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Role of C-terminus of Kir7.1 potassium channel in cell-surface expression

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Abstract

Inward rectifier K^+ channel Kir7.1 is predominantly expressed on the plasma membrane of a variety of ion-transporting epithelia. The electrophysiological property of Kir7.1 has been well characterized but the mechanism underlying the plasma-membrane targeting remains elusive. To address this issue, we examined the effect of deletion and site-directed mutagenesis on the plasma-membrane localization of Kir7.1 in Madin-Darby canine kidney cells by immunofluorescence microscopy and cell-surface biotinylation. Although deletions of up to 37 amino acid residues from the C-terminus had no effect, further deletion resulted in accumulation of the mutant proteins in intracellular membranes. No sequence motif for subcellular targeting was found in the distal C-terminal region. The cell-surface expression of the deletion mutant lacking 38 or 40 C-terminal residues was restored by addition of one or three alanine residues, respectively, to the C-terminus end. These results suggest that the C-terminal length plays an important role in the plasma-membrane localization of Kir7.1.

Keywords: Ion channel; Cell surface biotinylation; Mutagenesis; Transmembrane protein; Subcellular localization

1. Introduction

K^+ channels play important roles in cell physiology such as neuronal excitability, muscle contraction, hormone secretion and cell volume (Shieh et al., 2000). Sorting of K^+ channels according to their final destination, e.g. the plasma membrane or intracellular organelle, is tightly regulated. It is becoming increasingly clear that this process is mediated by specific sequence motifs found in the cytoplasmic domains of channel proteins (Deutsch, 2002). Several K^+ channels, e.g. Kv1.4, contain at their C-terminal ends the short peptide sequences recognized by PSD-95/Dlg/ZO-1 (PDZ) domains. Interaction with PDZ-containing scaffold proteins, e.g. PSD-95, allows the K^+ channels to cluster at high densities at the post-synaptic sites on neurons, thereby ensuring efficient synaptic transmission (Craven and Bredt, 1998; Sheng and Sala, 2001).

Inward rectifier K^+ (Kir) channels belong to a superfamily of the channel protein consisting of a pore-forming hairpin loop (H5), two transmembrane spans (M1 and M2) and cytoplasmic N- and C-terminal tails. In mammals, 15 genes encoding Kir channel proteins have been identified and classified into seven subfamilies (Kir1 to Kir7) with distinct electrophysiological properties, cellular localization and physiological functions (Abraham et al., 1999; Reimann and Ashcroft, 1999). Kir7.1, the most recently identified member, consists of 360 amino acid residues and exhibits unique pore properties, including a very low unitary conductance and a very low dependence of its current on extracellular K^+ concentration (Döring et al., 1998; Krapivinsky et al., 1998; Partiseti et al., 1998). Kir7.1 is highly expressed in the epithelial cells of small intestine, choroid plexus, renal tubules, thyroid and retina (Derst et al., 2001; Döring et al., 1998; Döring and

Abbreviations: CK, casein kinase; ER, endoplasmic reticulum; HA, hemagglutinin; Kir, inward rectifier K^+; MDCK, Madin-Darby canine kidney; PDZ, PSD-95/Dlg/ZO-1; RIPA, radioimmunoprepicitation assay.

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Karschin, 2000; Kusaka et al., 2001; Nakamura et al., 1999, 2000; Ookata et al., 2000; Shimura et al., 2001; Suzuki et al., 2003; Yang et al., 2003; Yasuda et al., 2003; Yuan et al., 2003). In all of these ion-transporting epithelia, Kir7.1 colocalizes with Na^+,K^+-ATPase at the plasma membrane. Based on the unique pore properties and subcellular localization, Kir7.1 is proposed to be involved in transepithelial solute and fluid transport in functional association with Na^+,K^+-ATPase. However, it is poorly understood how Kir7.1 is targeted and stably localized to the plasma membrane. In this study, we performed a mutational analysis of Kir7.1 and investigated whether there are changes in the subcellular localization. We now show that the C-terminal length of Kir7.1 is important for its plasma membrane expression.

2. Materials and methods

2.1. Plasmid construction

N-terminally hemagglutinin (HA)-tagged wild-type and deletion constructs of rat Kir7.1 were generated by cloning appropriate cDNA fragments synthesized by polymerase chain reaction into the *Eco*RI and *Not*I sites of pCMV-HA (BD Biosciences Clontech, Palo Alto, CA, USA). 1–308 + 15, the Δ324–360 construct in which the last 15 amino acid residues were replaced with an unrelated polypeptide (STEISRGTAAAGIQT), was generated as follows: the cDNA fragment encoding residues 1–308 of Kir7.1 was amplified with the primers SA-EcoF (5′-gc*gaattc*tggacagcaggaattgtaaagttaat-3′) and SA-K308-SalR (5′-tcc*gtcgac*ttggaacctcgagtattaga-3′), and then inserted into the *Eco*RI and *Sal*I sites of pCMV-HA. 1–323-FLAG, the Δ324–360 construct C-terminally tagged with 3 × FLAG (QDLISVPVDSRGSRA DYKDHDGDYKDHDIDYKDDDK), was generated as follows: the fragment encoding Δ324–360 was amplified with the primers HA-HindF (5′-ccc*aagctt*-caccatgtacccatacgatgt-3′) and SA-P323-BglIIR (5′-ggg*agatct*gaggaa-cagtcttgtcaaaa-3′), and then inserted into the *Hin*dIII and *Bgl*II sites of p3 × FLAG-CMV-14 (Sigma-Aldrich, St. Louis, MO, USA). Point mutations were created by the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All constructs were verified by sequencing with LI-COR Model 4000 (Aloka, Tokyo, Japan).

2.2. Cell culture

Madin-Darby canine kidney (MDCK) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in an atmosphere of humidified air with 5% CO_2. Transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and Opti-MEM I (Invitrogen) according to the manufacturer's instructions.

2.3. Cell-surface biotinylation

Cells were washed five times with ice-cold PBS and treated with 0.5 mg/ml EZ-link sulfo-*N*-hydroxysuccinimide (sulfo-NHS)-LC-biotin (Pierce, Rockford, IL, USA) in PBS for 30 min on ice. After rinsing with 100 mM Tris buffer (pH 7.5) to quench any unreacted reagent, cells were lysed in 500 μl of radioimmunoprepicitation assay (RIPA) buffer [10 mM sodium phosphate (pH 7.4), 1% (v/v) Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.15 M NaCl, 2 mM ethylenediaminetetraacetic acid and 50 mM NaF] containing protease inhibitors (10 mM leupeptin, 1 mM pepstatin and 1 mM phenylmethanesulfonyl fluoride). After centrifuging at 100,000 × *g*, the supernatants were incubated with Immunopure immobilized streptavidin (Pierce) at 4 °C overnight. After washing the beads with RIPA buffer, the biotinylated proteins were eluted by addition of Laemmli sample buffer containing 5% 2-mercaptoethanol.

2.4. Western blot analysis

Transfected cells were washed three times with PBS, scraped from culture dishes and pelleted by centrifuging at 1000 × *g* for 10 min. Cells were homogenized in 500 μl of RIPA buffer containing protease inhibitors and centrifuged at 20,000 × *g* for 1 h at 4 °C. The resulting supernatants (10 μg of protein) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane by the tank-blotting method. The blot was incubated with rat monoclonal anti-HA antibody (1:2000, Roche Diagnostics, Penzgerg, Germany) followed by secondary antibodies conjugated with horseradish peroxidase (1:10,000). The blot was developed with the ECL Plus kit (Amersham Biosciences, Piscataway, NJ, USA).

2.5. Immunofluorescence microscopy

All procedures were performed at room temperature. Cells grown on glass coverslips were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 in PBS for 15 min. After blocking with 1% fetal bovine serum in PBS for 30 min, cells were incubated with anti-HA antibody (1:2000) or rabbit polyclonal anti-calreticulin antibody (1:1000, Affinity Bioreagents, Golden, CO, USA) for 30 min followed by appropriate secondary antibodies conjugated with Alexa Fluor 488 or 546 (1:2000, Molecular Probes, Eugene, OR, USA) for 30 min. Signals were observed using an LSM510 confocal microscope system (Carl Zeiss, Thornwood, NJ, USA) or an Axioskop microscope (Carl Zeiss) equipped with an ORCA-ER CCD camera (Hamamatsu, Bridgewater, NJ, USA), a CSU10 confocal scanner unit (Yokogawa Electric, Tokyo, Japan) and an argon/krypton ion laser.

2.6. Sequence analysis

Multiple sequence alignments were generated by the Genetyx software (Software Development, Tokyo, Japan). The secondary structure of Kir7.1 was predicted by the PredictProtein server (Rost, 1996).

3. Results

To investigate the molecular basis for the subcellular localization of Kir7.1, a series of N- or C-terminal deletion mutants of Kir7.1 (Fig. 1A) were constructed and evaluated for their cell-surface expression by cell-surface biotinylation. The N-terminally HA-tagged deletion constructs were transiently transfected into MDCK cells. One day after transfection, cell-surface proteins were biotinylated extracellularly, and cells were subsequently extracted with RIPA buffer. The cellular expression of mutant proteins was confirmed by Western blotting of the extracts with anti-HA antibody (Fig. 2A). Biotinylated proteins were precipitated with streptavidin beads and subjected to Western blotting with anti-HA antibody. As shown in Fig. 2B, the cell-surface expression of Kir7.1 was not affected by deletions of up to C-terminal 37 residues (Δ355–360, Δ345–360, Δ333–360, Δ325–360 and Δ324–360, lanes 2–6) whereas it was abolished by further C-terminal deletions (Δ323–360, Δ322–360 and Δ321–360; lanes 8–10). These findings were also observed 2 and 3 days after transfection (Fig. 2C), suggesting that the loss of surface expression was unlikely due to delayed protein transport to the plasma membrane. However, all N-terminal deletions had no effect (Δ1–23, Δ1–47 and Δ1–54; Fig. 2B, lanes 12–14). These results suggest that the cytoplasmic C-terminal tail has a role in the surface expression of Kir7.1.