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Popeye domain containing proteins and stress-mediated modulation of cardiac pacemaking

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Abstract

An intricate network of ion channels and pumps are involved in generating a diastolic pacemaker potential, which is transmitted to the working myocardium with the help of the cardiac conduction system. The principles of cardiac pacemaking are reasonably well understood, however, the mechanism by which the heart increases its beating frequency in response to adrenergic stimulation has not been fully worked out. The Popeye domain containing (Popdc) genes encode plasma membrane-localized proteins that are able to bind cAMP with high affinity and mice with null mutations in *Popdc1* or *-2* have a stress-induced pacemaker dysfunction. The phenotype in both mutants develops in an age-dependent manner and thus may model pacemaker dysfunction in man, as well as providing novel mechanistic insights into the process of pacemaker adaptation to stress.

1 Introduction

The Popeye domain containing (Popdc) gene family consists of three genes, *Popdc1* (also known as *Bves* or *Pop1*), *Popdc2* (*Pop2*), and *Popdc3* (*Pop3*) (Andrée et al., 2000 and Reese et al., 1999). They encode membrane proteins found at the plasma membrane in cardiac and skeletal muscle cells (Figure 1). Antibodies directed against any of the three Popdc proteins label the plasma membrane as well as the transverse tubular system. Popdc1 protein, which is the best characterized Popdc protein contains three transmembrane helices by which it is anchored to the plasma membrane (Knight et al., 2003). In the cytoplasm an evolutionary conserved Popeye domain is present (Figure 1). The extracellularly-localized amino terminus is short (20–40 amino acids), while the carboxyl terminus is variable in length and displays only limited sequence conservation. Popdc1 forms protein dimers, which are stabilized by a disulfide bridge (Knight et al., 2003). A dimerization motif was mapped to the carboxyl terminus of the Popeye domain (Kawaguchi et al., 2008). Others have suggested alternative sequences essential for protein dimerization (Russ et al., 2011). The Popdc1 protein is extensively glycosylated, however, the functional significance of this protein modification has not yet been analyzed (Andrée et al., 2000 and Knight et al., 2003).

2 The Popeye domain functions as a cAMP-binding domain

The Popeye domain displays limited sequence homology to other proteins, while sequence conservation amongst Popeye proteins is high and amounts to approximately 40-60% (Andrée et al., 2000). The bacterial CAP or CRP proteins are the closest related non-Popdc proteins. CRP proteins function as cyclic nucleotide regulated transcription factors that modulate the expression of genes encoding enzymes involved in carbohydrate metabolism. The cyclic AMP binding domain of these proteins display approx. 25% identity and 60% similarity to the Popeye domain (Froese et al., 2012). Interestingly, under certain physiological conditions the Popdc proteins can be found in the nucleus, suggesting that apart from their main function at the plasma membrane Popdc proteins may have transcriptional control functions as well (Schindler et al., 2012). The similarity of the Popeye domain to bacterial CRP proteins and the availability of structural data in the Protein Data Bank made it possible to model the structure of the Popeye domain (Froese et al., 2012) (Figure 1). Based on this model significant structural similarity is evident between the Popeye domain and cAMP-binding domains of protein kinase A (PKA), EPAC, and HCN channels (Froese et al., 2012 and Rehmann et al., 2007). Using the model of the Popeye domain, evolutionary-conserved amino acids were found to cluster around the putative phosphate-binding cassette (PBC), which suggests that these amino acids are essential for biological function (Figure 1). The PBCs of cyclic nucleotide binding proteins displays significant sequence conservation (Kannan et al., 2007). The canonical PBC sequence is FGE[L/I/V]AL[L/I/M/V]XXX [P/V]RAA, where X is any amino acid. Key residues within this motif are glutamic acid and arginine, which form hydrogen bonds with the phosphate and sugar groups of cAMP. These hydrogen bonds anchor cAMP in a hydrophobic environment and mutations of the arginine and glutamic acid residues in PKA are causing a massive loss in binding affinity. Interestingly, the putative PBC of Popdc proteins lacks this conserved arginine residue and the PBC sequence does not resemble the sequence of the canonical PBC. However aspartate and glutamic acid residues (D200/E203 in human POPDC1) are present and were found to be essential for nucleotide binding (Froese et al., 2012) (Figure 1). Several mammalian proteins with a domain that structurally resembles a cAMP binding domain but lacking the arginine residue in the PBC do not bind cyclic nucleotides at physiologically relevant concentration (Kannan et al. 2007). It was therefore important to experimentally test the ability of the Popeye domain to bind cyclic nucleotides and specific binding was demonstrated with the help of cAMP-agarose precipitation experiments (Froese et al., 2012) (Figure 1). A radioligand-binding assay using the recombinant Popeye domain of Popdc1 established a binding affinity (IC_{50}) for cAMP of 120 nM, which is comparable to the affinities reported for PKA (100 nM) (Christensen et al., 2003) and HCN4 (240 nM) (Xu et al., 2010). We therefore conclude, that the affinity for cAMP is indeed in the physiological range, while the affinity for cGMP is 50-fold lower (Froese et al., 2012) (Figure 1). In another attempt to unequivocally demonstrate cAMP binding, we designed a bimolecular FRET assay, which is based on the interaction of Popdc proteins with the ion channel TREK-1 (Figure 1). A reduction in FRET signal was obtained after the addition of isoproterenol (raises cytoplasmic levels of cAMP) but not in response to nitroprusside (raises cytoplasmic level of cGMP) (Froese et al., 2012). The FRET assay was also utilized to functionally characterize the importance of a conserved aspartate residue

(D200) in the Popeye domain of *Popdc1*. This mutant does not show any change in the FRET signal after isoproterenol stimulation, which suggest that D200 is essential for cAMP binding (Figure 1). Such a mutant will be valuable to further assess the biological importance of the cAMP binding property of *Popdc* proteins.

3 *Popdc* mutants in mice display a stress-induced bradycardia phenotype

Null mutants for *Popdc* genes were engineered with help of a knock-in of β -galactosidase and simultaneous deletion of the first coding exon of *Popdc1* and *Popdc2* (Andrée et al., 2002, Froese et al., 2008 and Froese et al., 2012). Both genes revealed strong expression in muscle cells (smooth, cardiac and skeletal muscle cells). Correspondingly, a regeneration phenotype was observed in skeletal muscle of *Popdc1* null mutants (Andrée et al., 2002). In the heart, *Popdc1* and *Popdc2* are exclusively expressed in cardiac myocytes (Froese et al., 2008 and Froese et al., 2012) (Figure 2). There were significantly higher levels of expression in cells of the cardiac conduction system (CCS), which prompted us to investigate whether the CCS might be affected in *Popdc1* and *Popdc2* null mutants. Telemetric ECG devices were implanted into mice when they were 2 months of age and ECG measurements were performed at the age of 3, 5.5 and 8 months of age. This analysis revealed a normal ECG pattern at rest, however, when the animals were subjected to physical (5 min. of swimming), mental (hot air), or pharmacologically induced stress, significant ECG abnormalities were observed in an age-dependent manner. When the animals were tested at young age no difference was observed between control and mutant animals, however at the age of 8 months, the heart frequency after stress induction was abnormally low due to the presence of sinus pauses, which were numerous and variable in length (Froese et al., 2012) (Figure 2). Significantly, an identical phenotype was observed when *Popdc1* mutant animals were analyzed, suggesting that both genes work in the same molecular context in a non-redundant manner.

The age-dependent phenotype development in both mouse mutants is reminiscent of the sick sinus syndrome (SSS) (Rodriguez and Schocken, 1990). This syndrome occurs in about 1/600 cardiac patients aged 65 years or older. A total of 400,000 patients are diagnosed with SSS every year and 50% of all pacemaker implantations are due to this diagnosis. Several genes have been linked to SSS including *SCN5A*, which encodes the major cardiac sodium channel $\text{Na}_v1.5$ (Benson et al., 2003) *HCN4*, which encodes a member of the hyperpolarization-activated and cyclic-nucleotide gated channels (HCN) (Schulze-Bahr et al., 2003), and *ANK2*, which encodes the adaptor protein Ankyrin-B and anchors ion channel proteins and pumps to the plasma membrane (Mohler and Bennett, 2005) (Figure 3).

Apart from mutations in channel and adaptor proteins there is also evidence that electrical remodeling is part of the pathology of SSS. In rats, an age-dependent down-regulation of $\text{Na}_v1.5$ causes slow action potential upstroke velocity in the periphery of the SAN (Yanni et al., 2010). Likewise, a down-regulation of the *Ryr2* gene, which is an important part of the Ca^{2+} -clock mechanism of cardiac pacemaking has been observed in the aged rat (Tellez et al., 2011). In guinea pigs, an age-dependent down-regulation of Cx43 and Cav1.2 in the SAN has been described (Jones et al., 2007). Thus multiple changes at the transcriptional level are caused by age.

Histopathological findings suggests that another cause for SSS is a degenerative fibrosis, which causes a replacement of pacemaker myocyte tissue and the surrounding myocardium by fibroblasts (Rodriguez and Schocken, 1990). An animal model for such a condition has been recently engineered by genetic ablation of SAN cells, which is associated with severe pacemaker dysfunction (Herrmann et al., 2011). Analysis of the SAN of *Popdc1* and *Popdc2* mutants revealed significant structural alterations, suggesting that the SAN dysfunction was accompanied by a loss of SAN tissue in the aged animals (Froese et al., 2012). In particular the inferior part of the node showed a loss of HCN4-labeled pacemaker cells. In the superior part of the sinus node a reduction in non-muscle cell content was observed. Moreover pacemaker myocytes of null mutants displayed morphological changes characterized by a reduction in cell extensions. Presently it is unclear whether the observed morphological changes are the result of pacemaker dysfunction, or alternatively, are responsible for it. The preferential loss of pacemaker tissue in the inferior part of the sinuatrial node might explain why pacemaker function at baseline is unaffected. Different areas of the sinus node are acting as the primary pacemaker center under different physiological conditions (Ophthof et al., 1987). It is possible that the loss of inferior SAN myocytes causes pacemaker failure due to the shift of the primary pacemaker into the inferior part of the SAN. Further work using isolated node preparations will reveal the importance of the structural alterations observed in the mutant node.

4 Cardiac arrhythmia in zebrafish *popdc2* morphants

Three *Popdc* genes are also present in the zebrafish and expression is again mostly confined to muscle and heart (Kirchmaier et al., 2012). Loss-of-function analysis of *popdc2* was accomplished by morpholino injection. The morphants displayed a severe defect in skeletal muscle morphogenesis, most prominent in head muscles, which were reduced in size and aberrant in morphology. The morphants displayed abnormal circular swimming patterns, probably due to the misalignment of the myofibrillar apparatus in the trunk musculature. Myofibers were often ruptured, due to the aberrant formation of myotendinous junctions, which were irregular in shape and thickness (Kirchmaier et al., 2012). Additionally, the heart was dysfunctional and pericardial edema was present in many morphants. Upon reduction of the morpholino concentration, pericardial edema and skeletal muscle dysmorphogenesis were diminished or even normalized. However the heart still was abnormal and cardiac arrhythmia phenotypes were seen, which were characterized by an AV-block. Initially, episodes of a 2:1 or 3:1 heart block were observed. As development advanced also long pauses and even non-beating hearts were observed (Kirchmaier et al., 2012). Importantly in this species the phenotype was present at baseline and isoproterenol administration did not affect it. High-resolution optical analysis of calcium release using a calcium reporter gene revealed variations in action potential duration due to a variability in the length of cardiac repolarization (Kirchmaier et al., 2012).

It will be interesting to find out whether the phenotypes seen in zebrafish morphants and in mutant mice are related. There are significant differences in beating frequency between both species, the zebrafish heart beats at a rate of 120-180 beats per minute (bpm), while the mouse heart has a beating frequency of 300-600 bpm. Likewise, the cardiac repolarization phase is 300-440 msec in zebrafish, while it is significantly shorter in mice (83-96 msec)

(Leong et al., 2010). The fish and mouse differ in their utilization of repolarization currents (Nemtsas et al., 2010). Moreover, there are differences in the morphology of the cardiac conduction system. All these differences may have an impact on the phenotypic representation of Popdc morphants in zebrafish and Popdc mutants in mice. Since the human and the zebrafish heart have similar heart rates and make use of identical currents for cardiac repolarization, it will be interesting to see what phenotypes might be present in patients with loss-of-function mutations in POPDC genes.

5 Popdc proteins interact with the K2P channel TREK-1

In an attempt to identify interacting channel proteins, a screen for interacting proteins was devised in *Xenopus* oocytes by co-injection analysis. A specific interaction of the two-pore potassium (K2P) channel TREK-1 with Popdc proteins was observed (Froese et al., 2012) (Figure 1). In the presence of Popdc proteins, TREK-1 current was increased 2-fold. This increase was based on an enhanced membrane representation of TREK-1, suggesting a modulation of channel trafficking by Popdc proteins. There are additional K2P channels present in the heart such as TASK-1, TREK-2 and TWIK-2 but only TREK-1 was found to specifically interact with Popdc proteins. While the role of TREK-1 is well understood in neuronal cells, its role in cardiac cells has only been poorly characterized. In *Drosophila*, the related K2P channel ORK1 has been implicated in pacemaker regulation (Lalevee et al., 2006).

It is likely that other channel proteins or ion pumps are also interacting with Popdc proteins. A strong candidate is HCN4, which is abundantly expressed in SAN cells and is responsible for generating I_f . HCN channels have a cAMP-binding domain where binding causes an increased open probability. Despite these properties of HCN channels, genetic experiments in mice do not give support for an essential role of this current in heart rate-adaptation, while it is essential for proper pacemaker function in the embryo (Herrmann et al., 2007). Consistent with these data, I_f current under baseline conditions and after raising cAMP levels in SAN cells was unaffected in *Popdc2* null mutant mice (Froese et al., 2012). Many more ion channels are participating in cardiac pacemaking, which collectively are called the M-clock and include for example the L-type and T-type calcium currents (Figure 3). Loss of function alleles for $Ca_v1.3$ (Platzner et al., 2000) and $Ca_v3.1$ (Mangoni et al., 2006) are associated with a sinus bradycardia suggesting an important role in pacemaker function, however stress-induced sinus pauses have not been reported.

An alternative hypothesis proposes that stress-mediated heart rate adaptation is initiated by the Ca^{2+} -clock, which comprises a network of sarcoplasmic reticulum-localized proteins involved in calcium transport (Lakatta et al., 2010) (Figure 3). Evidence for an important role of the Ca^{2+} -clock stems from the observation that ryanodine treatment causes a slowing of the cardiac pacemaker. A local calcium release occurs in SAN myocytes independently of the membrane voltage and drives a net inward sodium current due to the activity of the Na^+ - Ca^{2+} -exchanger (NCX), which therefore represents a link between both clocks (Lakatta et al., 2010). SAN-specific loss of NCX affects specifically the ability of SAN to display a chronotropic response after adrenergic stimulation (Gao et al., 2012). Likewise, mice harboring a catecholaminergic polymorphic ventricular tachycardia (CPVT) - inducing point

mutation of the *Ryr2* gene (RyR2(R4496C)) display sinus pauses in response to adrenergic stimulation (Neco et al., 2012). It has been proposed that the M-clock has a major role in maintaining basal pacemaking, while the Ca^{2+} -clock specifically regulates chronotropism in response to adrenergic stimulation (Gao et al., 2012). If this holds true in further investigations it is likely that Popdc proteins are somehow linked to the Ca^{2+} -clock mechanism given the specific defect of pacemaker failure in mutant hearts after adrenergic stimulation and normal basal pacemaker activity. Adrenergic stimulation causes increased PKA activity, which results in phosphorylation and activity modulation of a number of proteins being part of the membrane and voltage clock networks (Figure 3). In addition, CaMKII has also been implicated as another mediator of pacemaker adaptation to stress (Wu et al., 2009). It will be interesting to find out whether Popdc proteins are substrates for PKA or CaMKII and whether such a phosphorylation affects their subcellular localization, cAMP binding ability, or protein interaction. In particular its role in the context of the membrane and Ca^{2+} -clocks needs to be further analyzed, which may help to explain the stress-induced pauses in the *Popdc1* and *Popdc2* null mutants.

Despite high levels of expression throughout the heart, our analysis of 3-8 month old mouse mutants only revealed stress-induced pacemaker dysfunction, while atrial and ventricular chamber myocardium or the ventricular conduction system appeared to be unaffected. Overlapping expression and function of the members of the Popdc gene family may prevent arrhythmic events in the working myocardium. Consistent with this interpretation is the observation of atrial fibrillation, polymorphic ventricular tachycardia, extrasystole, AV block and sinus pauses in *Popdc1/Popdc2* double null mutants (Simrick et al., 2012). Moreover in isolated ventricular myocytes delayed afterdepolarizations, spontaneously generated action potentials and shortening of the action potential durations were observed after isoproterenol stimulation. These data suggest that proarrhythmic changes are present in the working myocardium but may manifest later in life or require the loss of more than one member of the Popdc gene family.

6 Concluding remarks

This review has mainly focused on the role of the Popdc gene family in the heart. However Popdc genes are also expressed in other organs and tissues. A strong expression domain for example is present in skeletal muscle and several organs with a high content in smooth muscle tissue (bladder, intestine and uterus). At present the role of Popdc genes in these organs has not been studied apart from the role of *Popdc1* in skeletal muscle regeneration (Andrée et al., 2002). A large body of work suggests that Popdc proteins also perform essential functions in epithelial tissues. Loss of function analysis of *Popdc1* in *Drosophila* (Lin et al., 2005), *Xenopus* (Ripley et al., 2005), and zebrafish (Wu et al., 2012) point to an essential function of this gene during gastrulation. It is however possible that this function is not essential in mammalian development since no embryonic lethality has been observed in murine *Popdc1* or *Popdc2* null mutants (Andrée et al., 2002 and Froese et al., 2012). Functions of Popdc genes in epithelium formation and function is also suggested by the finding that a loss-of *Popdc1* function impairs tight junction formation (Osler et al., 2005) and signaling (Russ et al., 2011). Epithelial cell migration and cell adhesion are affected by a loss of *Popdc1*, and may involve specific interactions of Popdc1 with GEFT (Smith et al.,

2008) and Vamp3 (Hager et al., 2010). Popdc genes may also be involved in tumor development. Silencing of *POPDC1* and *POPDC3* by hypermethylation is associated with colorectal, gastric and non-small cell lung cancer (Williams et al., 2011).

Our understanding of Popdc gene function and its association with cardiac arrhythmias has just started to emerge. An important question for the near future is whether aside from TREK-1 other cardiac ion channels and pumps are directly or indirectly interacting with Popdc proteins and how these interactions are affected by cAMP binding. It is tempting to speculate that there are familial cases of cardiac pacemaker dysfunction or other cardiac arrhythmias, which are linked to mutations in Popdc genes. A study on the expression levels of Popdc genes in heart failure reported a specific loss of *POPDC1* and *POPDC3* in a large fraction of patients (Gingold-Belfer et al., 2010). The reduction of Popdc gene expression in the failing heart is likely to cause an increased risk of developing cardiac arrhythmia and sudden cardiac death. The function of Popdc genes in the heart is beginning to emerge, however much more work is needed to understand their role in the heart and beyond.

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Figure 1. Popdc1 is a plasma membrane-localized and cAMP-binding protein.

(A) Immunocytochemical detection of Popdc1 in an adult mouse cardiac myocyte. Note the presence of Popdc1 at the plasma membrane and in the T-tubular system. (B) Working model of Popdc proteins. Popdc proteins are extensively glycosylated and form a protein dimer at the plasma membrane, which is stabilized by a disulfide bridge. Popdc proteins are predicted to have three transmembrane domains and the conserved Popeye domain is found in the cytoplasmic part of the protein. (C) Structural model of the Popeye domain of human POPDC1. Conserved amino acids are colored in red and cluster mostly around the presumptive cAMP-binding site. The side chains of residues, which are believed to be involved in cAMP-binding are depicted and numbered. (D) Consensus sequence of the phosphate-binding cassette (PBC) of the Popeye domains. (E) Western blot detection of chick Popdc1 after affinity precipitation of cardiac tissue extracts incubated with cAMP-agarose. Bound Popdc1 protein was eluted with increasing amounts of cAMP. P, pellet; S, supernatant; T, total protein; C, control incubation using ethanolamine-agarose. (F,G) Bi-molecular FRET assay using YFP-tagged TREK-1 and (F) wildtype Popdc1, or (G) Popdc1-D200A mutant, both with a C-terminal CFP tag. Isoproterenol addition induces a rapid change in the FRET signal in case of wild type protein but does not affect the FRET signal in case of the D200A mutation. (H) Radioligand binding assay using [3 H]-cAMP and recombinant cytoplasmic domain of Popdc1. Panel E-H are reproduced from (Froese et al., 2012) with permission.

Figure 2. *Popdc2* null mutants develop a stress-induced bradycardia.

(A) Consecutive sections of a heterozygous *Popdc2* mouse heart were stained for β -galactosidase (LacZ) and acetylcholine esterase (AChE) activity. Arrowhead and arrow point to the enhanced enzyme activity in the SAN and His bundle, respectively. (B) Section through the vena cava region was LacZ stained for 10 (upper panel) or 80 minutes (lower panel) demonstrating high LacZ activity in the SAN and lower enzyme activity in the right atrium (RA). (C) Heart rate distribution of 8-month-old *Popdc2*^{-/-} and WT mice before (green), during (blue), and after (red) swim stress. (D) ECG recordings of 8 month old

Popdc2^{-/-} and wild type (WT) mice during swim stress test. (E) Number of pauses in WT and *Popdc2*^{-/-} mice during a 30 minute period after swim stress as a function of age. Panel A-E are reproduced from (Froese et al., 2012) with permission.

A network of membrane localized voltage-gated ion channels (M-clock) and the Ca^{2+} -clock, which controls the oscillatory local Ca^{2+} -release from the sarcoplasmatic reticulum via the ryanodine receptor (RyR2) and re-uptake by SERCA2, are interlinked by the Na-Ca^{2+} -exchanger (NCX), which causes an inward sodium current in response to a local Ca^{2+} -release. Ankyrin 2 (ANK2) is an adaptor protein, which controls the membrane localization of several proteins involved in pacemaking. Several inward currents, I_f and the L- and T-type Ca^{2+} -currents drive depolarisation of the pacemaker cell, while potassium currents (I_K) are involved in repolarization. Adrenergic stimulation causes a faster pacemaker rate. The binding of cAMP (C) causes an activation of protein kinase A (PKA), which phosphorylates phospholamban (Plb) and the Ca^{2+} -channels, resulting in an increase in the Ca^{2+} -inward current and a faster rate of Ca^{2+} - uptake. In addition HCN4 (I_f) is also modulated by cAMP. The Popdc proteins bind cAMP with high affinity and cAMP binding modulates the interaction with the K2P channel TREK-1. Another element in the complex network is CaMKII, which also phosphorylates several critical proteins of the pacemaking network and is also implicated in the chronotropic response after adrenergic stimulation..