Molecular cloning and functional characterization of the zebrafish ATP-gated ionotropic receptor $P2X_3$ subunit

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Abstract We describe a P2X subunit cloned from the zebrafish (*Danio rerio*) that is an orthologue of the mammalian $P2X_3$ subunit. Like the mammalian $P2X_3$, this receptor desensitizes rapidly in the presence of agonist. However, it differs in that αβ-meATP is a much less potent agonist than ATP and the antagonist TNP-ATP is not active at low nanomolar concentrations. Similar to the rat $P2X_3$ subunit, the zebrafish subunit forms hetero-oligomeric assemblies with the rat $P2X_2$ that possesses a phenotype distinct from either parent. This novel clone will provide an important basis for future experiments investigating the structure/function relationships of P2X subunit domains.

Key words: Transmitter-gated channel; Purinoceptor; Nucleotide; Zebrafish

1. Introduction

Accumulating evidence demonstrates that ATP can act as an extracellular signal in a number of tissues via its activation of cell surface receptors (for review, see [1]). There are at least two classes of ATP receptors; the G-protein-coupled receptors labeled P2Y and the ligand-gated ionotropic receptors named P2X [2]. The P2X receptors are non-selective cation channels that show appreciable permeability to Ca^{2+} [3]. Initial electrophysiological and pharmacological studies suggested the existence of multiple P2X receptor subtypes (for example, see [4]) and this has been borne out by the recent cloning of seven distinct mammalian genes encoding P2X receptor proteins [5]. Each of the seven mammalian subunits, when recombinantly expressed as homo-oligomers, displays a unique profile with regards to their pharmacological and biophysical profiles [6].

The predicted amino acid sequences of the seven mammalian P2X receptor subunits exhibit a moderate degree (approximately 35–50% when the intracellular C-terminus is excluded) of identity amongst themselves. The disparities in their amino acid sequences are thought to underlie their functional differences with regards to both pharmacological and biophysical properties. We sought to exploit this phenomenon by cloning orthologues of the mammalian proteins from more disparate species in anticipation that evolutionary divergence would be manifested in the primary sequences and that this in turn would yield subunit proteins with novel properties. Such differences could then be utilized for the elucidation of the determinants underlying such important receptor properties as ligand specificity and channel kinetics. To this end, we sought to identify subunit cDNAs from a lower vertebrate, the zebrafish (*Danio rerio*). We accomplished this through the use of virtual screening of the EST database. In this report, we show that a novel zebrafish cDNA encodes a $P2X_3$ subunit orthologue. This subunit is capable of forming both homo- and hetero-oligomeric receptors and does indeed possess functional properties quite different from the mammalian orthologue, suggesting that characterization of such evolutionarily divergent genes will provide a useful framework for future experiments investigating the structure/function relationships of P2X receptors.

2. Materials and methods

2.1. Molecular biology

A TBLASTN search of the GenBank EST database using the predicted amino acid sequence of the rat $P2X_5$ subunit identified the zebrafish EST clone fb99b08 (accession number AI588766) as possibly encoding a P2X receptor subunit. This plasmid was obtained from Research Genetics Inc. (Huntsville, AL, USA) and after sequencing both strands using the Terminator kit from US Biochemical (Cleveland, OH, USA) was found to contain the complete coding sequence for a zebrafish orthologue of the $P2X_3$ subunit (see Fig. 1). The sequence for this clone has been deposited in the GenBank and has the accession number AF238292. For engineering of an expression construct, the coding region was obtained by PCR using primers flanked by *Eco*RI and *Xho*I restriction sites and subcloned into the mammalian expression vector pCDNA-3.1 (Invitrogen, Carlsbad, CA, USA).

2.2. Transfection of HEK-293 cells

HEK-293 cells were transiently transfected with the P2X receptor cDNA(s) by incubating cells grown on 35 mm plates with 1 μg of total cDNA and 6 μl of lipofectamine (Gibco BRL, Gaithersburg, MD, USA) in 1 ml of serum-free medium (Opti-MEM, Gibco BRL). After 5 h at 37°C, the medium was replaced with complete minimal essential medium and cells were incubated for 40–48 h (as per [7]).

2.3. Electrophysiological recordings

Whole-cell current was recorded from single HEK-293 cells using the perforated-patch, whole-cell, voltage-clamp technique [8]. Low resistance (1–2 MΩ) electrodes were filled with 0.3 mg/ml amphotericin B dissolved in an intracellular solution of the following composition (in mM): 130 Cs methanesulfonate, 24 CsCl, 10 HEPES, 1 $CaCl_2$ and 1 $MgCl_2$. The extracellular solution was (in mM): 154 NaCl, 2.0 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, pH 7.3 with NaOH. Membrane current recording started when the series resistance fell to less than 10 MΩ. The typical holding voltage was −40 mV where the effect of ATP was generation of an inward current. Current–voltage curves were generated by measuring current during a 100 ms voltage ramp from −60 to 60 mV. Currents caused by voltage ramps

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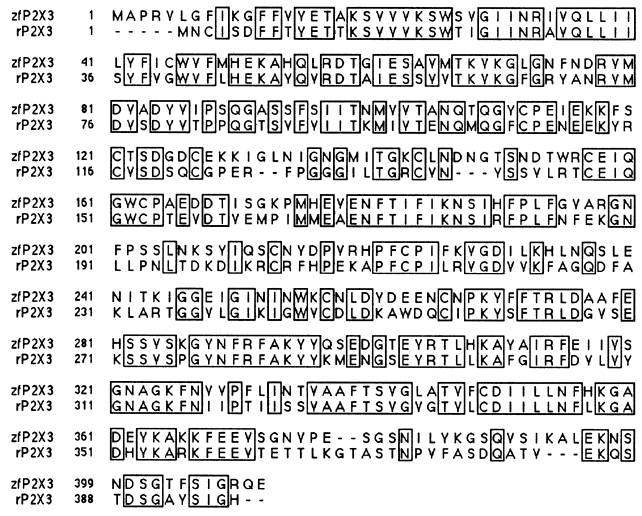

Fig. 1. Comparison of the primary sequences of the zebrafish and rat $P2X_3$ subunits. Alignment was carried out using the Clustal X program [9]. Common residues are boxed. Sequence is in GenBank, accession number AF238292.

recorded in the absence of ATP were subtracted from those recorded during ATP application to isolate the drug-induced current. In all experiments, drugs were applied by manually moving the electrode and attached cell into the line of flow of solutions exiting an array of inlet tubes.

3. Results and discussion

Screening of the EST database with the rat $P2X_5$ primary sequence identified a zebrafish EST clone (fb99b08) (Washington University Zebrafish EST Project), which when sequenced revealed an open reading frame encoding a protein of 410 amino acids. Alignment of the predicted amino acid sequence of this clone with those of the seven members of the rat subunit family using the Clustal X program [9] demonstrated that it was most similar to the $rP2X_3$ subunit (a comparison is shown in Fig. 1) and thus we tentatively designated this new clone as $zP2X_3$. The primary sequence exhibits many hallmarks of the mammalian P2X subunit sequences: the inferred topology is the same, the 10 conserved extracellular cysteine residues are present, and it has 63 of the 71 amino acids that are completely positionally conserved throughout the seven mammalian proteins. The nine residues that differ in the zebrafish are H54, R156, V179, N236, L239, T243, A312, E316 and V329, which are tyrosine, threonine, alanine, phenylalanine, alanine, glycine, aspartate and isoleucine residues, respectively, in the mammalian subunits.

Expression of $zP2X_3$ in HEK-293 cells resulted in ATP-induced inward currents, as seen in Fig. 2. The observed current was similar to those obtained at the rat $P2X_1$ and $P2X_3$ receptors [10,11] in two ways. First, $zP2X_3$ receptors desensitized rapidly in the continued presence of agonist with the time course of desensitization varying from cell to cell, although concentrations of ATP greater than about 1 μM always caused a near complete desensitization within a few (≥ 3) seconds. Second, closely spaced applications of ATP led to a progressive loss of ATP-gated currents (Fig. 2). Most importantly, the zebrafish clone exhibited a marked lengthening in resensitization time when compared to its mammalian orthologue, as it required a minimum of 30 min to recover completely from a single application of ATP versus approximately 15 min for the rat $P2X_3$ (data not shown).

The biophysical properties of $zP2X_3$ were examined using a low concentration of ATP in order to have a slower desensi-

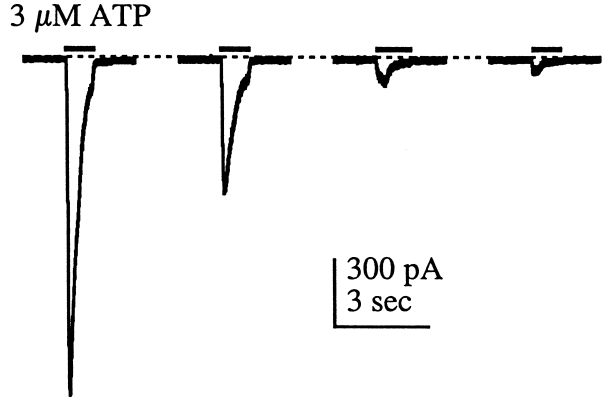

Fig. 2. Desensitization of ATP-gated current. ATP (3 μM) was applied every 5 min to an HEK-293 cell transiently expressing $zP2X_3$ receptors. Four consecutive traces are shown. The first application of ATP caused a large, rapidly desensitizing inward current. Subsequent applications evoked smaller currents. In this and all subsequent figures, the dotted line indicates the zero current level.

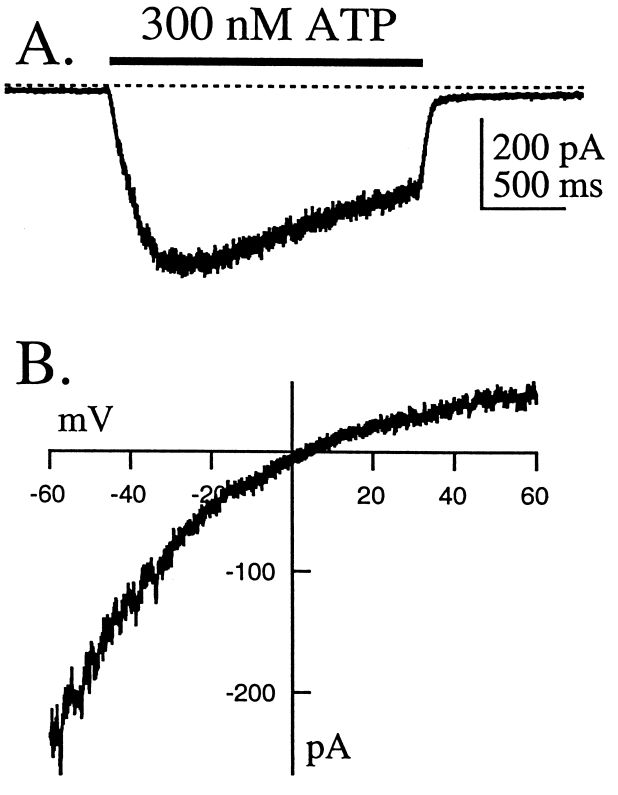

Fig. 3. Current–voltage relationship for the $zP2X_3$ receptor. A: A low concentration (300 nM) of ATP evoked an inward current that desensitized little during a short (about 2 s) application of agonist. The lack of desensitization permitted current–voltage curves to be generated using the voltage ramp protocol described in the text. B: Current–voltage relationship for the $zP2X_3$. The current reverses at 3 mV and shows pronounced inward rectification.

tization (Fig. 3A). Results from these experiments are consistent with the properties seen for the mammalian P2X subunits in that the channel exhibits non-selective cation permeability (reversal potential slightly positive of zero, Fig. 3B) and has a pronounced inward rectification (Fig. 3B).

The next step was to examine the basic pharmacological profile of $zP2X_3$. Due to the prolonged resensitization time of the receptor, dose–response curves were generated by measuring the effect of different concentrations of ATP applied at 30 min intervals. ATP often caused variable sized currents to the same concentration of agonist even when applied infrequently and therefore the data included in our estimation of the EC_{50} of ATP came only from those cells that showed a clear dose–response relationship. Data from individual cells were normalized to the effect of 3 μM ATP in that cell. The normalized data were then pooled and subsequently fit to the Hill equation using Igor (Wavemetrics, OR, USA). Analysis of dose–response experiments (Fig. 4A) gave an EC_{50} for ATP of 1.5 ± 0.5 μM with a Hill slope of 1.6 (pooled data obtained from eight cells), values similar to that seen for $rP2X_3$ ([11] and our unpublished results). Unexpectedly, $zP2X_3$ demonstrated a pharmacological profile different from its mammalian cognate as the ATP analogue αβ-meATP, which is equipotent to ATP at $rP2X_3$ [11], was much less potent at the zebrafish homo-oligomeric receptor (Fig. 4B). In addition, the analogue TNP-ATP, which is a potent antagonist at $rP2X_3$ (an IC_{50} of approximately 1 nM [12]), had no effect on 1 μM ATP-induced currents at $zP2X_3$ when co-applied at a concentration of 3 nM (Fig. 4C). In parallel experiments, we verified that this concentration of TNP-ATP was sufficient to block ATP's actions at the recombinant $rP2X_3$ receptor (data not shown).

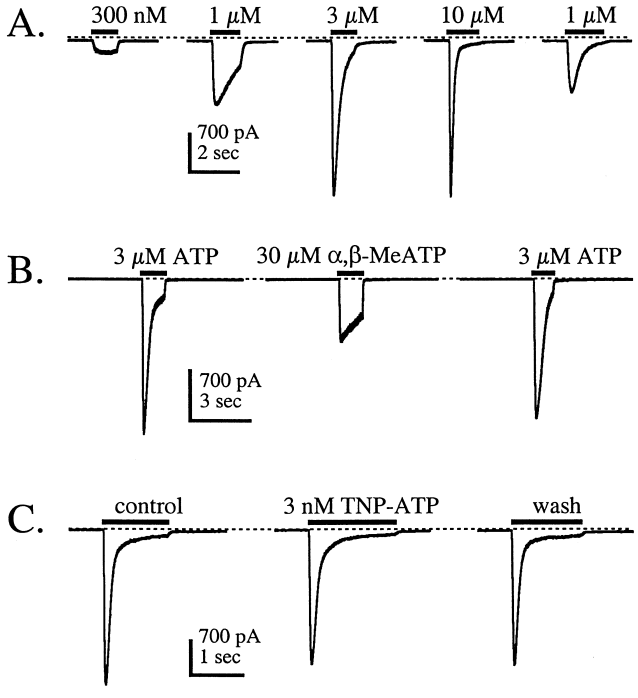

Fig. 4. Pharmacological profile of the $zP2X_3$ receptor. A: Inward currents evoked by four different concentrations of ATP. Lower concentrations evoked smaller currents that desensitize slowly. Higher concentrations yield larger currents that desensitize quickly. ATP was applied once every 30 min. B: A single application of 30 μM αβ-meATP was bracketed by applications of 3 μM ATP. The larger concentration of αβ-meATP evoked smaller currents than the lower concentrations of ATP. Drugs were applied at 30 min intervals. C: 3 nM TNP-ATP failed to block the current evoked by ATP. TNP-ATP was applied for 1 min before and then during an application of 3 μM ATP.

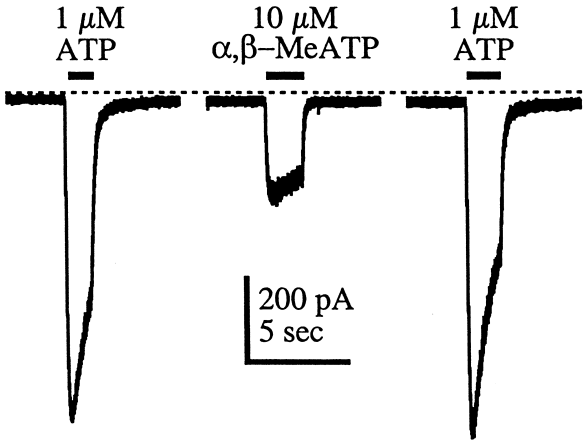

Fig. 5. The $zP2X_3$ subunit can co-assemble with the $rP2X_2$ subunit to form a novel hetero-oligomeric receptor. ATP (1 μM) was applied for 1 s to a cell co-transfected with both the $zP2X_3$ and $rP2X_2$ subunits (left trace). This was followed 2 min later by a 1 s application of 10 μM αβ-meATP (middle trace). A subsequent 1 s application of 1 μM ATP given an additional 2 min later (right trace) demonstrates that the $zP2X_3/rP2X_2$ hetero-oligomeric receptor recovers from desensitization much quicker than the homo-oligomeric $zP2X_3$ receptor.

Another attribute of rP2X subunits is their ability, in most part, to co-assemble with one another to form hetero-oligomeric receptors [13]. In some cases, the hetero-oligomers exhibit novel properties when compared to the parent homo-oligomers [11,7]. We therefore sought to determine if the $zP2X_3$ subunit could also participate in hetero-oligomeric receptor formation. With the mammalian receptors, a combination of special interest has been the $rP2X_{2/3}$ hetero-oligomer, which is thought to be a prevalent receptor subtype on sensory neurons [11,14]. A distinguishing characteristic of this particular hetero-oligomeric receptor is that it exhibits the pharmacological profile of the $rP2X_3$ subunit (αβ-meATP sensitive whereas the $rP2X_2$ subunit is insensitive to this analogue) but the desensitization kinetics of the $rP2X_2$ subunit (very slow in comparison to $rP2X_3$). As the $zP2X_2$ subunit is not yet available, we tested whether $zP2X_3$ could form a hetero-oligomeric receptor with $rP2X_2$. As seen in Fig. 5, a low concentration of ATP induced slowly desensitizing currents in cells co-transfected with $rP2X_2$ and $zP2X_3$ (left trace). When 10 μM αβ-meATP was applied 2 min following the ATP administration, a smaller slowly desensitizing current was observed (middle trace); a result which is in keeping with the relative potencies of these two drugs at the $zP2X_3$ homo-oligomeric receptor. Two minutes after the αβ-meATP treatment, 1 μM ATP was again administered and a current similar in size to that seen with the first ATP dose was observed (right trace). These results demonstrate that hetero-oligomeric receptors were formed and present. This interpretation is based on two facts: first, that the homo-oligomeric $zP2X_3$ desensitizes so quickly and resensitizes so slowly (see Fig. 1) that with only 2 min between drug applications neither the αβ-meATP response nor the second ATP current would have been present to any extent if co-assembly had not occurred, and second, that the αβ-meATP current could not arise from

homo-oligomeric $rP2X_2$ as that receptor is insensitive to αβ-meATP. Therefore, the last two responses are due to the co-assembly of $zP2X_3$ and $rP2X_2$ into a hetero-oligomeric receptor. Thus, like the combination of $rP2X_{2/3}$, it appears that the $zP2X_3/rP2X_2$ combination manifests the pharmacological profile of the $P2X_3$ subunit. Unlike the mammalian pair, the zebrafish subunit seems to retain the ability to exert some influence over the desensitization properties of the hetero-oligomer, a property that may be reflective of differences in the primary structures.

Although clearly the orthologue of the rat $P2X_3$, the zebrafish cDNA described in this report encodes a protein with enough differences in primary structure to cause phenotypic divergences as well. These findings help to lay the foundation for future structure/function investigation into the pharmacological and biophysical properties of P2X subunits, and validate the investigation of lower vertebrate gene products as a means of aiding in the elucidation of P2X receptor function.

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