Amino acid variations resulting in functional and nonfunctional zebrafish P2X₁ and P2X_{5.1} receptors

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Abstract Several zebrafish P2X receptors (zP2X₁, zP2X₂, and zP2X_{5.1}) have been reported to produce little or no current although their mammalian orthologs produce functional homomeric receptors. We isolated new cDNA clones for these P2X receptors that revealed sequence variations in each. The new variants of zP2X₁ and zP2X_{5.1} produced substantial currents when expressed by *Xenopus* oocytes, however the new variant of zP2X₂ was still nonfunctional. zP2X₂ lacks two lysine residues essential for ATP responsiveness in other P2X receptors; however introduction of these two lysines was insufficient to allow this receptor to function as a homotrimer. We also tested whether P2X signaling is required for myogenesis or synaptic communication at the zebrafish neuromuscular junction. We found that embryonic skeletal muscle expressed only one P2X receptor, P2X_{5.1}. Antisense knockdown of P2X_{5.1} eliminated skeletal muscle responsiveness to ATP but did not prevent myogenesis or behaviors that require functional transmission at the neuromuscular junction.

Keywords Zebrafish · P2X receptors · Skeletal muscle · Myogenesis

Introduction

P2X receptors are a family of ligand-gated ion channels activated by extracellular ATP [1]. Although P2X receptors

are widely expressed by both muscle and neural tissue in many organisms, only a handful of functions in these tissues have been attributed to members of this family. Most notably mouse knock outs have implicated P2X₁ in the neurogenic control of smooth muscle [2], P2X₂ and P2X₃ in nociception and as the post-synaptic receptors of neurons that receive input from taste cells [3], and P2X₄ in long-term potentiation [4]. These four examples represent the contribution of only four members of the family, in only a subset of cells known to express P2X receptors. Therefore other roles for P2X receptor signaling in the nervous system and muscle likely exist. In an attempt to identify potentially new roles for P2X receptors we turned to zebrafish, an organism amenable to both genetic manipulation and in vivo electrophysiology.

Our cloning of the P2X receptor family from zebrafish uncovered sequence variations in three subunits. One of these three subunits (zP2X₁) had been reported to be nonfunctional [5], and another (zP2X_{5.1}) had been reported to only produce very small currents [6]. In addition we characterized the in vivo responsiveness of skeletal muscle to applied ATP and determined through antisense knockdown that zP2X_{5.1} is required for the responsiveness of muscle to ATP. Finally we tested the hypothesis that signaling through P2X receptors is necessary for myogenesis.

Materials and methods

Animal care and use

Zebrafish were bred and raised according to approved guidelines set forth by the University Committee on Use and Care of Animals, University of Michigan. Developmental staging of embryos was performed either by

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counting somites prior to 24 h post-fertilization (hpf) or by assessing the migration of the lateral-line primordia as described previously [7].

Phylogenetic analysis of P2X sequences

The arrangement of P2X sequences into a phylogenetic tree was carried out for Family TF328633 on the TreeFam web page (www.treefam.org) using TreeView [8, 9] with all options at the default settings except “selected” species are shown.

Cloning of zebrafish P2X receptors

5′ and 3′ RACE ready cDNA (GeneRacer kit, Invitrogen, Carlsbad, CA) was synthesized from total RNA extracted from the AB genetic background between 24 and 48 hpf using TRIzol Reagent (Invitrogen, Carlsbad, CA). P2X receptors were PCR amplified (Pfu Turbo Polymerase, Stratagene, La Jolla, CA) using RACE primers and internal primers raised against published P2X sequences [5]. PCR products were A-tailed using Taq DNA Polymerase (Promega, Madison, WI) and subcloned into pGEM-Teasy (Promega, Madison, WI). Five individual clones for each zebrafish P2X (zP2X) receptor were sequenced and compared. Based on our analysis of sequences obtained from RACE, the following primers were used to clone full-length cDNAs:

zP2X ₁	Forward, 5′- ACACTGTGAAGACAGACTGAAGGAT-3′ Reverse, 5′- TCACTCCTTCTGTGACTCGCTGTCT-3′
zP2X ₂	Forward, 5′- TCACTTCACAGGAGCTTTATTTGT-3′ Reverse, 5′- GATTCAAATATGACATTTTATGATG-3′
zP2X _{3,1}	Forward, 5′- ATGGCTCCCAGAGTGCTGGGCT-3′ Reverse, 5′- TCACTCCTGACGCCCAATAGAA-3′
zP2X _{3,2}	Forward, 5′- ATGCAGCAGTGCTCTGGAAAAAT-3′ Reverse, 5′- TTAGCTGTAAACGTTTCGATGGAAA-3′
zP2X _{4,1}	Forward, 5′- GTGGAAGTGATTTAATGTTAAGTCG-3′ Reverse, 5′- CTATTTGTCTTCGTTGTGTAAAAGTC-3′
zP2X _{4,2}	Forward, 5′- ACAATGGGCTGTTCTGCTTATTG-3′ Reverse, 5′- ATCAAGGAGTCCCGTATGAGCTC-3′
zP2X _{5,1}	Forward, 5′- AGAGCTGCTTCCAGGCTGTCATT-3′ Reverse, 5′- TTCTACTGGTGGCGTGCTGCTCT-3′
zP2X _{5,2}	Forward, 5′- ATGGCTCGACGGTGCAGTAATCC-3′ Reverse, 5′- ATTTCACTGACTGAACCTTCTTTTC-3′

Site-directed mutagenesis

Mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All mutations were confirmed by DNA sequencing (University of Michigan DNA Sequencing Core) and are referred to using the one-letter code, such as S239A, which represents the substitution of serine 239 with an alanine.

Electrophysiological characterization of P2X receptors expressed by *Xenopus* oocytes and by zebrafish skeletal muscle

All reagents and drugs were purchased from Sigma-Aldrich (St. Louis, MO). Data acquisition was controlled by pClamp 8 or 9 software using a Digidata 1322A interface (Axon Instruments, Union City, CA). The initial data analysis was done in pClamp, and current-voltage relationships were fit using functions included in Sigma Plot 9.0 (SPSS, Chicago, IL).

For oocyte recordings, capped RNA encoding each zP2X receptor was synthesized using a mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX). RNA (5 ng) diluted to a volume of 50 nl in DEPC-H₂O was injected into stage V-VI defolliculated *Xenopus laevis* oocytes using a Nanoinject II system (Drummond Scientific Company, Broomall, PA). Oocytes were maintained in Barth's solution at 17°C for ~72 h before electrophysiological characterization. Two-electrode voltage-clamp recordings were made with an NPI Electronics (Tamm, Germany) TurboTec 3 amplifier at room temperature (22°C). The external recording solution was as follows (in mM): 90 NaCl, 1 KCl, 2 MgCl₂, and 10 HEPES, pH 7.6. In some experiments, the 2 mM MgCl₂ was replaced with 2 mM CaCl₂. Electrodes pulled from borosilicate glass were filled with 3 M KCl and had resistances of ~0.5 MΩ. Oocytes were held at −50 mV while ATP diluted from a stock solution (100 mM) in external solution was applied to each oocyte using a BPS-8 solution switcher (ALA Scientific Instruments, Westbury, NY).

Electrophysiological recordings were obtained from axial skeletal muscle as previously described [10]. In brief, embryos and larvae were anesthetized in 1× Evans recording solution (in mM): 134 NaCl, 2.9 KCl, 2.1 CaCl₂, 1.2 MgCl₂, 10 glucose, 10 HEPES, pH 7.8, containing 0.02% tricaine and pinned to a 35-mm dish coated with Sylgard through the notochord using 25 μm tungsten wires. The skin overlying the trunk and tail was removed with fine no. 5 forceps. The bath solution (1× Evans) containing 15 μM d-tubocurarine was continuously exchanged at ~1 ml/min throughout the recording session. The internal recording solution contained (in mM): 116 K-gluconate, 16 KCl, 2 MgCl₂, 10 HEPES, 10 EGTA, at pH 7.2 with 0.1% sulforhodamineB for muscle cell type identification. Electrodes pulled from borosilicate glass had resistances of 6–10 MΩ when filled with internal recording solution. Recordings were made with an Axopatch 200B amplifier (Axon Instruments, Union City, CA) low-pass filtered at 5 kHz and sampled at 10 kHz. The application of ATP (10 psi, 50 ms) was controlled by a Picospritzer II (Parker Hannifin, Fairfield, NJ).

Morpholino injections and behavioral experiments

An antisense morpholino oligonucleotide (Gene Tools, Philomath, OR) was synthesized against the following sequence for zP2X_{5.1}:

5′–TTGACACAGGAGCATGGCTCAGACC–3′

The underlined area designates the codon for the initial methionine. Embryos for morpholino injections were obtained from crosses of parents from the AB genetic background. zP2X_{5.1} morpholino diluted from a stock concentration of 2 mM to the indicated concentration in 5 nl of water containing 0.1% phenol red and 1× Deinhards solution was injected into embryos between the one- and four-cell stage using a Nanoinject II system. Thereafter morpholino-injected embryos were raised in a 28.5°C water bath. Prior to the beginning of an experiment, embryos were dechorionated with pronase and staged as described above. Locomotor behaviors were recorded (30 Hz) using a Panasonic CCD camera (wv-BP330) mounted to a Leica stereomicroscope at 10× magnification. Images were captured and analyzed off-line using Scion Image (www.scioncorp.com). Touch-evoked behaviors were elicited by touching the head, yolk sac, trunk, or tail region up to three times with a pair of no. 5 forceps.

Whole-mount in situ hybridization

In situ hybridization was performed using variations of a previously described approach [11]. The template was prepared by cutting 1.2 µg of plasmid DNA and phenol/chloroform precipitated by the following method. Following a 1- to 2-h digest, 2 mg of proteinase K plus SDS to a final concentration of 0.5% was added and incubated at 50°C for 30 min. Thereafter 200 µl of ddH₂O, 8 µl of 5 M NaCl, and 1 µl glycogen was added and mixture vortexed. Then 100 µl phenol and 100 µl chloroform were added and mixture vortexed. The mixture was then centrifuged at 14,000 rpm for 5 min at room temperature. The top phase was transferred to a fresh microfuge tube, and 200 µl of chloroform was added. The mixture was vortexed and centrifuged as before with the top phase again transferred to a fresh microfuge tube followed by the addition of ice-cold 100% ethanol (600 µl). The mixture was held at –80°C for 30 min and then centrifuged at 4°C for 20 min at 14,000 rpm. Supernatant was removed, and the precipitated template was resuspended in DEPC-H₂O. Riboprobes were synthesized and sheared to ~600 bp. Probe integrity was checked by gel electrophoresis.

Embryos were reared in 1-phenyl-2-thiourea (PTU) beginning ~22 hpf to prevent pigmentation and fixed with 4% paraformaldehyde in PBS for 1 h at room temperature (22°C). Embryos were then dehydrated with an increasing

percentage of methanol in PBS and stored at –20°C for 30 min. Thereafter embryos were rehydrated by decreasing the percentage of methanol in PBST (PBS containing 0.1% Tween) and incubated at 37°C with proteinase K for 12 min to increase the penetration of riboprobes. Antisense and sense control riboprobes (5 µg/ml) were hybridized for ~16 h at 65°C. Anti-DIG antibody conjugated to alkaline phosphatase (Roche Applied Science, Indianapolis, IN) was bound overnight at 4°C followed by chromogenic detection using a NBT/BCIP stock solution (Roche Applied Science). The chromogenic reaction was quenched at various time points for both antisense and sense control conditions. P2X receptor-specific staining was considered specific when corresponding staining was absent in sense control.

Results

Phylogenetic analysis of the zebrafish P2X receptor family

The cloning of P2X receptors from zebrafish (zP2X) has been reported previously [5, 6, 12–15]. Although mammals typically possess seven genes encoding P2X receptors, the partial duplication of the zebrafish genome has resulted in nine genes. The duplicated paralogs of P2X₃ and P2X₄ were originally designated as zP2X_{3a}, zP2X_{3b}, zP2X_{4a}, and zP2X_{4b}. However we will refer to these sequences as zP2X_{3.1}, zP2X_{3.2}, zP2X_{4.1}, and zP2X_{4.2} to distinguish them from the mammalian nomenclature for P2X receptors, which uses subscripted letters to indicate splice variants (as recommended by the guidelines established for zebrafish nomenclature, http://zfin.org/zf_info/nomen.html). In addition, phylogenetic analysis of the sequence originally referred to as zP2X_{5.14} [5], later renamed zP2X₈ [15], indicated that it is most likely a paralog of zP2X_{5.1} (Fig. 1), and therefore is referred to as zP2X_{5.2}. Finally, similar to other nonmammals, a gene encoding a P2X₆ ortholog has not been found in zebrafish.

Cloning of zebrafish P2X receptor cDNAs uncovered three new variants

We cloned cDNAs encoding each zebrafish P2X receptor (except zP2X₇) using RNA extracted from embryos during the first and second day of development. Sequence analysis revealed that while five zP2X receptor amino acid sequences matched the GenBank database, the sequences we obtained for zP2X₁, zP2X₂, and zP2X_{5.1} did not (Table 1). The previously reported variants will be referred to as zP2X_{1_tv1}, zP2X_{2_tv1}, and zP2X_{5.1_tv1}, and the new variants we isolated will be referred to as zP2X_{1_tv2}, zP2X_{2_tv2}, and zP2X_{5.1_tv2} according to established guidelines. Because

Fig. 1 Phylogenetic analysis of the zP2X receptor family. This tree produced by TreeFam shows the relationships among P2X receptors from selected chordates. The numbers next to the branches of this tree indicate the confidence level. Of note, the previously identified zP2X_{5.1}/P2X₈ receptor is most likely a paralog of zP2X_{5.1} and as such has been numbered zP2X_{5.2}. Also of note, zebrafish and three other fish with sequenced genomes (*Gasterosteus aculeatus*, *Oryzias latipes*, and *Takifugu rubripes*) lack a gene for P2X₆

these three zP2X receptors were previously reported either to be unresponsive to ATP or to produce extremely small currents [5, 6], we assessed whether these new variants produced currents more similar to P2X receptors from other species. To this end, each new zP2X receptor variant was expressed by *Xenopus* oocytes and exposed to ATP under two-electrode voltage-clamp.

Serine 239 is necessary for zP2X₁ channel activity

Application of ATP to *Xenopus* oocytes injected with RNA encoding zP2X_{1_tv2} evoked an inward current that rapidly desensitized (Fig. 2a). Repeated application revealed that the channel required several minutes to recover, making the estimation of an EC₅₀ for ATP problematic. The responsiveness

Table 1 Comparison of variations in zebrafish P2X receptor sequences

	Type of material	Accession number	Residues at									
			153	216	239	58	70–73	39		116	144	283–288
P2X ₁	zP2X _{1_tv1} cDNA	AAQ21194	M	N	Y							
	zP2X _{1_tv2} cDNA	This study	I	K	S							
	Genomic sequence	Zv7	M	K	S							
	Rat cDNA	NP_037129	V	K	S							
	Human cDNA	NP_002549	V	T	S							
P2X ₂	zP2X _{2_tv1} cDNA	AAQ21195				C	EMR					
	zP2X _{2_tv2} cDNA	This study				Y	EMR					
	EST	CO957065				Y	EMR					
	Genomic sequence	Zv7				Y	EMR					
	Rat cDNA	EDM14050				Y	KVK					
	Human cDNA	AAD42947				Y	KVK					
P2X _{5.1}	zP2X _{5.1_tv1} cDNA	AF500300						G	H	L	Y T A L G P	
	zP2X _{5.1_tv2} cDNA	This study						V	Y	H	S Q H S V T	
	EST	AA555532						Not in EST	Y	H	S Q H S V T	
	Genomic sequence	Zv7						V	Y	H	S Q H S V T	
	Rat cDNA	EDM05136						I	I	H	– – H S I S	
	Human cDNA	NP_002552						A	V	N	– – K S V S	

Fig. 2a–f Characterization of the newly identified zP2X₁_{tv2}, which contains a serine at position 239 (S239). **a** Response of an oocyte expressing zP2X₁_{tv2}. The bar above the trace indicates the application of 100 μ M ATP. **b** Schematic diagram of the location of S239 and sequence alignment of P2X₁ receptors from different species in the vicinity of S239. **c** Replacement of S239 with a tyrosine (S239Y) abolished responsiveness of zP2X₁_{tv2} to ATP. **d, e** Substitution of S239 with either an alanine or a cysteine also abolished responsiveness to ATP. **f** The substitution of a tyrosine at the homologous residue in rat P2X₂ (A236) was sufficient to abolish the responsiveness of rat P2X₂ to ATP

of zP2X₁_{tv2} to ATP is consistent with the reported activity of P2X₁ from other species [16]. To explore why zP2X₁_{tv1} was nonfunctional, we aligned zP2X₁_{tv1} with zP2X₁_{tv2} and P2X₁ sequences from other species which revealed a polymorphism at serine 239, a highly conserved amino acid (Fig. 2b). Substitution of serine 239 with a tyrosine in zP2X₁_{tv2} was sufficient to abolish responsiveness to 100 μ M ATP (Fig. 2c). Furthermore, substitution of serine 239 with an alanine, the residue present at the homologous position in rat P2X₂ (Fig. 2d), or a cysteine, the amino acid most chemically similar to serine (Fig. 2e) also produced nonfunctional receptors. Finally, substitution of the alanine found at the homologous position in rat P2X₂ with a tyrosine was also sufficient to abolish channel function (Fig. 2f). zP2X₁_{tv2} also differed from zP2X₁_{tv1} at two other sites (an I at position 153 rather than an M, and a K at position 216 rather than an N). The K at position 216 matches the amino acid at this position in the Zv7 genomic sequence, but the Zv7 genome has an M at position 153. I153 likely represents a polymorphism present in our line of AB fish, as it was found in cDNA prepared from two independent RNA isolations. We did not explore the functional significance of these differences, as I153M represented a conserved substitution, and position 216 is occupied

by a variety of residues in P2X₁ receptors from other species (Table 1).

zP2X₂_{tv2} does not form a functional homomeric receptor

The cDNA encoding zP2X₂_{tv1} possesses 11 cysteines in the extracellular domain in contrast to the 10 that characterize most members of the P2X family [17]. Sequence alignment indicated that the cysteine at position 87 failed to align with P2X₂ receptors across species (Fig. 3a). As the 10 extracellular cysteines are thought to form five disulfide bonds [18, 19], the inclusion of an additional cysteine might disrupt channel activity by altering normal disulfide bond formation. However zP2X₂_{tv2}, which has a tyrosine at position 87 and therefore contains only the 10 canonical cysteines (Table 1), was also found to be nonfunctional (Fig. 3b).

Another striking feature of both zP2X₂ variants was the sequence EMR at amino acid positions 70–72. In P2X₁_{–7} receptors from most other species, this site is occupied by a highly conserved KKK motif [17]. In human P2X₁, rat P2X₂, and rat P2X₄, mutation of either of these two highly conserved lysines severely impaired ATP potency [20–22].

Fig. 3a–c Characterization of the newly identified zP2X_{2_tv2}. **a** Membrane topology and sequence alignment of zP2X_{2_tv2} in relation to P2X₂ receptors from different species. Highlighted in the drawing of the topology is the location of the Y87C position in zP2X_{2_tv1}, and the EMR motif found in place of the KXX motif, which has been implicated in the binding of ATP in other P2X receptors. **b** Oocytes expressing the newly identified zP2X_{2_tv2} failed to respond to applied ATP. **c** Mutation of residues 70–73 of zP2X_{2_tv2} from EMR to KMK was not sufficient to produce sensitivity to ATP

However replacing E70 and R72 of zP2X_{2_tv2} with lysines (producing the sequence KMK) was insufficient to endow this subunit with the ability to respond to ATP when expressed alone in oocytes (Fig. 3c).

zP2X_{5.1_tv2} yields large currents when expressed by *Xenopus* oocytes

The initial identification and characterization of zP2X_{5.1_tv1} reported that, when expressed by HEK293 cells under the control of a promoter that gave robust expression of other zP2X receptors, the whole-cell current responses of zP2X_{5.1_tv1} were typically <20 pA [6]. We found that when

expressed by oocytes, zP2X_{5.1_tv1} failed to generate currents above background. zP2X_{5.1_tv2} differed from zP2X_{5.1_tv1} (AAM74150) at nine amino acid residues (Table 1). Among these were a string of six consecutive amino acids just prior to the second transmembrane domain that failed to match the sequence predicted for this region by the Zv7 zebrafish genome (www.ensembl.org/Danio_rerio/index.html). Upon closer examination of the nucleotide sequence encoding zP2X_{5.1_tv1} (AF500300), it appeared that a nucleotide insertion at position 848, followed by a nucleotide deletion at position 862, accounted for an in-frame six amino acid substitution at positions 283 through 288 (Fig. 4a).

When expressed by *Xenopus* oocytes and studied in our normal recording solution, zP2X_{5.1_tv2} exhibited a rapidly desensitizing component (I₁), followed by an entry into a long-lasting secondary phase (I₂), which reached its peak seconds after ATP removal (Fig. 4b). The nature of the I₂ phase of the response was reminiscent of the large-pore state reported for other members of the P2X family [23]. The large-pore state is characterized by (1) sensitivity to calcium and (2) a high relative permeability for the large cation NMDG, which is impermeable during the time the channels are in the I₁ state. We found that the inclusion of 2 mM Ca²⁺ in the recording solution blocked entry into the I₂ state (Fig. 4c), and that replacement of Mg²⁺ with Ca²⁺ at the peak of I₂ resulted in a rapid exit from the I₂ state (Fig. 4d). In addition, substantial inward current remained when NMDG was substituted for sodium in the extracellular solution (not shown). From these findings, we conclude that the I₂ state exhibited by zebrafish zP2X_{5.1_tv2} is similar to the large-pore state.

Localization of zP2X_{5.1}

The existence of an ion channel sensitive to ATP in skeletal muscle was first suggested many years ago [24]. Although subsequent studies confirmed the existence of ATP-sensitive ion channels in avian skeletal muscle, they suggested that the single-channel currents had a very low unitary conductance compared to the initial report [25, 26]. Following the cloning of the P2X family, the expression of multiple P2X receptors by skeletal muscle has been demonstrated in several different organisms [27–30]. Several roles for P2X receptors in muscle have been proposed including regulating myogenesis during development [28], regeneration of muscle following injury [31, 32], and roles in the regulation of muscle fiber contractility [33] and in muscle fiber type specification [34].

We used whole-mount in situ hybridization to explore which subunits were expressed by zebrafish skeletal muscle during the first two days of development. Only one subunit, zP2X_{5.1}, was found to be expressed by skeletal muscle at this time (Fig. 5a). Because antibodies for zebrafish

Fig. 4a–d Characterization of the newly identified $zP2X_{5.1_tv2}$. **a** Sequence comparison between $zP2X_{5.1_tv1}$ (AAM74150), $zP2X_{5.1_tv2}$ receptor, and the prediction for this region from the zebrafish genome assembly Zv7. **b** When calcium was not present in the recording solution, the response of $zP2X_{5.1_tv2}$ to a brief application of ATP was followed by a prolonged response. The arrows indicate the peak of the responses attributed to the small-pore state (I_1) and the large-pore state (I_2). **c** Entry into the I_2 state was prevented by the inclusion of 2 mM calcium in the recording solution. **d** Rapid exit from the I_2 state occurred when recording solution containing 2 mM magnesium was changed to a solution containing 2 mM calcium

$zP2X_{5.1}$ are lacking, we examined the subcellular localization of $zP2X_{5.1}$ by tagging it with EGFP and placing it under control of the heat-shock protein 70 promoter ($hsp70:zP2X_{5.1_tv2}$ -EGFP). Wild-type embryos were injected with the plasmid encoding $hsp70:zP2X_{5.1_tv2}$ -EGFP, or with $hsp70:EGFP$ as a control, at the one- to four-cell stage. Embryos were then raised at 28.5°C until 48 hpf at which time they were “heat-shocked” at 37°C for 30 min. In both control and

experimental animals, expression 2–3 h following heat shock was highly mosaic. However in animals injected with $hsp70:EGFP$, the expression of EGFP within muscle fibers was ubiquitous, while the fluorescence in animals injected with $hsp70:zP2X_{5.1_tv2}$ -EGFP could be observed in a pattern consistent with membrane localization (Fig. 5b). This finding indicates that $zP2X_{5.1}$ is most likely found on the plasma membrane of skeletal muscle.

Fig. 5a, b Expression analysis of $zP2X_{5.1}$. **a** Whole-mount in situ hybridization from a 24-hpf embryo revealed expression within the somites of the trunk and tail (*top*). The higher power image (*bottom*) shows labeling of axial skeletal muscle. **b** EGFP expression under the control of the $hsp70$ promoter (*top*) and when fused to the C-terminus $zP2X_{5.1_tv2}$ (*bottom*). The difference between the pattern of expression of unfused and fused EGFP indicated a pattern consistent with plasma membrane localization of $zP2X_{5.1}$ in 48-hpf embryos

P2X_{5.1} is required for muscle responses to ATP, but not for myogenesis or swimming

Two lines of evidence have suggested that P2X signaling may play an essential role in myogenesis: one study conducted using the mouse C2C12 cell line [35], and another using rat satellite cells, which are a population of myogenic precursors triggered to proliferate and then differentiate in response to injury in vivo [31]. In both studies, blocking purinergic signaling through P2X receptors disrupted the differentiation of myogenic precursors. These findings, coupled with the fact that P2X receptors are expressed during development by myogenic precursors in multiple organisms, has led to the hypothesis that P2X receptors might contribute to early myogenesis. However these reports have also indicated that myogenic precursors also express multiple P2X receptor subunits making the examination of P2X receptor function by knockout problematic. Because zebrafish express only one P2X receptor in muscle over the first 48 h (zP2X_{5.1}), we tested the hypothesis that P2X receptor signaling is required for myogenesis in zebrafish through the use of an antisense morpholino.

We found that the injection of a morpholino that targeted the 5' start site of zP2X_{5.1} had no observable effect on muscle morphology in injected embryos (Fig. 6a). The failure to alter muscle morphology was not due to the ineffectiveness of the morpholino. Muscle fibers from uninjected embryos held at -60 mV responded to applied ATP (100 μ M) with a transient inward current peaking at about -150 pA and lasting ~1–2 s, followed by a sustained outward current lasting tens of seconds (Fig. 6b–e). In contrast, morpholino at 100 μ M decreased the average ATP response to less than half of the control, and morpholino at 200 μ M resulted in a complete loss of ATP responsiveness. These results demonstrate that zP2X_{5.1} is required for muscle responsiveness to ATP at this developmental stage, but that P2X signaling is not required for myogenesis.

Finally ATP is known to be packaged in most, if not all synaptic vesicles. This finding, coupled with membrane expression of P2X receptors, led to the hypothesis that there might be an essential purinergic component to synaptic transmission at the neuromuscular junction. To examine this issue at the zebrafish neuromuscular junction, we assessed synaptic communication in the absence of P2X signaling behaviorally by assaying the ability of embryos to swim following morpholino-mediated blockade of zP2X_{5.1} expression. A swim was stimulated in control embryos by touching the head or tail with a pair of forceps to induce touch-evoked escape contractions and swimming [36]. Touch was found to induce similar responses in zP2X_{5.1} morpholino-injected embryos (Fig. 6f). Thus the neuromuscular junctions were functional in the absence of P2X signaling.

Discussion

The zebrafish family of P2X receptors is comprised of nine genes, one copy of P2X₁, P2X₂, and P2X₇, and two paralogous copies of P2X₃, P2X₄, and P2X₅. At this time there is no evidence for the existence of a gene encoding P2X₆ in the zebrafish genome. It should be noted that, in mammals, P2X₅ and P2X₆ are the most closely related subunits (see Fig. 1). However our phylogenetic analysis indicates that zP2X_{5.2} (zP2X_{5.14}/zP2X₈) is more closely related to other P2X₅ sequences than to the mammalian P2X₆ sequences. As zP2X_{1_tv2} and zP2X_{5.1_tv2} readily produce currents in *Xenopus* oocytes, eight of these nine subunits have now been shown to form functional homomeric receptors sensitive to ATP.

The subunit that remains nonfunctional as a homomeric receptor is zP2X₂. One contributing factor is that this subunit lacks two highly conserved lysine residues believed to form part of the ATP binding site of P2X receptors [37, 38]. However, there must be other differences from mammalian P2X₂ receptors as well, since substitution of EMR with KMK failed to result in ATP-induced currents above background. A previous report demonstrated that when zP2X_{2_tv1} was co-expressed with zP2X_{3.1} it was incorporated into ATP-responsive heteromeric complexes [5]. The overlapping expression pattern of zP2X₂ with other zP2X receptors in zebrafish is consistent with the idea that it can only play a role when co-assembled with another subunit. As any heterotrimeric complex containing one zP2X₂ subunit would have a nonfunctional ATP binding site, this also suggests that fewer than three ATP molecules are sufficient to activate the heteromeric channels in vivo.

Previous work had demonstrated that mRNA encoding P2X_{5.1} is expressed by embryonic zebrafish muscle [6, 15], but direct evidence as to whether functional receptors are present in embryonic zebrafish muscle was lacking. We demonstrated that embryonic zebrafish muscle responded to ATP with inward currents. We also found that zP2X_{5.1} channels expressed by *Xenopus* oocytes produced functional channels that exhibit a large-pore state (I₂) when experimental conditions were suitable. However 2 mM calcium was sufficient to suppress entry into the I₂ state, so this state may not be physiologically relevant under normal conditions. Evidence from other species had suggested that ATP responses would be found in zebrafish muscle. For instance, contractions of frog skeletal muscle in response to extracellular ATP were first reported in the 1940s [39], and the electrophysiological characterization of P2X receptor properties on cultured chick myoblasts and myotubes was explored in detail in the 1980s [24, 25, 40]. In the 1990s, cloning studies demonstrated that P2X₅ was expressed in chick skeletal muscle, and when chick P2X₅ was expressed in oocytes, the currents exhibited properties expected for the

Fig. 6a–f Morphology and responsiveness of zebrafish skeletal muscle to ATP. **a** *Left*: image of axial skeletal muscle above the yolk extension from an uninjected 48-hpf embryo. *Right*: image of axial skeletal muscle above the yolk extension from a 48-hpf embryo injected with 5 nl of zP2X_{5,1} morpholino at 200 μ M. **b–d** Responses of muscle fibers voltage-clamped at -60 mV to the application of ATP (100 μ M). The concentration of morpholino directed against zP2X_{5,1} is indicated in each panel. **e** Summary of a series of experiments performed as in **b–d**. Values represent the average $\pm$ SD, $n=10$ (one fiber per fish). Asterisks indicate conditions that differed from uninjected at $P<0.01$. **f** Response to a tap to the tail of an embryo injected with the control morpholino (*ConMO*), or of an embryo injected with the morpholino directed against zP2X_{5,1} (*P2X5 MO*). In some frames the embryo is seen in two positions because the movement was faster than the frame rate of the camera

endogenous chick receptors [41]. However the expression of other P2X receptors (P2X₁ and P2X₆) has been detected in chick skeletal muscle [27, 29]. Similarly, studies in mammals have revealed a complex expression pattern in skeletal muscle with P2X₁, P2X₂, P2X₄, P2X₅, and P2X₆ being detected [27, 28, 33, 42], but a clear role for P2X signaling in skeletal muscle has remained elusive.

One hypothesis is that P2X signaling is involved in regulating myogenesis. Support for this hypothesis comes from in vitro studies of the mouse C2C12 cell line [43] and rat satellite cells [31]. Consistent with an in vivo role of

P2X receptors during muscle regeneration, the up-regulation of several P2X receptors has been observed in muscle fibers of *mdx* mice [32, 42]. A report of alterations in skeletal muscle properties in P2X₂ knock-out mice has been reported [34], but the expression of multiple P2X receptor subunits by muscle complicates the interpretation of this work. The simplest interpretation of our ability to completely suppress ATP-evoked current with a morpholino designed specifically for P2X_{5,1} is that this is the only P2X receptor expressed at this developmental stage. In support of zP2X_{5,1} being the only P2X receptor expressed by

embryonic muscle, we and others failed to detect expression of any other P2X receptors by in situ hybridization [5, 6, 12–15]. An alternative explanation to the morpholino result is that zP2X_{5.1} is necessary for the movement of another zP2X subunit expressed in muscle cells at levels below the detectable limit of in situ hybridization to the membrane. However the finding that morpholino-injected embryos are morphologically normal and can swim away from a tactile stimulus when muscle fibers are completely unresponsive to ATP, demonstrated that P2X signaling is not essential during embryonic development for myogenesis or synaptic communication.

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