Toxicology and Applied Pharmacology 345 (2018) 57–65

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

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

ARTICLE INFO

Keywords:
Phosphodiesterase-6
Inhibitor
Inhibition constant
Retina
Photoreceptors
Ex vivo electroretinography

ABSTRACT

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 messenger molecules, cAMP and cGMP, 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

Abbreviations: A, activation coefficient; cAMP, adenosine 3′,5′ cyclic monophosphate; cGMP, guanosine 3′,5′ cyclic monophosphate; CNG channel, cyclic nucleotide-gated channel; ERG, electroretinography; Gt_{α}*, transducin α-subunit; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2 -ethanesulphonic acid]; IBMX, 3-isobutyl-1-methylxanthine; K_I, inhibition constant; $K_{I,dark}$, inhibition constant towards spontaneously activated PDE6; $K_{I,light}$, inhibition constant towards light-activated PDE6; LED, light emitting diode; LERG-OS, local electroretinography across rod outer segment layer; PDE, phosphodiesterase; RSE, relative standard error; SEM, standard error of the mean; TERG, transretinal electroretinography

* Corresponding author.
E-mail address: ari.koskelainen@aalto.fi (A. Koskelainen).

https://doi.org/10.1016/j.taap.2018.03.002
Received 3 January 2018; Received in revised form 1 March 2018; Accepted 2 March 2018
Available online 05 March 2018

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 relocalized during the light-activation of PDE6 but completely dismantled in 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 CO_2 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 (30K6A309I; 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; Mg^{2+}, 2.0; Ca^{2+}, 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 $BaCl_2$ 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). Leibovitz 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 (IQ5C (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^*rod^{-1})