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The barley two-pore K^+-channel HvKCO1 interacts with 14-3-3 proteins in an isoform specific manner

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Abstract

Members of the highly conserved family of 14-3-3 proteins play an important role in various cellular processes. Recent studies, using electrophysiological techniques, show that the 14-3-3 proteins also regulate plasma membrane and vacuolar K^+ conducting channels. The molecular mechanism behind the regulatory effect of 14-3-3 proteins on K^+ channels remains to be shown. One vacuolar channel down-regulated by 14-3-3 proteins is the slow-activating vacuolar (SV) channel. In *Arabidopsis*, the protein coded by the *KCO1* gene was recently shown to be present in the vacuolar membrane and identified as a component of the SV channel. These two observations raised the question whether the KCO1 protein does interact with 14-3-3 proteins. Therefore, we isolated the barley *HvKCO1* gene and the encoded protein indeed contains a canonical 14-3-3 interaction motif, which is conserved in all other KCO1 orthologues from other plant species. Using surface plasmon resonance (SPR) we determined in real-time the affinity between the phospho-peptide derived from the putative KCO1 14-3-3 interaction motif and three barley 14-3-3 proteins. The 14-3-3A protein showed the highest affinity, whereas the binding of all three isoforms was dependent on the presence of either Ca^{2+} or Mg^{2+}. Interestingly, the barley SV current was strongly reduced by 14-3-3B and C protein, but not by 14-3-3A. This difference between the SPR and patch-clamp data will be discussed, along with the role for Ca^{2+} in activation of the SV channel by direct interaction and inactivation of the channel by facilitating the binding of 14-3-3 to the channel.

Keywords: Barley; 14-3-3 proteins; KCO family; Slow vacuolar channel; Surface plasmon resonance; Patch-clamp

1. Introduction

First described in 1967 as a brain specific protein [1], the family of 14-3-3 proteins has evolved into a group of abundant regulatory proteins present in all eukaryotic tissues. These 14-3-3 proteins act as dimers and in most cases bind to distinct phosphorylated motifs, $RxxS^pT^pxP$ and $RxxxS^pT^pxP$ in which S^p indicates a phosphorylated serine, in their target proteins [2,3]. Furthermore, the presence of divalent cations, like Mg^{2+} and Ca^{2+}, seems to increase the affinity for some of those targets [4,5]. The highest number of family members, 12 expressed isoforms, has been found in *Arabidopsis* [6]. Monocots seem to contain fewer isoforms; three (A, B and C) have been described in barley so far [7,8] although our data suggest that at least five isoforms are present (Sinnige et al., unpublished results). The regulatory functions ascribed to 14-3-3 proteins are diverse; they include controlling metabolic enzymes [9], protein kinases [10], chloroplast import [11], transcription factors [12], the activity of ion transporters [13] and the correct assembling and targeting of ion channels [14].

In recent years, 14-3-3 proteins have emerged as novel regulators of plant ion homeostasis [13,15]. Besides modulating the P-type H^+-ATPase [16,17] and the mito-

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chondrial and chloroplast F-type ATP synthases [18], 14-3-3 proteins were shown to affect K^+ channels in the plasma and vacuolar membranes. Over-expression of Vf14-3-3a or Vf14-3-3b in tobacco enhanced outward K^+ currents [19] and addition of recombinant 14-3-3 to the cytosolic side of tomato suspension cells doubled the outward K^+ currents [20] of the plasma membrane. However, in the plasma membrane of barley embryonic root tissue, 14-3-3 proteins reduced outward K^+ currents and an inward rectifying K^+ current was found to be dependent on the presence of 14-3-3 [21]. Further, in the vacuolar membrane, two ion channels respond to the addition of recombinant 14-3-3 protein to the bath solution in a patch-clamp set-up. Whereas the current of the fast-activating vacuolar channel increased four-fold [15], the Ca^{2+} dependent slow-activating vacuolar (SV) current decreased up to 80% after adding recombinant barley 14-3-3B [22]. The mechanism through which 14-3-3 proteins affect the magnitude of the currents, without affecting the channel gating properties, is still poorly understood.

Recently, AtKCO1, a member of the two-pore domain K^+ channel family, was reported to be part of the vacuolar SV channel. A reduced SV current in an *Arabidopsis kco1* knockout plant led to the conclusion that AtKCO1 is a component of the SV channel [23]. In an earlier report, expression of *AtKCO1* in baculovirus-infected insect cells resulted in Ca^{2+} dependent outward rectifying currents [24]. Localization of the protein in the vacuolar membrane was confirmed using AtKCO1–GFP fusion proteins [23,25]. The SV channel has been subject of extensive, mainly electrophysiological, research. It is present in all studied plant species, conducts both K^+ and Ca^{2+} ions [26] and the activity is strongly dependent on cytosolic Ca^{2+} concentrations [27]. Besides the inhibition by barley 14-3-3B protein [22], the activity of SV is affected by calmodulin [28], the redox state [29], phosphorylation [30] and cytosolic Mg^{2+} [31]. A physiological role for the SV channel in Ca^{2+} induced Ca^{2+} release (CICR) was proposed [32], challenged [33] and is still a matter of debate [34–37].

Here, we report the isolation and analysis of *HvKCO1*, member of the KCO1 family of K^+-channels. Using a conserved motif in the KCO1 family, we demonstrate that 14-3-3 proteins are able to interact with HvKCO1 in the presence of divalent cations. To assess whether 14-3-3 regulation of the SV channel is mediated through interaction with the KCO1 component, we compare the binding properties of 14-3-3 isoforms to HvKCO1 with the effect of the different 14-3-3 isoforms on the SV current in barley leaf vacuoles.

2. Methods

2.1. Isolation and characterisation of HvKCO1

AtKCO1 was used to search the BLAST results of a barley EST library (http://pgrc.ipk-gatersleben.de/cr-est/index.php) and clone HK03F06u was identified as a KCO family member. Using primary leaf of *Hordeum vulgare* cv. Alexis (Josef Breun Saatzucht, Herzogenaurach, Germany), the cDNA fragment was extended to the 5′ using the Universal GenomeWalker Kit (BD Biosciences, Palo Alto, CA, USA) with two gene specific primers (1: 5′-CTCCACGAGA-TAATCCGCCGACTTG-3′ and 2: 5′-CGACGACGCCCGC-TAGCAGG-3′). The complete cDNA sequence of *HvKCO1* was obtained using 3′ RACE (BD Biosciences) with gene specific primers (3: 5′-GCGGGCGTCGTCGTCTTTTACC-3′ and 4: 5′-GCTGCTCGCTTGTGTGTTCGTC-3′). For expression experiments, total RNA from barley tissues was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) and first-strand cDNA was produced using SuperScript reverse transcriptase (Invitrogen). Barley radicles were isolated and maintained as described previously [21]. Quantitative RT-PCR (DNA Engine Opticon, MJ Research, Inc., Waltham, MA, USA) was done using primers KCOf (5′-GTTTGA-GAAACTCGATGTTGACCA-3′), KCOr (5′-CAAGCCCA-TATGTTCATCACTGAC-3′), Actinf (5′-GTATGGAAAC-ATCGTGCTCAGTGG-3′) and Actinr (5′-CTTGATCTT-CATGCTGCTCGGA-3′). GenBank accession nos.: AtKCO1 (X97323), HvKCO1 (AY770627), actin (AY145451). All kits were used according to the manufacturers protocol.

2.2. Expression and purification of recombinant 14-3-3 proteins

Barley 14-3-3 isoforms A, B and C (GenBank accession nos.: X62388, X93170 and Y14200, respectively) were cloned into the pRSETC vector (Invitrogen) and the orientation and reading frame checked by sequencing. Transformed BL21 (DE3) pLysS cells (Invitrogen) were grown O/N at 37 °C in 2xYT, 1% glucose and 50 μg/ml ampicillin until the OD_{600} was 0.8–0.9. Expression was induced by replacing the medium by 2xYT, 1 mM isopropyl-β-D-thiogalactoside (AppliChem, Darmstadt, Germany) and 50 μg/ml ampicillin and incubation at 28 °C for 4 h. Cells were pelleted at 5000 × *g* for 15 min and dissolved in 30 ml 20 mM Hepes/KOH pH 7.5, 0.5 M NaCl, 1 mM PMSF and l0 mM imidazol (Merck, Darmstadt, Germany) at 4 °C. Once dissolved, 1 mg/ml lysozyme (Sigma–Aldrich, St. Louis, MO, USA) and 4 μg/ml deoxyribonuclease 1 (Sigma–Aldrich) were added and incubated for 30 min. The cell lysate was centrifuged for 30 min at 100,000 × *g* and supernatant filtered through a 0.45 μM filter (Schleicher & Schuell, Dassel, Germany). The filtrate was applied to a Ni^{2+} charged HiTrap Chelating HP column (Amersham Pharmacia, Uppsala, Sweden) and subjected to a 10 mM to 500 mM imidazol gradient. The 14-3-3 peak was pooled, desalted to 1 mM Hepes/KOH pH 7.5 using a HiPrep 26/10 desalting column (Amersham) and concentrated by freeze drying. Quantity and quality of the recombinant proteins were checked using the Bradford protein assay (Bio-Rad, Hercules, CA, USA), Coomassie stained SDS-PAGE gel and far UV circular dichroism (not shown).