Published in final edited form as:

Prostaglandins Other Lipid Mediat. 2017 September ; 132: 31–40. doi:10.1016/j.prostaglandins.2016.09.002.

FUNCTIONAL SCREENING FOR G PROTEIN-COUPLED RECEPTOR TARGETS OF 14,15-EPOXYEICOSATRIENOIC ACID

Xuehong Liu⁴, Zu-yuan Qian⁴, Fuchun Xie², Wei Fan⁴, Jonathan W. Nelson⁴, Xiangshu Xiao^{2,4}, Sanjiv Kaul⁴, Anthony P. Barnes^{3,4}, and Nabil J. Alkayed^{1,2,4}

¹Departments of Anesthesiology & Perioperative Medicine, Oregon Health & Science University, Portland, OR

²Departments of Physiology & Pharmacology, Oregon Health & Science University, Portland, OR

³Departments of Pediatrics, Oregon Health & Science University, Portland, OR

⁴Knight Cardiovascular Institute, Oregon Health & Science University, Portland, OR

Abstract

Epoxyeicosatrienoic acids (EETs) are potent vasodilators that play important roles in cardiovascular physiology and disease, yet the molecular mechanisms underlying the biological actions of EETs are not fully understood. Multiple lines of evidence suggest that the actions of EETs are in part mediated via G protein-coupled receptor (GPCR) signaling, but the identity of such a receptor has remained elusive. We sought to identify 14,15-EET-responsive GPCRs. A set of 105 clones were expressed in *Xenopus* oocyte and screened for their ability to activate cAMP-dependent chloride current. Several receptors responded to micromolar concentrations of 14,15-EET, with the top five being prostaglandin receptor subtypes (PTGER₂, PTGER₄, PTGFR, PTGDR, PTGER_{3-IV}). Overall, our results indicate that multiple low-affinity 14,15-EET GPCRs are capable of increasing cAMP levels following 14,15-EET stimulation, highlighting the potential for cross-talk between prostanoid and other eicosanoid GPCRs. Our data also indicate that none of the 105 GPCRs screened met our criteria for a high-affinity receptor for 14,15-EET.

Keywords

Epoxyeicosatrienoic acid; EET; GPCR; CFTR; Prostaglandin E₂; vasodilation

INTRODUCTION

Epoxyeicosatrienoic acids (EETs) are endogenous vasodilators in multiple vascular beds [1,2]. Potency of 14,15-EET as a vasodilator varies depending on the tissue and vessel size. For example, in bovine coronary arteries and rat mesenteric arteries, 14,15-EET has been shown to induce vasodilation in micromolar range [2–8] while high potency (low picomolar)

Co-Corresponding Authors: Nabil J. Alkayed, MD, PhD, alkayedn@ohsu.edu, Anthony P. Barnes, PhD, barnesan@ohsu.edu, Knight Cardiovascular Institute, Oregon Health & Science University, 3181 S.W. Sam Jackson Park Road, Portland, Oregon 97239.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

vasodilation by EETs has been observed in canine and porcine coronary microvessels [1,9]. We have previously demonstrated that 14,15-EET is abundant in brain tissue, playing critical roles in both normal function and disease [10].

Multiple ligand binding studies suggest the existence of a high affinity (low nanomolar) receptor for 14-15-EET [8,11–14]. To identify this putative high affinity receptor, we selected 150 candidate GPCRs based on their similarities to other lipid-sensing GPCRs. Only a subset of 105 receptors were successfully cloned and shown to display cell surface expression in *Xenopus* oocyte, which were subsequently screened for their ability to increase cAMP-dependent chloride current.

We focused on cAMP-based activity because it has been suggested that 14,15-EET exerts its vasodilator effect via Gs-coupled signaling leading to increased cAMP [8,12]. To monitor changes in cAMP in real time, we used the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) channel as a functional reporter of increased cAMP and activation of Protein Kinase A (PKA), as opening of CFTR channel requires phosphorylation by PKA [15,16]. In this case, the CFTR channel was co-expressed in an oocyte expression system along with each GPCR of interest. The utility of this method in studying GPCR signaling has been demonstrated previously [17–19]. We also employed ERK activation assay and a β -arrestin recruitment assay as alternative methods to detect GPCR activation by 14,15-EET. Our results indicate that while none of the candidate receptors tested met our criteria for a high affinity Gs-coupled receptor for 14,15-EET, we were able to identify several previously unknown low affinity 14,15-EET receptors.

MATERIALS AND METHODS

Mouse mesenteric artery diameter measurement

Mouse mesenteric arteries were isolated, cut into 1–2 mm in length and mounted on a single vessel chamber (CH-1 Living Systems Instrumentation, Burlington, VT), secured between two glass micropipettes and tied with two knots on each end. Vessels were maintained at a constant pressure (80 mmHg) using a pressure servo system (Living Systems Instrumentation, Burlington, VT), and superfused continuously with MOPS buffered solution (in mM: 144 NaCl, 3.0 KCl, 2.5 CaCl₂, 1.5 MgSO₄, 2.0 MOPS, 5.0 glucose, 2.0 Pyruvate, 0.02 EDTA, 1.2 NaH₂PO₄) with a flow rate of 2.5 mL/min. The temperature of the perfusion chamber was maintained at 37°C using both stationary (TC-095, Living Systems Instrumentation, Burlington, VT) and inline heater (TC-344B, Warner Instruments, Hamden, CT). Vessel diameter was continuously monitored using a video dimension analyzer (Living Systems Instrumentation, Burlington, VT), digitized and recorded using AxonScope data acquisition software (Molecular Devices, Sunnyvale, CA)

HEK293 cell culture and transfection

Human Embryonic Kidney-293 cells (HEK 293; American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS). Cells were thawed, maintained in 5% CO₂ at 37°C and used through passage 12. Cells grown in 12-well plates were transfected

with 2.5 µg GPCR plasmids and Lipofectamine 2000 reagent (Invitrogen) at 50–70% confluency in media lacking antibiotics. Cells transfected with transfection solution alone without the plasmid served as controls.

GPCR vector construction

GPCR clones were obtained from DNASU Plasmid Repository (The Biodesign Institute, Arizona State University, Tempe, AZ) and UMR cDNA Resource Center (University of Missouri-Rolla, Rolla, MO). They were subcloned into a modified pcDNA3.1 vector containing both CMV and T7 promoters to permit both mammalian expression and *in vitro* transcription of each candidate receptor for oocyte expression. The expression vector also contains an in-frame hemagglutinin (HA) epitope tag that resulted in amino terminus tagging of each candidate receptor.

cAMP detection assay

cAMP concentrations of oocytes were detected using the Cyclic AMP XP® Assay Kit from Cell Signaling Technology (Danvers, MA) based on the manufacture's protocol with a slight modification where 50 µM IBMX [18] was included in the stimulatory cocktail and 100 mM HCl [20] was included in the lysis buffer to minimize PDE activity in oocytes.

Oocyte expression system

The cRNAs for human CFTR (hCFTR) and candidate GPCRs were generated using Ambion mMessage mMachine T7 Ultra transcription kit (Ambion, Foster City, CA). Follicular membranes of *Xenopus laevis* oocytes were removed using 0.2 Wünsch units/mL Liberase TL (Roche Molecular Biochemicals, Indianapolis, IN) for 2 h in a Ca²⁺-free solution containing (mM): 82.5 NaCl, 2 KCl, 1 MgCl₂, 5 HEPES, pH 7.5. Oocytes were sorted and maintained in a modified Barth's solution containing (mM): 88 NaCl, 1 KCl, 0.82 MgSO₄, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 2.4 NaHCO₃, 5 HEPES, 5 HEPES-Na, and 250 mg/L Amikacin plus 150 mg/L Gentamicin at pH 7.5. Stage V to VI oocytes were injected with 50 nL cRNA mix containing CFTR RNA and GPCR RNA.

Whole-cell recordings were carried out using Oocyte 725 amplifier (Warner Instruments, Hamden, CT) and pClamp 8 software (Molecular Devices, Sunnyvale, CA). Individual oocytes were placed in a modified recording chamber (RC-1Z, Warner Instruments, Hamden, CT) and continuously superfused with standard Frog Ringer's (FR) solution (in mM: 98 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 2.5 HEPES, 2.5 HEPES-Na) where they were maintained in the open circuit condition and the membrane potential was periodically ramped from –120 to +60 mV over 1.8 sec to obtain current-voltage relationships.

Detection of cell surface expression of GPCRs

Oocytes or HEK cells expressing candidate GPCRs were incubated for 1 h at 4°C in the dark in Frog Ringer's solution and PBS containing 1 µg/mL Alexa Fluor 488 conjugated anti-HA antibody (ThermoFisher®, Grand Island, NY), respectively. Cells were washed three times prior to imaging using laser scanning confocal microscopy. Fig. 1 contains examples of surface labeling in oocytes. Oocytes expressing hCFTR that lacks the HA-tag was used as a control. We confirmed surface expression of 105 candidate receptors in oocytes (Table 1).

ERK phosphorylation (ERKp) assay

HEK cells transfected with vector alone or with candidate receptors were serum starved for 1 hour prior to exposure to either vehicle (DMSO) or 1 μ M 14,15-EET for 15 min. Cells were then lysed in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM ethylenediaminetetraacetic acid and protease inhibitor (Roche, Nutley, NJ). HEK proteins in SDS sample buffer (2% SDS, 10% glycerol, 80 mM Tris, pH 6.8, 0.15 M β -mercaptoethanol, 0.02% bromophenol blue) were separated on 4–12% SDS–polyacrylamide gels (20 μ g/lane) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 30 minutes at room temperature and incubated overnight at 4°C with 1:1000 rabbit anti-ERK (Cell Signaling, Danvers, MA) primary antibodies in dry milk.

The antigens were detected by the luminescence method (ECL-plus Western blotting detection kit) with peroxidase-linked anti-rabbit antibodies (GE Amersham, Lafayette, CO). The intensity of immunoblot bands was detected and quantified using the Fluor Chem FC2 Image Analysis System (Alpha Innotech, Wallingford, CT). Levels of total ERK and phosphorylated ERK were normalized using levels of beta-actin.

PathHunter β -arrestin enzyme fragment complementation (EFC) assay

The high throughput β -arrestin screening assay developed by DiscoveRx Corporation (Fremont, CA) makes use of two inactive complementary fragments of β -galactosidase (β -Gal) that are fused to β -arrestin and target GPCR, respectively. Receptor activation restores β -Gal activity when the two complementary β -Gal fragments merge as a result of formation of β -arrestin-G-protein complex. The restored β -Gal activity was measured by chemiluminescent PathHunter® Detection Reagents following stimulation by 1 μ M 14,15-EET. A total of 241 GPCRs, including 73 orphan GPCRs were screened (<https://www.discoverx.com/services/drug-discovery-velopment-services/gpcscan-gpcr-profiling>).

Standard procedures employed by DiscoveRx Corporation (Fremont, CA) are summarized as follows: **Cell Handling**. PathHunter cell lines expressing each receptor were expanded from freezer stocks were seeded in a total volume of 20 μ L into white walled, 384-well microplates and incubated at 37°C for 90 min prior to testing. **Agonist Activity Assay**. 5 μ L of 5 $\times$ 14,15-EET diluted in assay buffer was added to cells and incubated at 37°C for 90 min. Final assay vehicle concentration was 1% for all experiments. **Antagonist Activity Assay**. 5 μ L of 5 $\times$ 14,15-EET stock diluted in assay buffer was added to cells and incubated at 37°C for 30 min. Then, 5 μ L of 6 $\times$ EC₈₀ agonist diluted in assay buffer was added to the cells and incubated at 37°C for 90 min. **Signal Detection**. Assay signal was generated through a single addition of 50% v/v of PathHunter Detection reagent cocktail. Two data points were collected for each receptor per assay. After one hour incubation at room temperature, microplates were read with a PerkinElmer Envision™ instrument for chemiluminescent signal detection. **Data Analysis**. Compound activity was analyzed using CBIS data analysis suite (ChemInnovation, CA). The activity was reported as the mean of two replica. Agonist activity for orphan receptors was determined as percentage activity (%Act) calculated using Eq (1):

$$\%Act = 100\% * \frac{RLU_{EET} - RLU_{vehicle}}{RLU_{vehicle}} \quad (1)$$

Agonist activity for receptors with known ligands was determined as percentage activity (%Act) calculated using Eq (2):

$$\%Act = 100\% * \frac{RLU_{EET} - RLU_{vehicle}}{RLU_{max} - RLU_{vehicle}} \quad (2)$$

Antagonist activity was determined as percentage inhibition (%Inh) calculated using Eq (3):

$$\%Inh = 100\% * \left(1 - \frac{RLU_{EET} - RLU_{vehicle}}{RLU_{EC80} - RLU_{vehicle}} \right) \quad (3)$$

where RLU_{EET} is the relative mean luminescent unit determined for 14,15-EET, $RLU_{vehicle}$ is the mean RLU for the vehicle control, RLU_{max} is the mean max RLU for control agonist and RLU_{EC80} is the mean RLU of control agonist EC_{80} .

Strategy for oocyte-based receptor screening

To efficiently screen a large number of receptors using the oocyte assay, we first confirmed surface expression of the receptors, then co-expressed each candidate receptor with CFTR in *Xenopus* oocytes and assayed receptor activity using an electrophysiological method (two-electrode-voltage clamp, TEVC). Oocytes were first exposed to 10 μ M forskolin. The response to forskolin served as an internal control for the level of cAMP-induced CFTR conductance given that forskolin activates adenylate cyclase directly and its action is independent of receptor activation. Oocytes were then exposed to 1 μ M 14,15-EET. If 1 μ M 14,15-EET induced an increase in conductance, the same oocyte would then be exposed to lower concentrations 14,15-EET.

Reagents

Forskolin, IBMX (Isobutylmethylxanthine), 14(R),15(S)-EET, 14,15-EEZE and prostaglandin E2 (PGE_2) were purchased from Cayman Chemical (Ann Arbor, MI); 14,15($\pm$)-EET was synthesized in-house [21]. Although the majority of the experiments were performed using 14,15($\pm$)-EET, 14(R),15(S)-EET was occasionally used, with no qualitative differences observed between the two compounds thus they were labeled throughout non-discriminately as 14,15-EET or EET; CF172 (CFTR_{inh}-172, 4-[4-Oxo-2-thioxo-3-(3-trifluoromethyl-phenyl)-thiazolidin-5-ylidenemethyl]-benzoic acid) was kindly provided by Dr. Robert Bridges (Rosalind Franklin University, Chicago, IL) and The Cystic Fibrosis Foundation (CFF).

Statistical analysis

Results were analyzed using the Student's t-test or two-way ANOVA when appropriate. Differences with $p < 0.05$ were considered statistically significant. Data are presented as means $\pm$ SEM.

RESULTS

14,15-EET induces vasodilation in U46619-precontracted mouse mesenteric arteries

The vasodilatory effect of EETs has been reported in vessels from multiple species, including bovine, rat, porcine and canine vessels [1–9]. Similarly, we find that 14,15-EET induces vasorelaxation with an EC₅₀ of 510 nM and maximal dilation to 68% in isolated mouse mesenteric arteries precontracted to 26–56% of their baseline diameter by 20–40 nM U46619, a thromboxane A₂ receptor agonist [22] (Fig. 2).

14,15-EET increases conductance of oocytes co-expressing CFTR and prostaglandin receptors

To determine which GPCR mediates the increase in intracellular cAMP by 14,15-EET, we co-expressed candidate GPCRs with CFTR, and used the latter as a reporter for GPCR/Gs/cAMP activation. As shown in Fig. 3, we first confirmed that in oocytes expressing CFTR alone, 14,15-EET had no effect on conductance. Vehicle (ethanol) also had no effect on conductance of uninjected oocytes or those injected with CFTR alone or together with receptors (not shown).

Thorough screening of each candidate receptor resulted in the identification of several EET-responsive receptors with the top five being prostaglandin receptor subtypes (PTGER₄, PTGER₂, PTGFR, PTGDR and PTGER₃IV) (Fig. 4). Because conductance changes in response to 14,15-EET were only induced when these receptors were co-expressed with CFTR, we conclude that stimulation of CFTR conductance by 14,15-EET is solely due to the activation of the receptors. However, we also found that none of these receptors meet our criteria for a high affinity Gs-coupled receptor because micromolar concentrations are needed to achieve maximum stimulation.

14,15-EET increases ERK phosphorylation in HEK293 cells expressing PTGER₂ or PTGER₄

To complement the results from the oocyte assay, we implemented another measure used extensively to study GPCR activation [23,24], namely ERK phosphorylation (pERK). As shown in Fig. 5, significantly affected levels of pERK were only seen in 14,15-EET treated cells transfected with either PTGER₂ or PTGER₄. The smaller effects of 14,15-EET on cells transfected with PTGDR, PTGFR and PTGER₃IV might have been compromised due to reduced surface expression in HEK cells (Fig. 6). In this regard, it has been shown previously that oocytes are capable of expressing exogenous proteins that are poorly expressed in mammalian cells such as HEK cells [25].

14,15-EET is a less efficacious agonist than PGE₂ in stimulating CFTR conductance in oocytes co-expressing CFTR and prostaglandin receptors

Our finding that 14,15-EET activates several prostaglandin receptors raises the question of whether the effect of 14,15-EET is comparable to that induced by prostaglandin E₂ (PGE₂). We compared the effects of 1 μ M 14,15-EET and 10 nM PGE₂ in the same oocytes co-expressing CFTR and prostaglandin receptors. As shown in Fig. 7, we first confirmed that oocytes expressing CFTR alone had no conductance response following application of 10 nM PGE₂. However, in oocytes co-expressing CFTR and the receptors, a 100 fold lower concentration of PGE₂ was capable of eliciting a larger or similar increase in conductance compared to that elicited by 1 μ M 14,15-EET (Fig. 8). Because PGE₂ only induced changes in conductance when these receptors were co-expressed with CFTR, our results indicate that the stimulation of CFTR conductance by PGE₂ was solely due to the activation of the receptors (the order of exposure to the reagents did not alter outcome, data not shown).

Effect of PGE₂ on intracellular cAMP levels

Using forskolin stimulated increase in CFTR conductance as an internal control for the level of CFTR expression and activation due to elevation of intracellular cAMP level, we found the average ratio between the conductance induced by 10 nM PGE₂ and that induced by 10 μ M forskolin was 18 ± 4 (n=4), 13 ± 9 (n=3), 3.5 ± 0.6 (n=4), 0.7 ± 0.5 (n=4) and -0.4 ± 0.1 (n=4) in oocytes co-expressing CFTR with PTGER₂, PTGER₄, PTGFR, PTGDR, or PTGER₃IV, respectively. This result is consistent with idea that PGE₂ is a preferred agonist that activates PTGER₂ and PTGER₄ and PTGDR via Gs-coupled receptors signaling via cAMP pathway [26–29].

PTGER₃ has multiple isoforms that couple to different signaling pathways [27,30]. A synthetic EP₃ agonist M&B28767 has been shown to increase cAMP in COS-7 expressing PTGER₃IV with a concentration greater than 1 μ M and in CHO cells transfected with PTGER₃IV with a concentration greater than 100 nM [30]. However, we are not aware of any study demonstrating the stimulatory effect of PGE₂ on cAMP in cells transfected with either PTGER₃IV or PTGFR. We therefore, used a commercial assay to determine if PGE₂ increases levels of cAMP in oocytes expressing PTGER₂, PTGFR, and PTGER₃IV. As shown in Fig. 9, however, we did not observe a change in cAMP in oocytes expressing PTGFR and PTGER₃IV. We were also unable to detect changes of cAMP in HEK cells transfected with PTGFR and PTGER₃IV (data not shown). The only increase in cAMP by PGE₂ we observed was in oocytes expressing PTGER₂. Because electrophysiological recording in oocytes may have a higher sensitivity in detecting changes in oocyte intracellular cAMP (note an 18 fold increase detected by TEVC in oocytes coexpressing CFTR and PTGER₂ vs. < 2 fold increase detected by the cAMP assay in oocytes expressing PTGER₂), and to further investigate the role of cAMP in PGE₂-mediated effects, we combined electrophysiological recordings with pharmacological inhibitors of cAMP pathway. Specifically, we compared the effects of PGE₂ on the conductance of oocytes co-expressing CFTR and receptors of interest with and without pretreatment with a known PKA inhibitor, H89. Using a concentration previously shown to partially block PKA activity in oocytes [31], we found that pretreatment with 50 μ M H89 eliminated more than 90% of PGE₂-induced conductance in each paired experiment (Fig. 10).

EEZE (14,15-EE-5(Z)-E) does not behave as a selective antagonist for 14,15-EET at the PTGER₂ receptor

14,15-EEZE was initially identified as a selective antagonist for 14,15-EET [32]. In bovine coronary arteries and rat mesenteric artery, 14,15-EEZE blunted vasorelaxation induced by 14,15-EET [6,32]. However, it has also been shown that 14,15-EEZE can induce vasorelaxation [32,33]. Because PTGER₂ has been suggested as a receptor for 14,15-EET [6], we carried out experiments to test the effect of 14,15-EEZE on PTGER₂. We first determined that 14,15-EEZE had no effect on oocytes expressing CFTR alone (Fig. 11A). However, in oocytes co-expressing CFTR and PTGER₂, 14,15-EEZE induced a robust increase in CFTR conductance, and pretreatment of 14,15-EEZE prevented additional stimulation by 14,15-EET in a concentration dependent manner (Fig. 11B&C), suggesting that 14,15-EEZE, like 14,15-EET, could be an agonist or partial agonist for PTGER₂. To confirm that the increase in conductance was due to stimulation of CFTR channels, we also exposed oocytes to 10 μ M CF172 [34], a specific inhibitor for CFTR channel. As shown towards the end of the experiments, CF172 induced a rapid decrease in conductance, demonstrating that activation of PTGER₂ by 14,15-EEZE was solely due to the stimulation of CFTR channels.

The stimulatory effect of 14,15-EET and 14,15-EEZE are not due to PGE₂ synthesis

An alternative explanation to direct binding and activation of the prostaglandin receptors by 14,15-EET is that it instead exerts its effect by stimulating PGE₂ synthesis in oocytes. We therefore investigated the effect of the prostacyclin inhibitor indomethacin on PGE₂-stimulated CFTR conductance. As shown in Fig. 12, pre-exposure of oocytes co-expressing CFTR and PTGER₂ to indomethacin had no significant effect on the changes in conductance induced by 14,15-EET (Fig. 12A) or 14,15-EEZE (Fig. 12B), suggesting that PGE₂ synthesis is unlikely to mediate the stimulatory effect of 14,15-EET or 14,15-EEZE on PTGER₂.

Putative GPCR screening based on β -Arrestin recruitment

To expand our search for a putative receptor for 14,15-EET, we took advantage of a high-throughput β -arrestin screening assay using 1 μ M 14,15-EET as a probe. The DiscoverX receptor library contained a total of 241 candidate receptors, including 174 candidate receptors not screened in the oocyte assay. All receptors were screened for the agonist activity of 14,15-EET. Those with known ligands were also screened for the antagonist activity of 14,15-EET. Based on the mean values of two replicas, none of the receptors exhibited more than 30% increase in activity following stimulation by 1 μ M 14,15-EET in the agonist assay. Receptors exhibiting an increase in activity for at least 15% are shown in Table 2. Similarly, none of the receptors exhibited more than 50% decrease in activity when 1 μ M 14,15-EET was employed as an antagonist and those receptors displaying more than 28% inhibition by 1 μ M 14,15-EET are indicated in Table 3.

14,15-EET increases ERK phosphorylation in HEK293 cells expressing CXCR4 or CMKLR1

A subset of receptors exhibiting responsiveness to 14,15-EET in the DiscoverX screen were found not to be responsive to 14,15-EET in the oocyte system (marked in *Italic Bold* in

Tables 2 and 3). Therefore, we further examined these candidates using the ERK phosphorylation assay. As shown in Fig. 13, 1 μ M 14,15-EET significantly increased pERK in HEK293 cells expressing CXCR4 or CMKLR1.

DISCUSSION

Identification of low affinity receptors for 14,15-EET

Greater insight into the molecular mechanisms underlying EETs' vasodilatory actions are critical to understanding their roles in vascular physiology and disease. Several studies have shown that 14,15-EET can dilate isolated vessels within the micromolar range [2–8]. One study found that the vasodilatory effect of 14,15-EET in rat mesenteric arteries was mediated through PTGER₂ via Gs-coupled signaling [6].

Our current findings support the idea that PTGER₂ is involved in mediating the micromolar effects of 14,15-EET [6]. It is not clear why these investigators did not observe a role for PTGER₄ in mediating the vasodilatory effect of 14,15-EET, although their Western blots showed similar abundance for PTGER₂ and PTGER₄ in primary cultured rat mesenteric artery smooth muscle cells. Both PTGER₂ and PTGER₄ are known to couple to G_s, causing an increase in intracellular cAMP in response to agonist [28,35].

Our observation that PGE₂ treatment of oocytes co-expressing CFTR and PTGDR results in a stimulatory effect on CFTR conductance is consistent with the notion that PTGDR is a Gs-coupled receptor [29,36]. PGE₂ has been shown to stimulate cAMP production in HEK cells stably expressing human PTGDR with an EC₅₀ of 84 nM [29]. However, in another study, it was showed that EC₅₀ was greater than 500 nM for PGE₂ in stimulating cAMP in HEK293 cells stably expressing human PTGDR [36]. It is not obvious why the difference in EC₅₀ varied by nearly tenfold between the two studies. It is possible that difference in the sources for the cells and detection reagents used may attribute to such difference.

PTGER₃ is known to have multiple subtypes that couple to different signaling pathways [28,30]. Our current observation that PGE₂ stimulation of PTGER₃IV increased CFTR conductance in oocytes co-expressing CFTR and PTGER₃IV suggests that PGE₂ activation of PTGER₃IV may increase intracellular cAMP. Inhibition of PGE₂ effect by PKA inhibitor H89 is also consistent with such hypothesis. Although H89 is known to inhibit other kinases in some cell types [37–39], we are not aware of any study linking these kinases to the activation of CFTR channels.

It is unclear why we were not able to detect a change in cAMP due to PTGER₃IV activation using an conventional cAMP assay. It is possible that the sensitivity of our electrophysiological approach (TEVC), detects small changes that were not detected by conventional ELISA assays. On the other hand, although the change in CFTR conductance due to PGE₂ activation of PTGER₃IV were significantly different from the controls, the extend of the change due to activation of PTGER₃IV is much smaller than that due to activation of PTGER₂ as demonstrated when their effects were compared with that induced by forskolin. Therefore, even if activation of PTGER₃IV increases intracellular cAMP, the physiological significance of such effect can not be evaluated without additional studies.

Our finding that activation of PTGFR stimulated CFTR conductance raises an interesting question regarding the signaling pathways associated with PTGFR, which is best known as a Gq-coupled receptor that increases intracellular Ca^{2+} when activated by its specific ligand, $\text{PGF}_{2\alpha}$ [40]. We are not aware of any previous study specifically examining the effect of activation of PTGFR by PGE_2 on intracellular cAMP concentration. As an agonist, PGE_2 is not as potent as $\text{PGF}_{2\alpha}$ for PTGFR based on a competitive binding assay against [^3H] $\text{PGF}_{2\alpha}$ ($K_d=1$ nM) [40]. Using membranes from COS cells expressing PTGFR, these authors found that the IC_{50} for PGE_2 was 85 nM. In oocytes expressing PTGFR, they observed a dose-dependent increase in intracellular Ca^{2+} stimulated by PGE_2 , although PGE_2 did not induce any detectable change in intracellular Ca^{2+} at 10 nM, unlike $\text{PGF}_{2\alpha}$ [40].

The increase in CFTR conductance due to activation of PTGFR by PGE_2 was significant as detected by monitoring using TEVC in real time. The inhibitory effect of PKA inhibitor H89 on PGE_2 stimulated CFTR conductance is also consistent with the idea that activation of PTGFR may increase cAMP. However, the extent of the increase in CFTR conductance due to activation of PTGFR is much smaller than that due to activation of PTGER_2 as demonstrated when their effects were normalized to forskolin-induced conductance. Furthermore, since we were not able to detect a change in cAMP directly using a conventional cAMP assay, additional studies using different cells type and different techniques may be necessary to validate whether PTGFR can couple to cAMP dependent signaling pathway.

Overall, our current study has raised interesting questions regarding the interaction between 14,15-EET and prostaglandin receptors. Given the relatively low plasma concentration of EETs [41,42], the physiological relevance of the micromolar dilatory effect of EETs remains unclear [1].

An argument for the existence of a high affinity receptor for 14,15-EET

A high affinity receptor for 14-15-EET was implicated in membrane binding studies (low nanomolar) [3,8,11–14]. High potency EET-dependent vasodilation has been reported in canine and porcine coronary microvessels [1,9]. We found that 14,15-EET induced vasorelaxation in mouse mesenteric arteries with an EC_{50} of 510 nM. Although EET-responsive receptors discovered in the current study are all low affinity, our results do not necessarily exclude the possibility of the existence of a high affinity receptor for 14,15-EET.

EEZE as a Partial Agonist

Although 14,15-EEZE has been suggested initially as a selective antagonist for EETs [32], additional studies have also shown that 14,15-EEZE can act as a partial agonist for endothelial derived hyperpolarizing factor (EDHF), exerting vasodilatory effect on Bovine coronary artery [32] or mouse mesenteric arteries [33]. In agreement with these studies, we found that 14,15-EEZE has a stimulatory effect on the conductance of oocytes co-expressing CFTR and PTGER_2 . Our observation is consistent with the notion that 14,15-EEZE is an agonist or partial agonist for PTGER_2 .

PGE₂ synthesis is not responsible for the stimulatory effects of 14,15-EET and 14,15-EEZE

Because PGE₂ is a naturally occurring physiological molecule, it is possible that 14,15-EET and 14,15-EEZE treatment could stimulate PGE₂ production in oocytes rather than directly activate the receptors. However, our results demonstrate that 14,15-EET and 14,15-EEZE directly activate the receptor because their stimulatory effects were unaffected by pre-treatment with the PGE₂ synthesis inhibitor indomethacin [43]. In addition, it has been shown that 14,15-EEZE partially blocked the dilatory effect of PGE₂ in rat mesenteric arteries [6]. Furthermore, the time course of the effects of 14,15-EET and 14,15-EEZE observed in oocytes was much more rapid than PGE₂ synthesis through the COX enzyme that has a half time in the scale of hours [44]

Importance of assay selection in receptor screening

We employed three different assays in the current study to screen for a putative receptor for 14,15-EET. Among the five low affinity EET-responsive receptors we identified using the oocyte expression assay, only two were significantly stimulated by 14,15-EET in the pERK assay. In addition, among the five receptors tested using both β -arrestin and pERK assays, only two were significantly stimulated by 14,15-EET in the pERK assay. There are at least three possible explanations for the discrepancies in results obtained by different assays. First, oocytes tend to be a more robust system to express some exogenous proteins than mammalian cells [25] and it has very low background noise, which makes it a very sensitive assay. Oocytes assay has the additional advantage of allowing real time studies under physiological conditions as demonstrated in the current study. Second, different methods are likely to have different detection limit for monitoring receptor activation. Third, the three assays used in the current study were performed in different cell types, which may utilize different G-protein coupling and signal transduction pathways. In our view, in cases when the coupling mechanisms are unknown, it is crucial to employ multiple assays for receptor screening.

CONCLUSION

Our current study confirms the existence of several low affinity receptors for 14,15-EET. None of the receptors screened in our study met the criteria for a high affinity Gs-coupled receptor for 14,15-EET. However, our results do not rule out the existence of a putative high affinity receptor for 14,15-EET. Our results also do not rule out the potential involvement of other signaling pathways in mediating the effect of 14,15-EET.

Acknowledgments

FUNDING

This work is supported by the Knight Cardiovascular Institute at Oregon & Health & Science University, Portland, OR.

ABBREVIATIONS

14,15-EET (EET)	14,15-Epoxyeicosatrienoic acids
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14,15-EEZE	14,15-Epoxyeicosa-5(Z)-enoic Acid
VSMCs	vascular smooth muscle cells
GPCR	G protein-coupled receptor
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
PKA	Protein Kinase A
PTG	prostaglandin
PGE₂	prostaglandin E2
IMC	indomethacin
CF172	CFTR _{inh} -172 (5-[(4-Carboxyphenyl)methylene]-2-thioxo-3-[(3-trifluoromethyl)phenyl]-4-thiazolidinone)
IBMX	3-isobutyl-1-methylxanthine
TEVC	two- electrode-voltage clamp

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Figure 1.

Examples of surface detection of prostaglandin receptors expressed in oocytes. Receptors were labeled with a fluorophore (Alexa Fluor® 488 dye) and detected by confocal microscopy. Gray scale was used for clarity. All receptors showed above-background labeling when oocytes were injected with equal or greater amount of RNA than that used for electrophysiological experiments described below.

Figure 2. 14,15-EET (EET)-induced vasodilation. Dose-response curve obtained from mouse mesenteric arteries pre-constricted with thromboxane A2 agonist U46619 (n=4–6).

Figure 3.

14,15-EET (EET) had no effect on conductance of oocytes expressing hCFTR alone. An oocyte expressing hCFTR is exposed to 10 μ M forskolin (white bars and circles). After a wash, it was exposed to 10 nM, 100 nM and 1 μ M EET (gray bars and circles), sequentially.

Figure 4.

14,15-EET (EET) increased conductance in oocytes co-expressing hCFTR and (A) PTGER₂, (B) PTGER₄, (C) PTGFR, (D) PTGDR, or (E) PTGER₃IV. In each case, oocytes co-expressing hCFTR and a candidate receptor were exposed to 10 μ M forskolin (white bars and circles), 1 μ M EET (dark gray bars and circles) and 100 nM 14,15-EET (gray bars and circles), sequentially with washes in-between. Normalized data with respect to forskolin-induced changes are summarized on the right side of each panel.

Figure 5.
Summary of 14,15-EET-induced pERK in cultured HEK293 cells expressing prostaglandin receptor subtypes (n=3 each).

Figure 6.

Examples of surface detection of PGE₂ receptors transfected in HEK cells. Receptors were labeled with an anti-HA antibody coupled to the fluorophore Alexa 488 and detected by confocal microscopy. Images were obtained under identical conditions. Scale bar in upper left image is 10 microns and applies to all images.

Figure 7.

PGE_2 had no effect on conductance of oocytes expressing hCFTR alone. An oocyte expressing hCFTR was exposed to 10 μ M forskolin (white bars and circles). After a wash, it was exposed to 10 nM PGE_2 .

Figure 8.

14,15-EET (EET) is less efficacious than PGE₂ to increase conductance in oocytes co-expressing hCFTR and (A) PTGER₂, (B) PTGER₄, (C) PTGFR, (D) PTGDR or (E) PTGER₃IV. In each case, oocytes co-expressing hCFTR and a candidate receptor were exposed to 10 μ M forskolin (white bar and circles) followed by 1 μ M and 100 nM EET (gray bar and circles) and 10 nM PGE₂ (dark gray bar and circles) sequentially with washes in between. Normalized data in respect to forskolin-induced changes are summarized on the right side of each panel.

Figure 9.

Effect of PGE_2 on cAMP levels in oocytes expressing PTGER₂, PTGFR and PTGER₃IV. Oocytes were injected with 20 ng RNA and divided into experimental (black bars) and control groups (white bars). Three oocytes were pooled as one data point. On day 4 following injection, oocytes in experimental groups were treated with 10 nM PGE_2 plus 50 μM IBMX for 15 min. cAMP levels in both groups were then assayed per manufacture's protocol.

Figure 10.

Effect of PKA inhibitor H89 on conductance of oocytes co-expressing hCFTR and (A) PTGER₂, (B) PTGFR, or (C) PTGER₃IV. Oocytes were pretreated with 50 μ M H89 for a minimum of 10 min. For each pair, control and H89-pretreated oocytes were exposed to 10 nM PGE₂ (black bars) as soon as stable background conductance was established (white bars). 50 μ M H89 was present at all times in the superfusate for H89-pretreated oocytes. P-values were less than 0.05 for panels A and C and it was 0.06 for panel B.

Figure 11.

14,15-EEZE (EEZE) is an agonist/partial agonist for PTGER₂. (A) An oocyte expressing hCFTR was exposed to 10 μ M forskolin (white bar and circles), vehicle (white bar and triangles), 1 μ M and 10 μ M EEZE. Summary of normalized data in respect to forskolin-induced changes are shown at right. (B) An oocyte co-expressing hCFTR and PTGER₂ was exposed to 1 μ M EET (gray bar and circles), 1 μ M EEZE (dark gray bar and circles, and 1 μ M EET in the presence of 1 μ M EEZE. Followed by exposure to 10 μ M CF172. (C) A similar experiment carried out in an oocyte co-expressing hCFTR and PTGER₂ as shown in panel B, except 10 μ M EEZE was used in place of 1 μ M EEZE. Summary of normalized data in respect to EET-induced changes for panel B&C are shown on the right for each panel.

Figure 12.

Effects of 14,15-EET (EET) and 14,15-EEZE (EEZE) were not due to PGE₂ synthesis. **(A)** An oocyte co-expressing hCFTR and PTGER₂ was exposed to 1 μM EET (gray bar and circles), 10 μM indomethacin (IMC) (dark gray bar and circles, and 1 μM EET in the presence of 10 μM IMC, followed by exposure to 10 μM CF172. **(B)** A similar experiment carried out in an oocyte co-expressing hCFTR and PTGER₂ as shown in **panel B**, except 10 μM EEZE was used in place of 1 μM EET.

Figure 13.

Summary of 14,15-EET-induced pERK in cultured HEK293 cells expressing receptors that exhibited highest activity in β -arrestin assay (n=3 each).

Table 1**GPCRs with Confirmed Expression in Oocytes**

ADRB2	CNR2	CCKBR	LPAR3
CXCR4	CNR1	CCR1	B1AR
CYSLTR1	GPR68 (oGPR1)	CCR3	Cmklr1
PTGER2	LPAR4	CHRM2	Mrgprf
PTGER4	GPR35	CHRM3	Galr2
GPCR132	PTGFR	CHRM4	Opn3
PAR2	PTGDR	GPCR4	Kiss1r
LTB4R	PTGER3	HRH2	Gpr135
EDG4 (LPAR2)	PTGER1	HTR1A	Ccr10
P2RY2	GPCR17	HTR4	Gpr19
PTAFR	GPCR18	HTR6	Lgr4
EDG3	GPCR20	LTB4R2	Sucnr1
EDG6	GPCR63	NPFFR1	Mrgpre
TBXA2R	GPCR161	OPRL1	Gpr162
GPR31	P2RY1	GPCR21	Gpr107
GPR3	P2RY4	GPCR52	Gpr146
GPR61	P2RY6	GPCR141	Gpr137B
GPR85	SSTR2	GPCR148	Tpra1
GPR173	SSTR3	GPCR176	Gpr39
PGIR	SSTR4	GPCR34	Npy1r
LPAR2	ADORA2	GPCR81	Adora2b
LPAR1	ADRB3	GPCR116	GPR32
S1P3	AGTR1	GPCR125	C5L2/C5AR2
S1P1	APLNR	GPCR26	GPR101
S1P5	AVPR1A	GPCR62	FPR3
S1P2	C5AR1	GPCR78	BONZO/CXCR6
			ADORA1

Table 2Top Ranked Receptors Based on Stimulation of β -Arrestin Recruitment by 14,15-EET

Receptor	%activity
<i>CCKBR</i>	26%
<i>CNR2</i>	20%
<i>CXCR4</i>	17%
GHSR1B	19%
<i>GPR61</i>	15%
GPR139	15%
HTR1B	15%
P2RY8	15%

Table 3

Top Ranked Receptors based on Inhibition by 14,15-EET

Receptor	%inhibition
FSHR	−47%
GPR109A	−41%
BRS3	−32%
PROKR2	−32%
HRH4	−30%
<i>CMKLR1</i>	−28%
CALCR-RAMP3	−28%
AVPR1B	−28%
PRLHR	−28%