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Cellular and molecular effects of unoprostone as a BK channel activator

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Abstract

Effects of unoprostone isopropyl (unoprostone), a prostaglandin metabolite analog; latanoprost, a $PGF_{2\alpha}$ analog; and $PGF_{2\alpha}$ were examined in HCN-1A cells, a model system for studies of large conductance Ca^{2+} activated K^+(BK) channel activator-based neuroprotective agents. Unoprostone and latanoprost, both used as anti-glaucoma agents, have been suggested to act through FP receptors and have neuroprotective effects. Ion channel activation, plasma membrane polarization, $[Ca^{2+}]_i$ changes and protection against long-term irreversible glutamate-induced $[Ca^{2+}]_i$ increases were studied. Unoprostone activated iberiotoxin (IbTX)-sensitive BK channels in HCN-1A cells with an EC_{50} of 0.6±0.2 nM and had no effect on Cl^- currents. Unoprostone caused IbTX-sensitive plasma membrane hyperpolarization that was insensitive to AL8810, an FP receptor antagonist. In contrast, latanoprost and $PGF_{2\alpha}$ activated a Cl^- current sensitive to $[Ca^{2+}]_i$ chelation, tamoxifen and AL8810, and caused IbTX-insensitive, AL8810-sensitive membrane depolarization consistent with FP receptor-mediated Ca^{2+} signaling Cl^- current activation. Latanoprost and $PGF_{2\alpha}$, but not unoprostone, increased $[Ca^{2+}]_i$. Unoprostone, $PGF_{2\alpha}$ only partially, but not latanoprost protected HCN-1A cells against glutamate-induced Ca^{2+} deregulation. These findings show that unoprostone has a distinctly different mechanism of action from latanoprost and $PGF_{2\alpha}$. Whether unoprostone affects the BK channel directly or an unidentified signaling mechanism has not been determined.

Keywords: HCN-1A; Latanoprost; $PGF_{2\alpha}$; IbTX; Neuroprotection; Glaucoma

1. Introduction

The goal of this study was to determine whether unoprostone exhibited properties consistent with neuroprotective agents, including protection against neuroexcitatory agent-induced increases in intracellular $[Ca^{2+}]_i$ and activation of Ca^{2+}- and voltage-activated K^+ channels (maxi K^+ or BK channels). These studies are relevant to the outcomes of unoprostone use in glaucoma therapy in protecting against vision loss. Primary open-angle glaucoma (POAG) is a primary cause of blindness and ocular hypertensive medication delays or prevents the onset of POAG [1]. Unoprostone causes a reduction in intraocular pressure (IOP) [2]. It has been shown that unoprostone has direct effects on the trabecular network thereby lowering IOP by maintaining aqueous outflow reduced by endothelin-1 (ET-1) [3]. These effects were shown to arise from preventing ET-1-induced contraction of the trabecular meshwork by preventing ET-1 induced $[Ca^{2+}]_i$ changes without affecting basal $[Ca^{2+}]_i$ levels and activation of large conductance Ca^{2+} activated (BK) channels [3]. Effects of unoprostone on Ca^{2+} entry pathways including L-type Ca^{2+} channels and Ca^{2+} release activated Ca^{2+} channels have been shown [4–6].

However, it is accepted that IOP reduction alone is not sufficient to protect against vision loss [7–10]. Rather, the loss of visual field due to glaucoma is directly associated with retinal ganglion cell death [11]. Several clinical studies suggest that unoprostone is neuroprotective [12–16]. In vitro studies have shown that unoprostone prevented apoptosis caused by serum deprivation [17] or glutamate in retinal progenitor cells [11], and protected against light induced photoreceptor damage in intact eyes [18]. A framework for understanding the neuroprotective effects of unoprostone has been put forward [2]. In this model, neuroexcitatory agents cause Ca^{2+} dysregulation and unoprostone prevents these effects by activating BK channels and preventing Ca^{2+} dysregulation that ultimately leads to apoptosis. Glutamate can cause neuronal cell delayed Ca^{2+} deregulation (DCD) [19,20].

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Human neuronal cortical cells (HCN-1A cells) are known to express ibertiotoxin (IbTX) sensitive BK channels [21–25]. They contain BK channels in a natural milieau and they are responsive to glutamate, but they do not readily allow determination of whether unoprostone directly or indirectly activates the BK channel. Single channel studies would be required for this.

Unoprostone has been suggested to act through the FP receptor [26–28] like $PGF_{2\alpha}$ [27] and latanoprost [27–29]. These agents bind to the FP receptor [28] and cause $[Ca^{2+}]_i$ mobilization [26,27] and stimulate phosphoinositide hydrolysis [27]. The EC_{50} for activation of BK channels by unoprostone was approximately 0.6 nM, and the other effects of unoprostone were evident over the range 1–100 nM. These concentrations are well below the reported EC_{50} values for FP receptor binding (5.9 μM) [2] and Ca^{2+} mobilization (approximately 1 μM) [26] by unoprostone. The highest concentrations achieved in treatment with unoprostone was approximately 100 nM [30], also well below the EC_{50} for FP receptor occupation or functional effects such as Ca^{2+} mobilization [26–28]. There is one report that unoprostone does not act as an FP receptor agonist [31]. The basis for this controversy may reside in the very high EC_{50} of unoprostone for occupation of FP receptors [28], and PI turnover [27] and the rapid transient unoprostone-mediated Ca^{2+} mobilization compared to other agents [26,27].

The reported findings suggest that unoprostone will activate BK channels and exhibit membrane hyperpolarization [22–25]. In contrast, $PGF_{2\alpha}$ or latanoprost occupation of FP receptors would activate Ca^{2+}-activated Cl^- currents through mobilization of $[Ca^{2+}]_i$ resulting in depolarization [26,27,32–35]. This depolarization should be blocked by tamoxifen, an anti-estrogen, which also inhibits Ca^{2+}-activated Cl^- channels [35] and would be sensitive to AL8810, an FP receptor antagonist [27]. Effects on Ca^{2+} homeostasis [26–28,31] could be the key to understanding the paradox of unoprostone being effective in glaucoma therapy despite being much less effective than $PGF_{2\alpha}$ analogs on FP receptor signaling [26–28,31], and might shed light on possible neuroprotective effects of unoprostone [8–11,17,18].

Whole cell patch clamp [36] of HCN-1A cells was used to measure K^+ and Cl^- currents. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$), a potential sensitive dye, was used for membrane potential studies [37–40]. The acetoxy-methyl ester of indo 1 (indo-1/AM) [41,42] was used to measure $[Ca^{2+}]_i$.

2. Materials and methods

2.1. Materials

Hank's balanced salt solution (HBSS) with and without phenol red, with and without Ca^{2+} was from Invitrogen (Carlsbad, California). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and human cortical neuronal cells (HCN-1A cells) were from ATCC (Manassas, VA). Valinomycin, bis-(1,3-diethylthiobarbituric acid) trimethine oxonol ($DiBAC_4(3)$), the acetoxymethyl ester (AM) of indo-1 (indo-1/AM) and of BAPTA (BAPTA/AM) were from Molecular Probes (Eugene, OR). Latanoprost, $PGF_{2\alpha}$ and unoprostone [(isopropyl(+)-(z)-7-[(1R,2R,3R,5S)-3,5-dihydroxy-2-(3-oxodecyl) cyclopentyl]-5-heptenoate)] were from R-Tech Ueno, Ltd. (Sanda, Japan) as frozen aliquots of 2 mM solutions in 100% DMSO. DMSO, also from R-Tech Ueno, Ltd. was used to dilute the compounds. IbTX was from Tocris Cookson (Ellisville, MO); tamoxifen-4-hydroxy was from Calbiochem (La Jolla, CA). Tetrodotoxin (TTX), AL8810, glutamate, Na_2ATP and ionomycin were from Sigma-Aldrich (St. Louis, MO). 2 mM unoprostone, latanoprost and $PGF_{2\alpha}$ were diluted with DMSO to give a final DMSO concentration of 0.1%. 0.1% DMSO controls were always carried out. IbTX and TTX were dissolved in water; AL8810, tamoxifen and BAPTA/AM were dissolved in DMSO and indo-1/AM was dissolved in ethanol.

2.2. Cell culture

HCN-1A cells were grown in DMEM medium with 10% FBS to 85–90% confluence on autoclaved 9x22 mm glass cover slips (Bellco Co. NJ). After 48 h in culture, these cells were used for experiments for 1 week and then fresh cells were plated. For patch clamp HCN-1A cells were seeded in 35 mm plastic petri dishes.

2.3. Patch clamp measurement of whole cell K^+ and Cl^- currents

Patch-clamp and analytical methods were as described previously [36]. K^+, currents were elicited by voltage clamp pulses (200 ms duration) between −70 and +130 mV in 20 mV steps from a beginning holding potential of −70 mV. Cl^- currents were elicited by voltage-clamp pulses (200 ms duration) between +40 and −140 mV in 20 mV increments from a beginning holding potential of −30 mV. Currents were averaged over a 50-ms time course starting at 50 ms and ending at 100 ms. For measurement of K^+ currents, the external solution contained (mM): 135 Na-methanesulfonate, 5 K-methanesulphonate, 2 $MgCl_2$, 5 glucose, and 10 HEPES pH 7.35. Tetrodotoxin (100 nM) was present to inhibit Na^+ channel currents. The pipette solution contained (mM): 140 K-methanesulphonate 0.1 $CaCl_2$, 2 $MgCl_2$, 0.1 Na_2-ATP, 0.1 Na_2-GTP, 0.145 EGTA and 10 HEPES pH 7.35. These solutions were also used to determine the resting membrane potential. Free Ca^{2+} in the pipet was calculated [43] to be 112 nM. For measurements of Cl^- currents, the external solution contained (mM): 150 tetraethyl ammonium Cl^-, 1.8 $CaCl_2$, 2 $MgCl_2$, 5 glucose and 10 HEPES pH 7.3. The pipette contained (mM): 150 tetraethyl ammonium Cl^-, 1 MgATP (1:1), 2 $MgCl_2$ and 10 HEPES pH 7.3. The resting membrane potential at 24 °C was determined by current clamp to be −67.3±0. 2 mV (n=5) in the following solutions. The external solution contained (mM): 135 NaCl. 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 glucose and 10 HEPES pH 7.35. The pipette contained (mM): 140 KCl, 2 $MgCl_2$ and 10 HEPES pH 7.35.

Pipettes were prepared from borosilicate glass and pulled by a two-stage Narashige puller to produce 1- to 1.5-MΩ resistance. The average cell capacitances for K^+ channel experiments were 57.5±2.9 pF (n=20) and for Cl^- channel experiments was 45.8±3.7 pF (n=3). Data were acquired with an Axopatch CV-4 headstage, a Digidata 1200 digitizer, and an Axopatch 1D amplifier. The sampling frequency was 1 kH, filter setting was 1 kH, and seal resistances were 10 gΩ. Data were analyzed using pClamp 6.04 (Axon Instruments, Union City, CA), Lotus 1-2-3 (IBM, White Plains, NY), and Origin 5 (OriginLab, Northampton, MA).

2.4. Measurement of plasma membrane potential

HCN-1A cells grown on cover slips were incubated with 100 nM $DiBAC_4$ (3) in HBSS for 5 min in the dark. $DiBAC_4(3)$ is a slow response membrane potential fluorescent probe. To ensure that the dye was at steady state after 5 min, the scans were taken from the same cover slip at 5 min and at 15 min (n=3 coverslips) after adding $DiBAC_4(3)$ and the apparent change was 1.25± 0.26 mV, which was small compared to the changes observed with test compounds. With test compounds cells were incubated for 3–5 min in the dark. Coverslips were placed in a stirred cuvette in an ISS K2 fluorometer and the signal was maximized by positioning the coverslip. Scans of 130 s were recorded with excitation at 540 nm and emission at 579–580 nm and then normalized to emission at 560 nm. For calibration, valinomycin (1 μM) was added to cells with different concentrations of external KCl. All of the compounds were tested under cell free conditions with 100 nM $DiBAC_4(3)$. No effects were observed at the highest concentrations of agents used here. In