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Molecular and pharmacological characterization of zebrafish '*relaxant*' prostanoid receptors

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ABSTRACT

Prostanoids comprising prostaglandins (PGs) and thromboxanes have been shown to play physiological and pathological roles in zebrafish. However, the molecular basis of zebrafish prostanoid receptors has not been characterized to date. Here, we demonstrate that there exist at least six '*relaxant*' (Gs-coupled) prostanoid receptors in zebrafish; one PGI_2 receptor IP and five PGE_2 receptors comprising two EP2 (EP2a and EP2b), and three EP4 receptors (EP4a, EP4b and EP4c). In contrast, we failed to find a zebrafish PGD_2 receptor with any structure and/or character similarities to the mammalian DP1 receptor. [^{3}H]iloprost, a stable IP radioligand, specifically bound to the membrane of cells expressing zebrafish IP with a *Kd* of 42 nM, and [^{3}H]PGE_2 specifically bound to the membranes of cells expressing zebrafish EP2a, EP2b, EP4a, EP4b and EP4c with a *Kd* of 6.9, 6.0, 1.4, 3.3 and 1.2 nM, respectively. Upon agonist stimulation, the '*relaxant*' prostanoid receptors showed intracellular cAMP accumulation. The responsiveness of these zebrafish receptors to subtype-specific agonists correlated with their structural conservation to the corresponding receptor in mammals. RT-PCR analysis revealed that the six zebrafish prostanoid receptors show unique tissue distribution patterns; each receptor gene may hence be under unique transcriptional regulation. This work provides further insights into the diverse functions of prostanoids in zebrafish.

1. Introduction

Prostanoids comprising prostaglandins (PGs) and thromboxanes (TXs) are 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 [2–4]. The prostanoid receptors are sub-grouped into three clusters on the basis of their structure, signal transduction and actions: '*contractile*', '*relaxant*', and '*inhibitory*' receptors [5,6]. 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 analysis suggested that the COX pathway was first initiated as a system composed of PGE and its receptor, and the PGE receptor subtypes then evolved from this primitive PGE receptor to mediate different signal transduction pathways, and subsequently the receptors for the other PGs and TXs evolved from functionally related PGE receptor subtypes by gene duplication [5,7].

Zebrafish is a vertebrate model organism that has been widely used for genetic and pharmacological analyses of embryogenesis because its fertilization and embryo development occur outside the maternal body under a transparent condition [8–10]. Furthermore, many disease models have been developed in zebrafish, and such models in combination with *in vivo* imaging of particular cells enabled the monitoring of specific pathological processes such as cardiovascular disease and cancer invasion [11,12]. Indeed, it has been demonstrated by using zebrafish as a model that prostanoids play critical roles in developmental processes such as gastrulation and hematopoietic stem cell expansion [13–16]. Moreover, it was recently suggested in a zebrafish model that leukocyte-derived PGs exert a trophic effect on tumor invasion [17].

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Nevertheless, there is no literature to date that systematically characterizes the pharmacological properties of zebrafish prostanoid receptors. Here, we characterized the pharmacological and signal transduction properties of the zebrafish '*relaxant*' prostanoid receptors. Such molecular knowledge will assist not only our understanding of the molecular evolution of prostanoid receptors, but also contribute towards the discovery of novel PG actions and mechanisms in embryogenesis and disease progression.

2. Materials and methods

2.1. Materials

The following materials were obtained from the sources indicated: [^{3}H]PGE_2 and [^{3}H]iloprost from PerkinElmer (Waltham, MA), PGD_2, PGE_2, $PGF_{2\alpha}$, iloprost, carbacyclin, cicaprost and U-46619 from Cayman Chemical (Ann Arbor, MI), cyclic AMP kit from Yamasa (Choshi, Japan). ONO-AE1-259 (an EP2-specific agonist), ONO-AE1-329 (an EP4-specific agonist) and ONO-AE3-208 (an EP4-specific antagonist) were generous gifts from Ono Pharmaceutical Co. (Osaka, Japan) [18,19]. All other chemicals were commercial products of reagent grade.

2.2. cDNA cloning

Total RNA was isolated from zebrafish embryos at 24 h post fertilization (h.p.f.), 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 '*relaxant*' prostanoid receptors were amplified and cloned into the pTA2 vector (TOYOBO, Osaka, Japan). Primer sequences used in the PCR are shown in Table S1. The cDNAs were then subcloned into the hemagglutinin- (HA-) tagged pcDNA3 expression vector. The resultant cDNA constructs were verified by dideoxy sequencing.

2.3. Cell culture and transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated (56 °C for 30 min) fetal bovine serum at 37 °C in a fully humidified 5% CO_2 atmosphere. Cells were transfected with each cDNA construct using FuGENE HD (Promega, Madison, WI) according to the manufacturer's instructions.

2.4. Binding assay

cDNA-transfected cells were harvested and homogenized in 0.25 M sucrose containing 25 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. After centrifugation at 100,000 × *g* for 1 h, the pellet was suspended in 20 mM 2-(N-morpholino) ethanesulfonic acid (pH 6.0) containing 10 mM $MgCl_2$, 1 mM EDTA and was used for the binding assay as crude membranes. The membranes (30 μg) were incubated with 20 nM [^{3}H]iloprost or 4 nM [^{3}H]PGE_2 with various concentrations of unlabeled ligands for 1 h. The reaction was terminated by addition of ice-cold K–P buffer (1.32 mM K_2HPO_4, 8.68 mM KH_2PO_4, 10 mM $MgCl_2$, 1 mM EDTA), and the mixture was rapidly filtered through a Unifilter-96-GF/C (Whatman, Florham Park, NJ). Radioactivity was measured using a LS6500 scintillation counter (Beckman, Miami, FL). The specific binding was calculated by subtracting the nonspecific binding from the total binding.

2.5. Measurement of cAMP formation

cDNA-transfected COS-7 cells were preincubated with HEPES-buffered saline consisting of 140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose, 15 mM HEPES (pH 7.4) and 100 μM indomethacin for 10 min at 37 °C. Reactions were started by the addition of test reagents along with 100 μM indomethacin and 100 μM Ro-20-1724 (an inhibitor of type IV phosphodiesterase). After incubation at 37 °C for 10 min, the reaction was terminated by the addition of ice-cold 10% trichloroacetic acid. The content of cAMP was measured by radioimmunoassay using a cAMP assay kit (Yamasa).

2.6. RT-PCR

Total RNA was extracted from various tissues in adult zebrafish with Sepasol RNA I Super G (Nacalai Tesque, Kyoto, Japan), subjected to the RT reaction with PrimeScript RT Master Mix (Takara Bio, Shiga, Japan), and subjected to PCR with Quick Taq HS DyeMix (Toyobo, Osaka, Japan). Primer sequences for each gene are shown in Table S2. The specificity of the PCR products was confirmed by gel electrophoresis.

2.7. Molecular phylogenetic analysis

A phylogenetic tree was constructed with amino acid sequences of zebrafish and human '*relaxant*' prostanoid receptors using the "AllAll program" at The Computational Server at Eidgenössische Technische Hochschule Zürich (ETHZ) (http://www.cbrg.ethz.ch/services/AllAll).

2.8. Statistical analysis

Data are represented as means ± SEM of at least three independent experiments. Two sets of data were compared by Student's *t* test. $P < 0.05$ was considered to be significant.

3. Results and discussion

3.1. Molecular cloning of cDNAs for zebrafish 'relaxant' prostanoid receptors

To obtain functional cDNAs for Gs-coupled prostanoid receptors in zebrafish, we searched for zebrafish cDNA sequences showing high homology to the human '*relaxant*' prostanoid receptors from the NCBI database. We identified six different sequences as candidates for cDNAs encoding zebrafish Gs-coupled prostanoid receptors; GI:292621348, GI:82659770, GI:71834635, GI:88900452, GI:190358593 and GI:189515727. We amplified full-length cDNAs corresponding to GI:292621348, GI:82659770, GI:71834635, GI:88900452 and GI:190358593, cloned them into an expression vector, and named them as zPRS1 (zebrafish prostanoid receptor, Gs-coupled-1), zPRS2, zPRS3, zPRS4 and zPRS5, respectively (Table S3). The cDNA sequence deposited as GI:189515727 appears to lack the N-terminal region of the coding region, since the predicted amino acid sequence had only six hydrophobic regions. We therefore searched for an 'in-frame' ATG in the 5′-upstream adjacent region of the zebrafish gene, and found a 'likely' initiation codon. Indeed, we successfully amplified this hypothetically full-length cDNA from the first strand pool of the zebrafish embryo, cloned it into an expression vector, and named the cDNA as zPRS6 (Genbank accession No.: AB776993).