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Cell Calcium 39 (2006) 187–196

cell calcium

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Ectopic expression of a *Drosophila* $InsP_3R$ channel mutant has dominant-negative effects in vivo

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Received 9 December 2004; received in revised form 5 August 2005; accepted 26 October 2005
Available online 1 December 2005

Abstract

The inositol 1,4,5-trisphosphate ($InsP_3$) receptor is a tetrameric intracellular calcium channel. It is an integral component of the $InsP_3$ signaling pathway in multicellular organisms, where it regulates cellular calcium dynamics in many different contexts. In order to understand how the primary structure of the $InsP_3R$ affects its functional properties, the kinetics of Ca^{2+}-release in vitro from single point mutants of the *Drosophila* $InsP_3R$ have been determined earlier. Among these, the Ka901 mutant in the putative selectivity-filter of the pore is of particular interest. It is non-functional in the homomeric form whereas it forms functional channels (with altered channel properties) when co-expressed with wild-type channels [S. Srikanth, Z. Wang, H. Tu, S. Nair, M.K. Mathew, G. Hasan, I. Bezprozvanny, Functional properties of the *Drosophila melanogaster* inositol 1,4,5-trisphosphate receptor mutants, Biophys. J. 86 (2004) 3634–3646; S. Srikanth, Z. Wang, G. Hasan, I. Bezprozvanny, Functional properties of a pore mutant in the *Drosophila melanogaster* inositol 1,4,5-trisphosphate receptor. FEBS Lett. 575 (2004) 95–98]. Here we show that due to its changed functional properties the Ka901 mutant protein has dominant-negative effects in vivo. Cells expressing Ka901:WT channels exhibit much higher levels of cytosolic Ca^{2+} upon stimulation as compared with cells over-expressing just the wild-type $DmInsP_3R$, thus supporting our in vitro observations that increased Ca^{2+} release is a property of heteromeric Ka901:WT channels. Furthermore, ectopic expression of the Ka901 mutant channel in aminergic cells of Drosophila alters electrophysiological properties of a flight circuit and results in defective flight behavior.
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Keywords: Ion selectivity filter; Muscarinic acetylcholine receptor; Aminergic neurons; Flight

1. Introduction

Ionic calcium (Ca^{2+}) functions as an intracellular signaling molecule to regulate processes as diverse as fertilization, cell differentiation, acquisition and storage of memory, and cell death (reviewed in [3]). Mechanisms by which the concentration of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) can undergo changes, include entry from the extracellular milieu and release from intracellular stores. The inositol 1,4,5-trisphosphate receptor ($InsP_3R$) is a tetrameric ligand gated Ca^{2+} channel present on membranes of intracellular calcium stores. It releases Ca^{2+} into the cytosol in response to extracellular signals that generate $InsP_3$. Unlike the mammalian genome, which has three genes for the $InsP_3R$, the *Drosophila* genome has a single gene for the $InsP_3R$ (*itpr*). This has allowed for the analysis of $InsP_3R$ function in vivo using well-characterized methods of *Drosophila* molecular genetics [4–6].

The *Drosophila* $InsP_3R$ is ∼60% homologous to the mammalian type I $InsP_3R$, both of which comprise of an amino-terminal ligand binding domain, a central modulatory domain and a carboxy-terminal channel domain. The structure–function relationships of the $InsP_3Rs$ have recently begun to be elucidated. Amino acid residues important for ligand binding and modulation by ATP as well as regions responsible for Ca^{2+} regulation and ion selectivity of the $InsP_3Rs$ have been identified (reviewed in [7]). Random single point mutants identified from genetic screens in *Drosophila* can provide important insights into the fundamental mechanisms of $InsP_3R$ function. In this context, the functional properties

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0143-4160/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ceca.2005.10.013

of three single amino acid mutants of the *Drosophila* $InsP_3R$ have been described recently [1,2]. One of the mutants, Ka901, is a Gly2630 → Ser mutation in the putative selectivity filter and pore region of the $InsP_3R$ [5]. The mutated Glycine is conserved among all known vertebrate $InsP_3Rs$. *itpr*^{Ka901} animals in combination with a deficiency for the *itpr* locus (*itpr*^{90B0}) show the same phenotype of molting delay and lethality as *itpr* deficient animals (*itpr*^{90B0}/*itpr*^{90B0}; [5]) indicating that mutation of the Ka901 residue affects a critical function of the $InsP_3R$. Interestingly though, in Ca^{2+} release assays, microsomal vesicles made from adult heads of *itpr*^{Ka901}/+ heterozygotes release over two-fold higher Ca^{2+} than wild-type flies [1]. Thus in this context Ka901 functions as a dominant gain-of-function mutation. Homomeric Ka901 tetramers do not show any currents in single channel analysis, indicating that these are non-functional, supporting the genetic observations. However, heteromeric channels of Ka901:WT are functional and show increased conductance, higher mean open times and altered ion selectivity consistent with our observation of increased Ca^{2+} release in microsomal vesicles of *itpr*^{Ka901}/+ heterozygotes [2].

Both the microsomal vesicle release assay and single channel analysis are in vitro assays requiring either extraction of microsomes from *itpr*^{Ka901}/+ heterozygotes or from Sf9 cells heterologously expressing the Ka901:WT heteromeric channels. Here we demonstrate the altered properties of Ka901:WT channels in a cellular context. We have used the well characterized *Drosophila* S2 cell line [8] and established, by biochemical means, the formation of Ka901:WT heteromeric channels in these cells. Subsequently from co-transfection experiments with the *Drosophila* muscarinic acetylcholine receptor (mAchR) and either wild-type *Drosophila* $InsP_3R$ or Ka901 constructs we show that the Ka901 mutation brings about a significant increase in the levels of cytosolic calcium upon stimulation by a muscarinic agonist. The physiological significance of this observation, in the context of the whole organism, was assessed by over-expression of the Ka901 transgene in wild-type *Drosophila* or flies heterozygous for Ka901 (*itpr*^{Ka901}/+). Earlier work has shown that aminergic neurons are most sensitive to altered signaling through the $InsP_3R$. In adult *Drosophila* this manifests as defects in flight behavior and physiology [6]. Significant levels of flight and associated electrophysiological defects were observed in *Ka901* heterozygous flies over-expressing a *Ka901* transgene in aminergic neurons.

2. Materials and methods

2.1. Generation of UASitpr-GFP, UASitprKa901-HA and UASmAchR constructs

To generate a fusion product of $InsP_3R$ with GFP, a fusion PCR strategy [9] was adopted. A 5′ $InsP_3R$ primer (DT 2071 5′ ATCTGTACTTCATCGTCCTTG 3′ at position 9155), a 3′ EGFP primer (5′ TTACTTGTACAGCTCGTCCATGC 3′) and a linking/fusion primer (5′ CACCATGGTGGCCACCG-GTGGATCCTGAAACGGCAGGAGGCTGTTG 3′) were used. The fusion primer contained 22 base pairs of the 3′ end of the *itpr* cDNA just preceding the stop codon followed by 18 base pairs of linking nucleotides, coding for six amino acids (to allow for independent folding of the $InsP_3R$ C-terminal peptide and GFP protein), followed by six nucleotides coding for the first two amino acids of the GFP protein. A PCR was set up using the two template DNA molecules, one encoding the GFP gene (pEGFP-N1, Stratagene) and another (pBSK-EM5), encoding the C-terminal region of the *Drosophila* $InsP_3R$ [10]. The PCR was carried out according to standard conditions using Pfu DNA polymerase with 1 ng of each of the template DNAs in a PCR mix containing 1 μM each of DT 2071 and EGFP 3′ primers and 0.01 μM *itpr*-GFP fusion primers. The PCR was terminated after 30 cycles, and the fusion product ethanol precipitated and digested with *Nhe*I restriction enzyme. The digested product was then ligated to pBSK-EM5 digested with *Nhe*I and *Sma*I, such that the C-terminal of the *itpr* cDNA following the *Nhe*I site was replaced with the fusion PCR fragment. The ligation mix was transformed and presence of the desired recombinant clone (pBSKEM5-GFP) was confirmed by appropriate restriction digests. Plasmid DNA from the clone was sequenced to rule out PCR errors in the coding region of either the $InsP_3R$ or GFP. A 2.3 kb *Xba*I fragment (using a unique *Xba*I site from $InsP_3R$ cDNA and a second one from the pBluescript-SK vector) was purified after restriction digestion of pBSKEM5-GFP clone. The purified fragment coding for the C-terminal 539 amino acids of the $InsP_3R$ protein fused to full-length GFP protein was ligated with the *UASitpr*$^+$ plasmid, digested with *Xba*I so as to remove the native 3′ *Xba*I fragment. The *UASitpr*$^+$ plasmid consists of the entire *itpr*$^+$ cDNA cloned into the *Drosophila* germline transformation vector *pUAST* [11]. The ligated and transformed products were checked by appropriate restriction digests for their orientation and a *UASitpr-GFP* clone in the correct orientation was used for the experiments described here.

For generation of Ka901-HA, the sequence encoding the hemaglutinin (HA) tag, comprising of the amino acids YPYDVPDYA, was incorporated in the 3′ end of an *itpr* primer just before the stop codon, along with an *Xba*I site (*itpr*HA primer 5′ CGTCTAGAGGCGTAATCGGGCA-CATC GTAGGGGTACTGAAACGGCAGGAGGC 3′). The PCR was performed using the pFASTBAC–Ka901 clone (described in [2]), using a 5′ *itpr* primer (5′ GTTCGAAAC-GATCGCACTCTA 3′) at position 7837 which lies just upstream of the single *Xba*I site in the *itpr* cDNA (at position 7854) and the 3′ *itpr*HA primer. The amplified product was digested with *Xba*I and ligated to *UASitpr*$^+$ digested in parallel with *Xba*I. The resultant clone was sequenced in order to verify presence of the Ka901 mutation and absence of any PCR errors.

The *Drosophila* muscarinic acetylcholine receptor (mAchR) clone in *pRMHA3* vector was a kind gift of Dr. Raghu Padinjat (Cambridge, UK). It has been described ear-

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