Developmental Biology 425 (2017) 109–129

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Original research article

Ca^{2+} release via two-pore channel type 2 (TPC2) is required for slow muscle cell myofibrillogenesis and myotomal patterning in intact zebrafish embryos

Jeffrey J. Kelu[a], Sarah E. Webb[a], John Parrington[b], Antony Galione[b], Andrew L. Miller[a,c,*]

[a] Division of Life Science & State Key Laboratory of Molecular Neuroscience, HKUST, Clear Water Bay, Hong Kong, PR China
[b] Department of Pharmacology, University of Oxford, Mansfield Road, Oxford, UK
[c] Marine Biological Laboratory, Woods Hole, MA, USA

ARTICLE INFO

Keywords:
Two-pore channel 2
Slow skeletal muscle cell differentiation
Myofibrillogenesis
Zebrafish
Morpholino oligonucleotides
CRISPR/Cas9
STED super-resolution microscopy
Ca^{2+} signaling

ABSTRACT

We recently demonstrated a critical role for two-pore channel type 2 (TPC2)-mediated Ca^{2+} release during the differentiation of slow (skeletal) muscle cells (SMC) in intact zebrafish embryos, via the introduction of a translational-blocking morpholino antisense oligonucleotide (MO). Here, we extend our study and demonstrate that knockdown of TPC2 with a non-overlapping splice-blocking MO, knockout of TPC2 (via the generation of a *tpcn2*^{dhkz1a} mutant line of zebrafish using CRISPR/Cas9 gene-editing), or the pharmacological inhibition of TPC2 action with bafilomycin A1 or *trans*-ned-19, also lead to a significant attenuation of SMC differentiation, characterized by a disruption of SMC myofibrillogenesis and gross morphological changes in the trunk musculature. When the morphants were injected with *tpcn2*-mRNA or were treated with IP_3/BM or caffeine (agonists of the inositol 1,4,5-trisphosphate receptor (IP_3R) and ryanodine receptor (RyR), respectively), many aspects of myofibrillogenesis and myotomal patterning (and in the case of the pharmacological treatments, the Ca^{2+} signals generated in the SMCs), were rescued. STED super-resolution microscopy revealed a close physical relationship between clusters of RyR in the terminal cisternae of the sarcoplasmic reticulum (SR), and TPC2 in lysosomes, with a mean estimated separation of ~52–87 nm. Our data therefore add to the increasing body of evidence, which indicate that localized Ca^{2+} release via TPC2 might trigger the generation of more global Ca^{2+} release from the SR via Ca^{2+}-induced Ca^{2+} release.

1. Introduction

There is a growing interest in the role played by two-pore channels (TPCs) and their link with nicotinic acid adenine dinucleotide diphosphate (NAADP) signaling with regards to their combinatorial contribution to the Ca^{2+}-mediated regulation of differentiation and development (Calcraft et al., 2009; Patel et al., 2010; Galione, 2011; Morgan and Galione, 2014; Parrington and Tunn, 2014; Parrington et al., 2015; Brailoiu and Brailoiu, 2016). The various roles played by the Ca^{2+} mobilizing messengers inositol 1,4,5-trisphosphate (IP_3) and cyclic adenosine diphosphate ribose (cADPR), and their respective receptors in the endoplasmic/sarcoplasmic reticulum (ER/SR) membrane, are well established (Berridge, 1993; Lee, 1993; Berridge et al., 2003; Mikoshiba, 2007; Lanner et al., 2010). However, the discovery of a third Ca^{2+} mobilizing messenger, NAADP, which releases Ca^{2+} from alternative Ca^{2+} stores, acidic endosomes and lysosomes, via TPCs (Patel, 2004; Yamasaki et al., 2005; Lee, 2005), has resulted in new challenges to our current understanding of the complexity of Ca^{2+}-mediated signaling pathways during differentiation and development.

TPCs are members of the superfamily of voltage-gated ion channels, and they have been reported in many cell types and in a wide range of circumstances to act as an endogenous NAADP receptor (Calcraft et al., 2009; Zong et al., 2009; Brailoiu et al., 2010; Morgan and Galione, 2014). Three isoforms of TPCs are present in most vertebrates, i.e., TPC1 to 3. TPC1 and TPC3 are suggested to localize to the endosomes and other compartments along the endo-lysosomal system; whereas TPC2 is specifically expressed in the membrane of late endosomes and lysosomes (Ruas et al., 2014). It has been suggested that in some cases TPCs are NAADP-insensitive; instead they are activated by the lipid phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$), and they conduct Na^+ rather than Ca^{2+} (Wang et al., 2012; Cang et al., 2013). However, recent reports have confirmed that NAADP does indeed bind to TPCs, perhaps via accessary proteins (Lin-Moshier et al., 2012; Walseth et al., 2012a, 2012b; Morgan and Galione, 2014), and that they are able to mediate Ca^{2+} release from acidic organelles (Jha et al., 2014; Jentsch et al., 2015; Ruas et al., 2015; Pitt et al., 2016). An intriguing property of TPCs is that in addition to NAADP, their activity might also be mediated by other regulators, such as $PI(3,5)P_2$, Mg^{2+}, P38 and JNK

* Corresponding author at: Division of Life Science, HKUST, Clear Water Bay, Kowloon, Hong Kong, PR China.
E-mail addresses: almiller@ust.hk (A.L. Miller).

http://dx.doi.org/10.1016/j.ydbio.2017.03.031
Received 7 December 2016; Received in revised form 30 March 2017; Accepted 31 March 2017
Available online 06 April 2017
0012-1606/

(Jha et al., 2014). They might, therefore, represent a form of signaling hub with some additional properties and functions that are still to be identified.

It has been shown that TPCs are important mediators of differentiation and function of a variety of cell types during early development. These include neurons (Brailoiu et al., 2005, 2006), skeletal muscle cells (Aley et al., 2010; Kelu et al., 2015), smooth muscle cells (Kinnear et al., 2008; Pereira et al., 2014), cardiac muscle cells (Collins et al., 2011; Capel et al., 2015), osteoclasts (Sun et al., 2003; Notomi et al., 2012), hematopoietic cells (Orciani et al., 2008), keratinocytes (Park et al., 2015), and embryonic stem cells (Zhang et al., 2013; Hao et al., 2016). In addition, for several decades, investigators have reported the essential role played by Ca^{2+} signaling during the embryonic development of skeletal muscle from a number of systems (for reviews see Webb and Miller (2011), Tu et al. (2016)). These include chick (David et al., 1981); frog (Ferrari et al., 1996, 1998; Ferrari and Spitzer, 1999); mouse (Lorenzon et al., 1997; Pisaniello et al., 2003); and zebrafish (Brennan et al., 2005; Cheung et al., 2011). These studies focused on the role played by extracellular Ca^{2+} as well as that of the then known intracellular Ca^{2+} mobilizing messengers, IP_3 and cADPR. More recently, however, the discovery of TPCs has led to a surge in interest of NAADP-mediated Ca^{2+} release from acidic vesicles, and how this integrates with the other Ca^{2+} mobilizing agents to initiate and mediate a variety of signaling pathways. Several recent reports from both in vivo (Kelu et al., 2015) and in vitro (Aley et al., 2010) studies have indicated that TPCs play a necessary role in the differentiation and function of skeletal muscle.

Here, we report the combined use of gene knockdown, gene knockout, and pharmacology to explore the function of TPC2 during the differentiation of non-muscle pioneer slow skeletal muscle cells during zebrafish development (Devoto et al., 1996; Du et al., 1997). Morpholino (MO) antisense oligonucleotide technology has been the main reverse genetic approach to study gene function in zebrafish for the past decade (see review by Blum et al. (2015)). MOs can work either by blocking the translation or splicing of the mature transcript and pre-mRNA, respectively, and thus are able to attenuate the expression of specific genes. Furthermore, following the recent advancement in genome-editing technologies, gene function can now be studied in vivo in the context of gene-knockout using clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas9 endonuclease. CRISPR/Cas9 is currently considered to be the most cost-effective and efficient genome-editing method, and it has been successfully applied to zebrafish for the generation of a number of loss-of-function mutants (Chang et al., 2013; Hwang et al., 2013; Jao et al., 2013; Irion et al., 2014; Varshney et al., 2015). By combining the transient-knockdown approach using MOs and the persistent-knockout approach using CRISPR/Cas9, we present in vivo evidence that TPC2-mediated Ca^{2+} release from lysosomes plays a key role in non-muscle pioneer slow muscle cell (SMC) differentiation, myofibrillogenesis and myotomal patterning in developing zebrafish embryos.

Specifically, here we report, via whole-mount immunohistochemistry and fluorescent-labeling, the disruption of the structure and organization of two of the main non-muscle pioneer SMC proteins (i.e., myosin heavy chain and F-actin), as well as a decrease in the number of $prox1^+$ SMC nuclei following inhibition of TPC2 by MO-based knockdown and CRISPR/Cas9-mediated knockout of TPC2. Moreover, a partial rescue of the normal phenotype was obtained via the co-injection of a mutant *tpcn2* mRNA that is not recognized by the translation blocking *TPCN2*-MO-T. Furthermore, a complementary pharmacological approach with bafilomycin A1 and *trans*-ned-19, which deplete the lysosome acidic Ca^{2+} stores and antagonize TPCs, respectively, also phenocopied the TPC2 morphants and mutants. To test the hypothesis that TPC2 triggers the release of additional Ca^{2+} from the ER/SR by activating IP_3Rs and/or RyRs, we treated the TPC2 morphants with either IP_3/BM or caffeine, and then investigated the effect on the development of the TPC2-depleted myotome by up-regulating Ca^{2+} release from the ER/SR. These rescue results following TPC2-knockdown suggest that the release of Ca^{2+} from IP_3Rs has a more profound role with regards to regulating the morphology of the non-muscle pioneer SMC than does release from RyRs.

Furthermore, using dual-colour STED super-resolution microscopy, we were able to resolve and hence report the presence of ~52–87 nm 'gaps' between clusters of fluorescently-labeled RyRs and TPC2 in mature myofibers prepared from the trunk muscles of ~48 hpf embryos. These RyR-TPC2 clusters were located (in a striated pattern) in the terminal cisternae of the SR, adjacent to the sarcomeric I-bands. Taken together, our new data suggest that in the differentiated non-muscle pioneer SMCs of zebrafish, TPC2 in the lysosomal membrane forms an intimate relationship with RyRs in the SR membrane. It has been proposed that TPC-RyR clusters act as "trigger zones" where TPCs are stimulated to release highly localized elementary Ca^{2+} signals that subsequently lead to the opening of RyR in the SR/ER membrane resulting in global signals via Ca^{2+}-induced Ca^{2+} release (CICR; Kinnear et al., 2004, 2008; Galione, 2011). We suggest, therefore, that Ca^{2+} release via TPC2 plays an essential role during excitation-contraction (EC)-coupling in mature non-muscle pioneer SMC, as well as an earlier necessary role in excitation-transcription (ET)-coupling during non-muscle pioneer SMC differentiation (Kelu et al., 2015).

2. Materials and methods

2.1. Zebrafish husbandry and embryo collection

Wild-type zebrafish (*Danio rerio;* AB and ABTU strains), the α-actin-apoaequorin-IRES-EGFP (α-actin-aeq) transgenic line (AB background; Cheung et al., 2011), and the mutant line $tpcn2^{dhkz1a}$ (ABTU background) were maintained, and their fertilized eggs collected, as previously described (Webb et al., 1997; Cheung et al., 2011). The AB strain was obtained from the Zebrafish International Resource Center (University of Oregon, Eugene, OR, USA), and the ABTU strain was a generous gift from Prof. Han Wang (Soochow University, Suzhou, China). Fertilized eggs were maintained at ~28 °C for most experiments, but sometimes they were kept at room temperature (i.e., ~23 °C), to slow development until the desired stage was reached.

2.2. Design and injection of morpholino oligomers

All the morpholino oligomers (MOs; synthesized by Gene Tools LLC, Philomath, OR, USA) were prepared at a 1 mM stock concentration in Milli-Q water and kept at room temperature. The expression of TPC2 was attenuated using the translation-blocking MO described previously (Kelu et al., 2015; *TPCN2*-MO-T) as well as a splice-blocking MO (*TPCN2*-MO-S). As some MOs are known to induce p53 activity and thus result in non-specific apoptosis (Robu et al., 2007), *TPCN2*-MO-T and -MO-S were co-injected with a previously characterized *p53*-MO (Robu et al., 2007; Kelu et al., 2015) at a ratio of ~1:1.5. The *p53*-MO (injected alone) and a standard control-MO were also used as specificity controls. Thus, ~1.5 nL of the diluted *TPCN2*-MO-T (~2.5 ng), or ~3 nL of the *TPCN2*-MO-S (~5 ng), the standard control-MO (~5 ng) or *p53*-MO (~7.5 ng) were injected into the yolk of embryos at the 1- to 4-cell stage and subsequently carried into the blastodisc/blastoderm by ooplasmic streaming (Leung et al., 1998). Embryos were microinjected using equipment and methods described by Webb and Miller (2013). In some experiments, sub-optimal doses of *TPCN2*-MO-T (i.e., ~1.3 ng) and *TPCN2*-MO-S (i.e., ~2.5 ng), both mixed with *p53*-MO at a 1:1.5 ratio, were individually injected into the yolk of embryos to further validate the specificity of the *TPCN2*-MOs. The sequences for the *TPCN2*-, *p53*- and standard control-MOs used, are as follows:

TPCN2(ATG)-MO-T: 5'-CAGCCAGCAGCGGTTCTTCTTCCAT-3'
TPCN2(Splice)-MO-S: 5'- TGATTGTGTTTTTACCTTAATCGCA-3'