Review

Ahnak, a new player in β-adrenergic regulation of the cardiac L-type Ca$^{2+}$ channel

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Abstract

Ahnak, originally identified as a giant, tumour-related phosphoprotein, has emerged as an important signalling molecule in a wide range of physiological activities. In this article, current knowledge will be reviewed that places ahnak into the context of cardiac L-type Ca$^{2+}$ channel function by its interaction with the β2 subunit. Beginning with an overview on structural and functional properties of ahnak, basic features of β subunits are highlighted. The review characterizes multiple ahnak/β2 subunit binding sites and focuses on recent progress in understanding their functional role in Cav1.2 channel conductance (ICaL). Three main aspects of ahnak function in ICaL of cardiomyocytes emerge from available experimental data. First, ahnak acts as repressor towards ICaL by β2 subunit sequestration. Second, PKA phosphorylation relieves the inhibition imposed by the C-terminal ahnak domain, ahnak-C1. Third, this action is mimicked by ahnak-derived fragments carrying a naturally occurring missense mutation Ile5236Thr. This paradigm introduces ahnak as a player in beta-adrenergic control of ICaL and sheds new light upon the molecular mechanism underlying this fundamental process of Cav1.2 channel physiology.

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1. Introduction

The influx of calcium ions (Ca$^{2+}$) through the L-type Ca$^{2+}$ channel (ICaL) is one major determinant of the plateau phase of the cardiac action potential and is crucial for excitation–contraction coupling in the heart [1–3]. The ICaL triggers the release of Ca$^{2+}$ from sarcoplasmic reticulum stores, and the resulting Ca$^{2+}$ transient drives the contraction. Structurally, the cardiac L-type Ca$^{2+}$ channel is a multi-subunit protein in which the pore-forming α1C subunit is physically associated with two obligatory auxiliary subunits: α2/δ and β [3]. The α1C gene (Cav1.2) encodes L-type Ca$^{2+}$ channels in cardiac and smooth muscle [4]; alternative splicing produces multiple isoforms which are differently distributed among different tissues [5].

In the adult mammalian myocardium, ICaL is primarily regulated by the sympathetic (beta-adrenoceptor) signalling cascade that ultimately controls the level of contractile force [6–10]. Defective ICaL regulation with blunted beta-adrenergic responsiveness is one major cause for myocardial contractile dysfunction in both animal models and in human syndromes [11,12]. The textbook view of adrenergic signalling is that cAMP-dependent protein kinase (PKA) activation transduces the “turn-on” signal from the G-protein coupled beta-adrenoceptor via phosphorylation of the Ser-1928 located within the long intracellular tail region of the α1C subunit [13–15]. Additionally, phosphorylation of the intracellularly located, regulatory β2 subunit was reported to play a crucial role in the PKA-mediated up-regulation of ICaL [16–18]. The underlying molecular mechanisms how phosphorylation of sites located either on α1C or β2 results in increased channel opening probability is still incompletely understood.

Early on, the postulated direct link between channel phosphorylation and enhanced ICaL was questioned by studies demonstrating the absence of a cAMP-mediated increase in ICaL in some heterologous expression models [19–22]. Therefore, the hypothesis was drawn that PKA-dependent regulation may require additional associated proteins [20]. The
identification of a family of A-kinase-anchoring proteins (AKAPs) has greatly enhanced the understanding how the cardiac muscle cell is able to coordinate different PKA signalling events in space and time. Adrenergic regulation of ICaL has been shown to require direct anchoring of PKA to the C-terminal of the α1C via a leucine zipper interaction with AKAP15 [15,23]. However, PKA-anchoring alone is obviously not sufficient to reconstitute robust PKA-regulation of ICaL in heterologous expression models. Searching for a further "missing link" we identified the ahnak protein in cardiomyocytes that is tightly bound to the Ca2+ channel β2 subunit [24].

2. Overview of ahnak properties and function

2.1. Molecular structure

Ahnak has been originally identified in 1992 as tumour-related gene by Shitivelman and coworkers [25]. These authors introduced the name “AHNAK” (meaning giant in Hebrew) to convey the large size of the encoded ~700 kDa protein. The ahnak gene was independently identified in 1993 by Hashimoto and coworkers [26], who demonstrated that this gene also codes for desmoyokin, a protein first isolated in 1989 as a desmosomal plaque protein from bovine muzzle epidermis [27]. Ahnak/desmoyokin is encoded by an intronless gene located on human chromosome 11q12-13 [28]. Hence, no ahnak isoforms arise from alternative splicing processes. The deduced amino acid sequence of human ahnak predicts a protein of ∼4400 amino acids composed of highly conserved repeated units, and a unique C-terminus of 1002 amino acids [25].

2.2. Structure/function: the ahnak central repeated units

The repeated units are believed to form a propeller-like structure that enables ahnak to "associate with Ca2+ channels of cardiomyocytes and other cells" [29]. This notion is not supported by data from our group, since we defined multiple Ca2+ channel interaction sites within the C-terminal ahnak domain [30]. Further work is needed to elucidate whether the propeller-like ahnak repeats interact also with the Ca2+ channel and what is the functional consequence. An interesting example for the action of ahnak’s central repeated units as a scaffolding motif has been recently described by Lee et al. [31]. The authors elaborated a PLC-γ1 activation pathway that includes the concerted interaction of four repeated ahnak units networking PLC-γ and PKC-α.

2.3. Structure/function: the ahnak C-terminal determines subcellular location

The C-terminal ahnak domain contains putative nuclear localization signals, and the ahnak protein identified by Shitivelman’s group located indeed primarily in the nucleus [32]. But, ahnak/desmoyokin distributes also to the plasma membrane and the cytoplasm. This ambiguity is explained by ahnak’s potential for subcellular translocation first described by Hashimoto et al. [33]. This group demonstrated that the C-terminus is necessary and sufficient to induce plasma membrane shuttling of ahnak from the nucleus [34]. Furthermore, Sussman et al. [35] reported on the nuclear exclusion of ahnak mediated through an export signal that involves the phosphorylation of Ser-5335 by PKB/Akt. The same study demonstrated an interesting cross-talk between the formation of cell-cell contacts and nuclear exclusion of ahnak in epithelial cells. Hence, the hypothesis was drawn that plasma-membrane-anchored ahnak is involved in the growth arrest of normal epithelial cells [35]. It is tempting to speculate that ahnak serves a similar function in other cells including terminally differentiated cardiomyocytes that withdraw from the cell cycle early during postnatal development.

Plasma membrane location of ahnak has been observed in rodent and human cardiomyocytes as well as in smooth muscle and skeletal muscle studied so far [24,30,36,37]. At the subcellular level, ahnak of cardiomyocytes is strictly localized to the inner aspect of all three sarcolemma compartments: surface sarcolemma, intercalated disc and T-tubular system [30]. Ahnak does not have membrane-spanning regions. Thus, ahnak is considered as plasma membrane support protein in muscle and lining epithelium [30,36].

2.4. Structure/function: the ahnak C-terminal interacts with the cytoskeleton

Ahnak’s C-terminal interacts with actin and bundles actin filaments into paracrystalline-like structures which we propose to serve structural and functional roles as an important link between T-tubular Cav1.2 channels and the actin-based cytoskeleton [30,37]. A further example for the formation of multi-protein complexes is the interaction of ahnak’s C-terminal domain with annexin2 via S100A as an adaptor which has been functionally related to the cytoskeleton of Madin–Darby canine kidney cells. In that cellular model, specific ahnak down-regulation prevented the cortical actin cytoskeleton reorganization required to support the cell height [38].

2.5. Ahnak knockout mice

Given ahnak’s implication in fundamental biological processes, such as Ca2+ signalling [31–34], plasma membrane support [30,36–38], regulated exocytosis [39], and blood brain barrier [40], one would expect that targeted ablation of ahnak gene results in a severe phenotype showing multi-organ damage. Surprisingly however, ahnak-deficient mice are viable, fertile, and show no cross-abnormalities suggesting that other molecules compensate for the loss of function of ahnak in mice [29,41]. A search performed by Komuro et al. [29] revealed an ahnak-like protein encoded on human chromosome 14q32 (designated as ahnak2). This protein displays an internal repeat structure like ahnak/
desmoyokin, while its N-terminal and C-terminal show essentially no similarities. Hence, ahnak2 is not considered to compensate for ahnak’s C-terminal, i.e. for interaction with the actin-based cytoskeleton and the β subunit. Presently, no experimental data are available on cardiomyocytes structure and function in ahnak-deficient mice.

3. Functional consequences of ahnak/β subunit interaction on Cav1.2 channel

3.1. Basic properties of Ca\(^{2+}\) channel β subunits

To understand ahnak actions on the Ca\(^{2+}\) channel, some background on the β subunit is necessary. The importance of β subunits for channel function has been highlighted by heterologous expression, antisense and gene knock-out experiments which are summarized by excellent reviews [3,42–45]. In fact, the β subunit is responsible for correct plasma membrane insertion of the α1 subunit and once α1 is inserted the β subunit becomes an allosteric modulator of channel gating properties. As such, membrane targeting and current modulation of the channel appear to be independent functions of the β subunit [43,46]. Furthermore, the various β subunit isoforms enhance the whole cell current density at different levels by increasing the channel opening probability [47,48], produce distinctive effects on channel inactivation kinetics [47–49], and induce hyperpolarizing shifts in the voltage-dependence of channel activation [50,51].

All known four Cavβ genes are expressed in the heart [52] and can in theory bind to the Cav1.2 subunit via the α1 interaction domain (AID), a highly conserved binding motif of 18 amino acid residues present in the cytoplasmic linker between repeat I and II of α1 subunits [53]. Although the identity of the assembled subunits is not entirely clarified, the β2 subunit isoform is generally believed to constitute the intracellular, accessory subunit of the Cav1.2 channel in adult mammalian myocardium [43,54].

Recent functional studies and crystallographic information have defined the β subunits of voltage-dependent Ca\(^{2+}\) channels as members of the membrane-associated guanylate kinase (MAGUK) family of proteins [55–57]. The MAGUK proteins are known to be scaffolding proteins with two well-conserved protein–protein interaction domains: a type-3 Sarc homology (SH3) domain and a guanylate kinase (GK) domain. Hence, it was suggested that the β subunit represents an ideal target for establishing molecular interactions of the channel with intracellular proteins required for channel regulation [55]. Indeed, proteins are emerging which interact with β subunits including ahnak [24,30,58,59] and various members of the Gem/kir family of small Ras-like GTPases [60].

3.2. The β subunit binds to multiple ahnak sites located at the C-terminal

β2 Subunit interaction with ahnak has been originally identified by co-immunoprecipitation with several cardiac preparations derived from rodent to human heart [24]. This observation suggested a tight physical association between ahnak and the β2 subunit. Consistent with this notion, subsequent in vitro binding studies disclosed a high affinity β2 subunit interaction site (Kd ≈ 50 nM) within distal C-terminal ahnak domain (designated as ahnak-C2), while apparently neither the ahnak head region nor the ahnak repeating units contribute to ahnak interaction [30]. Further in-depth equilibrium binding experiments reveal a complex picture for ahnak/β2 subunit interaction as multiple attachment sites have been defined within ahnak’s C-terminal. In fact, one additional β2 subunit binding site with ~160 nM affinity locates in the proximal tail region, designated as ahnak-C1 [59], while a second site with ~300 nM affinity occurs in the distal ahnak-C2 region [30]. At the level of the β2 subunit, ahnak interaction sites are less defined. A first step towards this direction is a pull-down assay with recombinant ahnak-C2 demonstrating that both cardiac Cav1.2 and skeletal muscle Cav1.1 channel complexes are recovered containing the β2 subunit and β1a subunit, respectively [30]. These findings suggest that the conserved β subunit modules, SH3 and/or GK, are important for ahnak/Cav1.x channel interaction.

3.3. Ahnak polymorphism, I5236T, interferes with adrenergic stimulation of I\(_{\text{Cal}}\)

In human ahnak, four polymorphic loci encoding amino acid substitutions have been identified so far [61]. The first characterized T/C polymorphism results in an Ile or Thr at amino acid 5236, whereby the Ile variant is clearly the wild-type allele. Comparison of allelic frequencies among a control Caucasian population and patients with hypertrophic or dilated cardiomyopathy (~100 individuals each) reveals that the majority of individuals are homozygous for Ile5236 in either group and only 2% is heterozygous Ile/Thr with no subjects to date identified as being Thr homozygous. As such, no direct correlation can be drawn between the heterozygous genotype and a cardiomyopathy phenotype. Nonetheless, the Ile5236Thr polymorphism is functional as recently demonstrated by us and collaborators [59]. A synthetic ahnak peptide carrying the Thr mutation mimicked the beta-adrenergic stimulation on I\(_{\text{Cal}}\), elicited under whole-cell patch-clamp conditions on ventricular rat cardiomyocytes using Ca\(^{2+}\) as charge carrier. Acute intracellular perfusion with Thr-containing ahnak peptide (10 μM) yielded a robust increase in current amplitude (1.7 fold at 0 mV depolarization), a leftward shift in current–voltage relationship and availability curve, and a slowing of current decay kinetics during the initial fast phase. All three effects are typical of adrenergic stimulation. Moreover, I\(_{\text{Cal}}\) enhanced by Thr5236-ahnak peptide does not anymore respond to the beta-adrenergic agonist, isoproterenol, nor to its counteracting agent, carbachol. These actions on I\(_{\text{Cal}}\) are specific for the Thr-containing ahnak peptide, while the Ile-containing (wild-type) ahnak peptide does essentially not modify basal I\(_{\text{Cal}}\), nor does it interfere with the isoproterenol response of the channel [59].
Thus, individuals with Thr5236 ahnak may exhibit defects in channel gating. Though Ile5236Thr ahnak is not considered to be a disease-causing mutation, it can substantially contribute to individual differences in the response to beta-adrenergic challenges or beta-blocker therapy.

A second ahnak polymorphism results in a Thr or Met at amino acid 5549, respectively. This mutation does not influence the specific features of recombinant ahnak-C2, i.e. strong actin binding and high-affinity β2 subunit interaction [61]. The consequence of this mutation on ICaL is not yet known. Likewise, information is lacking whether the additional ahnak polymorphisms reported are functional.

### 3.4. Tuning the ahnak-C1/β2 subunit interaction

As the Thr5236-ahnak peptide specifically mimics the isoprenaline actions on ICaL, we elucidated whether the proximal C-terminal ahnak region, ahnak-C1 (aa 4646-5288), plays a role in the signal transduction pathway between beta-adrenergic receptor and Cav1.2 channel. Binding assays reveal the ahnak-C1/β2 subunit binding is indeed phosphorylation-dependent. The binding affinity decreases by ~50% upon PKA phosphorylation of both protein partners. Surprisingly, the ahnak-C1/β2 subunit interaction is similarly attenuated by Thr5236-ahnak peptide, but not by the respective wild-type ahnak peptide [59]. The specific actions elicited by PKA phosphorylation and Thr5236-ahnak peptide are explained by the assumption that the ahnak-C1 domain operates as a phosphorylation-dependent suppressor of ICaL. Consequently, relief from ahnak-C1 suppression makes the β2 subunit more available for the ion-conducting Cav1.2 subunit leading to enhanced current density. Hence, a model is proposed in which Cav1.2 activity is reversibly regulated by tuning the ahnak-C1/β2 subunit interaction. Overall, the
actions exerted by PKA-mediated phosphorylation and Thr5236-ahnak peptide are ascribed to a functional uncoupling of the β2 subunit from the inhibitory ahnak-C1 domain leading to robust up-regulation of ICaL as seen in native cardiomyocytes upon sympathetic stimulation. (Fig. 1).

In general, functional uncoupling of inhibitory regulatory proteins upon sympathetic stimulation is in line with uncoupling of phospholamban (PKA phosphorylated) from SERCA 2a [62] or uncoupling of FKBP12.6 (PKA phosphorylated) from RyR2 [63]. Mice with targeted ablation of either phospholamban or FKBP12.6 are viable, fertile, and exhibit hyper-contraction cardiac phenotypes. Phospholamban-deficient mice show increased Ca2+ cycling capacity with no signs of Ca2+ overload or hypertrophy [64], while mice with targeted ablation of FKBP12.6 develop cardiac hypertrophy at higher age in a sex-specific manner due to Ca2+ overload in males [65]. Interestingly, phospholamban-deficient cardiomyocytes exhibit accelerated ICaL decay kinetics which is believed to protect the cell from Ca2+ overload [66]. This model demonstrates the close interrelated roles of ICaL and SERCA2a/ phospholamban in cardiomyocyte Ca2+ cycling.

3.5. Targeting the ahnak-C2/β2 subunit interaction

The functional role of the distal C-terminal ahnak domain, ahnak-C2, in Cav1.2 channel gating has been first examined in rat ventricular cardiomyocytes by Alvarez et al. [58]. Intracellular perfusion with ahnak-C2-derived fragments, which efficiently and reversibly bound to the β2 subunit, augmented ICaL predominantly through slowing the channel inactivation. Importantly, the effects of isoprenaline were fully retained and were additive to those of ahnak-C2 fragments. This implies an inhibitory role of ahnak-C2 on ICaL independent of the sympathetic signalling pathway [58].

4. Perspectives

Ahnak-derived peptides have emerged as potent modulators of ICaL in the adult mammalian heart [58,59] offering new therapeutic strategies for the treatment of cardiovascular diseases. For instance, the Thr5236-mutated ahnak peptide is able to enhance ICaL down-stream of PKA. This intervention seems beneficial for contractile support in heart failure in physiological and pathological conditions.

5. Conclusion

Beta-adrenergic stimulation of the cardiac Cav1.2 current (ICaL) is transmitted by PKA and is central to physiologic and pathologic events in the heart. However, a fundamental question of Cav1.2 channel physiology, the mechanisms responsible for beta-adrenergic enhancement of ICaL remain open since attempts to reconstitute the PKA modulation in heterologous expression systems are inconclusive. Experimental data, reviewed herein, implicate ahnak as new player in beta-adrenergic control of ICaL. Ahnak is abundantly expressed in cardiomyocytes, undergoes PKA phosphorylation, and associates to the intracellular regulatory β2 subunit of Cav1.2 channels. Our model proposes that ahnak binding controls the availability of the β2 subunit to increase ICaL. This paradigm implies that targeting the ahnak/β2 subunit interaction may be a novel therapeutic strategy to improve contractile function in the failing heart.

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References


