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Cd^{2+} sensitivity and permeability of a low voltage-activated Ca^{2+} channel with CatSper-like selectivity filter

Edgar Garza-López, Julio César Chávez, Carmen Santana-Calvo, Ignacio López-González, Takuya Nishigaki *

Departamento de Genética del Desarrollo y Fisiología Molecular, Instituto of Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62210, Mexico

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ABSTRACT

CatSper is a sperm-specific Ca^{2+} channel that plays an essential role in the male fertility. However, its biophysical properties have been poorly characterized mainly due to its deficient heterologous expression. As other voltage-gated Ca^{2+} channels (Ca_Vs), CatSper possesses a conserved Ca^{2+}-selective filter motif ([T/S]x[D/E]xW) in the pore region. Interestingly, CatSper conserves four aspartic acids (DDDD) as the negatively charged residues in this motif while high voltage-activated Ca_Vs have four glutamic acids (EEEE) and low voltage-activated Ca_Vs possess two glutamic acids and two aspartic acids (EEDD). Previous studies based on site-directed mutagenesis of L- and T-type channels showed that the number of D seems to have a negative correlation with their cadmium (Cd^{2+}) sensitivity. These results suggest that CatSper (DDDD) would have low sensitivity to Cd^{2+}. To explore Cd^{2+}-sensitivity and -permeability of CatSper, we performed two types of experiments: 1) Electrophysiological analysis of heterologously expressed human $Ca_V3.1$ channel and three pore mutants (DEDD, EDDD and DDDD), 2) Cd^{2+} imaging of human spermatozoa with FluoZin-1. Electrophysiological studies showed a significant increase in Cd^{2+} and manganese (Mn^{2+}) currents through the $Ca_V3.1$ mutants as well as a reduction in the inhibitory effect of Cd^{2+} on the Ca^{2+} current. In fluorescence imaging with human sperm, we observed an increase in Cd^{2+} influx potentiated by progesterone, a potent activator of CatSper. These results support our hypothesis, namely that Cd^{2+}-sensitivity and -permeability are related to the absolute number of D in the Ca^{2+}-selective filter independently to the type of the Ca_v channels.

1. Introduction

Sperm flagellar beating is essential for sexual reproduction in many eukaryotic species and Ca^{2+} is known to be a crucial factor to modulate its beating patterns. In mammals, a vigorous flagellar motility called hyperactivation is known to be induced by an intracellular Ca^{2+} increase [1,2]. Mammalian spermatozoa possess a sperm-specific Ca^{2+} channel, named CatSper, which is composed of four pore-forming α subunits and three auxiliary subunits (β, γ, δ) [3–9]. CatSper is an essential channel for male fertility, because it has been demonstrated that deletions of subunits (all four α and δ) in mouse [3,4,9–11] and recessive mutations in *CATSPER1* or *CATSPER2* in some human families [12,13] lead to male infertility due to a defect in hyperactivated flagellar motility [14]. In addition, it was recently demonstrated that specific loss of CatSper activity (absence of CatSper current in spermatozoa) is enough to cause male infertility even though apparent mutations are not found in the coding regions of the CatSper subunits in the patient [15]. These studies support the relevance of CatSper in the male fertility. According to the reduced number of positively charged residues in the fourth transmembrane segment in CatSper3 and CatSper4 [5], this channel exhibits moderate voltage-dependence [16,17]. In contrast, the activity of CatSper is highly dependent on intracellular alkalization [16,17]. It has been reported in the rhesus monkey that the pH in the lumen of the oviduct rises at ovulation [18], suggesting that this increase in pH of the oviduct fluid should stimulate CatSper. Moreover, progesterone and some types of prostaglandin, which are produced by cumulus cells of the oocyte, are now known to be potent activators for human CatSper [17,19] although these compounds have no effects on

* Corresponding author at: Departamento de Genética del Desarrollo y Fisiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México (UNAM), Avenida Universidad #2001, Colonia Chamilpa 62210, Cuernavaca, Morelos, México.
E-mail addresses: egarza@ibt.unam.mx (E. Garza-López), jcchavez@ibt.unam.mx (J.C. Chávez), csantana@ibt.unam.mx (C. Santana-Calvo), iglogo@ibt.unam.mx (I. López-González), takuya@ibt.unam.mx (T. Nishigaki).

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mouse CatSper. Curiously, many chemical compounds, including several endocrine-disrupting chemicals such as chlorophenothane (p,p′-DDE), 4-MBC and α-zearalenol, activate human CatSper and increase sperm intracellular Ca^{2+} [20–22], which could impair the human fertility and may partially explain the reduced reproductive ability in modern society. In addition, CatSper is a quite interesting channel from an evolutionary point of view. Despite its essential role in mammalian spermatozoa, some lineage of vertebrates such as birds, amphibians and teleosts do not possess this channel [23]. Also, in invertebrates, species that maintain CatSper are distributed heterogeneously [23]. Interestingly, CatSper is found in some fungi [24], algae and plants [25]. This discovery together with comparative analysis of ion selective filter of voltage-gated Na^+ and Ca^{2+} channels of prokaryotes and eukaryotes suggests that CatSper is one of the most primitive voltage-dependent Ca^{2+} channels in eukaryotes [26].

In spite of the primordial importance of CatSper in eukaryotic reproduction, the biophysical properties of this channel have been poorly characterized due to the difficulty to apply electrophysiological techniques to mature spermatozoa and to the lack of heterologous expression systems to study the recombinant CatSper channel. In this sense, alternative methods are required to advance our knowledge about biophysical properties of CatSper.

In this study, we intended to characterize Ca^{2+}-selective filter of CatSper. One of the interesting features of CatSper channel pore is a unique arrangement of negatively charged residues in the Ca^{2+}-selective filter [23]. As observed in other voltage-gated Ca^{2+} channels (Ca_Vs), all four alpha subunits of CatSper possess a typical motif of Ca^{2+}-selective filter, [T/S]x[D/E]xW [27]. In the case of high voltage-activated (HVA) channels such as L-type channels possess four glutamic acids, EEEE [28], while low voltage-activated channels (T-type channels) have two glutamic acids and two aspartic acids, EEDD [29]. On the other hand, in this locus CatSper have four aspartic acids, DDDD, in all species that have ever been studied, except for sea urchin (Catsper3 of *Strongylocentrotus purpuratus* and *Arbacia punctulata* has a glutamic acid in this locus) [23,30]. Previous studies based on site-directed mutagenesis of L- and T-type channels demonstrated that substitution of E for D (and vice versa) resulted in a decrease in Ca^{2+} selectivity against monovalent cations [28,29,31], indicating that each type of channel has a distinct pore structure and that they have evolved differently to have a particular composition of E or D to form a Ca^{2+} selective pore. However, Cd^{2+} sensitivity seems to have a simple negative correlation to the absolute number of D in the pore filter. For example, single replacement of E by D in any four subunits reduced the inhibitory effects of Cd^{2+} on the Ca^{2+} conductance in a L-type Ca^{2+} channel and all E substitution for D showed a largest decrease in Cd^{2+} sensitivity [28]. In T-type channels, the more substitution of D for E caused the higher inhibitory effects of Cd^{2+} on Ca^{2+} conductance [29,31]. These results suggest that CatSper (DDDD in the pore) would have low sensitivity to Cd^{2+}. Actually, it was reported in human sperm that IC_{50} of Cd^{2+} on progesterone-induced Ca^{2+} increases, basically mediated by CatSper, measured by fura-2 is 250 μM [32]; which is slightly higher than IC_{50} of Cd^{2+} on Ca_V3s reported previously, around 150 μM [33,34]. In sea urchin sperm *S. purpuratus*, it was reported that 300 μM Cd^{2+} did not inhibit the cytoplasmic Ca^{2+} fluctuations induced by sperm-activating peptide (SAP), speract [35]. Recent experimental evidence supports that the Ca^{2+} influx induced by SAP in sea urchin spermatozoa is mediated by CatSper [26,36]. In addition, CatSper may have different permeability of other divalent cations. For example, it has been reported that progesterone, a potent activator for human CatSper channel, increases Mn^{2+} permeability of human sperm plasma membrane [37,38]. Speract was also demonstrated to increase Mn^{2+} permeability in sea urchin sperm plasma membrane [39]. These results suggest that CatSper would have higher permeability to Mn^{2+} compared to the other Ca_Vs.

Here, we explored molecular basis of DDDD arrangement of Ca^{2+}-selective pore of CatSper channel using two alternative approaches: 1) Electrophysiological characterization of wild type of human Ca_V3.1 (EEDD) and three mutants that bore DEDD, EDDD and DDDD expressed in HEK 293 cells to measure Cd^{2+} sensitivity and Mn^{2+} permeability. 2) Fluorescence imaging of human spermatozoa to determine a Cd^{2+} influx through endogenous CatSper with a Cd^{2+}-sensitive (relatively Ca^{2+}-insensitive) indicator, FluoZin-1 [40]. Results of both approaches support our hypothesis, namely that Cd^{2+}-sensitivity is negatively correlated to and Cd^{2+}-permeability is positively correlated to the absolute number of D in the Ca^{2+}-selective pore. Although the physiological significance of Cd^{2+}-permeability through CatSper remains unknown, this feature could be a useful tool to distinguish the activity of CatSper from that of other Ca_Vs.

2. Materials and methods

2.1. Generation of CatSper-like pore mutants from Ca_V3.1 channels

The glutamates located at the pore region in the I and II domains of the human Ca_V3.1 channel were individually mutated using the PCR-based site-directed mutagenesis protocol previously described [41]. All PCRs were performed using Phusion High-Fidelity DNA Polymerase™ (Thermo Scientific, USA). The PCR-amplification products were evaluated by agarose gel electrophoresis, purified with DNA Clean & Concentrator™-5 kit (Zymo Research, USA), treated with DpnI restriction enzyme (Thermo Scientific, USA) and transformed into DH5α chemocompetent cells. We used the hα1Ga-pDsRed plasmid, a gift from Edward Perez-Reyes (Addgene plasmid # 45811) [42], as template of PCR. To make the Ca_V3.1/E354D (DEDD) mutant we used the following forward and reverse primers: 5′-GCT GGA TGG CTG GGT CGA CAT C-3′ and 5′-CCA GCC ATC CAG CGT GAT GAC-3′, respectively. For the Ca_V3.1/E923D (EDDD) mutant we amplified the plasmid using the following forward and reverse primers 5′-ACC CAA GAT GAC TGG AAC AAA GTC CTC TAC AAT G-3′ and 5′-CCA GTC ATC TTG GGT CAG GAT CTG AAA GAC AGT G-3′, respectively. To create the double mutant Ca_V3.1/E354D, E923D (DDDD) we used the plasmid encoding the Ca_V3.1 DEDD mutant as template and the pair of primers for the EDDD mutation. To confirm that all the plasmids had its respective mutations without further mutations in the rest of the channel, the entire open reading frames of all the plasmids were verified by sequencing.

2.2. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells (ATCC, Manassas, VA) were maintained in Advanced Dulbecco's Modified Eagle Medium (DMEM, Gibco Life Technologies) supplemented with 5% fetal bovine serum (FBS), 1% penicillin and streptomycin antibiotics, at 37 °C in a 5% CO_2-95% air humidified atmosphere. Gene transfer was performed using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA). Briefly, the medium of cell culture in 35-mm Petri dish was replaced with 800 μL of serum-free medium at 60% confluence (~800 cells/mm^2). According to the manufacturer's instructions, 1.2 μg of the plasmid encoding the WT human Ca_V3.1 channel or one of the pore mutants, 300 ng plasmid encoding the green fluorescent protein (pEGFP; Clontech, CA, USA) and 6 μL of lipofectamine were mixed in 100 μL of serum-free medium (transfection solution). Then, the transfection solution was added to the culture dishes and the cells were incubated for 3 h at 37 °C. Subsequently,