Regulation of Store-Operated Channels by Scaffold Proteins in A431 Cells

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ABSTARCT **Store-operated channels are major calcium influx pathways in nonexitable cells. Homer scaffold proteins are well known for their role in regulating calcium signaling. Here we report on a detailed single-channel level characterization of native store-operated channels regulated by Homer scaffold proteins in A431 carcinoma cells. By applying the single-channel patch-clamp technique, we found that different types of store-operated calcium channels have different sensitivities to Homer proteins.**
KEYWORDS **SOC, Homer, A431**
ABBREVIATIONS **SOC - store-operated calcium channels, mGluR - metabotropic glutamate receptors, IP_3 - inositol 1,4,5-trisphosphate, IP_3R - the inositol 1,4,5-trisphosphate receptor, RyR - ryanodine receptors, UTP - uridine triphosphate, GST - glutathione S-transferase.**

INTRODUCTION

Currently, calcium signaling in nonelectroexcitable cells is arousing substantial interest from researchers, because alterations of cytoplasmic Ca^{2+} regulate a plethora of intracellular events. The calcium concentration in cytosol increases upon the release of Ca^{2+} ions from intracellular stores either when extracellular Ca^{2+} enters the cell. In nonelectroexcitable cells, the entrance of Ca^{2+} is basically mediated by store-operated channels (SOC) [1]. These channels get activated upon the depletion of intracellular Ca^{2+} stores. SOCs from various tissues were shown to have different biophysical properties, indicating that there is a difference in their molecular composition [1]. Although the basic proteins which mediate store-operated Ca^{2+} influx are known, the mechanism underlying the colocalization of these proteins remains undiscovered.

Homer proteins that are found in neural tissue mediate the formation of the intermolecular complex, which includes the metabotropic glutamate receptor (mGluR) of the plasma membrane and inositol-1,4,5-trisphosphate receptor (IP_3R) [2]. It was shown later that Homer interacts with TRPC proteins [3,4], the Ca^{2+}-ATPase, ryanodine receptor (RyR), and other proteins [5]. Moreover, in neural cells, Homer and Shank proteins were shown to form a meshlike structure which acts to organize postsynaptic proteins [6]. Homer proteins contain the EVH1 domain, which is located on the N-terminus of the polypeptide chain and accounts for the interaction between Homer and its targets by binding to the consensus amino acid sequence PPXXF (proline, proline, any two amino acids, and phenylalanine). There are short and long isoforms of Homer proteins (Fig. 1A). The long isoform has a coiled-coil domain on its C-terminus which mediates oligomerization. The short isoform, which is produced by alternative splicing, lacks this domain [5]. The long isoforms were shown to form tetramers with the parallel arrangement of C-termini and four EVH1 domains, which makes it possible to mediate the colocalization of proteins of Ca^{2+} signaling [7]. Short Homer isoforms are believed to act as the negative regulators of the long one, since they are not able to form oligomers. It was shown that Homer proteins not only regulate protein colocalization, but they can also modulate the activity of mGluR [8], RyR [9], and TRPC [3, 4].

It was hypothesized that Homer proteins participate in the regulation of SOC. Previous studies confirming this suggestion were carried out by means of patch clamp in a whole-cell configuration and fluorescent imaging of Ca^{2+} signaling [3, 4]. However, these approaches make it possible to determine the net Ca^{2+} influx only. Since the cell contains various types of calcium–SOC, it has been unclear which of them are regulated by Homer proteins. In this study we set out to elucidate the role that Homer proteins play in the regulation of store-operated calcium channels in A431 cells.

EXPERIMENTAL PROCEDURES

Cells

Human epidermal carcinoma A341 cells (Cell lines collection, Cytology institute, Russian Academy of Sciences) were cultivated in a DMEM medium supplemented

with 10% fetal calf serum and antibiotics (100 μg/ml penicillin and 100 U/ml streptomycin). The cells were grown on microscope cover glasses 2–4 days prior to the experiment

Materials

DMEM medium (ICN), fetal calf serum (FCS, GIBCO BRL, United States), fetal bovine serum (FBS, GIBCO BRL, United States), and geniticin G-418 (Geniticin, GIBCO BRL, United States) were used in cell cultivation. Glutathion-sepharose and 1,5-isopropylthio-β-D-galactoside (IPTG) were from Pharmacia, Sweden; inositol-1,4,5-trisphosphate (IP_3) was from LC Laboratories, United States; uridine triphosphate (UTP) was from Cabiochem, Germany; EGTA was from Fluka, Switzerland; HEPES, Triton X-100 anti-GST antibodies, and secondary rabbit and mouse antibodies were purchased from Sigma, United States; and anti-Homer 1 bc antibodies were from Santa Cruz, United States. Anti- IP_3R T443 polyclone antibodies were described earlier [10]. PPKKFR and PPKKRR peptides were synthesized by Diapharm, Russia.

Patch Clamp

In all the experiments, the potential of extracellular solution was taken as zero.

In inside-out configuration experiments, the intracellular solution (chamber) contained (mM) 140 K-glutamate, 5 NaCl, 1 $MgCl_2$, 10 HEPES-K, pH 7.4, 2 EGTA-K, and 1.13 $CaCl_2$ (pCa 7). The pipette solution contained (mM) 105 $BaCl_2$ and 10 Tris-HCl (pH 7.3). The presence of Ba^{2+}-ions led to the inhibition of voltage-**dependent** Ca^{2+} channels and Ca^{2+}-dependent channels. The electric resistance of solution-filled pipettes was 8-20 MOm.

In whole-cell configuration experiments, the pipette solution contained (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). The intracellular solution contained (mM) 140 NMDG aspartate, 10 $BaCl_2$, 10 Cs-HEPES, and pH 7.3. In these experiments we used pipettes which had resistances of 3–5 MOm. In all the whole-cell experiments, the membrane potential was equal to 0 mV. The potential was changed according to the following scheme: in the beginning, the −100 mV potential was applied for 60 ms, which was followed by a voltage ramp in a −100- to 100-mV interval for 600 ms; after that, the 0 mV potential was restored. The whole-cell currents were normalized with respect to cellular capacity, which reflects the size of the cell. The average capacity value was 21±4 pF (25 experiments).

Currents were registered by means of an Axopatch 200B intensifier (Axon instruments, United States). The signal was digitized at a frequency of 5000 Gz with ADC L305 board (L-Card, Russia). For an analysis and presentation of data on currents of individual channels of low conductivity, additional filtration was performed (80–100 Gz). Amplitudes of currents through individual channels of low conductivity were determined from registered currents and an amplitude histogram. The level of channel activity was characterized by the NP_o value, which is the product of the number of conducting channels (N) and the probability of the open state (P_o), which is equal to $P_0 = I/(iN)$, where I is the average value of a current in the given membrane fragment at a certain time interval and i is the open channel current amplitude.

The registered data were digitized and analyzed by means of software developed by V.A. Alexeyenko, as well as pClamp 6.0.4, Microcal Origin 6.0, and Microsoft Excel.

Electrophoresis and Immunoblotting

Protein samples were analyzed in 8% polyacrylamide gels under denaturing conditions. Gels were stained with Coomassie or, alternatively, proteins were transferred to the membrane and visualized by specific antibodies against the proteins of interest.

Expression and Purification of Recombinant GST-Homer 1c and Homer 1a Proteins

Escherichia coli BL-21(DE3) cells were transformed with pGEX-2T-Homer1A and pGEX-2T-Homer1C plasmids (courtesy of M.M. Solovyev, University of Oxford, Great Britain). The expression of recombinant proteins was induced by the addition of 1 mM IPTG; then, bacterial cells were lysed, and chimeric GST-Homer proteins were purified on glutathion-sepharose. Proteins were stored at 4°C. The purity of protein preparations was checked by electrophoresis and immunoblotting with polyclone antibodies against GST and Homer.

Cellular Lysate

A431 cells were lysed for 10 min at 4°C in a 10-cm plate in 1 ml of solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP40, 10% glycerol, and 0.5 mM PMSF with the addition of a protease-inhibiting cocktail (PIC, Hoffmann–La Roche AG, Switzerland). The lysates were passed through a syrynge three times and spun at 22 000g for 30 min at 4°C. The supernatant was withdrawn and used in subsequent experiments.

Pull-Down Assay (PD)

Glutathione-sepharose (25 μl) with bound chimeric GST-Homer was mixed with A431 cellular lysates and incubated on a rocking platform for 12–24 h. The re-

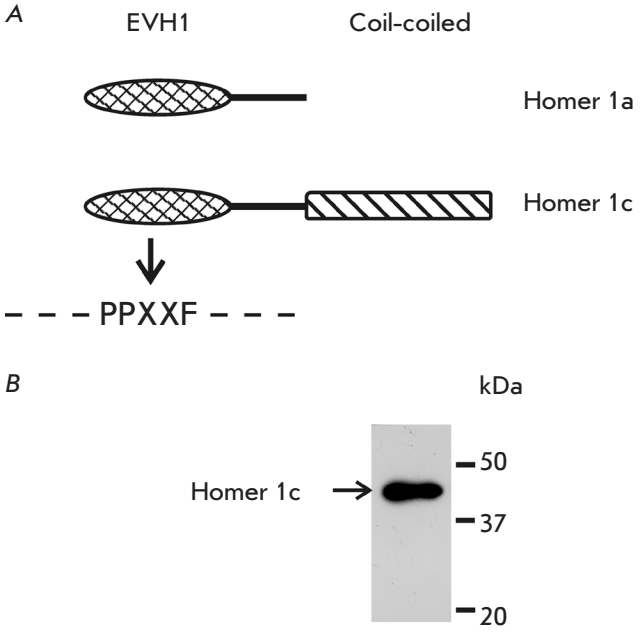

Fig. 1. Homer 1bc long isoform is expressed in the human epidermal carcinoma cell line A431. A – Domain organization of Homer 1 proteins. B – The immunoblotting of whole cell lysates from A431 cells was performed with polyclonal anti-Homer 1bc antibodies. The positions of molecular mass markers are shown on the right.

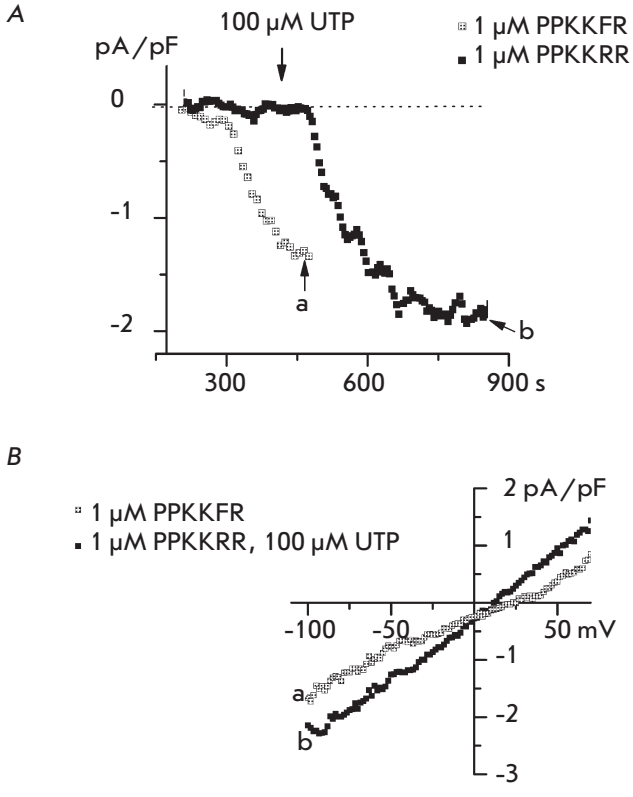

Fig. 2. Whole-cell recordings of currents induced by PPKKFR or PPKKRR peptides. A The development in time of a current from a cell dialyzed with 1 μM PPKKFR peptide at –80 mV potential (gray squares) and with 1 μM PPKKRR (black squares). The arrow at the top indicates when 100 μM UTP was added. Arrows *a* and *b* indicate the maximum amplitudes of the current. B Average current–voltage relationships of currents induced by dialysis with PPKKFR peptide or by the addition of UTP. The current–voltage relationships were measured when the inward currents reached the maximum (indicated by arrows *a* and *b* in panel A).

action was carried out in a PBS buffer containing 1% Triton X-100. In some cases, the incubation was conducted in the presence of IP_3; after that, sepharose was rinsed three times with 1 ml PBS containing 1% Triton X-100. The presence of IP_3R in the samples was confirmed by immunoblotting with polyclone antibodies against IP_3R.

RESULTS AND DISCUSSION

Uncoupling of Homer-Target Protein Interactions Caused by PPKKFR Interaction Activates Ca^{2+} Influx in A431 Cells

The results of immunoblotting show that A431 cells express long isoforms of Homer proteins (Fig. 1B). The EVH1 domain in Homer proteins recognizes the PPXXFR motif in target proteins, where X stands for any amino acid (Fig. 1A) [2]. In order to reveal the roles which Homer proteins play in regulating receptor-operated or store-operated calcium channels, we used a synthetic peptide PPKKFR, since it has been shown to promote the dissociation of the Homer-mGluR complex [2].

In control experiments we used peptide PPXXRR in which the substitution of phenylalanine with arginine cancelled the peptide's interaction with the EVH1 domain. In order to elucidate the role that Homer proteins play in the regulation of the Ca^{2+} influx in A431 cells, we applied a patch clamp technique in the whole-cell configuration. The net Ca^{2+} influx, which was measured under conditions of intracellular dialysis against PPKKFR and PPKKRR-containing solutions, was compared with the net store-operated Ca^{2+} influx, which was induced by uridine triphosphate.

Intracellular dialysis with a solution containing 1 μM PPKKFR led to the selective activation of a Ca^{2+} current with an average maximal amplitude of 1.3 ± 0.1 pA/pF (n = 5) (Fig. 2A). The absence of PPKKRR in the pipette solution led to no current (n = 10), but the

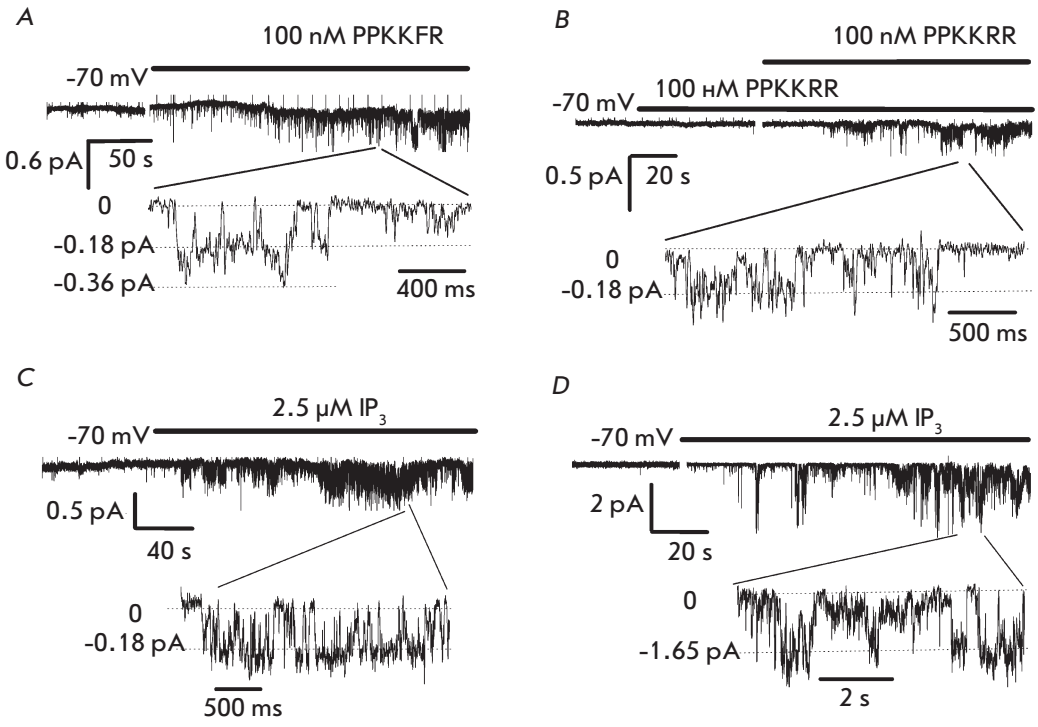

Fig. 3. Homer dissociation led to I_{min} activity, but it didn't lead to I_{max} activity. A– The application of 100 nM PPKKFR to the cytosolic surface of an inside-out patch held at a –70mV membrane potential activated I_{min}. The fragment of the current record is shown at the bottom on the expanded time scale. B – The application of 100 nM of the control peptide PPKKRR did not activate I_{min}, whereas the subsequent addition of 100 nM PPKKFR to the same patch induced I_{min} activity. C, D – The application of 2.5 μM IP_3 induced (C) I_{min} and (D) I_{max} activity. Examples of single-channel recordings from the isolated membrane at –70 mV potential.

addition of 100 μM UTP caused the current to appear (n = 5) (Fig. 2A). The amplitude of the UTP-induced current was equal to approximately 1.8 ± 0.3 pA/pF and, therefore, was bigger than that of the PPKKFR-induced current (Fig. 2B). The reversal potential of PPKKFR-induced currents was more positive than that of UTP-induced currents. Thus, PPKKFR-activated channels are more selective for Ca^{2+} than UTP-activated channels.

Our data are in agreement with previously published works by other authors who suggested an interconnection between Homer proteins and store-operated Ca^{2+} influx in nonelectroexcitable cells [3, 4].

In previous works we described several types of UTP-sensitive Ca^{2+} channels with different reversal potentials of the currents [11]. We assumed that the difference in amplitudes of currents and channel selectivity in the response of PPKKFR and UTP has the following explanation: UTP treatment activates various types of channels, whereas PPKKFR activates particular channels.

I_{min} (but Not I_{max}) Channels are Sensitive to PPKKFR-Induced Homer Dissociation from Protein Targets

In order to investigate which Ca^{2+} channels from A431 cells account for the currents induced by the dissociation of Homer proteins from their targets, we performed patch clamp experiments in an inside-out configuration. These experiments show that, in A431 cells, there are two types of store-operated channels (I_{min} and I_{max}) [11]. These channels have different electrophysiological characteristics, making it possible to identify them based on the registration of currents through them. I_{min} channels have low conductivity (1.2 pS) and high selectivity for bivalent cations. In comparison with I_{min} channels, I_{max} have higher conductivity (18 pS) but lower selectivity.

In an inside-out configuration, the addition of a 100-nM PPKKFR peptide from the cytoplasmic side of the membrane fragment led to the activation of the influx current (Fig. 3A). It turned out that the basic electrophysiological properties of the activated channels (kinetics, conductivity, and reversal potential) coincide with those of previously described I_{min} channels [11–18]. This observation leads to the conclusion that uncoupling Homer proteins and their protein targets activates I_{min} channels in A431 cells, which is in agreements with the results obtained in our laboratory on HEK293 cells [12]. I_{min} channels were activated by PPKKFR peptide in 43% of cases (n = 60) (Fig. 3A). Control peptide PPKKRR (which should not uncouple interactions between Homer and target proteins) did not activate channels in any of the 36 experiments, though the subsequent addition of PPKKFR led to the activation of I_{mim} channels in 42% of experiments (n = 26) (Fig. 3B). PPKKFR did not activate I_{max} channels, which are another group of store-operated channels in A431 cells (n = 60).

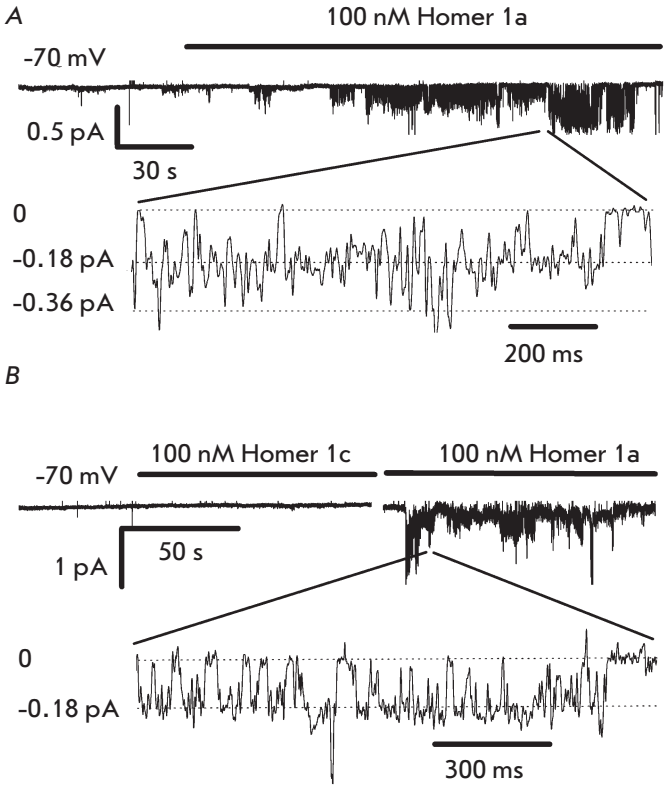

Fig. 4. Homer 1a protein activates I_{min} channels. A The application of 100 nM Homer 1a to the cytosolic surface of an inside-out patch held at a – 70mV membrane potential activated I_{min} channels. The fragment of the current record is shown at the bottom on the expanded time scale. B Homer 1c (100 nM) did not activate store-operated channels, whereas the subsequent application of Homer 1a was effective in the same experiment. The fragment of the current record is shown at the bottom on the expanded time scale.

The addition of 2.5 μM inositol-1,4,5-trisphosphate from the cytosolic side of the plasma membrane in inside-out experiments activated I_{min} channels in 32% and I_{max} channels in 8% of experiments (n = 80) (Figs. 3C, 3D), which is in agreement with our previous data [11]. At potential values of −70 mV, the amplitudes of I_{min} currents were 0.18 pA, while in case of I_{min} currents they were equal to 1.7 pA (Figs. 3A–3D).

These data allowed us to conclude that PPKKFR-induced uncoupling of interactions between Homer proteins and their protein targets leads to the activation of I_{min} channels, while I_{max} channels in A431 cells are insensitive to this peptide. It is unknown whether two other store-operated channels of A431 cells – I_{NS} and I_{CRAC} – are regulated by Homer proteins, since these channels are not present (or are indistinguishable) in an inside-out configuration.

Data on the activity of single channels are in good agreement with the results of the whole-cell experiments. UTP activates all kinds of store-operated channels in A431 cells, and PPKKFR peptide does not activate at least I_{max} channels, hence the peptide causes a partial activation of calcium channels in the whole-cell configuration. This fact explains the difference in values of the registered net currents. Since I_{max} channels are less selective than I_{min} channels, a UTP-induced net current exhibits lower selectivity.

I_{min} Channels Are Activated by Homer 1a, but Not Homer 1c

Homer proteins fall into two different groups [5]. Long isoforms (e.g., Homer 1c) have a coiled-coil domain on their C-termini and so are able to form homo-oligomers. The lack of a coiled-coil domain in short isoforms (e.g., Homer 1a) prevents the formation of oligomeric complexes (Fig. 1A). In order to investigate the effects that long and short isoforms have on the activity of store-operated calcium channels in A431, we used recombinant Homer proteins produced in *E.coli* transformed with a GST-Homer plasmid. The functional activity of Homer 1a- and Homer 1c-purified proteins was judged by their ability to bind IP_3R1 in a pull-down assay.

The monomer isoform Homer 1c at a concentration of 100 nM activated Ca^{2+} channels in 30% of inside-out experiments (n = 101) (Fig. 4A). The current-voltage characteristics of Homer 1a-activated channels coincided with those of I_{min} channels activated by UTP, store depletion, or IP_3 in A431 and HEK293 cells [11, 13–18]. The conductivity of Homer 1a-activated channels was equal to 1.3 pS. The long isoform Homer 1c at a concentration of 100 nM did not lead to channel activation (n = 58), while the subsequent addition of Homer 1a activated I_{min} channels in 27% of cases (n = 44) (Fig. 4B). Neither Homer 1a nor Homer 1c activated I_{max} channels.

The present data indicate that various Homer isoforms differ in their action on I_{min} channels in A431 cells: the monomeric Homer 1a protein causes activation, while Homer 1c does not. Various Homer isoforms similarly affect mGluR [8] and TRPC channels [3, 4], but not RyR channels [19, 20], which are activated by both long and short isoforms of Homer proteins, long isoforms being stronger activators of type-I RyR than short ones.

One explanation, which was suggested in the case of TRPC channels, is probably applicable to I_{min} channels as well. Since Homer 1c proteins can form oligomers and Homer 1a cannot because they are devoid of the coiled-coil domain, it was hypothesized that the oligomeric complexes block the channels, while uncoupling

with Homer oligomers activates them [3, 4]. Our experiments with peptides and recombinant proteins show that it is not Homer 1a itself but the uncoupling of the interaction of Homer oligomers and their protein targets that is necessary for the activation of I_{min} channels. We can suggest that the dissociation of Homer oligomeric complexes from their targets leads to the alteration of interactions of the Ca^{2+} channel and other proteins, including IP_3R. This uncoupling can be caused by the PPKKFR peptide, short isoform Homer 1a, and IP_3 (see below). It is known that Homer 1 proteins do not affect the phosphoinosotide metabolism. In particular, they do not cause the elevation of the IP_3 concentration and cannot promote the release of Ca^{2+} from IP_3-sensitive Ca^{2+} stores [21]. Therefore, we can conclude that Homer activates store-operated Ca^{2+} channels by means of direct interaction with the channel and not through releasing Ca^{2+} from the intracellular stores. Thus, despite the fact that I_{min} channels are store-operated in A431 cells, they apparently can also exploit a store-independent mechanism, which happens if complexes of Homer and its protein targets are dissociated.

The Effects That IP_3 and the Uncoupling of Native Homer Proteins with Their Protein Targets have on I_{min} Channels Are Nonadditive

Since I_{min} channels are regulated by both IP_3 and Homer proteins, the question arises as to whether their action is additive. Adding 2.5 μM IP_3 to membrane fragments with PPKKFR-induced activity most of the time did not lead to any further increase in channel activity (in 10 out of 13 experiments) (Fig. 5A). In similar experiments with recombinant Homer 1a, the protein application of IP_3 also did not alter the I_{min} channel activity (Fig. 5B). However, in several experiments, the addition of IP_3 activated I_{max} channels (Fig. 5B). When PPKKFR or the recombinant protein Homer 1a did not activate I_{min} channels, IP_3 also did not show any activation effect either. Therefore, we had demonstrated that the effects of IP_3 and the uncoupling of native Homer proteins with their protein targets on I_{min} channels are nonadditive. These data led us to suggest that the regulation of I_{min} channels by IP_3 and Homer proteins exploits the same signal pathway.

IP_3 Impairs Homer–IP_3R1 Interaction

A431 cells basically express type-1 IP_3R [Glushankova, unpublished data]. It has been reported that, in these cells, I_{min} channels are apparently regulated by means of conformational coupling with type-1 IP_3R [15, 17]. Here we show that Homer proteins participate in the regulation of I_{min} channels. X-ray diffraction data reveal that, in the IP_3R molecule, the Homer-recognizing motif and the IP_3-binding domain are positioned closely

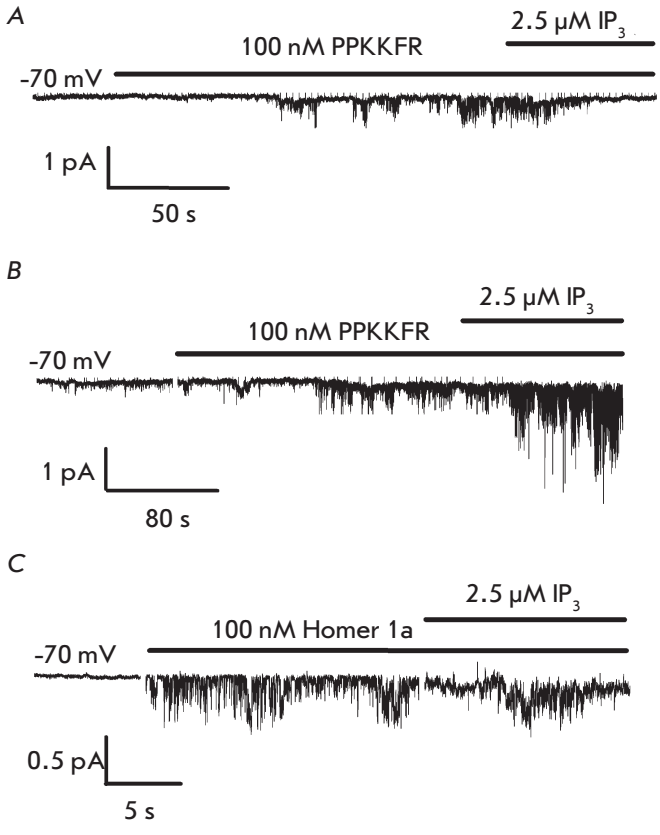

Fig. 5. Detachment of native Homer proteins and the IP_3 effect are not additive for I_{min}-channel activation. A – The subsequent application of 2.5 μM IP_3 to patches did not increase I_{min}-channel activity induced by 100 nM PPKKFR peptide. B – The application of 100 nM PPKKFR to the cytosolic surface activated I_{min} channels. The subsequent application of 2.5 μM IP_3 activated I_{max}. C – I_{min}-channel activity, induced by 100 nM Homer 1a, did not change after the addition of 2.5 μM IP_3.

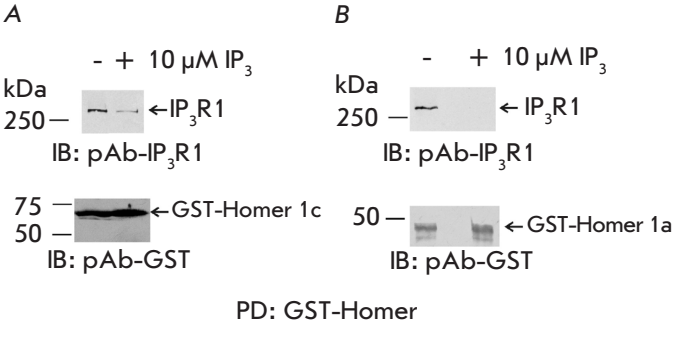

Fig. 6. IP_3 decreased the Homer interaction with IP_3R1. A, B - Results of pull-down experiments. IP_3 was incubated with GST-Homer 1c (A) or GST-Homer 1a (B) in the presence or absence of 10 μM IP_3.

[22]. It was also shown that IP_3 causes the dissociation of Homer from its complex with type-3 IP_3R [4]. Our pull-down experiments show that IP_3 can impair interactions between type-1 IP_3R and Homer 1a or Homer 1c proteins (Figs. 6A, 6B). These results are in good agreement with the data obtained in inside-out experiments, which show that the effects of IP_3, Homer 1a, and PPKKFR peptides are nonadditive.

Thus, the Homer-mediated regulation of native store-operated channels in A431 cells has been investigated in our work for the first time. These results deepen our knowledge of the organization of the components that mediate store-operated influx. ●

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