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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

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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).

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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).