11)

For competitive PDE6 inhibitors, Eqs. (10) and (11) can be combined to give

\[ \frac{[\text{cGMP}]_{\text{dark},i}}{[\text{cGMP}]_{\text{dark}}} = \frac{\beta_{\text{dark}}}{\beta_{\text{dark},i}} = 1 + \frac{[I]}{K_{I,\text{dark}}} \]  

(12)

where \( K_{I,\text{dark}} \) denotes the inhibition constant towards spontaneously active PDE6.

The fraction of open CNG channels and the circulating dark current obey the Hill’s equation

\[ \frac{J_{\text{GMP}}}{J_{\text{GMP,\text{max}}} \cdot \gamma_{\text{GMP}}} = \frac{[\text{cGMP}]_{\text{dark},i}}{[\text{cGMP}]_{\text{dark}} + K_{\text{GMP}} \cdot \gamma_{\text{GMP}}} \]  

(13)

The cGMP concentration producing half of the maximal current, \( K_{\text{GMP}} \), is typically around 20 \( \mu \)M, which is prominently larger than the rod intracellular [cGMP] (Pugh and Lamb, 2000). Because the change in the extracellular voltage across the rod outer segments is directly proportional to changes in the circulating dark current, the ratio of saturated response amplitudes in the presence and without a PDE6 inhibitor is

\[ \frac{I_{\text{max},i}}{I_{\text{max,control}}} = \frac{J_{\text{dark},i}}{J_{\text{dark,control}}} \approx \left( \frac{[\text{cGMP}]_{\text{dark},i}}{[\text{cGMP}]_{\text{dark}} + K_{\text{GMP}} \cdot \gamma_{\text{GMP}}} \right)^{\gamma_{\text{GMP}}} \]  

(14)

Hill coefficient for CNG channels, \( \gamma_{\text{GMP}} \), is generally assumed to be close to 3 for mouse rods (Gross et al., 2012; Pugh and Lamb, 2000). By combining Eqs. (12) and (14) we obtain a formula that allows the determination of the inhibition constant towards spontaneously activated PDE6, \( K_{I,\text{dark}} \):

\[ \left( \frac{I_{\text{max},i}}{I_{\text{max,control}}} \right)^{\gamma_{\text{GMP}}} = \frac{[I]}{K_{I,\text{dark}}} + 1 \]  

(15)

3. Results

3.1. Effects of PDE inhibitors on flash response kinetics and rod sensitivity

PDE inhibitors reduce the rate of cGMP hydrolysis by PDE6, which has various effects on rod flash responses as illustrated in Fig. 1A for the non-selective PDE inhibitor IBMX. The figure presents two sets of responses to increasing flash strengths, one in control solution (black traces) and the in the presence of 150 \( \mu \)M IBMX. Rod flash responses to dim flashes are linear, i.e. doubling the flash strength would increase the response amplitude by a factor of two without affecting the flash response kinetics. When stimulus strength is increased beyond linear range, the response amplitude grow less steeply and the flash response kinetics become faster (the middle-sized traces in Fig. 1A). Finally, when the stimuli are strong enough, all the CNG channels in photoreceptor outer segments close and the response saturates. The saturation amplitude can be determined from the plateau level of the response to a strong flash (Turunen and Koskelainen, 2017). Further increases in stimulus strength only lengthen the response saturation period. When IBMX was introduced, it slowed down the leading edge of dark-adapted rod responses both to dim and strong flashes, increased the saturated response amplitude, and delayed the flash response recovery. The slowdown of the flash response leading edge reflects a decrease in the molecular amplification of the phototransduction activation reactions. However, the decreased hydrolysis rate of cGMP by the inhibition of light-activated PDE6 cannot explain the increased flash response amplitudes or the slower flash response shutoff. These effects indicate that IBMX elevates the level of intracellular cGMP even in darkness, attesting that IBMX also decreases the spontaneous PDE6 activity, which is a key effector controlling flash response shutoff and amplitude (Astakhova et al., 2008; Gross et al., 2012). Fig. 1B demonstrates the effects of increasing concentrations of IBMX on dim flash responses normalized with their respective saturated response amplitudes. The stimulus strength was chosen to produce practically linear range
responses to emphasize the differences in flash response kinetics. A salient feature in the responses of Fig. 1B is that in spite of the strong deceleration of the response kinetics, the relative response amplitudes decreased only slightly by IBMX. After washout of IBMX, the responses recovered completely (see the inset of Fig. 1A). The effects of IBMX were concentration dependent and showed no signs of saturation with doses between 50 and 150 μM.

3.2. Inhibition constant towards light-activated PDE

The slowdown of the leading edge of the flash responses caused by the inhibitors was quantified by fitting the Lamb and Pugh (1992) activation model to the early phase of the sub-saturated flash responses as shown in Fig. 2A. The model was fitted from zero time point (time of the flash stimulus) to the time point where the response reached 50% of its peak amplitude or to 40 ms, if the 50% level was reached later. A constant delay, \( t_d \), of 7 ms was used in the fittings. The activation coefficient declined monotonically as a function of increasing inhibitor concentration (Fig. 2B).

We applied Eq. (9) to determine the inhibition constant of IBMX towards the light-activated PDE6 (\( K_{I, light} \)) at temperatures 25 °C and 37 °C, and for sildenafil and zaprinast at 37 °C. Fig. 3A shows the fitting of Eq. (9) to the data collected at 37 °C for IBMX and Fig. 3B for sildenafil and zaprinast, emphasizing the linear relationship between the inhibitor concentration and the relative decrease in the activation coefficient as predicted by the theory. The \( K_{I, light} \) of IBMX was 11.5 ± 0.6 μM (n = 7) at 25 °C and 13.4 ± 0.7 μM (n = 16) at 37 °C. This temperature dependence of \( K_{I, light} \) of IBMX was subtle but statistically significant. The inhibition constant of sildenafil was 0.56 ± 0.09 μM (n = 4) and for zaprinast 0.97 ± 0.07 μM (n = 4). The relative standard error (RSE) was 5% for IBMX, 16% for sildenafil, and 7% for zaprinast at 37 °C.

To examine whether the PDE6 activation level or the intracellular cGMP concentration affects inhibition efficiency, the PDE6 activity was elevated by steady background light suppressing on average 50% of the saturated response amplitude. This decrease in saturated flash response amplitude corresponds to ca. 20% decrease in the intracellular cGMP level (see Eq. (14)). The determined inhibition constant of IBMX at 37 °C was similar in light (13.9 ± 1.0 μM, n = 4) and in darkness, demonstrating that the \( K_{I, light} \) determination is not sensitive to the level of PDE6 activity or to moderate changes in the cGMP concentration in rods.

3.3. Inhibition constant towards spontaneously activated PDE

Previous studies on amphibian rod photoreceptors have shown that PDE inhibitors increase the photoreceptor cell circulating current in darkness (Astakhova et al., 2008; Capovilla et al., 1982; Nikonov et al., 2000; Rieke and Baylor, 1996; Zhang et al., 2005), indicating that PDE6 inhibitors can also reduce spontaneous (thermal) PDE6 activity. As shown in the Methods (Eqs. (10)–(15)), the change in the saturated flash response amplitude, corresponding to the increase in the circulating current, can be used to determine the \( K_I \) for spontaneously activated PDE6, \( K_{I, dark} \). However, the circulating current is partly carried by calcium ions, and an increase in the circulating current raises the intracellular calcium level that mediates negative feedback to guanylate

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**Fig. 1.** (A) Effect of 150 μM IBMX on ERG rod photoresponses from a single retina to dim (12.7 R*rod⁻¹), half-saturating (127 R*rod⁻¹), and saturating (4010 R*rod⁻¹) flashes of light at 37 °C. Responses without inhibitor are shown in black and with inhibitor in red. The inset shows the dim flash responses (12.7 R*rod⁻¹) in control medium (black), in inhibitor medium (red), and after the washout of the inhibitor (gray), normalized with the saturating flash amplitudes giving the relative dim flash responses. (B) Effect of IBMX concentration on the relative dim flash response amplitude and kinetics with a stimulus strength of 16.7 R*rod⁻¹ at 37 °C.

**Fig. 2.** (A) Fitting the Lamb and Pugh activation model to a set of sub-saturated responses from one retina at 37 °C with flash strengths ranging from 5.3 to 1670 R*rod⁻¹ at 3.2-fold increments. The saturation amplitude is determined from the plateau level of the strongest flash response after the fast transient component has ceased. For the presented responses, the determined activation coefficient was 5.4 s⁻¹. (B) Activation coefficients obtained with different IBMX concentrations at 37 °C. The activation model was always fitted to sub-saturated responses as shown in Fig. 2A. Each data point is an average from 6 to 17 experiments and the individual data points are overlaid on the background.
cyclase activity through the calcium sensor proteins GCAP1 and GCAP2. This feedback counteracts the PDE6 inhibition-induced increase in the intracellular cGMP level and thus in the circulating current (Tsang et al., 2012; Zhang et al., 2005). To eliminate the effect of negative feedback on guanylate cyclase, we used GCAPs−/− mice where this calcium feedback is absent (Mendez et al., 2001). In addition, while the activation phase of the sub-saturated TERG flash responses purely reflect the changes in the phototransduction cascade as assessed by local ERG in this preparation (Turunen and Koskelainen, 2017), the signal components originating from voltage sensitive channel currents and capacitive currents in photoreceptor inner segments can shape the response shut off and complicate the determination of the saturated response amplitude (Arden, 1976; Green and Kapousta-Bruneau, 1999; Robson and Frishman, 2014; Turunen and Koskelainen, 2017; Vinberg et al., 2009). To ensure that the changes in the saturated response amplitude correspond to the changes in the circulating dark current, the recordings with GCAPs−/− mice were conducted with local ERG across the rod outer segments (LERG-OS).

With GCAPs−/− mouse retinas, introduction of 40 μM IBMX increased the saturated rod flash response amplitude over 4-fold on average, while in some experiments > 6-fold increase was observed (Fig. 4A). In contrast, similar inhibitor treatments caused only a slight increase in saturated rod flash response amplitudes with WT mice retinas (data not shown). The effects of different inhibitors on the saturation amplitude are compared in Fig. 5. To facilitate comparison, the inhibitor concentration on the x-axis was normalized by the inhibition constants towards light-activated PDE6 determined separately for each inhibitor in GCAPs−/− background (for GCAPs−/− KI,light,IBMX = 14.1 ± 2.0 μM, n = 5, RSE = 14%; KI,light,sildenafil = 0.56 ± 0.01 μM, n = 3, RSE = 2%; KI,light,zaprinast = 0.97 ± 0.04 μM, n = 5, RSE = 4%). Thus, the x-axis shows the ratio between the concentration of enzyme-inhibitor complex and the free enzyme concentration. The inhibition constants in GCAPs−/− background were similar to those determined for WT mice. Saturated flash response amplitudes were determined when they had reached steady state after the medium change. The y-axis gives the cube root of the saturated flash response amplitude in the presence of the inhibitor (rmax, I), normalized with the saturation amplitude in control medium (rmax, control) corresponding to the relative change in the intracellular cGMP concentration when the Hill coefficient for CNG channels, nGMP, is assumed to be 3 (Eq. (14)) (Gross et al., 2012; Pugh and Lamb, 2000). It is evident that the inhibitors had very different effects on the intracellular cGMP level of...
4.5 \times \text{KI}_{\text{light}}$ for IBMX, sildenafl and zaprinast. The saturated response amplitudes were normalized by the saturation amplitude in control solution obtained with linear interpolation of the saturated response amplitude before the addition and after the removal of inhibitor. Each data point is an average from 3 to 5 experiments for IBMX, 3 experiments for sildenafl and 4–5 experiments for zaprinast. Inhibitor concentrations were normalized by the inhibition constant towards light-activated PDE, $\text{KI}_{\text{light}}$, of each inhibitor. Estimation of $\text{KI}_{\text{dark}}$ values from a slope of the exponential fit in zero inhibitor concentration was 1.6$\times \text{KI}_{\text{light}}$ for IBMX, 4.0$\times \text{KI}_{\text{light}}$ for sildenafl, and 9.2$\times \text{KI}_{\text{light}}$ for zaprinast (95% confidence bands [1.1; 2.0]$\times \text{KI}_{\text{light}}$, [3.5; 4.5]$\times \text{KI}_{\text{light}}$, [4.5; 14]$\times \text{KI}_{\text{light}}$ for IBMX, sildenafl, and zaprinast, respectively).

GCAPs$^{-/}$-mouse rods. IBMX caused the largest increase in the saturation amplitude while the effects of zaprinast and sildenafl were modest. Contrary to the theory (Eq. (15)), the cubic root of the saturated flash response amplitude did not increase linearly with the inhibitor concentration but showed downturn towards higher drug concentrations. This is also evident in Fig. 4A, where the recorded voltage signal increased after the introduction of 40 $\mu$M IBMX but then declined somewhat before settling to a steady state. This decline is profoundly smaller in 5$\mu$M IBMX (Fig. 4B).

To estimate the inhibition constant of drugs on the spontaneous PDE6 activity from Eq. (15), we fitted an exponential curve to the data presented in Fig. 5 and determined the slope of the fitted curve at zero inhibitor concentration (dashed lines in Fig. 5). The estimated $\text{KI}_{\text{dark}}$ values were 1.6$\times \text{KI}_{\text{light}}$ for IBMX, 4.0$\times \text{KI}_{\text{light}}$ for sildenafl and 9.2$\times \text{KI}_{\text{light}}$ for zaprinast. In addition, Fig. 5 presents the 95% confidence bands for the fits. The 95% confidence intervals of $\text{KI}_{\text{dark}}$ were 1.1–2.0$\times \text{KI}_{\text{light}}$ for IBMX, 3.5–4.5$\times \text{KI}_{\text{light}}$ for sildenafl, and 4.5–14$\times \text{KI}_{\text{light}}$ for zaprinast. This demonstrates that the inhibition efficiency ratio between the light-activated and spontaneously activated PDE6 varies between inhibitors: sildenafl and zaprinast inhibited spontaneous PDE6 activity less efficiently than light-induced PDE6 activity, while the inhibition efficiency of IBMX was approximately similar towards spontaneously activated and light-activated PDE6.

4. Discussion

In photoreceptor cells, PDE6 can appear in two active forms. Firstly, in the light-activated form in which the binding of activated transducin $\alpha$-subunit, $\text{Gt}_\alpha$, to the PDE6 $\gamma$-subunit has detached the $\text{Gt}_\alpha$-PDE6$\gamma$ complex from the active site of the PDE6 molecule but it still remains attached to the PDE6 body (Artemyev et al., 1992; Cote, 2006). Secondly, in the spontaneously activated form where thermal energy dislocates the PDE6 $\gamma$-subunit and causes momentary activations of PDE6. Rieke and Baylor (1996) estimated that in dark-adapted rod cells, one out of 5000 PDE6 molecules is spontaneously active at a given moment. In addition to the naturally occurring active forms of PDE6, a third, trypsin-activated form is generally used in biochemical research. In this form, the $\gamma$-subunits are enzymatically dissociated from the PDE6 holoenzyme and the PDE6 is maximally activated (Liu et al., 2009). The first method introduced in this study provides an apparent inhibition constant of PDE6 inhibitors when PDE6 is activated by light in its native cellular environment. As predicted by theory, the activation phase of flash responses decelerated linearly with respect to the inhibitor concentration (see Eq. (10) and Fig. 3). The second method combines genetic, pharmacological and electrophysiological approaches to determine the efficiency of the inhibitors towards spontaneously activated PDE6. Both methods were tested with three PDE inhibitors, 3-isobutyl-1-methylxanthine (IBMX), sildenafl and zaprinast. Our results demonstrate that the inhibition efficiency of various PDE inhibitors may be quite different towards these two naturally activated forms of PDE6.

4.1. Feasibility of the method

Our method for resolving the $\text{KI}$ of PDE inhibitors for light-activated PDE6 ($\text{KI}_{\text{light}}$) rests on the determination of the activation coefficient describing the molecular amplification of phototransduction without and in the presence of the inhibitor. The activation coefficient is, by definition, sensitive only to the changes in the mechanisms responsible for phototransduction activation and not affected by the mechanisms of phototransduction deactivation, negative feedbacks, changes in intracellular [cGMP] or changes in the level of PDE6 activity (Pugh and Lamb, 2000). In practice, phototransduction deactivation can cause minor underestimation of the activation coefficient determined by fitting, but only interactions of the inhibitor molecules with rhodopsin, transducin, or the CNG channels, constituting the phototransduction activation part, would cause substantial error in $\text{KI}_{\text{light}}$ determination. No signs of such interactions were observed with the three inhibitors tested. Additionally, in $\text{KI}_{\text{light}}$ determination, relative standard errors < 20% were achieved with a small number of experiments ($n \leq 5$) indicating that the method is precise, and as such, applicable for preclinical testing of potential drug compounds.

Our approach for determining the inhibition potency of the PDE inhibitors towards spontaneously active PDE6 is less straightforward than that towards light-activated PDE6, because it requires the use of GCAPs$^{-/}$-mouse retinas. The main source of error in this method is likely the decline in the steady state circulating current after the introduction of inhibitors at higher concentrations (see Fig. 4A). Thus, using the change in the steady state $r_{\text{max}}$ caused by a PDE inhibitor can yield to underestimation of the fitted slope in Eq. (15) and to overestimation of $\text{KI}_{\text{dark}}$. This error could be reduced with faster introduction of inhibitors into photoreceptors. Rapid introduction of an inhibitor, however, is hard to achieve with intact retinas. Further, the plasma membrane permeability of the inhibitors may also be low, which expands the time needed for the inhibitors to reach the intracellular space of photoreceptor outer segments. With our approach, we estimate that based on the confidence intervals for $\text{KI}_{\text{dark}}$, the error made in the determination is less than by a factor of 2.

4.2. Inhibition constants are smaller towards trypsin-activated than towards light-activated PDE6

PDE5 inhibiting drugs commonly used to treat erectile dysfunctions, e.g. sildenafl, vardenafl, and tadalafil, have been reported to cause various kinds of visual disorders in subgroups of patients (Azzouni and Abu samra, 2011). A likely source of these adverse effects is the inhibitory effect of the PDE5 inhibitors on PDE6 located in photoreceptor cells. However, in spite of the fact that the molecular amplification of phototransduction is a key factor in the absolute sensitivity of vision, to our best knowledge, prior to this study no attempts have been made to determine the inhibition constant of any PDE inhibitor towards the light-activated form of mammalian PDE6. Instead, the inhibition efficiencies have been determined mostly for trypsin-activated PDE6 in homogenized rod outer segment suspensions, with purified PDE6, or with recombinant PDE6.

In previous studies, the inhibition constant obtained with purified...
In conclusion, it appears that the KI of IBMX towards light-activated and competition for the common binding sites in the PDE6 activated form. One possible explanation for this difference might be the spontaneous activation of PDE6, which is lower than towards light-activated form in this study was 11.5 μM for mouse rods. This is only slightly larger than the KI (4.5 μM, Zhang et al., 2005) for trypsin-activated purified bovine IBMX and similar to that for native human PDE6 (12.2 μM, Zhang et al., 2004), all determined at room temperature. The reason for the difference between electrophysiologically (PDE6 in natural environment) and biochemically (PDE6 with γ-subunit enzymatically dissociated) determined KI values is unclear. A possible explanation is that the competitive PDE6 inhibitors, cGMP, and the inhibitory PDE6 γ-subunit all compete for binding to the same catalytic domain in the PDE6 apoenzyme (D’Amours et al., 1999; Zhang et al., 2005). In the light-activated state of PDE6, the γ-subunit still attached to the PDE6 GAFα domain may hinder the binding of sildenafil and zaprinast to the active site, while in trypsin-activated PDE6 this hindrance is abolished. For IBMX, the interaction with γ-subunit seems to be of lower significance since the KI towards light- and trypsin-activated PDE6 are closely similar. This may be due to the smaller size of IBMX in comparison to sildenafil and zaprinast. Another explanation could be that, in our experiments, the drug concentration inside the intact photoreceptors differ from that in the plasma membrane in the steady state would require active transport of sildenafil and zaprinast.

4.3. Inhibition constants can be substantially different for spontaneously activated and light-activated PDE6

To our knowledge, the inhibition constant of any PDE inhibitors towards spontaneously active and light-activated PDE6 has been investigated only in one study conducted on living amphibian (salamander) rod prs to this work (Cobb, 1991). Cobb found no difference in KI for IBMX (32 μM) towards light- and spontaneously activated PDE6. However, the KI, dark obtained by Cobb was more than fourfold higher than the KI, light (7 μM) in salamander rods determined by Hodgkin and Nunn (1988). This difference is partly explained by the choice of nGMP – value, which was assumed to be 2 by Cobb. With nGMP of 3 the KI estimate by Cobb increases to 17 μM. The KI, light for IBMX determined in our study, 11.5 μM in mice at room temperature (Zhang et al., 2005). In addition, our results on mice at body temperature support the result by Cobbs that the KI, light and KI, dark are closely similar.

In conclusion, it appears that the KI of IBMX towards light-activated and spontaneously active PDE6 are quite similar both in amphibians (salamanders) and in mammals (mice), and that in mice the KI for IBMX depends only weakly on temperature.

The inhibition behavior of sildenafil and zaprinast is qualitatively different from that of IBMX because their inhibition efficiency towards spontaneously activated PDE6 is clearly lower than towards the light-activated form. One possible explanation for this difference is the competition for the common binding sites in the PDE6 γβ-α2-α2-γ-subzyme of sildenafil and zaprinast with the γ-subunit. The γ-subunit is bound with high affinity to the GAFα domain in the PDE6 γβ-α2-α2-γ-α3-α3-γ-subzyme and with lower affinity to the amino acid residues Met759, Phe778 and Phe782 near the active site, thereby closing the entrance of cGMP to the catalytic core (Cote, 2004; Granovsky and Arterjmov, 2001a, 2001b).

Molecular modeling of the PDE6 catalytic site suggests that, in addition to binding to the catalytic core, zaprinast interacts with Met759 and sildenafil with Met759 and Phe778 (Simon et al., 2006). In the light-activated form of PDE6 the binding of the Gтγ to the PDE6 γ-subunit reveals the catalytic core as well as the amino acids Met759 and Phe778 needed for sildenafil and zaprinast binding. Although the mechanism of thermal activation of PDE6 is not known, it seems possible that the competition for the common binding sites with the γ-subunit decreases the apparent affinity of sildenafil and zaprinast. IBMX, being the smallest of the tested inhibitors, do not interact with Met759 and Phe778 and may escape the competition with the γ-subunit.

5. Conclusions

In this study, we introduced methods that allow precise determination of the inhibition constants of PDE6 inhibitors towards light- and spontaneously activated PDE6 in their natural environments, an intact retina. We utilized ex vivo ERG, where the photoreceptor component of the ERG signal was isolated either pharmacologically (transretinal ERG) or mechanically (local ERG across the rod outer segment layer). We demonstrated, using IBMX, sildenafil and zaprinast as model drugs, that the inhibitor efficiency towards the differently activated forms of PDE6, spontaneously activated, light-activated, and trypsin-activated, can differ substantially. We hypothesize that this may be due to a competition between the PDE inhibitors and the PDE6 γ-subunit for the common binding sites in PDE6 αβ-α2-α2-γ-β-γ-β-α2-α2-α2-γ-β-α2-γ-subzyme. Overall, the introduced methods are feasible for fast and precise preclinical testing of drugs targeting to PDE6.

Conflicts of interest

The authors declare they have no actual or potential competing financial interests.

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