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ATP-sensitive potassium channels in zebrafish cardiac and vascular smooth muscle

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Abstract

ATP-sensitive potassium channels (K_{ATP} channels) are hetero-octameric nucleotide-gated ion channels that couple cellular metabolism to excitability in various tissues. In the heart, K_{ATP} channels are activated during ischaemia and potentially during adrenergic stimulation. In the vasculature, they are normally active at a low level, reducing vascular tone, but the ubiquitous nature of these channels leads to complex and poorly understood channelopathies as a result of gain- or loss-of-function mutations. Zebrafish (ZF) models of these channelopathies may provide insights to the link between molecular dysfunction and complex pathophysiology, but this requires understanding the tissue dependence of channel activity and subunit specificity. Thus far, direct analysis of ZF K_{ATP} expression and functional properties has only been performed in pancreatic β-cells. Using a comprehensive combination of genetically modified fish, electrophysiology and gene expression analysis, we demonstrate that ZF cardiac myocytes (CM) and vascular smooth muscle (VSM) express functional K_{ATP} channels of similar subunit composition, structure and metabolic sensitivity to their mammalian counterparts. However, in contrast to mammalian cardiovascular K_{ATP} channels, ZF channels are insensitive to potassium channel opener drugs (pinacidil, minoxidil) in both chambers of the heart and in VSM. The results provide a first characterization of the molecular properties of fish K_{ATP} channels and validate the use of such

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Author contributions

S.S.S. carried out cellular isolation and electrophysiology studies, data analysis, as well as the design of the study and drafting of the manuscript; H.I.R. and G.v.H. contributed to the RT-PCR study; R.T. generated the 6.2-KO ZF and performed the isolation of β cells from zebrafish islets; J.I. procured the SUR1-KO ZF. C.M. and C.G.N. participated in the electrophysiology studies, design of the study and drafting of the manuscript. All authors gave final approval for publication and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed

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Competing interests

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Supporting information

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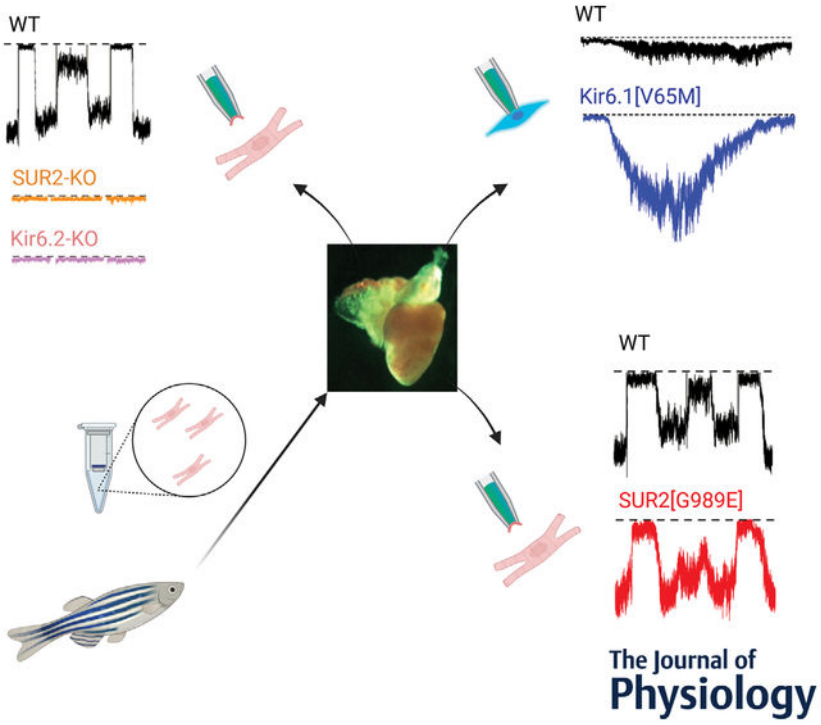
Statistical Summary Document

genetically modified fish as models of human Cantú syndrome and ABCC9-related Intellectual Disability and Myopathy syndrome.

Graphical Abstract



Patch-clamp analysis of atrial, ventricular, and vascular smooth muscle cells isolated from zebrafish reveal K_{ATP} channels of similar make-up and properties to those from mammalian cardiovascular tissues.

Keywords

ABCC9; Cantú syndrome; cardiomyocytes; cardiovascular; ion channels; K_{ATP}; KCNJ8; Kir6.2; SUR2; vascular smooth muscle cells; zebrafish

Introduction

Mammalian K_{ATP} channels are hetero-octameric potassium-selective ion channels composed of four pore-forming inwardly rectifying Kir6.x subunits (Kir6.1 or Kir6.2 encoded by *KCNJ8* and *KCNJ11*, respectively) and four regulatory sulfonylurea receptor SURx subunits (SUR1, SUR2 encoded by *ABCC8* and *ABCC9*), whose molecular heterogeneity is further increased by variable splicing of SUR2 into at least two distinct isoforms: SUR2A and SUR2B (Nichols, 2006). Regulated by intracellular nucleotides and membrane phospholipids, K_{ATP} channels serve as electrical transducers of the metabolic state of the cell by coupling cellular metabolism to the membrane potential (Nichols, 2006). K_{ATP} channels are widely expressed in plasma membranes throughout the body and serve a

diverse range of functions such as ischaemic preconditioning in cardiomyocytes, protection against fibre damage in skeletal muscle, vasomotor control in vascular smooth muscle (VSM), regulation of insulin secretion in pancreatic β cells and determination of nerve-fibre excitability in the central nervous system (Cole *et al.* 1991; Suzuki *et al.* 2002; Li *et al.* 2013; Barzegar *et al.* 2014; Tinker *et al.* 2014). K_{ATP} channels in different tissues exhibit distinct nucleotide sensitivities as a result of distinct subunit compositions. In mammals, Kir6.2 is coupled with SUR1 in the pancreas and in neurons, Kir6.2 is coupled with SUR2A in striated muscles and Kir6.1 is coupled with SUR2B in VSM. The discovery of gain-of-function (GOF) or loss-of-function (LOF) mutations in *KCNJ8* and *ABCC9* as causes of Cantú Syndrome (CS) (van Bon *et al.* 2012; Harakalova *et al.* 2012*a*; Brownstein *et al.* 2013; Cooper *et al.* 2014, 2017; McClenaghan *et al.* 2018) or *ABCC9*-related Intellectual Disability and Myopathy syndrome (AIMS) (Smeland *et al.* 2019), respectively, provides a clear illustration of the pathologic potential of K_{ATP} channels.

Zebrafish (ZF) have long been a model organism in biology and, in recent years, have gained popularity as models to study human cardiovascular (CV) disease mechanisms and potential therapies (Yoong *et al.* 2007). Their highly conserved amenable genome (Howe *et al.* 2013; Hodgson *et al.* 2018), together with their rapid development and fecundity (Hodgson *et al.* 2018), offers potentially significant advantages for modelling congenital CV disease progression and high-throughput drug screening. Their nearly transparent larvae that survive at high ischaemic conditions for up to 5 days-post-fertilization (dpf) (Poss, 2007; Hodgson *et al.* 2018) are well suited for *in vivo* imaging studies. Several studies suggest a close similarity between the electrical activity of ZF and human cardiomyocytes (Nemtsas *et al.* 2010; Vornanen & Hassinen, 2016; van Opbergen *et al.* 2018), but electrophysiological studies of fish vascular myocytes in general are lacking and there has been no molecular dissection of fish cardiovascular K_{ATP} channels. Because of the early genome duplication that occurred in the teleost lineage (Vornanen & Hassinen, 2016), ZF contain at least two orthologues for many human genes, including a novel K_{ATP} subunit-Kir6.3 (encoded by *kcnj11l*) (Zhang *et al.* 2006), and there could be significant differences between the molecular basis of K_{ATP} in ZF and human CV system (Hassinen *et al.* 2015).

In this study, we have developed approaches for efficiently identifying and isolating cells from ZF CV tissues and for electrophysiological analysis of isolated cardiomyocytes and vascular smooth muscle (VSM) cells. Using a comprehensive combination of genetically modified fish, electrophysiology and gene expression analysis, we demonstrate that ZF cardiac and vascular smooth muscles express functional K_{ATP} channels of similar subunit composition, structure and metabolic sensitivity to their mammalian counterparts, validating the use of genetically modified ZF K_{ATP} to model human CV K_{ATP} channelopathies.

Methods

Ethical approval

All animal experiments were conducted under the guidelines of the animal welfare committee of the Royal Netherlands Academy of Arts and Sciences (KNAW) and the Washington University Institutional Animal Care and Use Committee (IACUC Protocol no.

20-0308) and conform to the principles and regulations as described in the Editorial by Grundy (Grundy, 2015).

Animal lines and maintenance

The Kir6.1 and SUR2-knockout (SUR2-KO) and Cantú GOF zebrafish were generated previously, as described (Tessadori *et al.* 2018). In brief, CRISPR-Cas9 genome editing was combined with a short template oligonucleotide to introduce a single nucleotide mutation into *abcc9* (c.2969 GC>AA; Z.G983E and c.3176 G>A; Z.C1052Y) and *kcnj8* (c.204 G>A; Z.V65M) to mimic the human disease-causing amino acid substitution, H.G989E and H.C1043Y in SUR2 and H.V65M in K_{ir}6.1, respectively. We generated Kir6.2 (*kcnj11*) knockout fish using CRISPR-Cas9 genome editing. Two Cas9 target sites within the *kcnj11* open reading frame were chosen using the UCSC Genome Browser. Alt-R CRISPR crRNA molecules (IDT DNA) targeting the sites CGAACAGGGACGGTTTCTAC and GAGTGGATGTCCGTTACGCA within the first third of the open reading frame of *kcnj11* were produced and independently combined with Alt-R CRISPR tracRNA (IDT DNA) to generate functional gRNA molecules. Two nanolitres of solution containing both gRNA molecules (final concentration 25 pg/nl) and Cas9 protein (final concentration 322 pg/nl) were co-injected into one-cell stage zebrafish embryos of the wild type strain AB. Injected embryos were grown to adulthood to generate 'founder' fish (F0) and screened for large deletions by PCR across the kcnj11 locus with primers GTTGGCGGAGGATGTGTTAC and CAGTTATCACCGCGTGTTTG. Sequencing analysis confirmed a deletion of 129 bp, including portions of the pore-forming domain of the channel. The founder was outcrossed to wild-type AB to generate heterozygous carrier (F1) fish. Inbreeding of F1 fish generated homozygous (F2) fish, which were used for experiments.

SUR1 knockout fish were generated using ENU-mutagenesis at the Sanger Institute, as part of the Zebrafish Mutation Project, using *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis to identify knockout alleles for all protein-coding regions in the zebrafish genome (https://www.sanger.ac.uk/resources/zebrafish/zmp/). This project outcrosses ENU-mutagenized F0 males to create a population of F1 fish heterozygous for ENU-induced mutations, which were then obtained through the Zebrafish International Research Consortium (ZIRC). The *abcc8(sa15863)* nonsense mutant allele (K499-STOP, TT CTGGCTCCRGTGCAGTACTTTGTGGCAACCAAGT TATCAGATGCACAG**[A>T]**AAAGCACATTGGTGAG CTACTTTATTTTGGTTAATGTCCTAATGAGGCCA) generated K499-STOP mutants that were in-crossed as heterozygotes to generate homozygous progeny, genotyped by Transnetyx using restriction digest with the inserted digestion site for HpyCHRIII which is inserted into the mutant allele (forward primer: TTGTTGTTGTCTGCTTTTTGC; reverse primer: TTTACAAGCACAGCGCTCAC) to identify homozygotes.

Fish were maintained in the WU zebrafish facility (http://zebrafishfacility.wustl.edu/documents.html). All procedures were approved by the Washington University in St Louis IACUC.

Reverse-transcriptase PCR

Adult wild-type zebrafish were anaesthetized by transfer into ice water before they were decapitated. After removal of the skin and opening of the pericardial sac, the hearts were harvested. Atrium, ventricle and bulbous arteriosus from 15 fish were pooled separately. RNA was isolated using Trizol (Ambion) and cDNA was prepared using the High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific).

Isolation of zebrafish cardiomyocytes

Modified versions of published protocols (Brette *et al*. 2008; Louch *et al*. 2011; Sander *et al*. 2013) were used to isolate ZF ventricular and atrial cardiomyocytes (VCMs and ACMs). Briefly, fish were killed using cold-shock (8°C water immersion) followed by decapitation. The atria and ventricles were quickly excised, gently torn open using forceps to drain the blood and placed into separate 1.5 ml Eppendorf tubes containing perfusion buffer (PB; 10 mM HEPES, 30 mM taurine, 5.5 mM glucose, 10 mM BDM in PBS) and 5 ng/ml insulin. The PB in the tubes was then replaced by 750 μl of digestion buffer (DB; 5 mg/ml each of Collagenase Type II and Type IV (Worthington), 5 ng/ml insulin, 12.5 μM $CaCl_2$ in PB) each, for four pooled atria and three pooled ventricles per tube. The tissues were digested at 32°C, on a shaking table (800 rpm) for 25 min (for atria) to 40 min (for ventricles). The digestion was ended by replacing the DB with stopping buffer (SB; 10% FBS, 10 mg/ml BSA, 5 ng/ml insulin, 12.5 μM $CaCl_2$ in PB). The DB and SB were prepared freshly before each isolation using a fresh stock (<1 month) of PB. After 15 min of incubation at room temperature, the SB was replaced with PB, in which the tissue was gently triturated using a Pasteur pipette to disperse the cells, which were then viable for physiological measurements at room temperature for up to 12 h.

Isolation of zebrafish vascular smooth muscle cells

The bulbous arteriosus (BA) of teleost fish comprises vascular smooth muscle cells (VSMCs). By crossing with a previously reported *Tg(tagln:egfp)* smooth muscle cell transgenic reporter line (Liu *et al*. 2003; Seiler *et al*. 2010) and adapting existing protocols for isolation of mammalian arterial VSM (Huang *et al*. 2018), we successfully isolated and identified ZF VSMCs from BAs (Fig. 1A). BAs were excised from 4 ZF and placed in Solution 1 (S1; 0.1% BSA (w/v), 145 NaCl, 4 KCl, 10 HEPES, 10 glucose, 0.05 $CaCl_2$, 1 $MgCl_2$ (mM), adjusted to pH 7.4 using NaOH) on ice. S1 was then replaced by 400 μl Solution 2 (S2; 2 ml S1, 4 mg papain (Worthington), 2 mg DTT (Sigma)). The BA tissue was digested in S2 at 32°C on a shaking table (800 rpm) for 25 min. Following this, S2 was replaced by 500 μl Solution 3 (S3; 2 ml S1, 3 mg Collagenase Type H (Sigma), 2 mg trypsin inhibitor (Worthington), 1 mg elastase (Worthington)) and digested for additional 5 min. Digestion was terminated by replacing S3 with 500 μl S1. The tissue was triturated using Pasteur pipette and isolated cells were plated onto coverslips, then left to attach for at least 1 h before experiments and were used within 8 h.

Whole-cell voltage-clamp and excised inside-out patch-clamp experiments

After successful isolation of the ZF CMs and VSMCs, inside-out excised patch-clamp experiments were performed to characterize K_{ATP} channel expression and activity, using

a perfusion chamber equipped with oil gates and piezo-regulated flow control (Cannell & Lederer, 1986; Lederer & Nichols, 1989). The pipette solution for excised patch-clamp of CMs was K_{INT} buffer (140 mM KCl, 10 mM HEPES and 1 mM EGTA at pH 7.4) and the bath solution was the same, with or without additional nucleotides/drugs/Mg^{2+}, added depending on the study. For whole-cell patching, the bath solutions were either high-Na^+ (136 NaCl, 6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose (mM) at pH 7.4 adjusted with NaOH) or high-K^+ (140 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose (mM) at pH 7.4 adjusted with KOH). The pipette solution for whole-cell patch clamp of VSMCs was internal cell solution (ICS; 110 potassium aspartate, 30 KCl, 10 NaCl, 1 $MgCl_2$, 10 HEPES, 0.5 $CaCl_2$, 4 K_2HPO_4 and 5 EGTA (mM) at pH 7.2 adjusted with KOH) with or without 1 mM MgUTP or 0.1 mM ATP. The amount of $MgCl_2$ needed to achieve 0.5 mM free Mg^{2+} concentration was calculated using Webmaxc Standard website (WEBMAXC STANDARD (ucdavis.edu)).

Micropipettes for patch-clamping were pulled from soda-lime glass microhematocrit tubes (Kimble-Chase 2502) using a P-97 puller (Sutter Instruments). Pipette tips were coated with a molten mix of Parafilm in mineral oil to reduce the capacitance and had resistances of 1—2 MΩ (for excised patching) or 3–4 MΩ (for whole-cell) when filled with pipette solution.

A characteristic feature of K_{ATP} channels is rapid inhibition by intracellular ATP. To measure K_{ATP} channel activity and sensitivity to ATP via inside-out excised patching, the patch was exposed to different concentrations of MgATP (0 mM, 5 mM, 10 μM and 100 μM). In the case of whole-cell patching, the cells were patched in high-Na^+, mimicking the physiological extracellular solution, before switching the bath solution to high-K^+ based buffers to shift the Nernst potential. Excised-patch membrane currents were recorded at a constant membrane potential of −50 mV and whole-cell currents at −70 mV, using an Axopatch-1D amplifier and Axon pCLAMP software (Molecular Devices, LLC). Experiments were performed at room temperature. A minimum of three recordings in each condition was obtained for statistical analysis. Excised-patch channel currents in solutions of varying nucleotide concentrations were normalized to the basal currents in the absence of nucleotides for respective recordings and the dose-response curve was plotted using a four-parameter Hill fit according to the equation:

$$\text{Normalized current} = I_{\min} + (I_{\max} - I_{\min})/\left(1 + ([X]/\text{IC}_{50})^{H}\right), \quad (1)$$

where I_{max} is the maximum current (in zero ATP); I_{min} is the minimum current (in high, 5 mM ATP); [X] refers to the concentration of nucleotides/drugs; IC_{50} is the concentration of half-maximal inhibition; and H denotes the Hill coefficient.

Data analyses

All statistical analyses were performed using Microsoft Excel with the Real Statistics Resource Pack add-in (www.real-statistics.com). Significance values were calculated using the Kruskal-Wallis test and a subsequent *post hoc* Dunn's test for pairwise comparison. The Mann-Whitney U test (for two independent samples) was used to compare two groups,

with the Dunn/Sidak correction used when making multiple comparisons. All values are expressed as means ± SD and *P* values are provided in the figures.

Results

Zebrafish cardiac and vascular muscle express multiple K_{ATP} subunit transcripts

We performed PCR on cDNA generated from RNA isolated from the zebrafish CV system, to characterize Kir6 and SUR subunit expression in the zebrafish cardiac ventricle and atrium and from the bulbous arteriosus (Fig. 1A). SUR2A, SUR2B (*abcc9*), Kir6.1 (*kcnj8*) and Kir6.2 (*kcnj11*) were detected but not SUR1 (*abcc8*) or Kir6.3 (*kcnj11l*) (Fig. 1B). Notably, only Kir6.1 and SUR2B were detected in the bulbous arteriosus (Fig. 1C), which consists primarily of vascular smooth muscle cells (VSMCs), consistent with Kir6.1/SUR2B complexes forming vascular smooth muscle K_{ATP} channels, as in mammals. The additional presence of Kir6.2 and SUR2A expression in the heart (Fig. 1C) is consistent with cardiomyocyte K_{ATP} being formed of these subunits as in mammals, but the heart is innervated and permeated by capillaries and hence the presence of Kir6.1 and SUR2B transcripts in ventricle and atrium may reflect the presence of these other cell types.

Zebrafish cardiac muscle myocytes express functional K_{ATP} channels

As shown in Fig. 2A, both ventricular and atrial myocytes express robust ATP-sensitive potassium currents, with similar sensitivity to ATP in the absence or presence of Mg^{2+} (Fig. 2B), but at slightly higher density and lower sensitivity in the atrium (Fig. 2B). Similar to mammalian K_{ATP} channels, ADP in the presence of Mg^{2+} markedly activated atrial and ventricular K_{ATP} to a similar extent (Fig. 2C). As shown by ion replacement of K^+ by Na^+ (Fig. 3), the channels are highly K^+ selective in both the atrium and ventricle, with single-channel conductance of ~80 pS in 140 mM KCl on either side of the membrane (Fig. 3), essentially the same as seen in mammalian myocytes (Babenko *et al.* 1998*b*).

Ventricular and atrial K_{ATP} channels are generated by $K_{ir}6.2$ and SUR2 subunits

To define the subunit composition of the functional K_{ATP} channels in each of the different tissues, we characterized currents from multiple genetically modified fish. We have previously generated zebrafish knockouts for each of SUR2 (*abcc9*) and Kir6.1 (*kcnj8*) (Tessadori *et al.* 2018) and have now acquired or generated knockouts for SUR1 (*abcc8*) and Kir6.2 (*kcnj11*) (see Methods). Both atrial and ventricular K_{ATP} currents were indistinguishable from control, in terms of ATP sensitivity in SUR1 and Kir6.1 knockouts, although the density was slightly lower in SUR1 knockouts, but currents were abolished in SUR2 and Kir6.2 knockouts (Figs 4A–C and 5A–C). These data confirm that SUR2 and Kir6.2 are essential components of functional K_{ATP} channels in both chambers and that neither SUR1 nor Kir6.1 contribute significantly. SUR2[G898E], SUR2[C1043Y] and Kir6.1[V65M] mutations are associated with CS (Grange *et al.* 2019) and have been shown to cause GOF in recombinant channels (Cooper *et al.* 2015, 2017; McClenaghan *et al.* 2018). Strikingly and essentially confirming the contribution of SUR2 but not Kir6.1 to active cardiac channels, ATP sensitivity was also markedly reduced in patches from SUR2[G898E] and SUR2[C1043Y], but not, Kir6.1[V65M], myocytes from both ventricle (Fig. 4B and D) and atrium (Fig. 5B and D).

Vascular smooth muscle K_{ATP} channels are generated by Kir6.1 and SUR2 subunits

We were not able to detect K_{ATP} channels in excised membrane patches from bulbous arteriosus (BA, not shown). However, in whole-cell voltage-clamp recordings, measurable K conductance was present with zero ATP in the pipette but absent when 100 μM ATP was included (Fig. 6A), similar to what is seen in mammalian vascular smooth muscle (Li *et al.* 2013; Huang *et al.* 2018). In marked contrast to cardiac myocytes, K_{ATP} currents were absent in Kir6.1 knockout vascular smooth muscle myocytes (Fig. 6B). In addition, ATP-sensitive K^+ conductance was markedly increased in vascular smooth muscle myocytes from heterozygous Cantú SUR2[G898E] and Kir6.1[V65M] (Fig. 6B) and dramatically so in homozygous Kir6.1[V65M] (Fig. 6B) further confirming that VSM K_{ATP} channels are formed of SUR2 and Kir6.1 subunits, as in mammalian smooth muscle (Huang *et al.* 2018).

Zebrafish cardiac and vascular smooth muscle K_{ATP} channels are insensitive to K channel openers

Mammalian K_{ATP} channels are activated by a wide range of chemical K^+ channel openers (KCOs), including pinacidil, minoxidil and diazoxide (Flagg *et al.* 2010). There are indirect indications that fish K_{ATP} channels may lack KCO sensitivity (Paajanen & Vornanen, 2002), but this has not been studied directly. As shown in Fig. 7A, zebrafish cardiac myocytes in both atrium and ventricle show no response to 100 μM of pinacidil or minoxidil, under conditions that cause marked activation of mouse cardiac K_{ATP} channels. Although diazoxide causes activation of SUR1-containing K_{ATP} channels from zebrafish pancreatic β-cells (Fig. 7A), diazoxide also has no effect on cardiac K_{ATP}, further arguing that SUR1 is not a significant component of the latter. Furthermore, while pinacidil causes marked activation of whole-cell K_{ATP} currents in mouse vascular smooth muscle myocytes, again there is no activation of zebrafish bulbous arteriosus channels (Fig. 7C).

Discussion

Structure and functional properties of cardiovascular K_{ATP} channels are conserved between zebrafish and mammals

Mammalian K_{ATP} channels are formed by several combinations of Kir6.1/2 and SUR1/2 subunits (Nichols, 2006). K_{ATP} subunit orthologues are found in all vertebrate genomes, but few studies have investigated the structural or functional properties of K_{ATP} channels in non-mammalian vertebrates, including fish. Emfinger *et al.* (2017) demonstrated that zebrafish islet β-cells express functional K_{ATP} channels of similar subunit composition, structure and metabolic sensitivity to their mammalian counterparts (Emfinger *et al.* 2017). Using PCR on cDNA isolated from various cardiovascular tissues, we show that zebrafish cardiac (CM) and vascular smooth muscle (VSM) cells express K_{ATP} channels with very similar composition to their mammalian counterparts, with Kir6.1/SUR2B in VSM and Kir6.2/SUR2A in cardiomyocytes (Figs 1–6). This makeup in functional K_{ATP} channels is supported by electrophysiological studies on isolated myocytes, with the exception that we are unable to distinguish SUR2A and SUR2B dependence of functional channels. Although the activity of SUR2A and SUR2B are readily differentiated in mammals via KCO pharmacology, as SUR2B, but not SUR2A, is activatable by diazoxide (Babenko *et al.*

1998*a*), this is not possible in the fish myocytes, given the lack of KCO activation in both cardiac and vascular K_{ATP} (see below).

Excised-patch-clamp techniques confirm that zebrafish ventricular (VCM) and atrial (ACM) myocyte sarcolemmal K_{ATP} channel properties are essentially indistinguishable from those in mammalian ventricles, with two significant exceptions. Firstly, although the prominent SUR subunit in rodent cardiac myocytes is SUR2 in the ventricles, but SUR1 in the atria (Flagg *et al.* 2007; Glukhov *et al.* 2010), both atrial and ventricular channels are SUR2-dependent in the fish (Figs 4 and 5). In this regard, it is notable that in humans there is evidence for both subunits in both atrial and ventricles (Fedorov *et al.* 2011). In addition, although K_{ATP} was still present in SUR1 knockout CM, there was a non-significant ~50% reduction of current density in both chambers (Figs 4 and 5). Secondly, while ZF VCM and ACM channels are inhibited by ATP and MgATP, with similar sensitivity to those in mammalian myocytes, both are insensitive to the K_{ATP} channel openers (KCOs) pinacidil and minoxidil (Fig. 7). Although this has not been directly demonstrated previously, these data are consistent with prior findings that these KCOs are ineffective at eliciting K_{ATP} conductance in whole-cell patch clamp on myocytes from other teleost fish myocytes (Paajanen & Vornanen, 2002), suggesting that KCO insensitivity may be common to SUR2-dependent channels in all fish. Prior studies identified L1249 and T1253 as key residues conferring opener sensitivity in rat SUR2a (Uhde *et al.* 1999; Moreau *et al.* 2000) and mutation of residue M1290 in hamster SUR1 to Thr (equivalent to residue 1253 in SUR2a) renders it fully activated by the other KCOs (Moreau *et al.* 2000). Alignment of the zebrafish and rat SUR2A sequence shows that the equivalent Leu and Thr residues are already present in ZF. Thus, it appears that additional unidentified residues may be involved in KCO binding.

Zebrafish as a model organism for studying cardiovascular K_{ATP} pathophysiology

Recent studies have shown that GOF and LOF in KCNJ8 (Kir6.1) and ABCC9 (SUR2) underlie human Cantú syndrome and ABCC9-related Intellectual Disability and Myopathy syndrome (AIMS), respectively (van Bon *et al.* 2012; Harakalova *et al.* 2012*b*; Smeland *et al.* 2019). In previous studies, we have shown that zebrafish carrying disease-causing mutations in the equivalent *kcnj8* and *abcc9* loci reiterate essential features of these syndromes (Tessadori *et al.* 2018; Smeland *et al.* 2019) and here we have shown that each of these CS mutations causes increased VSM K_{ATP} conductance, but cardiac channel properties are only affected in the SUR2 mutants. In mammals and in zebrafish, the cardiac and vascular Cantú syndrome phenotypes are similar for both the KCNJ8 (kcnj8) and ABCC9 (abcc9) mutations at the organismal level (Brownstein *et al.* 2013; Cooper *et al.* 2014). This has been explained in mouse studies by the finding that cardiac pathology is a secondary response to decreased vascular resistance (Huang *et al.* 2018; McClenaghan *et al.* 2020*a*,b). Our finding that the molecular effects of SUR2 mutations are present in CM and VSM, whereas those of Kir6.1 mutants are only present in VSM is important, suggesting that cardiac enlargement in each disease model arises from the same compensatory mechanisms in the fish as in the mouse. Confirming that the subunit composition of cardiac and vascular myocyte K_{ATP} channels is essentially identical to the mammalian counterparts in each case is thus an essential validation of the use of zebrafish as a model organism for studying

the pathophysiological consequences of Cantú syndrome and of K_{ATP} activity changes in general.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Biography

Soma S. Singareddy is a PhD student in the Biomedical Engineering Program at Washington University in St Louis. His thesis work is focused on understanding the molecular make-up of cardiovascular K_{ATP} channels in zebrafish and their role in a fish model of Cantú syndrome, using electrophysiological and other techniques.

Data availability statement

All original data has been uploaded to the following repositories:

https://osf.io/v6yzx

https://osf.io/pn8tz

https://osf.io/htrpy

https://osf.io/a83b7. and will be made available to interested parties on request.

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Key points

- Zebrafish cardiac myocytes (CM) and vascular smooth muscle (VSM) express functional K_{ATP} channels of similar subunit composition, structure and metabolic sensitivity to their mammalian counterparts.
- In contrast to mammalian cardiovascular K_{ATP} channels, zebrafish channels are insensitive to potassium channel opener drugs (pinacidil, minoxidil) in both chambers of the heart and in VSM.
- We provide a first characterization of the molecular properties of fish K_{ATP} channels and validate the use of such genetically modified fish as models of human Cantú syndrome and ABCC9-related Intellectual Disability and Myopathy syndrome.

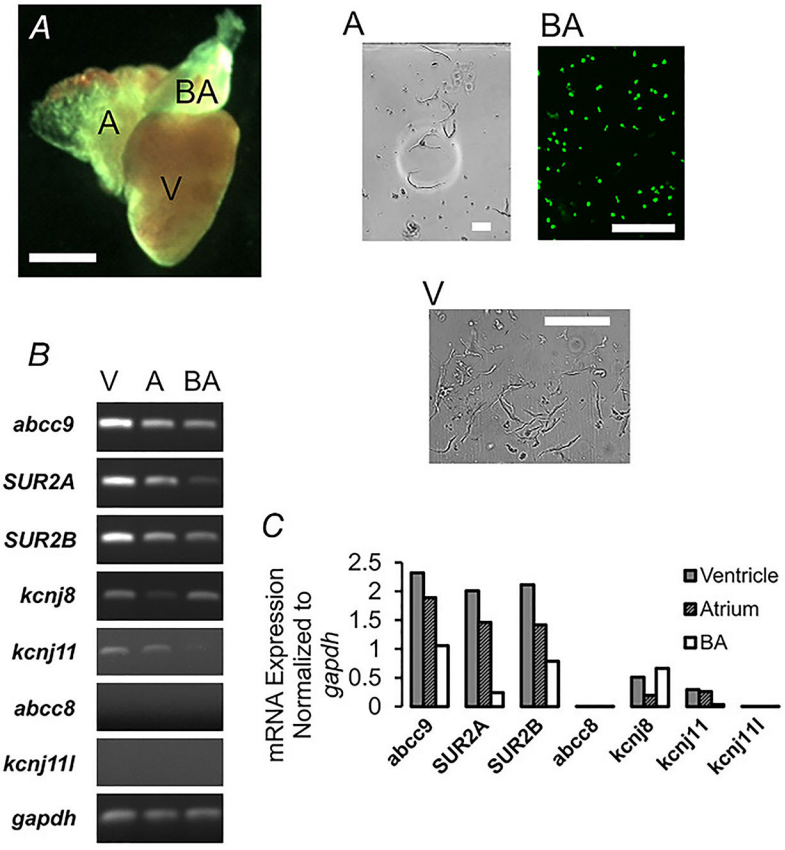

Figure 1. Zebrafish cardiovascular cell types and K_{ATP} channel subunit expression

A, photomicrograph (left) of isolated zebrafish heart (green filter lens) reveals atrium (A), ventricle (V) and bulbous arteriosus (BA). Scale bar, 1 mm. Images of isolated cells (right) from each tissue. BA cells expressing GFP are shown under UV illumination. Scale bars, 100 μm. *B*, RT-PCR analysis of K_{ATP} channel subunit expression, including SUR2a and SUR2b isoforms and gapdh. *C*, quantification of data from *B*.

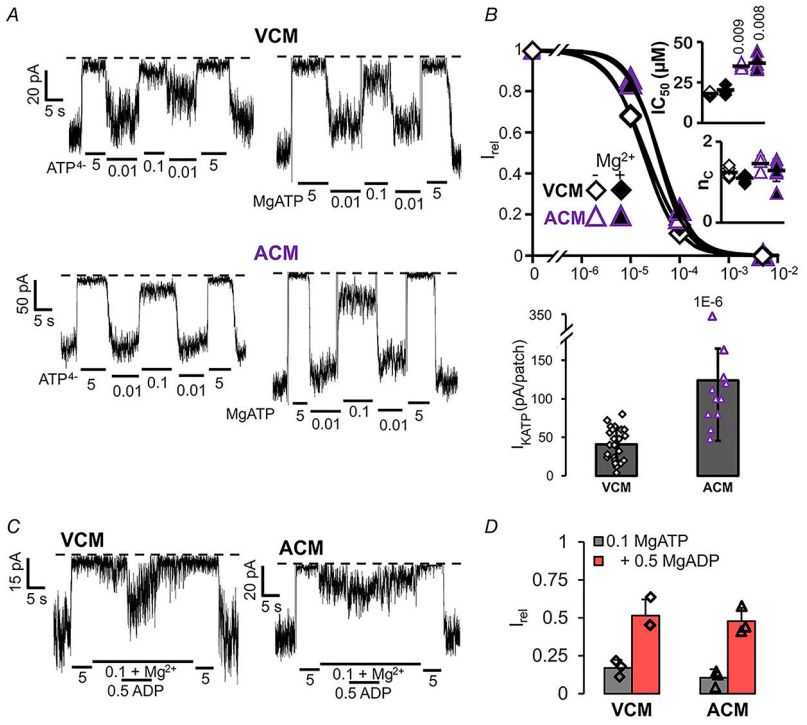

Figure 2. K_{ATP} channel current in ventricular and atrial myocytes

A, representative inside-out patch-clamp recordings from ventricular (VCM, above) and atrial (ACM, below) cardiac myocytes in the presence of differing [ATP], with (right) or without (left) Mg^{2+} ions. *B*, [ATP] dependence of channel activity (I_{rel}) for VCM and ACM, from recordings as in *A* (n = 3–7 recordings from n = 3 preparations (three animals per preparation) in each case). Upper panel, dose-response relationships were fitted with Hill plots (eqn (1)). Graph shows fit to averaged data; inset shows individual data, mean and SD for fits to individual recordings. Lower panel, measured K_{ATP} density (current in zero ATP). *C*, representative inside-out patch-clamp recordings from VCM and ACM, in the presence of differing [ATP] and [ADP], with Mg^{2+} ions. *D*, currents in 0.1 mM ATP, with or without ADP, relative to current in zero ATP (I_{rel}). Graph shows individual data, mean and SD.

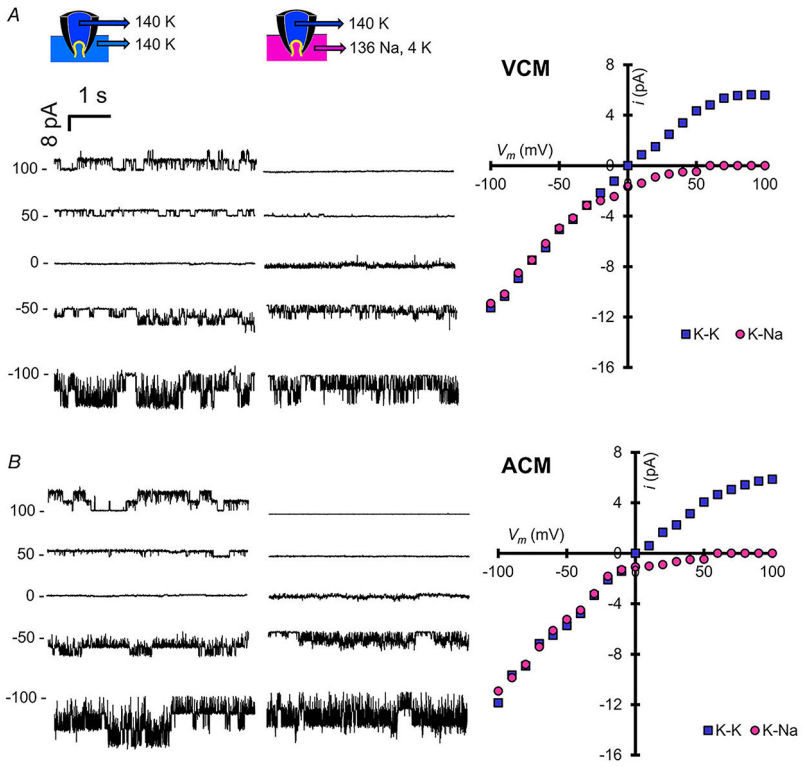

Figure 3. Single channel K_{ATP} currents in ventricular and atrial myocytes

A and *B*, left: representative inside-out current recordings from VCM (*A*) and ACM (*B*) in 140 mM K on both sides of the membrane and after replacement of cytoplasmic solution with 4 mM K^+/136 mM Na^+. Markers indicate zero current. Right: single channel *I-V* relationships from each condition.

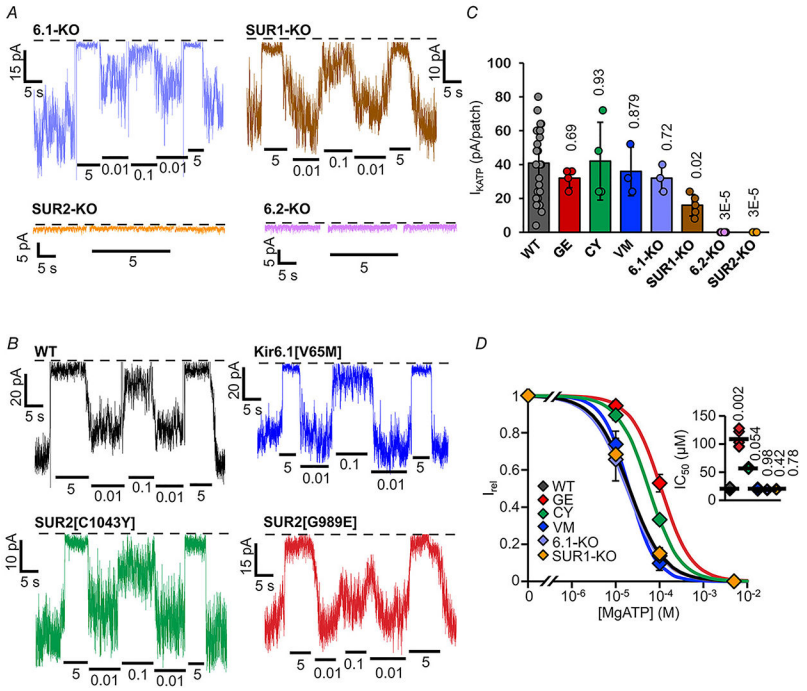

Figure 4. Subunit dependence of K_{ATP} channels in ventricular myocytes

A and *B*, representative inside-out patch-clamp recordings from VCM in the presence of differing [ATP], as in Fig. 2A, from K_{ATP} subunit knockout fish (*A*) and Cantú mutant fish (*B*). with (right) or without (left) Mg^{2+} ions. *C*, measured K_{ATP} density (current in zero ATP) from individual experiments as in *A* and *B* (n = 3–24 recordings from 1–3 preparations (three animals per preparation), in each case). *D*, [ATP] dependence of channel activity (I_{rel}) from recordings as in *A,B*. (above) Dose-response relationships were fitted with Hill plots (eqn (1)). Graph shows fit to averaged data; inset shows individual data, mean and SD for fits to individual recordings.

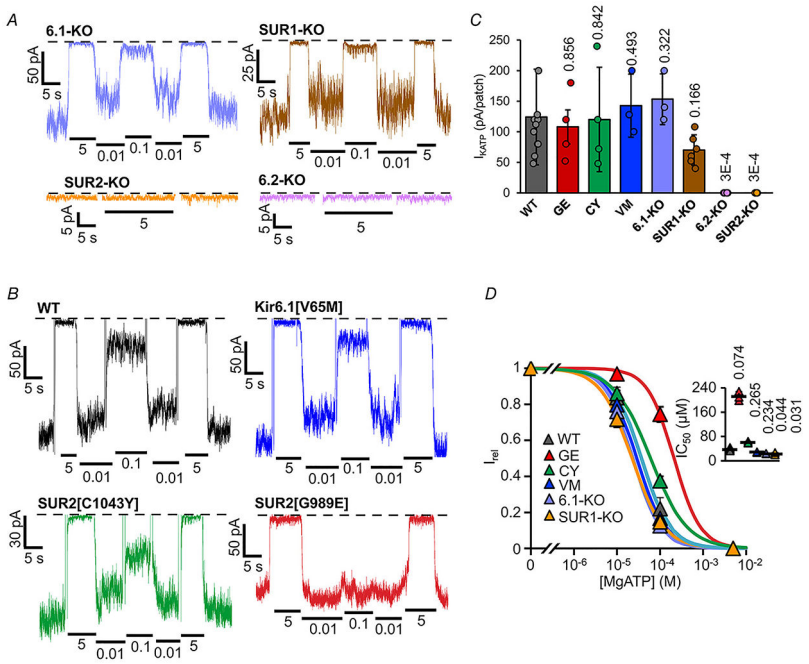

Figure 5. Subunit dependence of K_{ATP} channels in atrial myocytes

A and *B*, representative inside-out patch-clamp recordings from ACM in the presence of differing [ATP], as in Fig. 2A, from K_{ATP} subunit knockout fish (*A*) and Cantú mutant fish (*B*). with (right) or without (left) Mg^{2+} ions. *C*, measured K_{ATP} density (current in zero ATP) from individual experiments as in *A* and *B* (n = 3–12 recordings, from 1–3 preparations (four animals per preparation), in each case). *D*, [ATP] dependence of channel activity (I_{rel}) from recordings as in *A* and *B*. (above) Dose-response relationships were fitted with Hill plots (eqn (1)). Graph shows fit to averaged data; inset shows individual data, mean and SD for fits to individual recordings.

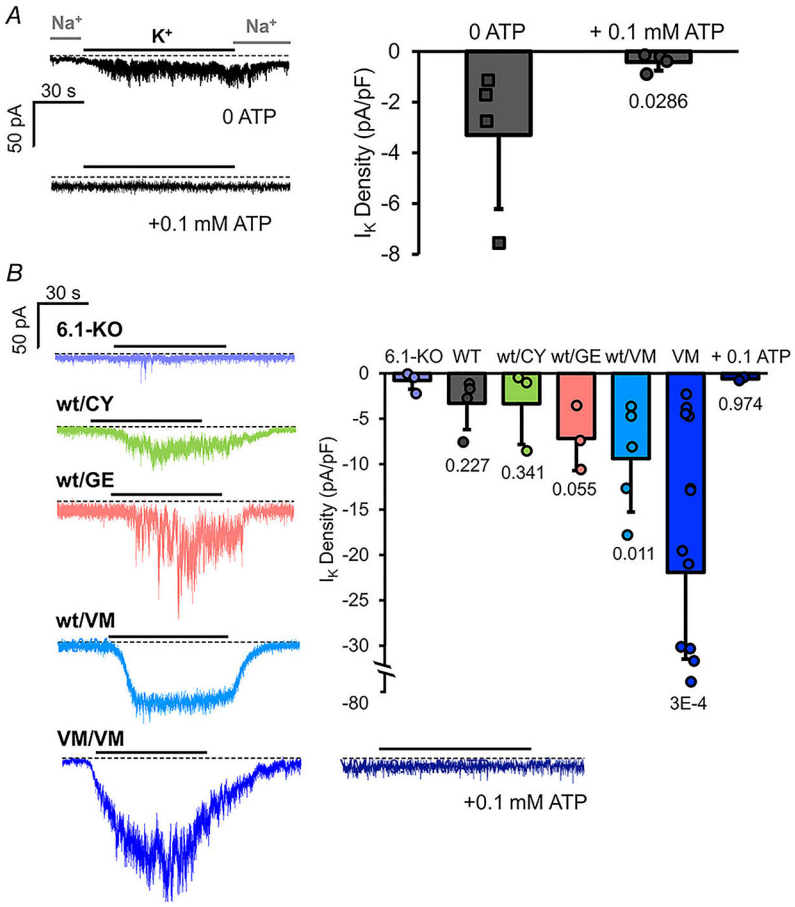

Figure 6. Subunit dependence of K_{ATP} channels in bulbous arteriosus myocytes

A, left: representative whole-cell voltage-clamp recordings from WT BA smooth muscle myocytes with zero 0.1 mM ATP in the recording pipette. Currents were recorded with 136 mM Na^+/6 mM K^+ (Na^+) in the bath or 0 Na^+/140 K^+ (K^+) as indicated. Right: measured K current density (current in K^+ – current in Na^+) from individual experiments as at left (n = 4 recordings from 2 preparations (five animals per preparation), in each case). *B*, left: representative whole-cell voltage-clamp recordings from Kir6.1 knockout and hetero- or homozygous Cantú mutant zebrafish as indicated. Right: measured K^+ current density (current in K^+ – current in Na^+) from individual experiments as at left (n = 3–10 recordings from 1–3 preparations (five animals per preparation), in each case).

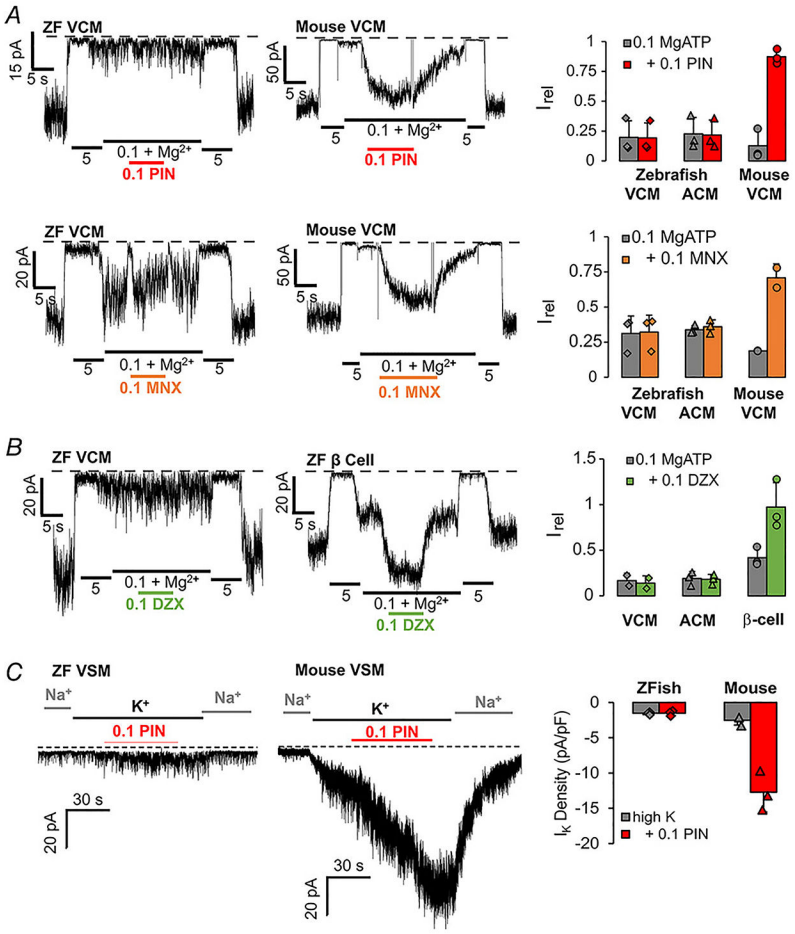

Figure 7. K^+ channel opener insensitivity in zebrafish cardiovascular myocytes

A, left: representative inside-out patch-clamp recordings from zebrafish and mouse VCM, as indicated, in the presence of differing [ATP], with or without pinacidil (0.1 mM, PIN) or minoxidil (0.1 mM MNX). Right: currents in 0.1 mM ATP with and without drug (I_{rel}). Graph shows individual data, mean and SD ($n = 3$, in each case). *B*, left: representative inside-out patch-clamp recordings from zebrafish VCM and pancreatic β-cell, as indicated, in the presence of differing [ATP], with or without diazoxide (0.1 mM, DZX). Right: currents in 0.1 mM ATP with and without DZX (I_{rel}). Graph shows individual data, mean and SD ($n = 3$ recordings from 1 preparation each), in each case). *C*, left: representative whole-cell voltage-clamp recordings from zebrafish BA (VSM) and mouse aortic VSM, as indicated, with or without addition of pinacidil (0.1 mM, PIN). Right: K^+ current density in high K^+ solution, with and without PIN. Graph shows individual data, mean and SD ($n = 3$ recordings from 1 preparation in each case).