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Addendum

Insights into the Roles of Conserved and Divergent Residues in the Ankyrin Repeats of TRPV Ion Channels

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TRP channels, TRPV subfamily, TRPV1, vanilloid receptor, ATP, capsaicin, calmodulin, tachyphylaxis

ABBREVIATIONS

TRP	transient receptor potential
TRPV	TRP channels of the vanilloid subfamily
ARD	ankyrin repeat domain
ATP	adenosine triphosphate

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Addendum to:

The Ankyrin Repeats of TRPV1 Bind Multiple Ligands and Modulate Channel Sensitivity

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ABSTRACT

Ion channels are often modulated by intracellular calcium levels. TRPV1, a channel responsible for the burning pain sensation in response to heat, acid or capsaicin, is desensitized at high intracellular calcium concentrations. We recently identified a multiligand-binding site in the N-terminal ankyrin repeat domain (ARD) of TRPV1 that binds ATP and sensitizes the channel. Calcium-calmodulin binds the same site and is necessary for calcium-mediated TRPV1 desensitization. Here, we examine in more detail the conservation of this TRPV1 multiligand-binding site in other species. Furthermore, using sequence analysis, we determine that the unusually twisted shape of the TRPV1-ARD is likely conserved in other TRPV channels, but not in the ARDs of other TRP subfamilies.

Transient Receptor Potential (TRP) proteins are a large family of ion channels involved in a number of sensory functions and divided into seven subfamilies: TRPA, TRPC, TRPM, TRPML, TRPN, TRPP and TRPV.[1] TRPV1, the vanilloid receptor, is probably the best studied mammalian TRP channel.[2] Activation of TRPV1 in peripheral nociceptor neurons by painful stimuli, like noxious heat or capsaicin from chili peppers, leads to a burning sensation. During prolonged or repeated stimulation, TRPV1 is inactivated by a Ca^{2+}-dependent mechanism.[3] At a molecular level, the transmembrane domain of TRPV1 and other TRPV-subfamily channels is flanked by large cytoplasmic N- and C-terminal domains that participate in channel gating and sense information about the cellular state—calcium and phosphoinositide levels, for example—to regulate channel sensitivity (reviewed in refs. 4 and 5). The N-terminal region of TRPV proteins contains six ankyrin repeats,[6,7] sequence motifs found in a broad spectrum of proteins involved in protein-protein interactions.[8]

In a recent report, we described the first crystal structure of a TRPV1 fragment, that of the ankyrin repeat domain (ARD).[9] In the TRPV1-ARD structure, we unexpectedly observed electron density corresponding to a bound ATP molecule. Biochemical studies demonstrated that, in addition to ATP, Ca^{2+}-calmodulin binds the same site. Providing intracellular ATP or non-hydrolyzable ATP analogs prevents desensitization to repeated applications of capsaicin, i.e., tachyphylaxis, while calmodulin plays an opposing role and is required for tachyphylaxis. The accumulated data lead to a model for the calcium-dependent regulation of TRPV1 via the competitive interactions of ATP and calmodulin at the N-terminal binding site.[9]

The ATP-bound TRPV1-ARD structure represents, to our knowledge, the first example of an ankyrin repeat domain binding a small molecule, expanding the functional roles for ankyrin repeats. This begs the question of whether some of these ankyrin repeat functions are conserved in other TRPV channels, and whether similar binding sites may be found in other TRP channel subfamilies. Here we consider what the structure of the TRPV1-ARD can teach us about: (i) the ARDs of other TRP channels, and (ii) what functions might be unique to the TRPV1-ARD.

Conserved residues in homologous sequences are typically important for two main reasons: (i) to preserve the structural integrity of the protein fold, and/or (ii) to preserve a given function of the protein. We first examined the ARD sequences present in several TRP channel subfamilies: TRPC, TRPA, TRPN and TRPV. Our phylogenetic analysis shows that the ARDs group together along the TRP subfamilies (Fig. 1A), with the TRPV channels clustered together. Hence, the ARD structure of the other TRPV proteins will be similar to that of TRPV2-ARD[6,7] and TRPV1-ARD.[9] However, the ARDs of the TRPC, TRPA and TRPN subfamilies may be significantly different and may not have the

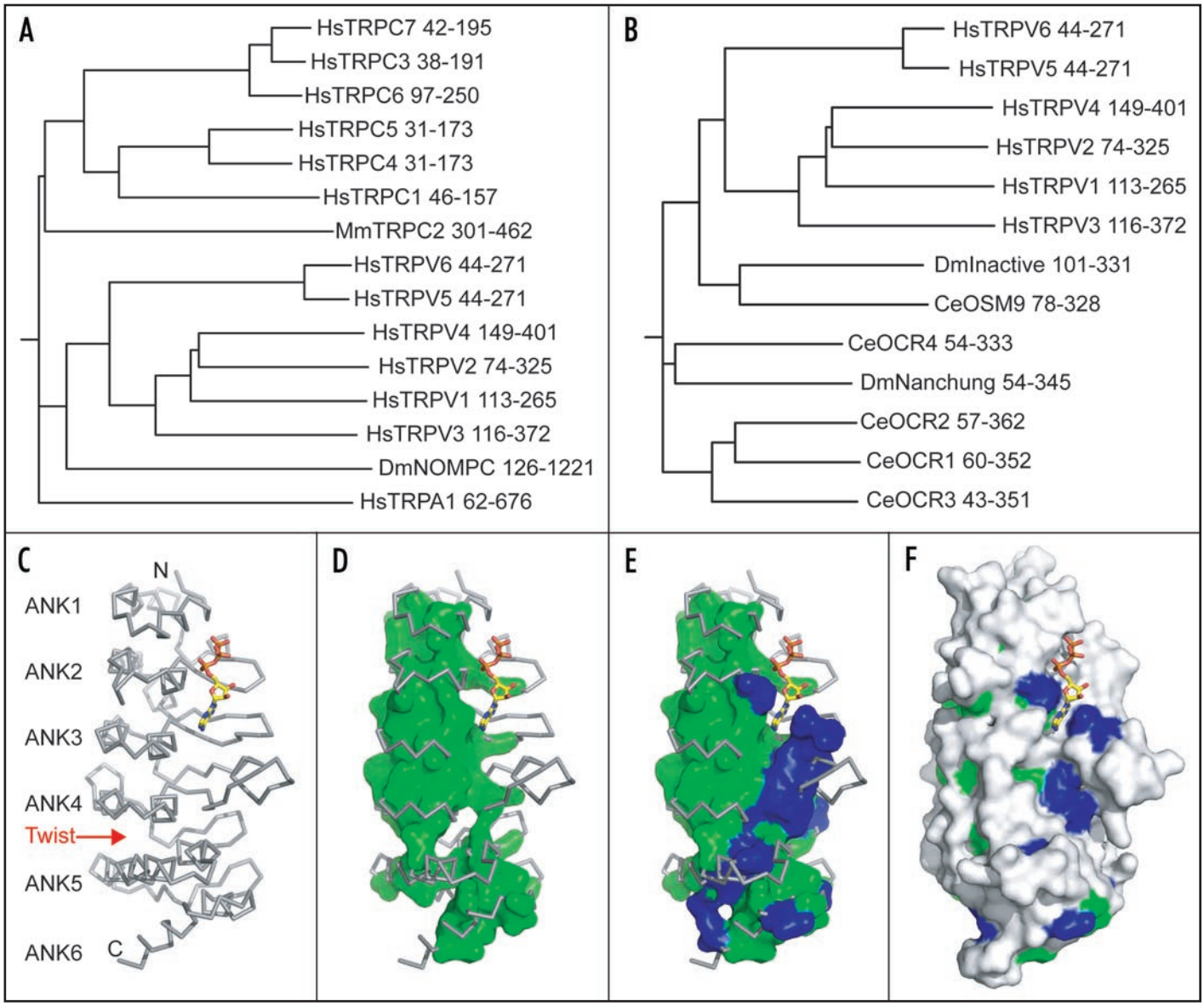

Figure 1. Mapping the sequence conservation of ankyrin repeats in TRP channels. (A) Phylogenetic analysis of the sequence segments that can readily be assigned as ankyrin repeats in TRP channels (residue numbers adjacent to sequence names). (B) Phylogenetic analysis of TRPV channels from humans, *Drosophila* and *C. elegans*. (C–F) Sequence conservation in mammalian TRPV proteins is mapped on the TRPV1-ARD structure. Green surfaces are consensus ankyrin repeat residues, blue residues are conserved in TRPV proteins but deviate from or are not part of the ankyrin repeat consensus, and white residues are not conserved in all mammalian TRPV proteins. ATP is shown in stick representation. Phylogenetic analyses were performed using ClustalW[13] and PHYLIP[14] as part of the Biology Workbench.[15] The species used are: Hs, *Homo sapiens*; Mm, *Mus musculus*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*.

characteristic long fingers and pronounced twist between repeats 4 and 5 observed in the TRPV1- and TRPV2-ARDs (Fig. 1C).[6,7,9]

Extending our analysis further, we examined the phylogenetic tree of the TRPV ARDs from a mammal (human), arthropod (*Drosophila*) and nematode (*C. elegans*) (Fig. 1B). While all these channels are clearly recognized as TRPV channels and their ARDs readily cluster together, a tree built with the ARD sequences indicates that the mammalian TRPV channels diverged after separation of vertebrates and invertebrates, as does a tree built from the full-length TRPV sequences.[10] Our analysis therefore indicates that all mammalian TRPV ARDs are more similar to each other in sequence, and likely in structure, than they are to the invertebrate TRPV ARDs.

Mapping conserved residues onto the TRPV1-ARD structure is also informative. In Figure 1C–F, the TRPV1-ARD residues are grouped into three different categories based on TRPV sequence conservation. In green are consensus ankyrin repeat motif residues, and Figure 1D illustrates that they form the inner hydrophobic core of the TRPV1-ARD structure. Blue residues either are not part of or deviate from the ankyrin repeat consensus, but are highly conserved in TRPV proteins. The blue diagonal wedge observed in Figure 1E suggests that these conserved TRPV residues are responsible for the pronounced twist observed between repeats 4 and 5 in both TRPV1- and TRPV2-ARD structures. Moreover, this unusual twist in the typically regular stacking of ankyrin repeats is most likely a shared

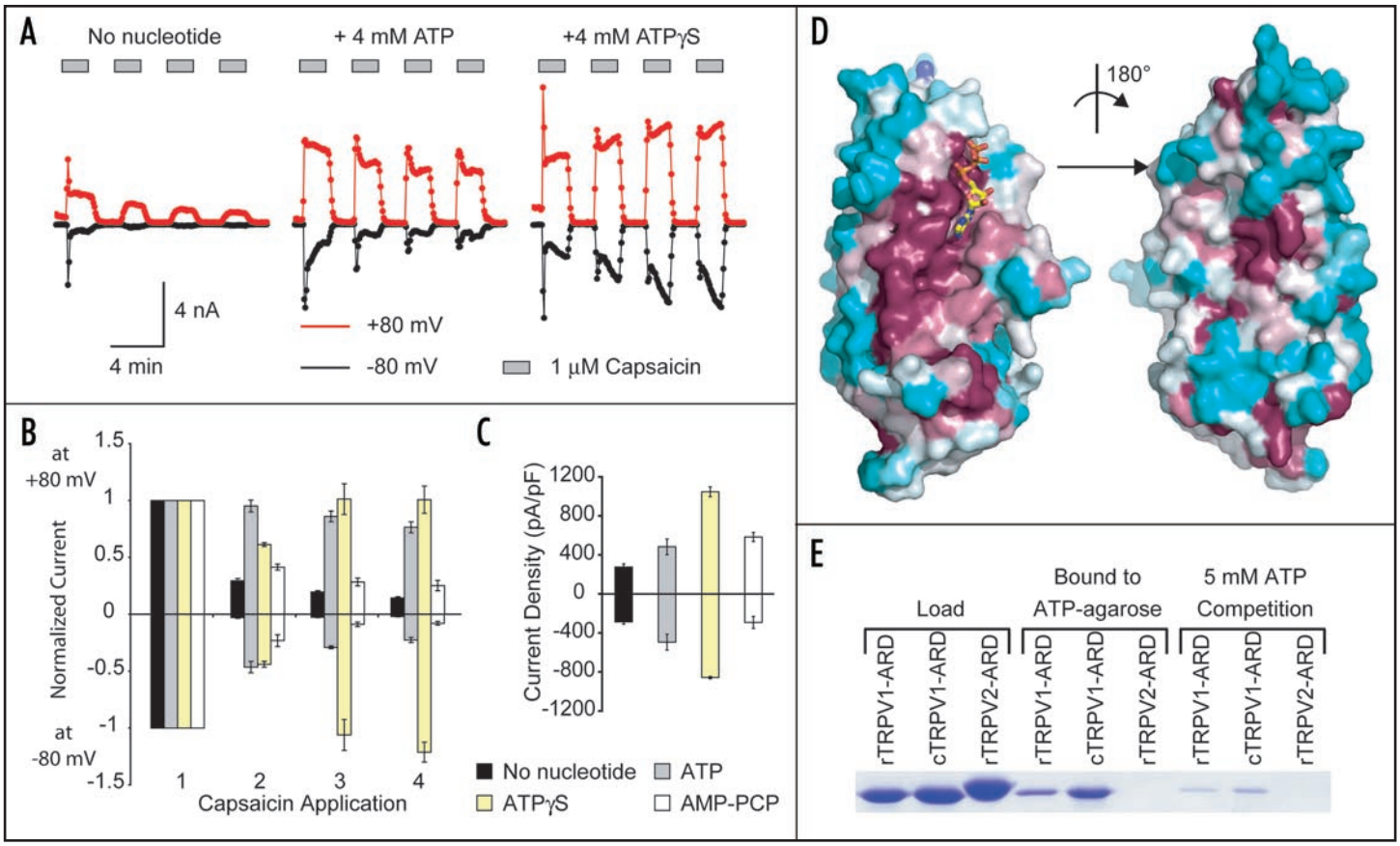

Figure 2. Robustness of the effects of ATP on TRPV1. (A) ATP and ATPγS have strong sensitizing effects and prevent TRPV1 tachyphylaxis in HEK293 cells, whereas AMP-PCP has a moderate effect. Cells were maintained and currents recorded under whole-cell patch-clamp mode as previously described.[9] (B and C) Mean current amplitudes for successive capsaicin applications, normalized to the current amplitudes obtained on the first application (B) and mean current density of the maximal response to the first capsaicin application normalized to the cell capacitance (C) in the absence (black) or presence of ATP (grey), ATPγS (yellow), or AMP-PCP (white) (n = 3, 5, 5 and 4 cells, respectively; error bars are standard errors of mean). (D) The sequence conservation of TRPV1 orthologues is mapped onto the TRPV1-ARD surface using ConSurf,[11] with the most conserved residues in purple, and the least conserved residues in cyan. On the left is the protein in the same orientation as in Figure 1C, and on the right it is rotated 180° around the vertical axis. ATP is shown in stick representation. The sequences used in the TRPV1 alignment are *Rattus norvegicus* (O35433), *Homo sapiens* (Q8NER1), *Mus musculus* (Q704Y3), *Cavia porcellus* (AAU43730), *Canis familiaris* (Q697L1), *Oryctolagus cuniculus* (Q6RX08), *Gallus gallus* (NP_989903), *Xenopus tropicalis* (e_gw1.354.37.1), *Macaca mulatta* (XP_001117609), *Danio rerio* (OTTDARP00000018832) and *Fugu rubripes* (e_gw2.511.7.1). (E) A Coomassie-stained gel showing that both rat and chicken TRPV1-ARD proteins bound to ATP-agarose (middle) and the binding was competed by free ATP (right), whereas rat TRPV2-ARD does not bind ATP-agarose. The binding experiments were performed as previously described.[9]

feature of all TRPV ARDs. Finally, the white residues that pepper the ARD surface are the variable residues in TRPV proteins. Since most of the surface consists of non-conserved residues, the ARDs of different mammalian TRPV proteins are likely to have different cellular interactions. In particular, the binding pocket occupied by the triphosphate moiety of ATP in the TRPV1 structure is clearly not conserved in all TRPV proteins. The TRPV2-ARD does not bind ATP[9] and while it remains to be determined whether any other TRPV ARD binds ATP, Figure 1F certainly suggests that several of them do not.

In our recent report, we used TRPV1-expressing baculovirus-infected Sf21 insect cells to show that ATP sensitizes TRPV1 and prevents tachyphylaxis to repeated capsaicin application.[9] Furthermore, the effect of non-hydrolyzable ATP analogs matched their relative affinity for the TRPV1-ARD, strongly suggesting that the effect of ATP on TRPV1 function is due to direct binding. In Figure 2A–C, we show that ATP and non-hydrolyzable analogs have similar effects on the TRPV1 response to capsaicin in mammalian HEK293 cells. This further supports our interpretation that the effect of ATP is due to direct binding to TRPV1 and further validates insect cells as a valuable heterologous expression system to study TRP channel function.

We also examined whether the ATP-binding site is conserved in TRPV1 from other species (Fig. 2D and E). We used ConSurf[11] to map the TRPV1 sequence conservation onto the TRPV1-ARD surface. The ATP-binding site and an adjoining region are clearly very well conserved in TRPV1 (Fig. 2D). Furthermore, the chicken TRPV1-ARD binds to ATP-agarose and its binding is efficiently competed by free ATP, similar to the rat TRPV1-ARD (Fig. 2E). Therefore, ATP binding is conserved in chicken TRPV1, a channel that is activated by heat, but not capsaicin.[12]

The modulation of ion channels and other cellular proteins by intracellular calcium levels is of broad interest, and our findings regarding the use of ATP within such a regulatory mechanism are surprising and novel. Our data on the chicken TRPV1-ARD suggest that ATP-mediated regulation of TRPV1 is conserved through

evolution. Although other TRPV channels may also share this mechanism, it is not conserved in all TRPV proteins. In contrast, the shape of the six-repeat ARD is most likely conserved in all TRPV channels, with its pronounced twist between the fourth and fifth repeat, and this structural feature is likely unique to the TRPV ARDs. Structural and biochemical analyses of the ARDs of other TRP channels are needed to determine their respective structural features and binding partners.

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