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The first ankyrin-like repeat is the minimum indispensable key structure for functional assembly of homo- and heteromeric TRPC4/TRPC5 channels

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Abstract

The closely related TRPC4 and TRPC5 proteins, members of the canonical transient receptor potential (TRPC) family, assemble into either homo- or heterotetrameric, non-selective cation-channels. To elucidate domains that mediate channel complex formation, we evaluated dominant negative effects of N- or C-terminal TRPC4/5 fragments on respective currents of full-length proteins overexpressed in HEK293 cells with whole-cell electrophysiology. Confocal Förster Resonance Energy Transfer (FRET) measurements enabled to probe the interaction potential of these CFP/YFP-labelled fragments in vivo. Only N-terminal fragments that included the first ankyrin-like repeat potently down-regulated TRPC4/TRPC5 currents, while fragments including either the second ankyrin-like repeat and the coiled-coil domain or the C-terminus remained ineffective. Total internal reflection fluorescence (TIRF) microscopy data suggested that the dominant negative N-terminal fragments led to a predominantly intracellular localisation of coexpressed TRPC5 proteins. FRET measurements clearly revealed that only fragments including the first ankyrin-like repeat were able to multimerise. Moreover a TRPC5 mutant that lacked the first ankyrin-like repeat was unable to homo-multimerise, failed to interact with wild-type TRPC5 and resulted in non-functional channels.

Keywords: TRPC; Transient receptor potential; Channel; Assembly; Ankyrin

1. Introduction

The canonical transient receptor potential (TRPC) proteins represent a mammalian family of *Drosophila* TRP that function as non-selective, cation-permeable ion-channels. TRPC4 and TRPC5 channels are highly expressed in brain and have been reported to function in both neurotransmitter release and growth cone extension [1]. Activation of TRPC4/5 mediated currents is dependent on phospholipase C pathways [2]. In hippocampal neurons vesicle fusion-mediated insertion led to plasma-membrane targeting of TRPC5 through increased cytosolic Ca^{2+} levels initiated via a phosphoinositol 3-kinase signaling cascade [3].

Functional TRP channels consist of a tetrameric structure [4–7], in expression systems predominantly characterised as an assembly of homomeric TRP proteins. Heteromeric assembly of TRP channels has been identified as well; for TRPCs within subfamilies containing TRPC1/4/5 and TRPC3/6/7 [8,9]. Furthermore, TRPC1 seems to enable inter-subfamily heteromultimerisation such as TRPC1/3 [10,11] via N-terminal interactions, as well as TRPC1/3/5 [12] and TRPC1/3/7 [13]. Additionally TRPC6/7 [14] and TRPC3/4 [15] have been reported to occur as endogenous

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heteromers in vascular smooth muscle cells and endothelial cells, respectively.

Common motifs within TRPC proteins are two to three ankyrin-like repeats and a coiled-coil domain (except of mTRPC2 or hTRPC7) in their N-terminal strands followed by six transmembrane domains and a C-terminus containing the TRP box and a Calmodulin binding site [16–18], see Fig. 1. The ankyrin repeat is one of the most common protein–protein interaction motifs enabling such diverse functions ranging from transcription initiation, cell cycle regulation, cytoskeleton integrity, ion transport to cell–cell signaling [19]. For vanilloid TRPV channels, ankyrin repeats have been reported as an essential oligomerisation domain, as mutations or deletion in single ankyrin repeats in TRPV4, TRPV5 and TRPV6 abolished channel assembly [20–24]. Only chimeras of TRPC6 with TRPC4 that included either the ankyrin-repeats and a following coiled-coil motif, or the pore region and part of the C-terminus of TRPC4 were able to interact with TRPC4 [25].

We report here that the first ankyrin-like repeat is a key domain for the assembly of TRPC4 and TRPC5 in homo- as well as heteromeric complexes. Only fragments including the first ankyrin-like repeat were able to multimerize, and deletion of the first ankyrin-like repeat abolished TRPC5 channel assembly and function further strengthening its indispensable key role.

2. Results

The canonical TRPC4 and TRPC5 have been reported to form both homo- or heteromeric channels [9,26]. To identify interaction domains within the cytosolic strands of these channels, we initially constructed various fragments of TRPC5, to study their potential dominant negative effect on functional channel formation. We and others [11,22,27] have previously used coexpression of fragments that include potential interaction domains to suppress the function of endogenous TRP channels by generation of non functional protein assemblies.

2.1. Fragments including the first ankyrin-like repeat of TRPC5 inhibit TRPC5 currents

TRPC5 proteins were overexpressed in HEK cells and the time course of their currents recorded in whole-cell configuration was monitored at −74 mV applying repetitive voltage ramps. After obtaining whole-cell configuration, TRPC5 expressing cells showed modest constitutively active TRPC5 currents (Fig. 1A–F) that were not detected in mock-transfected cells (Fig. 1B). Application of 100 μM carbachol (Cch) resulted in a further stimulation of TRPC5 currents (Fig. 1A–F) that were characterised [2] by their typical double-rectifying current–voltage relationship with a more pronounced inward component (Fig. 1A). The first two fragments examined, included either the whole N-terminal strand (N_{308}-TRPC5; first 308AA) or the whole C-terminal strand (C_{621}-TRPC5; AA621 to 966). Coexpression of C_{621}-TRPC5 with TRPC5 did neither affect the time-course of TRPC5 current development nor the maximum inward current reached following Cch stimulation in comparison to control expression of TRPC5 alone (Fig. 1B). In contrast, application of Cch failed to markedly activate TRPC5 currents when N_{308}-TRPC5 was coexpressed (Fig. 1C). To further pinpoint the domains essential for this dominant negative effect, three smaller fragments of the N-terminal strand were generated: N_{129}-TRPC5 that includes the first ankyrin-like repeat, $N_{130\text{-}308}$-TRPC5 containing the second ankyrin-like repeat together with the coiled-coil domain and N_{61}-TRPC5 which lacks all ankyrin-like repeats. Coexpression of N_{129}-TRPC5 together with TRPC5 in comparison to TRPC5 alone yielded substantially down-regulated TRPC5 currents (Fig. 1E). However, the $N_{130\text{-}308}$-TRPC5 (Fig. 1D) or N_{61}-TRPC5 (Fig. 1F) fragment each coexpressed with TRPC5 affected neither time-course nor maximum inward currents when compared to control TRPC5-derived currents.

We moreover utilised both epi-fluorescence in combination with total internal reflection fluorescence (TIRF) microscopy to examine if the cellular distribution of TRPC5 is affected by coexpression with dominant negative fragments (Fig. 1G–I). TRPC5 and N_{129}-TRPC5 were labeled at their N-termini with YFP and CFP, respectively. YFP-TRPC5 showed membrane localization in epi-fluorescence and a punctuate staining in TIRF microscopy at sites within <200 nm of the plasma-membrane (Fig. 1G). In contrast, CFP-N_{129}-TRPC5 was only visible with epi-fluorescence microscopy as expected for a cytosolic located fragment (Fig. 1H). Coexpression of YFP-TRPC5 and CFP-N_{129}-TRPC5 yielded intracellular distribution in epi-fluorescence and lacked punctuate staining in TIRF microscopy (Fig. 1I).

These initial experiments show that only N-terminal fragments that include the first ankyrin-like repeat displayed dominant negative interference with wild-type TRPC5 and retention of the protein in intracellular compartments.

2.2. N-terminal TRPC5 fragments that include the first ankyrin-like repeat display strong homomeric interactions

To address the question as to whether or not the dominant negative N-terminal fragments are able to interact in a homomeric manner, we used confocal FRET microscopy to examine for *in vivo* homomultimerization of various TRPC5 constructs (N_{308}-TRPC5, $N_{130\text{-}308}$-TRPC5, N_{129}-TRPC5, N_{61}-TRPC5) when expressed in HEK cells in comparison to full-length TRPC5. Based on images of CFP- (Fig. 2A–H, image 1) and YFP- (Fig. 2A–H, image 2) labelled proteins and their overlay that illustrated colocalisation (Fig. 2A–H, image 3), FRET values (Fig. 2A–H, image 4) were calculated as a measure for their binding capabilities (see Section 4 part: *FRET microscopy*). Coexpression of YFP-