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Homer regulation of native plasma membrane calcium channels in A431 cells

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ABSTRACT

Homers are adapter proteins that play a significant role in the organization of calcium signaling protein complexes. Previous functional studies linked Homer proteins to calcium influx in nonexcitable cells. These studies utilized calcium imaging or whole-cell current recordings. Because of limited resolution of these methods, an identity of Homer-modulated ion channels remained unclear. There are several types of plasma membrane calcium influx channels in A431 cells. In the present study, we demonstrated that Homer dissociation resulted in specific activation of I_{min} channels but not of I_{max} channels in inside-out patches taken from A431 cells. In contrast, inositol 1,4,5-trisphosphate activated both I_{min} and I_{max} channels in inside-out patches. Short (1a) and long (1c) forms of Homer had different effects on I_{min} channel activity. Homer 1a but not Homer 1c activated I_{min} in the patches. This study indicates that I_{min} channels are specifically regulated by Homer proteins in A431 cells.

1. Introduction

In non-excitable cells, activation of phospholipase C (PLC) mediates calcium (Ca^{2+}) release from inositol 1,4,5-trisphosphate (IP_3)-sensitive intracellular Ca^{2+} stores and Ca^{2+} influx through plasma membrane Ca^{2+} channels. Two distinct pathways for calcium influx have been identified: the receptor-operated pathway, which is directly activated by downstream signaling cascades of PLC, and the store-operated pathway, which is activated by depletion of intracellular calcium stores [1–3]. Several types of store-operated calcium currents have been characterized in various types of cells. Depending on the cell type, these currents display variability in biophysical characteristics and modes of regulation [4–8], suggesting that different proteins may be involved in forming store-operated calcium channels (SOCs) in the plasma membrane and/or in regulation of SOC activity.

Biochemical and microscopic data support the idea that Ca^{2+} signaling proteins are assembled in multiprotein complexes that are localized in distinct regions of the cells and that Ca^{2+} signaling events occur in spatially segregated domains of the cell. Homers participate in targeting and localization of Ca^{2+} signaling proteins in signaling complexes [9]. In neurons, Homer proteins are well known for their role as adapter molecules that enhance signaling at the postsynaptic density by altering receptor trafficking and clustering, Ca^{2+} flux, and actin organization. Homer proteins function via an N-terminal enabled/vasodilator-stimulated phosphoprotein homology domain (EVH1) and a C-terminal coil-coiled (CC) domain [9].

The amino-terminal EVH1-like domain (also called the target-binding or ligand-binding domain) interacts with the proline-rich sequences of the PPXXF motif (where X is any amino acid), which are found in many target and ligand proteins (Fig. 1A) [10]. Through the EVH1 domain, Homer binds signaling ligands, such as the metabotropic glutamate receptors, the inositol trisphosphate receptor (IP_3R), ryanodine receptors [11,12], anchoring proteins such as Shank [13], and transient receptor potential canonical (TRPC) channels [14,15]. Alternative splicing results in two essentially different groups of Homer proteins [10], including full-length transcripts known as long-form Homer and shorter transcripts lacking the CC domain at the C-terminus. Constitutively expressed long-form Homer proteins possess a CC domain, which mediates homophilic and/or heterophilic interactions with other members of the Homer protein family. Recent elegant biophysical and bio-

Abbreviations: PLC, phospholipase C; Ca^{2+}, calcium; IP_3, inositol 1,4,5-trisphosphate; SOC, store-operated calcium channels; EVH1, Homer N-terminal enabled/vasodilator-stimulated phosphoprotein homology domain; CC, Homer C-terminal coil-coiled domain; IP_3R, the inositol trisphosphate receptor; TRPC, transient receptor potential canonical channels; pAb, polyclonal antibodies; GST, glutathione S-transferase; WB, western blot; TPEN, N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylene diamine; IP_3R1, inositol trisphosphate receptor type 1; PD, pull-down.

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chemical experiments [16] have suggested that native long-form Homers exist as tetrameric hubs with the CC domains aligned in parallel. Upon binding of the EVH1 domain to specific partner proteins, tetrameric Homers crosslink the proteins into macromolecular complexes that display enhanced signaling properties [12,17]. In addition to the long forms of Homer, there are two activity-inducible short forms of Homer: Homer 1a (Vesl-1s) and Ania-3. These isoforms possess a fully functional EVH1 domain, but lack the CC domain and therefore, crosslinking potential. Homer 1a and Ania-3 are thought to act as naturally occurring dominant negative molecules that function by uncoupling long-form Homer-ligand interactions, resulting in molecular rearrangements [11,12,18,19].

Homer proteins couple key calcium signaling proteins such as TRPC channels [14,15]. A role of Homer 1 in the regulation of Ca^{2+} influx in non-excitable cells was further supported when Ca^{2+} signaling was analyzed in pancreatic acini prepared from Homer 1−/− mice [14]. Deletion of Homer 1 results in increased spontaneous Ca^{2+} influx, indicating that Homer plays an inhibitory role in controlling plasma membrane Ca^{2+} channels [14,15]. Previous studies linking Homer proteins with Ca^{2+} influx utilized Ca^{2+} imaging or whole cell current recordings, techniques that report combined Ca^{2+} influx via multiple channel types. Because of limited resolution of these methods, an identification of Homer-modulated channels remained unclear. Here, we utilized single-channel recordings to determine an identity of Homer-regulated Ca^{2+} channels supporting Ca^{2+} influx in A431 human carcinoma cells.

2. Experimental procedures

2.1. Cell culture

Human carcinoma A431 cells (Cell Culture Collection, Institute of Cytology, St. Petersburg, Russia; ATCC CRL-1555) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 80 μg/ml gentamicin, and 2 mM glutamine. The cells were maintained in 37 °C incubator (5% CO_2). For patch clamp experiments the cells were plated on glass coverslips and maintained in culture for 1–3 days before use.

2.2. Chemicals

HEPES, EGTA, were from Sigma-Aldrich, UTP and IP_3 were from Calbiochem. Polyclonal antibodies (pAb) anti-IP_3R type 1 T443 were described earlier [20], anti-glutathione S-transferase (Sigma), anti-Homer 1bc H174 (Santa Cruz). PPKKFR and PPKKRR peptides were from Diapharm, Saint-Petersburg, Russia.

2.3. Expression and purification of Homer proteins

Glutathione S-transferase (GST) fusion plasmids pGEX-2T-Homer1A and pGEX-2T-Homer1C were a gift from Dr. Soloviev (University of Oxford). GST fusion plasmids of Homer 1c and Homer 1a were transformed into *E. coli* BL21 cells and positive clone expanded for 12 h at 37 °C in LB containing 50 μg/ml ampicilin with shaking. Bacterial culture was diluted 1:100 with pre-warmed 100 ml LB medium containing 50 μg/ml ampicilin at 30 °C and shaken until A_{600} was reached 1. IPTG was added to a final concentration 0.1 mM and incubation continued for an additional 2 h. Cells were lysed in 5 ml PBS with 5 mg lysozyme, 1% Triton X-100 and protein inhibitors cocktail (PIC, Roche) by sonication, and the lysate was centrifuged at 12,000 × *g* at 4 °C for 15 min. Bacterial supernatant was added to 500 μl of glutathione-sepharose (Pharmacia) and incubated for 2–3 h at 4 °C, then washed thrice with 10 ml of PBS with 1% Triton X-100. Glutathion sepharose with GST fusion Homer proteins was used in pull-down experiments or fusion proteins were eluted with 500 μl 100 μM glutathione, 50 mM Tris, pH 8.0 buffer at 4 °C three times and probes were fused. The protein concentration was determined by using the Bradford method. Such procedure gave about 1 mg/ml of purified GST-Homer protein. Before being used for functional studies, the purity was analyzed by SDS-PAGE and staining of gels with Coomassie brilliant blue G-250 (Suppl. Fig. 1) or immunoblotting with polyclonal antibodies specific for Homer or for GST. In ultraviolet range spectrum of absorption of recombinant proteins was corresponded to folded protein. Absorbance at 280 nm was about 0.85 and 0.71 for Homer 1a and Homer 1c respectively, what gives concentration about 1 mg/ml, similar to result obtained by Bradford method. Further at 7.4 pH estimated charge of recombinant proteins was 3.7 and −8.9 for Homer 1a and Homer 1c, respectively (calculated with program Protein Calculator v3.3 by Chris Putnam). Given together this data consider solubility of Homer proteins in used conditions.

2.4. Pull-down

For pull-down experiments 10 cm dish with A431 cells were lysed in 1 ml of 150 mM NaCl, Tris–HCl pH 7.6 20 mM, EDTA 1 mM, EGTA 1 mM, Triton X-100 1%, NP40 0.5%, glycerin 10%, PMSF 0.5 mM, PIC (Roche). The lysate was run through cooled syringe trice and centrifuged at 22,000 × *g* at 4 °C for 30 min, the supernatant was separated. 25 μl of 1:1 glutathion sepharose slurry with GST-homer proteins were added to 0.5 ml of total A431 cell lysate and incubated in presence or absence of IP_3 for 12 h at 4 °C. Then probes were washed for three times with lysis buffer, and analysed by electrophoresis and immunoblotting.

2.5. SDS-PAGE and Western blotting (WB)

Protein probes were separated using SDS-PAGE electrophoresis in 6 or 8 percentage gel and then transferred to Immobilon P membrane (Millipore Inc.). Membranes were blocked and incubated with antibodies to proteins of interest. To detect the primary antibody, blots were incubated with peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (Sigma). Membranes were treated with SuperSignal Chemiluminescent Substrate (Pierce) or ECL reagents (Tris–HCl pH 8.5 187.5 mM, luminol 85 mM, coumaric acid in DMSO 270 mM, 0.012% H_2O_2) and exposed to films.

2.6. Electrophysiology

All electrophysiological experiments were performed with Axopatch 200B patch clamp amplifier (Axon Instruments). The pClamp6 software (Axon Instruments) was used for data acquisition and off-line data analysis. All experiments were carried out at room temperature (22–24 °C).

The whole cell recordings were performed with 3–5 MΩ fire-polished glass pipettes. The pipette solution contained (in mM) 145 NMDG aspartate, 10 Cs-EGTA, 10 Cs-HEPES pH 7.3, 1.5 $MgCl_2$ and 4.5 $CaCl_2$ (pCa 7.0). Extracellular solution contained (in mM): 140 NMDG aspartate, 10 $BaCl_2$, 10 Cs-HEPES, pH 7.3. The currents were sampled at the frequency of 5 kHz. In all experiments, the holding potential was 0 mV. Periodically (once every 5 s) the holding potential was shifted to −100 mV (for 60 ms) and a 600-ms voltage ramp to +100 mV was applied. The traces recorded before current activation were used as templates for leak subtraction. Whole cell currents were normalized to the cell capacitance. The mean value of cell capacitance was 21 pF ± 4 (total number of experiments $n = 25$).

The single channel recordings were performed with 8–15 MΩ sylgard-coated, glass pipettes. The pipette solution contained (in mM): 105 $BaCl_2$, 10 Tris–HCl (pH 7.3). In inside-out experiments,