Biochemical and Biophysical Research Communications 438 (2013) 353–358

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Molecular and pharmacological characterization of zebrafish *'contractile'* and *'inhibitory'* prostanoid receptors

Ryo Iwasaki [a], Kyoshiro Tsuge [a], Kazushi Morimoto [a,b], Tomoaki Inazumi [a,b], Osamu Kawahara [c], Atsuo Kawahara [d], Soken Tsuchiya [a], Yukihiko Sugimoto [a,b,*]

[a] Department of Pharmaceutical Biochemistry, Graduate School of Pharmaceutical Sciences, Kumamoto University, Chuo-ku, Kumamoto 862-0973, Japan
[b] Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan
[c] Institute of Resource Development and Analysis, Kumamoto University, Chuo-ku, Kumamoto 862-0973, Japan
[d] Laboratory for Cardiovascular Molecular Dynamics, Riken Quantitative Biology Center, Suita, Osaka 565-0874, Japan

ARTICLE INFO

Article history:
Received 16 July 2013
Available online 26 July 2013

Keywords:
Prostaglandins
Thromboxanes
GPCR
Pharmacology
Signal transduction

ABSTRACT

Prostanoids comprising prostaglandins (PGs) and thromboxanes (TXs) have been shown to play physiological and pathological roles in zebrafish. However, the molecular basis of zebrafish prostanoid receptors has not been established. Here, we demonstrate that there exist at least five '*contractile*' (Ca^{2+}-mobilizing) and one '*inhibitory*' (G_i-coupled) prostanoid receptors in zebrafish; five '*contractile*' receptors consisting of two PGE_2 receptors (EP1a and EP1b), two $PGF_{2\alpha}$ receptors (FP1 and FP2), and one TXA_2 receptor TP, and one '*inhibitory*' receptor, the PGE_2 receptor EP3. [^{3}H]PGE_2 specifically bound to the membranes of cells expressing zebrafish EP1a, EP1b and EP3 with a *Kd* of 4.8, 1.8 and 13.6 nM, respectively, and [^{3}H]$PGF_{2\alpha}$ specifically bound to the membranes of cells expressing zebrafish FP1 and FP2, with a *Kd* of 6.5 and 1.6 nM, respectively. U-46619, a stable agonist for human and mouse TP receptors, significantly increased the specific binding of [^{35}S]GTPγS to membranes expressing the zebrafish TP receptor. Upon agonist stimulation, all six receptors showed an increase in intracellular Ca^{2+} levels, although the increase was very weak in EP1b, and pertussis toxin abolished only the EP3-mediated response. Zebrafish EP3 receptor also suppressed forskolin-induced cAMP formation in a pertussis toxin-sensitive manner. In association with the low structural conservation with mammalian receptors, most agonists and antagonists specific for mammalian EP1, EP3 and TP failed to work on each corresponding zebrafish receptor. This work provides further insights into the diverse prostanoid actions mediated by their receptors in zebrafish.

1. Introduction

Prostanoids comprising prostaglandins (PGs) and thromboxanes (TXs) are the arachidonate metabolites synthesized by cyclooxygenase (COX) as the rate-limiting enzyme. The diverse actions of prostanoids are mediated by membrane-bound receptors on neighboring cells [1]. In mammals, there exist eight types and subtypes of prostanoid receptors; DP for PGD_2, FP for $PGF_{2\alpha}$, IP for PGI_2, TP for TXA_2, and four EP subtypes (EP1, EP2, EP3 and EP4) for PGE_2 [2,3]. The prostanoid receptors are sub-grouped into three clusters on the basis of their structure, signal transduction and actions, namely '*contractile*', '*relaxant*', and '*inhibitory*' receptors [3,4]. The '*contractile*' receptors consist of EP1, FP and TP, which mediate Ca^{2+} mobilization and induce smooth muscle contraction. The '*relaxant*' receptors, which consist of DP, IP, EP2 and EP4, mediate increase in cAMP and induce smooth muscle relaxation. EP3 is an '*inhibitory*' receptor that mediates decrease in cAMP and inhibits smooth muscle relaxation. Indeed, sequence homology among these functionally related receptors is higher than those between the receptors from the three separate clusters. Molecular evolution analyses suggested that the COX pathway was initiated as a system composed of PGE and its receptor, and the subtypes of the PGE receptor then evolved from this primitive PGE receptor to mediate different signal transduction pathways, and subsequently the receptors for other PGs and TXs evolved from functionally related PGE receptor subtypes by gene duplication [3,5].

Zebrafish is a vertebrate model organism that has been used widely for genetic and pharmacological analyses of embryogenesis, because its fertilization and embryo development occur outside the maternal body under a transparent condition [6,7]. Furthermore, many disease models have been developed in zebrafish, and such models in combination with *in vivo* imaging of particular cells enables the monitoring of specific pathological processes such as cardiovascular disease and cancer invasion [8,9]. Indeed, it has been demonstrated by using zebrafish as a model that prostanoids

* Corresponding author at: Department of Pharmaceutical Biochemistry, Graduate School of Pharmaceutical Sciences, Kumamoto University, Chuo-ku, Kumamoto 862-0973, Japan.
E-mail address: ysugi@kumamoto-u.ac.jp (Y. Sugimoto).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.07.075

play critical roles in developmental processes such as gastrulation and hematopoietic stem cell expansion [10,11]. Moreover, it was recently suggested in a zebrafish model that leukocyte-derived PGs exert a trophic effect on tumor invasion [12]. Nevertheless, pharmacological properties of zebrafish prostanoid receptors have been systematically identified only in the '*relaxant*' receptors [13]. Here, we established the pharmacological and signal transduction properties of zebrafish *'contractile'* and *'inhibitory'* prostanoid receptors. Such a molecular basis will be of help not only for understanding the molecular evolution of prostanoid receptors, but also for discovering novel PG actions and mechanisms involved in embryogenesis and disease progression.

2. Materials and methods

2.1. Materials

The following materials were obtained from the sources indicated: [^{3}H]PGE2, [^{3}H]PGF2α, [^{3}H]iloprost, [^{3}H]SQ-29,548 and [^{35}S]GTPγS from PerkinElmer (Waltham, MA), PGD_2, PGE_2, $PGF_{2\alpha}$, iloprost, carbacyclin, U-46619, sulprostone, fluprostenol and SQ-29,548 from Cayman Chemical (Ann Arbor, MI), pertussis toxin (PT) from Seikagaku Co. (Tokyo, Japan), calcium 5 Assay Kit from Molecular Devices (Sunnyvale, CA), cyclic AMP kit from Yamasa (Choshi, Japan). The mammalian EP-specific agonists ONO-DI-004 (EP1) and ONO-AE-248 (EP3), and the mammalian EP-specific antagonists ONO-8713 (EP1) and ONO-AE3-240 (EP3) were generous gifts from Ono Pharmaceutical Co. (Osaka, Japan) [13]. All other chemicals were commercial products of reagent grade.

2.2. cDNA cloning and RT-PCR analysis

Total RNA was isolated from zebrafish embryos at 24-h post fertilization, and cDNAs were synthesized using SuperScript III (Invitrogen, San Diego, CA) and an oligo (dT) primer, and used as a template for PCR. The coding regions of the *'contractile'* and *'inhibitory'* prostanoid receptors were amplified from the cDNAs and cloned into the pTA2 vector (Toyobo, Osaka, Japan). Primer sequences used in the PCR are shown in Table S1. Such cDNAs were subcloned into the hemagglutinin- (HA-) tagged pcDNA3 expression vector. The resultant cDNA constructs were verified by dideoxy sequencing. Construction of a phylogenetic tree and RT-PCR analysis for the tissue distribution study were performed as described previously [13]. Primer sequences for each gene are shown in Table S2.

2.3. Binding assay

Binding assay of the membranes of COS-7 cells transfected with each cDNA were performed as described previously [13]. For the [^{35}S]GTPγS binding assay, membranes (30 μg) were incubated in GTPγS binding buffer (20 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.3 μM GDP and 0.1% BSA) containing 0.5 nM [^{35}S]GTPγS with or without various concentrations of U-46619 for 30 min at 30 °C. Membrane-bound [^{35}S]GTPγS was separated, and radioactivity was measured using a Top-Count microplate scintillation counter (Beckman, Miami, FL). The specific binding was calculated by subtracting the nonspecific binding from the total binding.

2.4. Intracellular Ca^{2+} and cAMP Assay

Mobilization of intracellular Ca^{2+} was measured on FlexStation III (Molecular Devices) using the FLIPR Calcium 5 Assay Kit (Molecular Devices). Briefly, HeLa cells transfected with each of the zPRC1–5 cDNAs or zPRC6-transfected HEK293 cells were labeled with calcium 5 loading buffer for 1 h, and stimulated with various agonists. Fluorescence (excitation, 485 nm; emission, 515 nm) was monitored for 90 sec and the area under the curve (AUC, relative fluorescence units (RFU) × 90 sec) was evaluated as the agonist-induced intracellular Ca^{2+} mobilization. Establishment of CHO cells stably expressing zPRC3 and the cAMP assay were performed as described previously [14].

2.5. Statistical analysis

Data are shown as means ± SEM. Comparison of two groups was analyzed by the Student's *t* test. For comparison of more than two groups with comparable variances, one-way ANOVA was performed first, and the Tukey's test was used to evaluate the pairwise group difference. *P* values < 0.05 were considered to indicate a significant difference.

3. Results and discussion

3.1. Molecular cloning of cDNAs for zebrafish 'contractile' and 'inhibitory' prostanoid receptors

To obtain functional cDNAs for *'contractile'* and *'inhibitory'* prostanoid receptors in zebrafish, we searched for zebrafish cDNA sequences showing high homology to human EP1, EP3, FP, and TP receptors from the NCBI database. We identified six different sequences as candidates for cDNAs encoding zebrafish orthologues; GI:260619540 (46.2% homology with human EP1), GI:260619542 (43.0% with human EP1), GI:292615058 (63.5% with human EP3), GI:297374752 (51.7% with human FP), GI:260619544 (37.9% with human FP) and GI:189535646 (53.9% with human TP). We successfully amplified full-length cDNAs corresponding to the former five sequences, cloned them into an expression vector, and named them zPRC1 (zebrafish Prostanoid Receptor, Ca^{2+}-mobilizing-1), zPRC2, zPRC3, zPRC4 and zPRC5, respectively (Table S3). Since the cDNA sequence deposited as GI:189535646 appeared to be incomplete, we designed a forward primer along the 5′-upstream adjacent region of the initiation ATG in the zebrafish gene, and successfully amplified a full-length cDNA, which was subcloned into an expression vector, and named this as zPRC6 (Genbank accession No; AB776994; 53.2% homology with human TP) (Table S3).

3.2. Ligand binding properties of zebrafish 'contractile' and 'inhibitory' prostanoid receptors

To identify their endogenous ligands, we transfected each full-length cDNA (zPRC1-zPRC6) into COS-7 cells and subjected the membranes to binding assays. No significant specific bindings of the PGs tested were detected in the membranes of mock-transfected cells (data not shown). The membranes of zPRC1-, zPRC2- and zPRC3-transfected cells showed specific binding for [^{3}H]PGE_2. The *Kd* values of zPRC1-, zPRC2- and zPRC3-transfected cell membranes for [^{3}H]PGE_2 were 4.8, 1.8 and 13.6 nM, respectively, which were comparable to the *Kd* values of mammalian EP receptors [15,16]. Furthermore, the membranes of zPRC4- and zPRC5-transfected cells exhibited specific binding for [^{3}H]$PGF_{2\alpha}$ with *Kd* values of 6.5 and 1.6 nM, respectively, which were also comparable to the *Kd* values of mammalian FP receptors [17]. However, the membranes of zPRC6-transfected cells failed to show any significant levels of specific binding for [^{3}H]PGE_2, [^{3}H]$PGF_{2\alpha}$, [^{3}H]iloprost (a radiolabeled stable IP agonist) or [^{3}H]SQ-29,548 (a radiolabeled TP antagonist).

We next examined the ligand specificities of the [^{3}H]PGE_2 binding (zPRC1–3) and the [^{3}H]$PGF_{2\alpha}$ binding (zPRC4–5) (Fig. 1A–E).

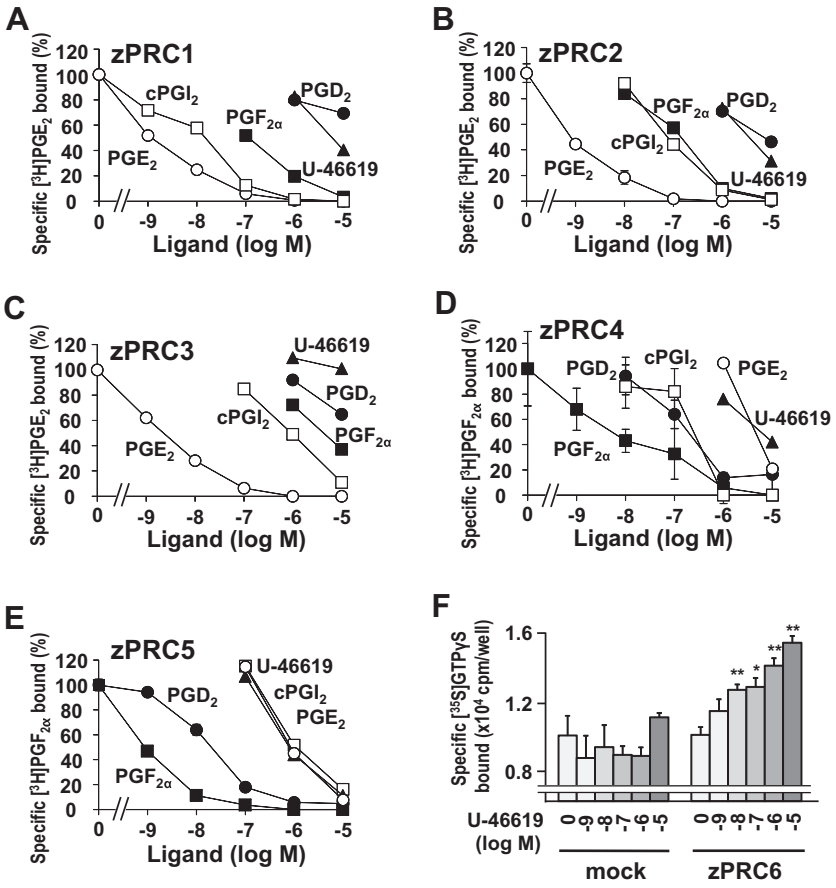

Fig. 1. Identification of prostanoid ligands for zebrafish receptors encoded by each full-length cDNA. A–E, [^{3}H]PGE$_2$ binding to zPRC1- (A), zPRC2- (B) and zPRC3-transfected cell membranes (C), or [^{3}H]PGF$_{2\alpha}$ binding to zPRC4- (D) and zPRC5-transfected cell membranes (E) were determined in the presence or absence of various concentrations of unlabeled PG analogues. *cPGI$_2$*, carbacyclin. *F*, Membranes of zPRC6- or mock-transfected COS-7 cells were incubated with 0.5 nM [^{35}S]GTPγS and the indicated concentrations of U-46619 for 30 min at 30 °C. Values represent the means ± SEM (n = 3). $^*P < 0.05$, $^{**}P < 0.01$ *vs.* vehicle.

The specific [^{3}H]PGE$_2$ binding to the zPRC1-transfected cell membranes was inhibited most effectively by unlabeled PGE$_2$ (IC$_{50}$, 1×10^{-9} M), followed by carbacyclin (IC$_{50}$, 1×10^{-8} M) (Fig. 1A). The specific [^{3}H]PGE$_2$ binding to zPRC2-transfected cell membranes was also displaced most potently by PGE$_2$ (IC$_{50}$, 1×10^{-9} M), followed by carbacyclin and PGF$_{2\alpha}$ (IC$_{50}$ for both compounds; 1×10^{-7} M) (Fig. 1B). [^{3}H]PGE$_2$ binding to zPRC3-transfected cell membranes was inhibited most potently by PGE$_2$ (IC$_{50}$, 3×10^{-9} M), but was minimally inhibited by the other PG analogues up to 10^{-6} M (Fig. 1C). The specific [^{3}H]PGF$_{2\alpha}$ binding activities to zPRC4- and zPRC5-transfected cell membranes were inhibited most potently by PGF$_{2\alpha}$ (IC$_{50}$ for zPRC4 and zPRC5; 3×10^{-9} and 1×10^{-9} M, respectively) (Fig. 1, D and E). [^{3}H]PGF$_{2\alpha}$ binding to zPRC4-transfected membranes was inhibited weakly by PGD$_2$ and carbacyclin (IC$_{50}$ for both compounds, 3×10^{-7} M). [^{3}H]PGF$_{2\alpha}$ binding to zPRC5-transfected membranes was inhibited modestly only by PGD$_2$ (IC$_{50}$, 3×10^{-8} M). These results suggest that zPRC1, zPRC2 and zPRC3 have the characters of EP receptors and zPRC4 and zPRC5 have the characters of FP receptors.

To further characterize the ligand binding specificities of zebrafish EP and FP receptors, we investigated which EP-selective agonists were able to bind to each receptor. [^{3}H]PGE$_2$ binding activities to zPRC1-, zPRC2- and zPRC3-transfected cell membranes were inhibited by sulprostone, an EP1/EP3 agonist, as potently as PGE$_2$ (IC$_{50}$, 1×10^{-8}, 1×10^{-9}, and 1×10^{-9} M, respectively), indicating that the products of zPRC1, zPRC2 and zPRC3 possess characters of the EP1 and EP3 receptors. [^{3}H]PGE$_2$ binding activities to zPRC1- and zPRC2-transfected cell membranes were weakly inhibited by ONO-DI-004, an EP1-specific agonist (IC$_{50}$ for both zPRC1 and zPRC2, 3×10^{-6} M). These results suggest that the products of zPRC1 and zPRC2 possess characters of the EP1 receptor (Fig. S1, A and B). On the other hand, the [^{3}H]PGE$_2$ binding to zPRC3-transfected cell membranes was inhibited weakly by ONO-AE-248 (IC$_{50}$, 3×10^{-6} M). These results suggest that the zPRC3 product possesses characters of the EP3 receptor (Fig. S1C). Both [^{3}H]PGF$_{2\alpha}$ binding activities to zPRC4- and zPRC5-transfected cell membranes were inhibited by fulprostenol, an FP-specific agonist (IC$_{50}$, 3×10^{-8}, 3×10^{-7} M, respectively) (Fig. S1, D and E). These results suggest that both zPRC4- and zPRC5-encoded receptors possess characters of the FP receptor.

Since the primary sequence of the zPRC6 product showed highest homology with the mammalian TP receptor (~54%), the zPRC6 product was suspected to be the zebrafish TP receptor. Currently [^{3}H]SQ-29,548 is the only commercially available radiolabeled ligand for the TP receptor, but this compound is quite different from TXA$_2$ especially in its ω-chain structure [1]. Therefore, we were unsure as to whether SQ-29,548 would bind to the zebrafish TP receptor as a ligand, and hence instead examined the effects of U-46619, a stable TP agonist, on [^{35}S]GTPγS binding to the membranes (Fig. 1F). As expected, the specific [^{35}S]GTPγS binding was significantly augmented by U-46619 in a concentration dependent manner in the membranes of zPRC6-transfected cells, but not in those of mock-transfected cells. Such an increase in [^{35}S]GTPγS binding was not detected by other PGs (data not shown). These results suggest that the zPRC6-encoded receptor possesses characters of the TP receptor.

3.3. Signal transduction properties of zebrafish 'contractile' and 'inhibitory' prostanoid receptors

In mammals, both *'contractile'* EP1, FP and TP receptors and the *'inhibitory'* EP3 receptor have been shown to stimulate intracellular Ca^{2+} mobilization upon agonist stimulation [15,17–19]. To characterize which signal transduction pathways each zebrafish receptor is coupled to, we first investigated whether the potential agonists induce Ca^{2+} mobilization in the cells expressing each zebrafish receptor (Fig. 2). In cells transfected with zPRC1, PGE_2 and sulprostone dose-dependently increased intracellular Ca^{2+} levels, and the agonist potency order was PGE_2 = sulprostone (EC_{50}, 3×10^{-9} M) (Fig. 2A). Moreover, ONO-DI-004 at 10^{-6} M induced a Ca^{2+} response, but ONO-AE-248 failed to do so at this dose (data not shown). In cells transfected with zPRC2, PGE_2 and sulprostone significantly induced intracellular Ca^{2+} mobilization (EC_{50}, 3×10^{-8} M), but the degrees of Ca^{2+} mobilization induced by both agonists were much less than those in zPRC1-transfected cells (Fig. 2B). These results may be because the receptor encoded by zPRC2 can be weakly coupled to Ca^{2+} mobilization, but is preferably coupled to signaling pathways other than Ca^{2+} mobilization. On the other hand, in the cells transfected with zPRC3, PGE_2 and sulprostone dose-dependently increased intracellular Ca^{2+} levels, and the agonist potency order was PGE_2 = sulprostone (EC_{50}, 1×10^{-7} M) (Fig. 2C). Moreover, ONO-AE-248 at doses of more than 10^{-6} M induced a Ca^{2+} response, but ONO-DI-004 failed to do so even at 10^{-5} M (data not shown). In cells transfected with zPRC4 and zPRC5, $PGF_{2\alpha}$ most potently increased Ca^{2+} levels (EC_{50} for zPRC4 and zPRC5, 3×10^{-8} and 3×10^{-9} M, respectively). In cells transfected with zPRC4, fluprostenol was as effective as $PGF_{2\alpha}$ (EC_{50}, 3×10^{-8} M), but this FP-agonist was less effective than $PGF_{2\alpha}$ against the zPRC5-encoded receptor (EC_{50}, 1×10^{-7} M) (Fig. 2, D and E). In cells transfected with zPRC6, U-46619 stimulated intracellular Ca^{2+} mobilization in a dose-dependent manner (EC_{50}, 3×10^{-9} M) (Fig. 2F). No such responses were observed in mock-transfected cells (data not shown).

The mammalian EP3 receptor, the sole *'inhibitory'* prostanoid receptor, has been shown to be coupled to inhibition of cAMP formation and Ca^{2+} mobilization via G_i, and G_i coupling can be characterized by its sensitivity to pertussis toxin (PT) [16,18,20]. To survey the potential G_i coupling of zebrafish receptors, we examined the PT sensitivity of each receptor-mediated Ca^{2+} response (Fig. 2, A–F, right panels). PT treatment prominently abolished the zPRC3 receptor-mediated Ca^{2+} response, but failed to affect responses mediated by any of the other receptors. Thus, only the zPRC3-encoded receptor appears to possess characters of the EP3 receptor. We further investigated whether the zPRC3-encoded receptor has the ability to inhibit adenylyl cyclase. In CHO cells stably expressing the zPRC3 product, PGE_2 as well as sulprostone suppressed forskolin-induced cAMP formation in a dose-dependent manner (IC_{50} for both agonists, 1×10^{-9} M) (Fig. S2A). Such an inhibitory effect of sulprostone on cAMP production was completely abolished by PT treatment (Fig. S2B). No such responses were observed in mock-transfected CHO cells (data not shown). These results indicate that the zPRC3-encoded receptor inhibits adenylyl cyclase in a PT-sensitive manner as observed with the mammalian EP3 receptor [16]. Based on the characters of ligand binding, GTPγS binding, signal transduction and PT sensitivity, we finally named the zebrafish receptors encoded by zPRC1, zPRC2, zPRC3, zPRC4, zPRC5 and zPRC6 as EP1a, EP1b, EP3, FP1, FP2 and

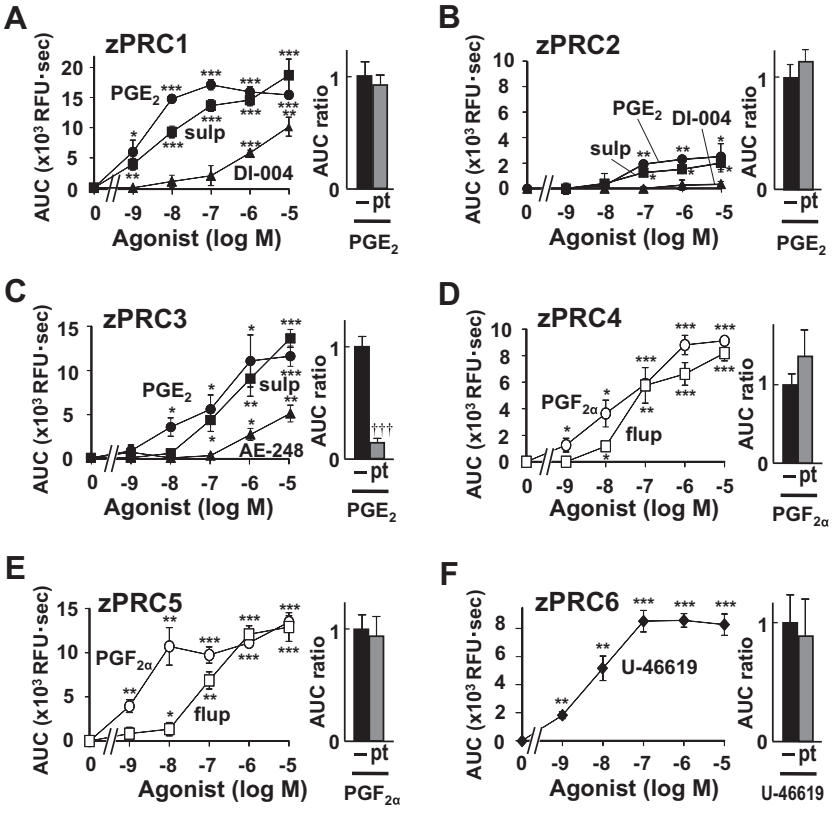

Fig. 2. Effects of various agonists and PT treatment on intracellular Ca^{2+} levels in cells transfected with each receptor cDNA. Cells transfected with each receptor cDNA were incubated with loading buffer for 1 h and stimulated with the indicated concentrations of agonists (A–F, left panels). Alternatively, the cells were incubated with PT (*pt*, 20 ng/ml) for 12 h, followed by stimulation with the indicated agonists (10^{-6} M each) (A–F, right panels). The change in $[Ca^{2+}]_i$ level was presented by the AUC (RFU·sec). *sulp*, sulprostone. *flup*, fluprostenol. Values represent the means ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* vehicle. $^{\dagger\dagger\dagger}P < 0.001$ *vs.* without PT (–).

TP, respectively. The characters revealed for each zebrafish receptor are summarized in Table S4.

ONO-8713, ONO-AE-240, and SQ-29,548 were developed as antagonists for the mammalian EP1, EP3 and TP receptor, respectively. Indeed, each antagonist effectively inhibited mouse receptor-mediated responses (data not shown). We investigated whether each antagonist could also function as a blocker against the corresponding zebrafish receptor (Fig. S3). ONO-8713 failed to inhibit both zebrafish EP1a- and EP1b-mediated Ca^{2+} responses (Fig. S3, A and B). ONO-AE-240 also failed to inhibit the zebrafish EP3-mediated response (Fig. S3C). SQ-29,548 again failed to show any antagonisms against the zebrafish TP-mediated response (Fig. S3D), which is consistent with the absence of any specific [^{3}H]SQ-29,548 binding to the cell membranes expressing the zebrafish TP receptor.

3.4. Gene expression patterns of zebrafish 'contractile' and 'inhibitory' prostanoid receptors among adult tissues

We next investigated the gene expression patterns of *'contractile'* and *'inhibitory'* prostanoid receptors among adult tissues of zebrafish by RT-PCR (Fig. 3). The mRNA for EP3 was detected in every tissue examined except for kidney and testis. Such ubiquitous expression may reflect the wide range of roles played by PGE_2-EP3 signaling in the nervous, respiratory, digestive and reproductive systems, since mouse EP3 receptor mRNA is also widely expressed in central and peripheral neurons [21], lung epithelia [22], gastric epithelia [23] and uterine myometrium [24]. Although mouse EP3 mRNA is most abundantly expressed in the tubules of the kidney [25], zebrafish EP3 was hardly detected in the kidney. TP mRNA was most abundantly expressed in the gill, and also showed modest expression in many tissues. Since it was suggested that TXA_2-TP signaling plays a role in venous blood flow in zebrafish [26], the wide distribution of TP mRNA may reflect its universal expression in blood vessels throughout the body. Both EP1a and EP1b mRNAs exhibited similar distributions; they were abundantly detected in the gill and muscle, modestly in the eye and slightly in the brain, and only EP1a-mRNA was detected in the ovary. Since the EP1 receptor was originally identified as a receptor mediating contractile actions of PGE_2 on airway smooth muscles [27], the abundant expression of EP1a and EP1b in the gill may suggest similar regulatory effects on smooth muscle contraction of zebrafish respiratory organs. FP1 and FP2 showed quite different tissue distributions; FP2 mRNA was detected in many tissues, but FP1 mRNA was detected only in the gill. Since the FP receptor has been shown to play a role in proliferation and collagen production in fibroblasts [28], the wide distribution of FP2 mRNA may reflect its expression in fibroblast-like cells within various connective tissues.

3.5. Primary structure of zebrafish 'contractile' and 'inhibitory' prostanoid receptors and their molecular evolutions

Fig. S4 shows the alignment of amino acid sequences of human, mouse and zebrafish EP1, EP3, FP and TP receptors. In all four receptor types, the sequences within the transmembrane (TM) regions are highly conserved among species. Moreover, in addition to the TM regions, the sequences within the 2nd intracellular (i2) loop between TM3 and TM4 and the sequences within the 2nd extracellular (e2) loop between TM4 and TM5, which are considered to be important for selective coupling of G proteins [29] and ligand binding [30] of prostanoid receptors, respectively, are also highly conserved. Although potential N-linked glycosylation sites are also observed in zebrafish receptors, there is no conservation of the positions among species.

Fig. 4 shows the phylogenic tree of human and zebrafish *'contractile'* and *'inhibitory'* prostanoid receptors. This picture illustrates that the *'inhibitory'* EP3 receptors separated first from the main branch, and then the different types of *'contractile'* receptors, TP, FP and EP1 emerged. Compared to the *'relaxant'* prostanoid receptors [13], the distances between the human and zebrafish receptors belonging to the same type are greater. Indeed, subtype-specific agonists and antagonists for human *'inhibitory'* and *'contractile'* receptors did not work well on the corresponding zebrafish receptors (Fig. 2 and Fig. S3). It should be noted that zebrafish FP2 is branched from EP1 cluster. The database also predicted this receptor as an *'EP1-like receptor'*, although FP2 did not show specific [^{3}H]PGE_2 binding at all. Indeed, fluprostenol, an agonist designed for human FP, exhibits excellent agonist activity against zebrafish FP1, but this compound shows less potent activity on the FP2 receptor (Fig. 2, D and E). The responsiveness of FP1 and FP2 receptors to fluprostenol appears to be associated with its structural conservation with the human FP receptor.

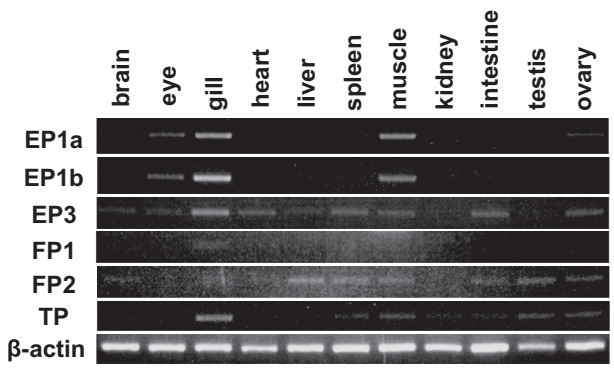

Fig. 3. Zebrafish Ca^{2+}-mobilizing prostanoid receptors show unique gene expression patterns among adult tissues. RT-PCR products were visualized in ethidium bromide-stained agarose gels. β-actin was amplified as an internal control for each sample (lowest panel).

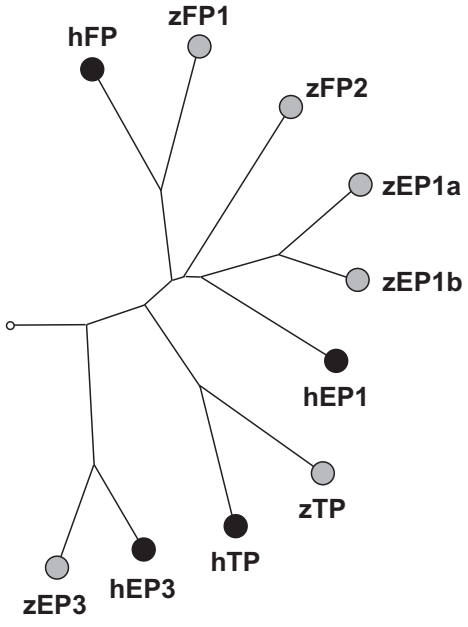

Fig. 4. Phylogenic tree of zebrafish and human '*contractile*' and '*inhibitory*' prostanoid receptors. Filled circles indicate human *'contractile'* and *'inhibitory'* prostanoid receptors, and grey circles indicate zebrafish receptors. Each branch length indicates the phylogenic distance.

Acknowledgments

K.M. was supported by a research fellowship from the Japan Society for the Promotion of Science for Young Scientists. This work was supported in part by Grants-in-Aid for Scientific Research and on Priority Areas 'Machineries of bioactive lipids in homeostasis and diseases' from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We thank Drs. Kazuhisa Nakayama and Shuh Narumiya (Kyoto University) for continuous support. We are grateful to Dr. H. Akiko Popiel for careful reading of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.075.

References

[1] R.A. Coleman, I. Kennedy, P.P.A. Humphrey, et al., Prostanoids and their receptors, in: P.G. Sammes, J.B. Taylor (Eds.), Comprehensive Medicinal Chemistry, Pergamon Press, Oxford, 1990, pp. 643–714.
[2] Y. Sugimoto, S. Narumiya, Prostaglandin E receptors, J. Biol. Chem. 282 (2007) 11613–11617.
[3] S. Narumiya, Y. Sugimoto, F. Ushikubi, Prostanoid receptors; structures, properties, and functions, Physiol. Rev. 79 (1999) 1193–1226.
[4] T. Hirata, S. Narumiya, Prostanoids as regulators of innate and adaptive immunity, Adv. Immunol. 116 (2012) 143–174.
[5] H. Toh, A. Ichikawa, S. Narumiya, Molecular evolution of receptors for eicosanoids, FEBS Lett. 361 (1995) 17–21.
[6] A.H. Meijer, H.P. Spaink, Host-pathogen interactions made transparent with the zebrafish model, Curr. Drug. Targets. 12 (2011) 1000–1017.
[7] A.V. Gore, K. Monzo, Y.R. Cha, et al., Vascular development in the zebrafish, Cold Spring Harb. Perspect. Med. 2 (2012) a006684.
[8] T. Tanaka, T. Oka, Y. Shimada, et al., Pharmacogenomics of cardiovascular pharmacology: pharmacogenomic network of cardiovascular disease models, J. Pharmacol. Sci. 107 (2008) 8–14.
[9] M. Mione, A.H. Meijer, B.E. Snaar-Jagalska, et al., Disease modeling in zebrafish: cancer and immune responses, Zebrafish 6 (2009) 445–451.
[10] Y.I. Cha, S.H. Kim, D. Sepich, et al., Cyclooxygenase-1-derived PGE_2 promotes cell motility via the G-protein-coupled EP4 receptor during vertebrate gastrulation, Genes Dev. 20 (2006) 77–86.
[11] W. Goessling, T.E. North, S. Loewer, et al., Genetic interaction of PGE_2 and Wnt signaling regulates developmental specification of stem cells and regeneration, Cell 136 (2009) 1136–1147.
[12] Y. Feng, S. Renshaw, P. Martin, Live imaging of tumor initiation in zebrafish larvae reveals a trophic role for leukocyte-derived PGE_2, Curr. Biol. 22 (2012) 1253–1259.
[13] K. Tsuge, R. Iwasaki, K. Morimoto, et al., Molecular and pharmacological characterization of zebrafish *'relaxant'* prostanoid receptors, Biochem. Biophys. Res. Commun. 436 (2013) 685–690.
[14] Y. Sugimoto, T. Namba, A. Honda, et al., Cloning and expression of a cDNA for mouse prostaglandin E receptor EP3 subtype, J. Biol. Chem. 267 (1992) 6463–6466.
[15] A. Watabe, Y. Sugimoto, A. Honda, et al., Cloning and expression of cDNA for a mouse EP1 subtype of prostaglandin E receptor, J. Biol. Chem. 268 (1993) 20175–20178.
[16] Y. Sugimoto, M. Negishi, Y. Hayashi, et al., Two isoforms of EP3 receptor with different C-terminal domains; identical ligand binding properties and different coupling properties with Gi proteins, J. Biol. Chem. 268 (1993) 2712–2718.
[17] Y. Sugimoto, K. Hasumoto, T. Namba, et al., Cloning and expression of a cDNA for mouse prostaglandin F receptor, J. Biol. Chem. 269 (1994) 1356–1360.
[18] A. Irie, E. Segi, Y. Sugimoto, et al., Mouse prostaglandin E receptor EP3 subtype mediates calcium signals via Gi in cDNA-transfected Chinese hamster ovary cells, Biochem. Biophys. Res. Commun. 204 (1994) 303–309.
[19] T. Namba, Y. Sugimoto, M. Hirata, et al., Mouse thromboxane A_2 receptor: cDNA cloning, expression and Northern blot analysis, Biochem. Biophys. Res. Commun. 184 (1992) 1197–1203.
[20] M. Negishi, Y. Sugimoto, Y. Hayashi, et al., Functional interaction of prostaglandin E receptor EP3 subtype with guanine nucleotide-binding proteins, showing low-affinity ligand binding, Biochim Biophys Acta. 1175 (1993) 343–350.
[21] Y. Sugimoto, R. Shigemoto, T. Namba, et al., Distribution of the messenger RNA for the prostaglandin E receptor subtype EP3 in the mouse nervous system, Neurosci. 62 (1994) 919–928.
[22] T. Kunikata, H. Yamane, E. Segi, et al., Suppression of allergic inflammation by the prostaglandin E receptor subtype EP3, Nat. Immunol. 6 (2005) 524–531.
[23] K. Morimoto, Y. Sugimoto, M. Katsuyama, et al., Cellular localization of mRNAs for the prostaglandin E receptor subtypes in mouse gastrointestinal tract, Am. J. Physiol. 272 (1997) G681–G687.
[24] M. Katsuyama, Y. Sugimoto, K. Morimoto, et al., Distinct cellular localization of the mRNAs for prostaglandin E receptor subtypes in the mouse uterus during pseudopregnancy, Endocrinology 138 (1997) 344–350.
[25] Y. Sugimoto, T. Namba, R. Shigemoto, et al., Distinct cellular localization of the mRNAs for three subtypes of prostaglandin E receptor in kidney, Am. J. Physiol. 266 (1994) F823–F828.
[26] H. Teraoka, A. Kubota, W. Dong, et al., Role of the cyclooxygenase 2-thromboxane pathway in 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced decrease in mesencephalic vein blood flow in the zebrafish embryo, Toxicol. Appl. Pharmacol. 234 (2009) 33–40.
[27] R.A. Coleman, I. Kennedy, Characterisation of the prostanoid receptors mediating contraction of guinea-pig isolated trachea, Prostaglandins 29 (1985) 363–375.
[28] T. Oga, T. Matsuoka, C. Yao, et al., Prostaglandin $F_{2\alpha}$ receptor signaling facilitates bleomycin-induced pulmonary fibrosis independently of transforming growth factor-β, Nat. Med. 15 (2009) 1426–1430.
[29] Y. Sugimoto, T. Nakato, A. Kita, et al., A cluster of aromatic amino acids in the i2 loop plays a key role for Gs coupling in prostaglandin EP2 and EP3 receptors, J. Biol. Chem. 279 (2004) 11016–11026.
[30] B.A. Stillman, L. Audoly, R.M. Breyer, A conserved threonine in the second extracellular loop of the human EP2 and EP4 receptors is required for ligand binding, Eur. J. Pharmacol. 357 (1998) 73–82.