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Proteolytic cleavage in the S1–S2 linker of the Kv1.5 channel does not affect channel function

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ABSTRACT

Kv1.5 channels mediate the ultra-rapidly activating delayed rectifier potassium current (I_{Kur}), which is important for atrial repolarization. It has been shown that cell-surface Kv1.5 channels are sensitive to cleavage by the extracellular serine protease, proteinase K (PK). Here, we investigated the effects of extracellular proteolytic digestion on the function of Kv1.5 channels stably expressed in HEK 293 cells. Our data demonstrate that PK treatment cleaved mature membrane-bound (75 kDa) Kv1.5 channels at a single locus in the S1–S2 linker, producing 42-kDa N-terminal fragments and 33-kDa C-terminal fragments. Interestingly, such PK treatment did not affect the Kv1.5 current ($I_{Kv1.5}$) recorded using the whole-cell patch clamp technique. Analysis of cell-surface proteins isolated using biotinylation indicated that the PK-generated N- and C-terminal fragments were both present in the plasma membrane. Co-immunoprecipitation (co-IP) experiments indicated that the N- and C-terminal fragments are no longer associated after cleavage. Furthermore, following PK digestion, the N- and C-fragments degraded at different rates. PK is frequently used as a tool to analyze cell-surface localization of membrane proteins, and cleavage of cell-surface channels has been shown to abolish channel function (e.g. hERG). Our data, for the first time, demonstrate that cleavage of cell-surface channels assessed by Western blot analysis does not necessarily correlate with an elimination of the channel activities.

1. Introduction

Synchronized ionic currents control regular heart rhythm. Accordingly, disruption of ion channel function causes cardiac arrhythmias [1]. Kv1.5 channels, encoded by the *KCNA5* gene, conduct the ultra-rapidly activating delayed rectifier potassium current (I_{Kur}), which is important for atrial repolarization [2,3]. The atria-specific nature of cardiac I_{Kur} has sparked considerable interest in linking Kv1.5 channels to atrial arrhythmias, such as atrial fibrillation (AF) [3]. Indeed, several loss-of-function mutations in *KCNA5* have been identified in AF patients [4,5].

Ion channels must be expressed in the plasma membrane to serve their function. To determine the cell-surface localization of channels, extracellular application of proteases including proteinase K (PK) has been widely used [6–10]. It has been shown that, in Western blot analysis, extracellularly applied PK cleaves mature human ether-a-go-go-related gene (hERG) protein into two fragments. Such cleavage is associated with a complete loss of hERG function as revealed by patch clamp analysis [10]. Although it has been shown via Western blot analysis that PK also cleaves cell-surface Kv1.5 channels [7], it is not known whether such cleavage leads to an elimination of Kv1.5 channel function, as observed in hERG channels.

Here, we demonstrate that PK cleaves the cell-surface Kv1.5 (75 kDa) channels into 42-kDa N-terminal fragments and 33-kDa C-terminal fragments. Surprisingly, such cleavage did not affect Kv1.5 current ($I_{Kv1.5}$) recorded by the whole-cell patch clamp method. The two fragments derived from mature Kv1.5 channels did not associate, as revealed by co-immunoprecipitation (co-IP) analysis, and they seemed to degrade at different rates. We conclude that cleavage of cell-surface channels, detected by Western blot analysis, does not necessarily lead to a diminished channel function. In addition, our observation provides novel insights into the structure–function relationship of Kv1.5 channels.

2. Materials and methods

2.1. Molecular biology

Kv1.5 cDNA was obtained from Dr. Michael Tamkun (Colorado State University, Fort Collins, CO). hERG cDNA was provided by Dr. Gail A. Robertson (University of Wisconsin–Madison, Madison, WI). Human embryonic kidney (HEK) 293 cell lines stably expressing Kv1.5 (Kv1.5-HEK) or hERG (hERG-HEK) were established using G418 for selection

Abbreviations: CHX, Cycloheximide; co-IP, Co-immunoprecipitation; hERG, Human ether-a-go-go-related gene; I_{Kur}, The ultra-rapidly activating delayed rectifier potassium current; PK, Proteinase K.

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(1 mg/ml) and maintenance (0.4 mg/ml). Kv1.5-HEK and hERG-HEK cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, and 1% sodium pyruvate (Invitrogen, Waltham, MA). To generate NΔ209 and NΔ303 truncation mutant Kv1.5 channels, the codon for methionine at position 210 (NΔ209) or at position 304 (for NΔ303) was used as the starting point for protein translation, respectively. Thus, the NΔ209 mutant lacks the N-terminal amino acids up to 209, and the NΔ303 mutant lacks the N-terminus and S1 amino acids up to 303. Original mutations were generated in a pBK-CMV vector via PCR and transferred to a pcDNA3 vector using BamH1 and EcoR1 restriction enzymes. The mutations in the Kv1.5 constructs were verified through sequencing (GENEWIZ, Inc.). WT, NΔ209, or NΔ303 Kv1.5 plasmid was co-transfected with GFP plasmid (pIRES2-EGFP, Clontech) into HEK cells using Lipofectamine 2000. Twenty-four hours after transfection, cells were collected following trypsinization, and GFP-positive cells were selected for patch clamp experiments.

2.2. Cleavage of cell-surface proteins

Live HEK cells stably expressing Kv1.5 or hERG channels were treated with PK (200 μg/ml in MEM) (Sigma–Aldrich, St. Louis, MO) in the cell culture medium at 37 °C for 20 min. Phosphate-buffered saline (PBS) containing 6 mM phenylmethylsulfonyl fluoride and 25 mM EDTA was used to quench the reaction. Biochemical and electrophysiological experiments were performed either immediately after PK digestion (0 h post-cleavage) or after re-culture of PK-treated cells for various periods.

2.3. Western blot analysis and Co-IP

Whole-cell protein lysates from Kv1.5-HEK or hERG-HEK cells were used for Western blot analysis using the procedure described previously [11]. Proteins were separated on 8 or 10% SDS–polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes. The BLUeye Prestained Protein Ladder (FroggaBio, Toronto, ON) was used to identify the mass of proteins. Nonspecific interactions were blocked using 5% non-fat milk and 0.1% Tween 20 in Tris-buffered saline. The membranes were then incubated with N-terminal specific rabbit anti-Kv1.5 (H-120) (Santa Cruz Biotechnology, Dallas, TX), C-terminal specific rabbit anti-Kv1.5 (Alomone, Jerusalem, Israel), or goat anti-hERG (N-20) (Santa Cruz Biotechnology) polyclonal primary antibodies for 1 h. Actin was probed as a loading control using monoclonal mouse anti-actin (AC-40) antibody (Sigma–Aldrich). The blots were incubated with goat anti-rabbit, mouse anti-goat, and/or goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h. Protein bands were visualized using an enhanced chemiluminescence detection kit (GE Healthcare, Little Chalfont, United Kingdom) and X-ray films (Fujifilm, Tokyo, Japan). For the quantification of Western blot data, the band intensities of proteins of interest in each gel were first normalized to their respective actin (loading control) intensities and then expressed as values relative to the control.

For each co-IP analysis, 0.5 mg whole-cell protein in 0.5 ml lysis buffer was incubated with the appropriate primary antibody at 4 °C overnight. The protein complexes were mixed with Protein A/G Plus agarose beads (Santa Cruz Biotechnology) at 4 °C for 4 h prior to precipitation by centrifugation at 10,000×*g* for 1 min. The immunoprecipitates were washed four times with cold lysis buffer to remove unbound protein. 2× Laemmli sample loading buffer was added to resuspend the pelleted immunoprecipitates. The samples were boiled for 5 min and centrifuged at 20,000×*g* for 5 min. The supernatants were loaded into SDS–polyacrylamide gels and analyzed by Western blot analysis. GAPDH was precipitated with rabbit anti-GAPDH antibody (Santa Cruz Biotechnology) as a negative control.

2.4. Electrophysiological recordings

All currents were recorded using the whole-cell patch clamp method. Aliquots of cells were allowed to settle on the bottom of a 0.5 ml cell bath mounted on an inverted microscope (Eclipse Ti–U, Nikon). Cells were superfused with the bath solution. Patch electrodes were fabricated using thin-walled borosilicate glass (World Precision Instruments, Sarasota, FL). The pipettes had inner diameters of approximately 1.5 μm and resistances of about 2 MΩ when filled with solution. An Axopatch 200B amplifier was used to record membrane currents. Computer software (pCLAMP10; Molecular Device) was used to generate voltage clamp protocols, acquire data, and analyze current signals. Data were filtered at 5–10 kHz and sampled at 20–50 kHz. Typically, 80% series resistance (Rs) compensation was used and leak subtraction was not used. The bath solution consisted of (in mM) 135 NaCl, 5 KCl, 10 HEPES, 10 glucose, 1 $MgCl_2$, and 2 $CaCl_2$ (pH 7.4 with NaOH). The pipette solution contained (in mM) 135 KCl, 5 EGTA, 1 $MgCl_2$, and 10 HEPES (pH 7.2 with KOH). All chemicals used in the solutions for patch clamp experiments were purchased from Sigma–Aldrich. $I_{Kv1.5}$ and I_{hERG} were evoked in their HEK stable cell lines from the holding potential of −80 mV by depolarizing steps from −70 to +50 or from −70 to +70 in 10 mV increments, respectively. Peak pulse currents at the end of the +50 mV depolarizing step were used to analyze Kv1.5 current amplitudes. hERG tail currents upon a repolarizing step to −50 mV after a depolarization to 50 mV were used to analyze hERG current amplitudes. All patch clamp experiments were performed at room temperature (22 ± 1 °C).

2.5. Isolation of cell-surface proteins

A Cell Surface Protein Isolation Kit (Pierce, Waltham, MA) was used as per the manufacturer's protocol. In brief, Kv1.5-HEK cells were grown in 100 mm culture dishes to 90% confluence. Extracellularly exposed cell-surface proteins were bound with 250 μg/ml of the membrane impermeant thiol-cleavable amine-reactive biotinylation reagent, Sulfo-NHS-SS-biotin, for 30 min at 4 °C. The reaction was terminated via addition of the Quenching Solution. Biotin-labeled cells were then collected and lysed with lysis buffer containing 1% protease inhibitor cocktail. Following centrifugation at 10,000×*g* for 2 min at 4 °C, biotin-bound proteins were isolated with Immobilized NeutrAvidin Gel. After washing away non-biotinylated proteins (cytosolic proteins), the bound proteins were eluted by incubating the gel in a Tris buffer (62.5 mM Tris–HCl, pH 6.8, 1% SDS, 10% glycerol) containing 50 mM DTT. The purified cell-surface proteins were subjected to Western blot analysis. To ensure equal protein loading, Na^+/K^+ ATPase expression was detected with mouse anti-Na^+/K^+-ATPase α1 antibody (Santa Cruz Biotechnology) and the corresponding goat anti-mouse HRP-conjugated secondary antibody.

2.6. Immunofluorescence microscopy

Kv1.5-HEK cells were treated with cycloheximide (10 μg/ml) (Sigma–Aldrich) for 24 h to eliminate immature 68-kDa Kv1.5 channels via inhibition of protein biosynthesis. The live cells were treated with PK (200 μg/ml, in MEM) at 37 °C for 20 min to cleave cell-surface channels. The PK-treated cells were then reseeded onto coverslips and cultured in the presence of cycloheximide (10 μg/ml) for 2 h. Live cell membranes were stained using red-fluorescent Texas Red Wheat Germ Agglutinin (5 μg/ml) (Invitrogen) for 1 min in Hank's Balanced Salt Solution (Invitrogen). The cells were fixed with 4% ice-cold paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 (Sigma–Aldrich) for 10 min, and blocked with 5% bovine serum albumin in PBS for 1 h. Kv1.5 channels were labeled with N- or C-terminal recognizing rabbit anti-Kv1.5 primary antibodies. Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (Invitrogen) was then used to