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Homer Binds TRPC Family Channels and Is Required for Gating of TRPC1 by IP_3 Receptors

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Summary

Receptor signaling at the plasma membrane often releases calcium from intracellular stores. For example, inositol triphosphate (IP3) produced by receptor-coupled phospholipase C activates an intracellular store calcium channel, the IP_3R. Conversely, stores can induce extracellular calcium to enter the cell through plasma membrane channels, too. How this "reverse" coupling works was unclear, but store IP_3Rs were proposed to bind and regulate plasma membrane TRP cation channels. Here, we demonstrate that the adaptor protein, termed Homer, facilitates a physical association between TRPC1 and the IP_3R that is required for the TRP channel to respond to signals. The TRPC1-Homer-IP_3R complex is dynamic and its disassembly parallels TRPC1 channel activation. Homer's action depends on its ability to crosslink and is blocked by the dominant-negative immediate early gene form, H1a. Since H1a is transcriptionally regulated by cellular activity, this mechanism can affect both short and long-term regulation of TRPC1 function.

Introduction

The TRP family of ion channels is involvement in diverse physiological functions, including osmoregulation, epithelial Ca^{2+} transport, and temperature and metabolism sensing. In addition, members of the family are associated with human diseases such as polycystic kidney disease, mucolipidosis, and hypomagnesemia (Venkatachalam et al., 2002). Accordingly, it is important to understand the mechanism by which these channels are regulated and gated in vivo. A subgroup of the TRP channels, the TRPC members, encode nonspecific cation channels that are activated in response to G protein-coupled receptor activation and/or depletion of intracellular Ca^{2+} stores (Minke and Cook, 2002; Montell et al., 2002). In a native context, several TRPC channels are believed to participate in replenishment and regulation of intracellular Ca^{2+} pools in a process termed capacitive calcium entry (CCE) (Vennekens et al., 2002). The mechanism of regulated channel gating remains an area of intense investigation (Putney, 2001). Several lines of evidence support a conformational coupling model with an essential role for interaction between TRPC channels and intracellular Ca^{2+} release channels. TRPC3 interacts with the inositol trisphosphate receptor (IP_3R), and the N-terminal fragment of the IP_3R can induce channel opening (Kiselyov et al., 1998, 1999). Moreover, expression of interacting regions of IP_3R and TRPC4 in HEK cells (Boulay et al., 1999) and of IP_3R and TRPC1 in CHO cells (Vaca and Sampieri, 2002) can modify native CCE and TRPC1 function.

The TRP family members encode a conserved proline-rich sequence LP(P/X)PFN in their C termini (Montell, 2001) that is similar to the consensus binding site for Homer (PPXXF; (Tu et al., 1998). Homer family proteins bind to proline rich sequences in group 1 metabotropic glutamate receptors (Brakeman et al., 1997), IP_3Rs (Tu et al., 1998), ryanodine receptors (Feng et al., 2002), and the Shank family of scaffolding proteins (Tu et al., 1999). Homer's binding to these proteins is mediated by a unique EVH1 domain (Beneken et al., 2000). Most Homer proteins also encode a coiled-coil domain that mediates specific self-association (Xiao et al., 1998). Upon binding of the EVH1 domain to specific partner proteins, Homer crosslinks the proteins into macromolecular complexes that display enhanced signaling properties (Tu et al., 1998; Xiao et al., 1998). A noncrosslinking form of Homer, termed Homer 1a (H1a), is regulated as an immediate early gene (Brakeman et al., 1997) and appears to function as a dominant-negative for crosslinking forms of Homer (Xiao et al., 2000). H1a is also expressed in non-neural tissues (Soloviev et al., 2000a, 2000b). The dynamic expression of H1a suggests that Homer crosslinking is precisely regulated by cell-specific stimuli.

Based on the presence of a consensus Homer ligand in TRPC channels and on the precedent that Homer can couple membrane receptors and intracellular Ca^{2+} channels, we examined the contribution of Homer to TRPC channel function. Here, we report that Homer proteins bind multiple TRPC family members, and this interaction is required for regulated gating of TRPC1. The TRPC1-Homer interaction is more complex than that described for other targets of Homer in that it requires interactions with both the TRP domain sequence and a second Homer binding site in the N terminus. Disruption of binding blocks the assembly of a TRPC1-Homer-IP3R complex and, surprisingly, results in channels that are

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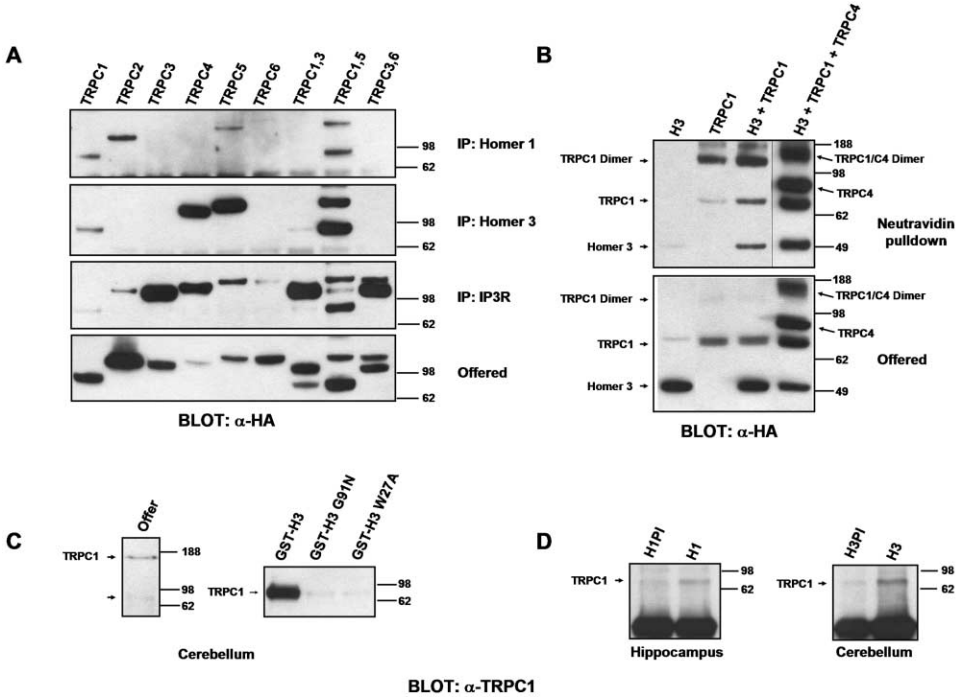

Figure 1. Homer and IP_3R Coimmunoprecipitate TRPC1 and Other TRPC Family Members

(A) HEK293 cells were transfected with TRPC constructs as shown. All constructs were HA-tagged, and their expression is detected with α-HA antibody (offered). Native Homer 1 co-IPs TRPC1, C2 and C5, and TRPC1/5, while native Homer 3 co-IPs TRPC1, C4, C5, and TRPC1/5. IP_3R co-IPs TRPC1-6; TRPC1/3; TRPC1/5; TRPC3/6.

(B) TRPC1 is present at the plasma membrane and associates with Homer. HEK293 cells were transfected with Homer 3 (H3), TRPC1, H3 + TRPC1, or H3 + TRPC1 + TRPC4. Membrane proteins were biotinylated and precipitated mixed with neutravidin beads. TRPC1 alone is detected at the membrane surface while H3 alone is not. Combined expression of TRPC1 and H3 results in precipitation of both proteins.

(C) Native TRPC1 from cerebellum binds GST-Homer 3 EVH1 in a pull-down assay but not GST-Homer 3 EVH1 point mutants: G91N and W27A. Monomeric TRPC1 is enriched in the pull-down assay relative to lysates in which TRPC1 appears to run as a predominant dimer.

(D) Homer 1 or Homer 3 co-IPs TRPC1 from detergent lysates of hippocampus or cerebellum, respectively.

spontaneously active. These studies define molecular determinants of TRPC1 channel activation and indicate that Homer proteins dynamically regulate TRPC gating.

Results

Homer Binds Multiple TRPC Family Members

The proline-rich sequence LP(P/X)PFN in the C terminus of the TRPC channels resembles a canonical Homer binding motif. To examine the association of Homer with TRPC family members, we expressed TRPC 1–6 in HEK cells either separately or in combinations that are reported to occur in vivo (Hofmann et al., 2002). TRPC constructs were identically tagged with N-terminal HA to facilitate comparisons. TRPC1, TRPC2, and TRPC5 co-IPed with native Homer 1 when expressed singly (Figure 1). TRPC1 and TRPC5 co-IPed when coexpressed. Homer 3 co-IPed TRPC1, TRPC4, TRPC5, and coexpressed TRPC1/5. By contrast, Homer 2 co-IPed only TRPC1 (data not shown). In these assays, TRPC3 and TRPC6 did not co-IP with any native Homer. All TRPC family members co-IPed with native anti-IP_3R. The anti-IP_3R antibody was generated against native rat cerebellum IP_3R and recognizes all three IP_3R family members (Nucifora et al., 1996). TRPC1, TRPC2, and TRPC6 showed less robust co-IP with the IP_3R than TRPC3, TRPC4, and TRPC5. The co-IP of TRPC1 with IP_3R was augmented when coexpressed with TRPC5. These studies indicate selective interactions of native Homer with multiple TRPC family members. They also indicate that TRPC family members all associate with the IP_3R but do so to a variable degree.

In anticipation of electrophysiological and biochemical studies of TRPC1, we examined its presence at the plasma membrane using a nonmembrane permeant agent [sulfo-NHS-SS-biotin (Pierce)] that biotinylated surface proteins, and precipitated proteins with avidin-linked beads [neutravidin (Pierce)]. In the same experiment, we assessed whether membrane TRPC channels associate with Homer. When expressed singly, TRPC1 is precipitated with avidin beads while Homer is not (Figure 1B). When coexpressed, both Homer and TRPC1 precipitate, consistent with the notion that Homer binds TRPC1 at the plasma membrane surface. It is notable that the dimer species is enriched in this assay of membrane receptors. Coexpression of Homer did not substantially change the amount of TRPC1 expression or the fraction at the plasma membrane. In previous studies, combined expression of TRPC1 with TRPC4/5 was reported to be important for detection of TRPC1 at the plasma membrane (Hofmann et al., 2002). Consistent with this report, coexpression of TRPC1 with TRPC4

resulted in ~10-fold increase of TRPC1 at the membrane surface. In our experiments, the ability to detect TRPC1 at the plasma membrane is likely due to the efficiency of our expression construct (see Experimental Procedures).

Homer Associates with TRPC1 in Brain and Binding Requires the Homer EVH1 Domain

Since TRPC1 interacts with all native Homers, we focused on this interaction. The crystal structure of the Homer EVH1 domain defines two surfaces that are critical for binding to mGluR5 peptide (Beneken et al., 2000). To examine the molecular determinants of Homer interaction with native TRPC1, we used GST-Homer in pull-down assays with detergent lysates from brain. Native TRPC1 from cerebellum bound GST-Homer 3 EVH1 (GST-H3) and migrated on SDS-PAGE as a single band of the anticipated size for a monomer (Figure 1C). TRPC1 immunoreactivity was identically detected with two independent antibodies (Alomone Labs Ltd. and gift from Dr. Ambudkar, NIH). The GST-pull-down provided enrichment of TRPC1 relative to lysates in which TRPC1 immunoreactivity migrated as a high mw species consistent with an SDS-stable dimer (Figure 1C). The interaction with Homer appears to utilize binding surfaces that are identical to those used in binding to mGluR5 (Beneken et al., 2000), since TRPC1 did not bind to either GST-H3(W27A) (interrupts proline binding surface) or GST-H3G91N (interrupts binding pocket for phenylalanine; Beneken et al., 2000).

To determine whether Homer and TRPC1 associate in vivo, we assayed for co-IP of TRPC1 from brain (Figure 1D). TRPC1 is reported to be expressed in both forebrain and cerebellum (Strubing et al., 2001). Homer 3 is the predominant Homer protein in cerebellum (Xiao et al., 1998), and TRPC1 specifically co-IPed with Homer 3 Ab. Homer 1 is the predominant Homer protein in forebrain (Xiao et al., 1998), and TRPC1 co-IPed with Homer 1 specific Ab from hippocampus (Figure 1D).

Homer Binding to TRPC1 Is Dependent on Interaction Sites in Both the N and C Termini of TRPC1

We examined the Homer-TRPC1 interaction using point mutants of the predicted Homer ligand site, as well as C-terminal deletion mutants that removed sequence either immediately after or before the proline-rich domain (PRD). TRPC1(F648R) and TRPC1(P646L) failed to bind Homer (Figure 2A). These results are consistent with prior mutational analysis of mGluR5 (Tu et al., 1998) and are rationalized by loss of binding energy from the phenylalanine interaction and by reduction of the proline-dependent secondary structure (Beneken et al., 2000), respectively. Unexpectedly, we found that TRPC1(P645L) showed markedly enhanced binding to GST-H3. In Figure 2A, we used 10-fold less GST-H3 for the pull-down yet still see substantially more binding than with wt TRPC1. This result was not consistent with our understanding of Homer binding to other proteins, since the equivalent P-to-L mutation of mGluR5 destroys Homer binding (Tu et al., 1998). Identical results were obtained with GST-Homer1 EVH1 (data not shown).

Analysis of GST-H3 binding to TRPC1 deletion mutants yielded results that also appeared paradoxical. As expected, deletion of the C terminus after the PRD, TRPC1(1–650), did not reduce binding in GST-H3 pull-down assays (Figure 2B). However, deletion of the PRD resulted in a TRPC1(1–644) mutant that retained binding to Homer. Indeed, Homer binding to TRPC1(1–644) is substantially more robust than to wt TRPC1. Moreover, both TRPC1 deletion mutants showed binding to GST-H3(G91N) (Figure 2B, middle image), which is not evident with wt TRPC1. This binding appeared specific since the deletion mutants did not bind to GST-H3(W27A). These results indicate that TRPC1 possesses a second Homer binding site that may be allosterically influenced by its C terminus.

The second site was identified by sequential deletion analysis and point mutagenesis. We determined that an N-terminal fragment, which includes the ankyrin repeats [TRPC1(1–316)], binds GST-H3, and mapped the region essential for Homer binding to the N-terminal 23 aa (Figure 2C). This region was noted to encode a proline-rich sequence (aa 19–28; LPSSPSSSSP) but lacked a phenylalanine typical of conventional Homer binding sites. In the context of the TRPC1 NT fragment, point mutations P20A or P23A, but not P28A, destroyed binding to GST-H3 (Figure 2D). The N-terminal TRPC1 fragment did not bind GST-H3(W27A) but did bind GST-H3(G91N) (Figure 2D). This is consistent with binding specificity of the deletion mutants TRPC1(1–650) and TRPC1(1–644) (Figure 2B). TRPC1(1–316) binding to GST-H3(G91N) mutant was less robust than to wt GST-H3 but much greater than to GST-H3(W27A). In this assay, the L19A mutant also showed reduced binding. The binding specificity of the LPSSP ligand suggests that a proline interaction with W27 of Homer is energetically important; however, the phenylalanine binding pocket of the EVH1 domain is not essential (Beneken et al., 2000).

In Vivo Association of Homer and TRPC1 Is Cooperatively Dependent on C- and N-Terminal Binding Sites

A panel of full-length TRPC1 proteins with point mutations in either the N- or C-terminal Homer binding sites was expressed in HEK293 cells and assayed for co-IP with native Homer (Figure 2E). Of these, only wt and P645L co-IPed with Homer. Identical results were obtained with Homer 1 and 3 antibodies. In contrast to the GST-H3 pull-down experiments in which the P645L mutant showed dramatically increased binding (Figure 2A), the Homer co-IP of this mutant was substantially reduced compared to wt TRPC1. This difference indicates that GST-pull-down assays do not entirely reconstitute native interactions. Nevertheless, these assays did confirm an essential role of the TRPC1 N-terminal binding site. Thus, both the N-terminal and C-terminal binding sites are essential for in vivo association of full-length TRPC1 with Homer.

TRPC1 Mutants that Do Not Bind Homer Are Spontaneously Active

TRPC1 constructs were transiently expressed in HEK293 cells and channel activity was assayed by Fura 2 imaging of Ca^{2+} or Sr^{2+} and by whole-cell current

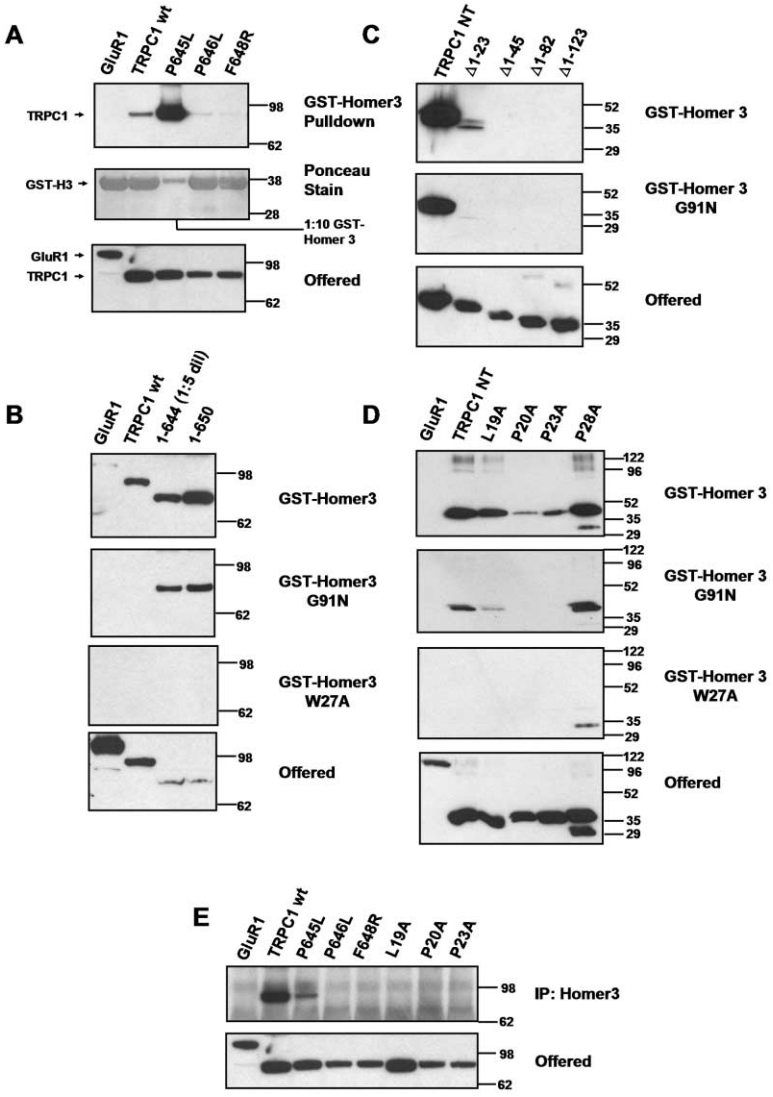

Figure 2. Homer Binding to TRPC1 Is Dependent on PPPF Motif in C Terminus but Also Involves a Second Proline-Rich Sequence in the N Terminus

The Homer binding sites on TRPC1 were mapped using a combination of GST-pulldown (A–D) and co-IP assays (E).

(A) HEK293 cells were transfected with wt TRPC1 and point mutants in the C-terminal PPPF motif. GST-H3 pulls down wt TRPC1 and P645L but not P646L or F648R.

(B) TRPC1 deletion mutants that retain (1–650) or eliminate (1–644) the PPPF site were expressed in HEK293 cells. Both deletion mutants show robust binding to GST-H3 but not GST-H3 W27A. TRPC1(1–644) lysate was diluted 1:5 to provide comparable levels of binding. Note that both deletion mutants bind GST-G91N H3, while wt TRPC1 does not.

(C) Deletion mapping of N-terminal Homer binding site. TRPC1 N-terminal fragment (NT; aa 1–316) and serial N-terminal deletions were expressed in HEK293 cells and assayed for binding to GST-H3 and GST-H3 G91N. The Δ1–45 and more extensive deletion fragments did not bind Homer, while the Δ1–23 showed marked reduced binding, suggesting the second Homer binding site resides in this region.

(D) Point mutation mapping of N-terminal Homer binding site. Point mutants of TRPC1 NT sequence aa 19–28 (**LPSSPSSSSP**) were expressed in HEK293 cells. Binding to GST-H3 was reduced for P20A and P23A but not P28A. Binding to GST-H3 G91N showed a similar pattern and additionally shows reduced binding of the L19A mutant. None of the constructs bound GST-H3 W27A.

(E) Mutations of either N- or C-terminal binding sites disrupt TRPC1 co-IP with native Homer 3. HEK293 cells were transfected with full-length TRPC1 containing point mutations in the N- or C-terminal binding sites. Homer 3 co-IPs wt TRPC1 and TRPC1(P645L) but not other point mutants. All TRPC constructs were HA-tagged and probed with α-HA antibody.

measurements. UTP was used to activate native P2Y2 receptors, which are G protein coupled, and release Ca^{2+} from intracellular stores (Baltensperger and Porzig, 1997). Channel activity was also assayed after store depletion with thapsigargin or ionomycin. Current recordings are shown in Figures 3A–3D, but similar results were obtained with Fura 2 imaging. TRPC1 functions as a nonselective cation channel (Vaca and Sampieri, 2002). Therefore, TRPC1 current was estimated by recording the inward current at a holding potential of −100 mV and in cells incubated in media containing Na^+ (permeant) or NMDG (not permeant for TRPC channels). Cells expressing wt TRPC1 showed a small spontaneous nonselective cationic current that was blocked by NMDG. This current was absent from nontransfected cells. Stimulation of cells expressing TRPC1 with UTP resulted in a rapid increase in current that was abolished by replacing Na^+ with NMDG. By contrast, cells expressing TRPC1 with point mutations of the C-terminal Homer binding site showed increased basal current (Figures 3C and 3D). In these cells, application of UTP did not induce additional current. In control experiments, we found that mutations did not alter the current/voltage relationship of the channel. Figures 3E and 3F illustrate that wt TRPC1 in cells stimulated with UTP, and TRPC1(P645L) in unstimulated or stimulated cells show similar current/voltage characteristics. Images 3G and 3H summarize the results of multiple experiments to show effect of mutations on spontaneous and UTP-induced currents. Deletion of the entire C terminus up to, but not including, the C-terminal Homer ligand [(TRPC1(1–650)] results in coupled channel that can be activated by agonist stimulation. Deletion of the Homer ligand [TRPC1(1–644)] and all point mutations in the Homer binding ligand that eliminate Homer binding result in spontaneously active channels. Importantly, the converse situation was observed when the fraction of current activated by agonist stimulation was calculated. Stimulation with UTP further increased the current only of WT TRPC1 and TRPC1(1–650). Finally, similar results were obtained when the internal stores were passively depleted with ionomycin [See example in Figure 4G below for TRPC1(P646L)]. As expected, the spontaneously active TRPC1 mutant in this and subsequent figures

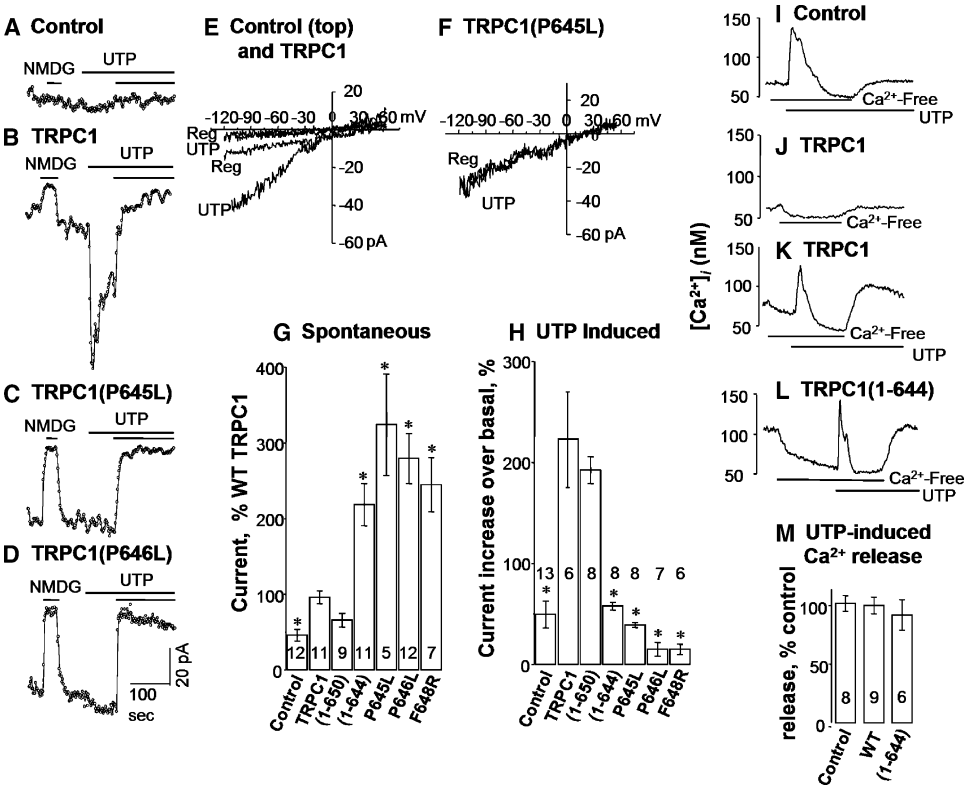

Figure 3. Effect of Mutations in the TRPC1 C-Terminal Homer Binding Site on Channel Activity

Control HEK293 cells (A) and cells transfected with wt-TRPC1 (B), TRPC1(P645L) (C), or TRPC1(P646L) (D) were used to measure the whole-cell current before and after stimulation with 100 μM UTP. Current was evaluated by replacing bath Na^+ with impermeant $NMDG^+$. In (B), note small spontaneous inward current that is blocked by $NMDG^+$, and a larger inward current induced by UTP and blocked by $NMDG^+$. In (C) and (D), note spontaneous inward current that is blocked by $NMDG^+$ and is not substantially increased by UTP. See Results for further description.

(E) shows the I/V relations recorded from control cells (upper traces) and cells transfected with TRPC1 before (Reg) and after (UTP) stimulation with UTP.

(F) shows the I/V curves for TRPC1(P645L).

(G) and (H) show the summary of the spontaneous current and the current induced by UTP stimulation, respectively, for the indicated constructs.

(I–L) show measurement of $[Ca^{2+}]_i$ in cells transfected with the indicated constructs. As indicated by the bars, the cells were bathed in Ca^{2+}-free medium and stimulated with UTP. In (J) the cells were not stimulated to show the effect of TRPC1 expression on spontaneous influx. After the incubation in Ca^{2+}-free, medium Ca^{2+} was restored to evaluate Ca^{2+} influx. The time scale is the same as (A–D). Cells expressing wt TRPC1 show a reduction of $[Ca^{2+}]_i$ upon removal of Ca^{2+} from the medium and a ~2-fold increase in $[Ca^{2+}]_i$ upon stimulation with UTP. By contrast, $[Ca^{2+}]_i$ is elevated in (L) and influx does not increase further with addition of UTP.

(M) shows the summary of peak $[Ca^{2+}]_i$ following UTP and confirms that Ca^{2+} stores are not altered in cells expressing TRPC1(1–644). The results are expressed as the mean ±S.E.M of the number of experiments listed in (G), (H), and (M), respectively. * denotes $P < 0.05$.

caused a sustained increase in $[Ca^{2+}]_i$. Resting $[Ca^{2+}]_i$ of control, nontransfected cells, cells transfected with WT TRPC1, and cells transfected with spontaneously active mutants averaged 55 ± 6 nM (n = 8), 63 ± 4 nM (n = 14), and between 105–120 with S.E. of between 3–7 nM (n = between 9–12), respectively.

Since TRPC1 and Homer interact with the IP_3Rs, a possible mechanism for the spontaneous activity of TRPC1 mutants is constitutive store depletion due to spontaneous activation of the IP_3Rs. To exclude this possibility, we measured store Ca^{2+} content by their discharge with UTP or ionomycin. Examples of such traces for UTP are shown in Figures 3I–3L. The cells were first incubated in Ca^{2+}-free medium, which revealed the increased Ca^{2+} permeability of the plasma membrane of cells transfected with the TRPC1(1–644) mutant. After stabilization of $[Ca^{2+}]_i$ the cells were stimulated with UTP or ionomycin (see Figure 4). No difference in the amplitude of Ca^{2+} increase was found between control cells and cells transfected with WT or the TRPC1 mutants.

Mutations of TRPC1 N-terminal Homer binding site were also examined and, as shown in Figure 4, produce similar increase in spontaneous current and loss of current activation by either UTP stimulation (Figures 4A–4D) or passive store depletion with ionomycin (Figures 4E–4I). Also, for these mutants we verified that spontaneous activity was not due to spontaneous store depletion. Figures 4J–4L show examples of how Ca^{2+} store content was evaluated by rapidly and maximally discharging the stores with ionomycin. Incubating the cells in Ca^{2+}-free medium revealed the increased plasma membrane Ca^{2+} conductance and ensured that only Ca^{2+} in the stores is being measured. None of the mutants used in this figure significantly reduced the amplitude of the Ca^{2+} signal evoked by ionomycin. The TRPC1(P23A)(1–644)

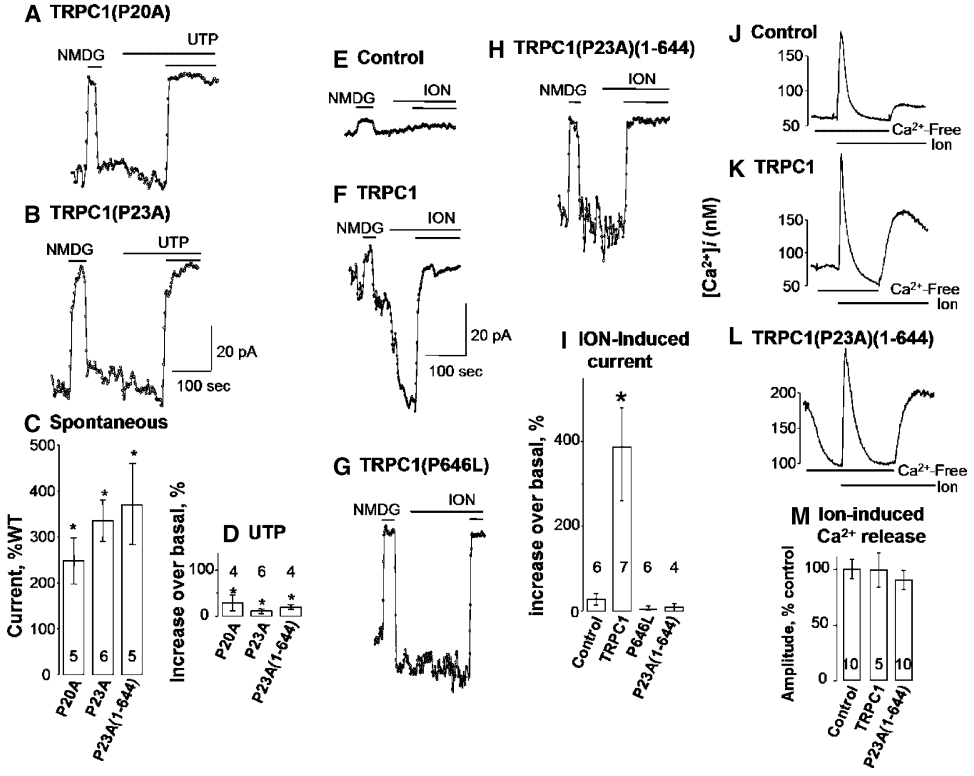

Figure 4. Effect of Mutations in the TRPC1 N-Terminal Homer Binding Site on Channel Activity

In (A–D), HEK293 cells were transfected with the indicated mutants of the N-terminal Homer binding site in TRPC1, and the spontaneous and UTP-activated currents were measured.

(C) and (D) show the summary of the effects of the mutations on spontaneous and UTP-induced currents, respectively. Control (E) cells and cells transfected with wt-TRPC1 (F), TRPC1(P646L) (G), or TRPC1(P23A) with a truncation of the C terminus that deletes the type 1 Homer ligand TRPC1(P23A)(1–644) (H) were used to measure the spontaneous current. Cells were incubated with 2 μM ionomycin to completely deplete the stores to estimate the extent of residual store-operated current. The results of multiple experiments are summarized in (I). Note ionomycin induces an inward current with wt TRPC1 (F) but produces little additional current with mutants (G and H). In (J–M), cells transfected with the indicated TRPC1 constructs were used to measure $[Ca^{2+}]_i$ and evaluate Ca^{2+} content in the stores released upon addition of 2 μM ionomycin (Ion). Subsequent addition of Ca^{2+} to the medium was used to evaluate Ca^{2+} entry and confirms TRPC1 channel expression. * denotes $P < 0.05$

mutant is illustrated since this construct includes changes in both the N and C termini that disrupt Homer binding and yet does not alter calcium stores in expressing cells. Thus, for both N- and C-terminal mutants, the spontaneously active state of TRPC1 channels is not a consequence of store depletion.

Expression of Homer 1a Versus 1b Differentially Modulates Gating of Wild-Type TRPC1

Homer is expressed in both crosslinking (Homer 1b/c, Homer 2a/b, Homer 3) and noncrosslinking (Homer 1a and Ania3) forms (Xiao et al., 2000). To examine the differential effect of these forms of Homer on TRPC1 function, we coexpressed Homer 1b or H1a with wt and mutant TRPC1 and estimated channel activity by measurement of Sr^{2+} entry detected by Fura 2 imaging. Several studies show that Sr^{2+} and Ba^{2+} entry specifically probe TRPC channels-mediated divalent ion permeability and distinguish it from native CCE [for example see Venkatachalam et al., 2001]. Coexpression of H1b and wt TRPC1 did not substantially alter basal channel permeability (Figure 5D) or the response to UTP (data not shown). By contrast, H1a coexpression resulted in increased basal channel activity as reflected in increased resting $[Ca^{2+}]_i$ and increased Sr^{2+} entry rates (Figures 5A and 5B). Thus, H1a coexpression with wt TRPC1 produced a channel that mimics TRPC1 mutants that do not bind Homer. These observations implicate the cross linking activity of Homer in establishing a state of the channel that can respond to agonist. This point was tested further by measuring the ability of Homer 1b to recouple the TRPC1-Homer mutants. Interestingly, H1b reduced basal channel activity when expressed with TRPC1(P645L) but not with other point mutants that disrupt Homer binding (Figures 5C, 5F, and 5G). By contrast, coexpression of H1a did not restore wt properties to TRPC1(P645L). The TRPC1(P645L) mutant is notable in that it retains some ability to bind Homer (Figure 2E).

Homer Contributes to the Association of TRPC and IP_3Rs

Previous studies indicate that interaction of the IP_3R with TRPC3 can activate the TRPC3 channel (Kiselyov et al., 1999). Accordingly, we examined the role of Homer interaction with TRPC1 in its association with the IP_3R.

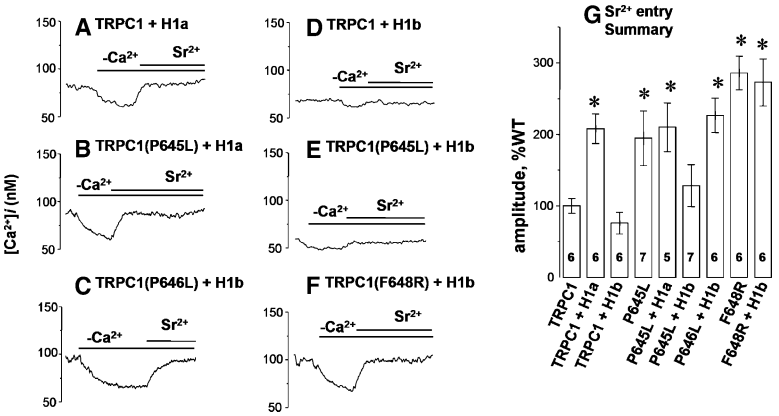

Figure 5. Opposing Effects of Homer 1a Versus 1b on Spontaneous Permeability of TRPC1 Mutants

HEK293 cells were cotransfected with the indicated TRPC1 mutants and either Homer 1a (A and B) or Homer 1b (C–F). After loading with Fura 2 the permeability to divalent ions was estimated, first incubating the cells in Ca^{2+}-free medium and recording the reduction of fluorescence and then incubating the cell in Ca^{2+}-free medium supplemented with 2 mM Sr^{2+}. Sr^{2+} does not pass native ion channels but is conducted by TRPC1 channels and detected by Fura 2. The summary of all experiments performed is listed in (G). Note that Homer 1a increases the spontaneous activity of wt-TRPC1 (see also Figure 5A) while having no further effect of the passive permeability of TRPC1(P645L) (B). By contrast, Homer 1b blocks spontaneous activity of TRPC1(P645L) (E) but not that of the other mutants.
* denotes $P < 0.05$

TRPC1 wt, and TRPC1 C-terminal point mutants were expressed in HEK293 cells and assayed for co-IP with native IP_3R. IP of TRPC1 resulted in co-IP of the IP_3R for wt and TRPC1(P645L) but not TRPC1(P646L) or TRPC1(F648R) (Figure 6A). These associations were confirmed in the reverse manner by IP with the anti-IP_3R Ab and blot for TRPC1 co-IP. The co-IP of IP_3R with TRPC1(P645L) was less robust than of wt TRPC1. This result recapitulates the observed reduction in co-IP of Homer and TRPC1(P645L) (Figure 2E). Thus, TRPC1 mutations produce parallel effects on association with Homer and IP_3R, suggesting that Homer contributes to

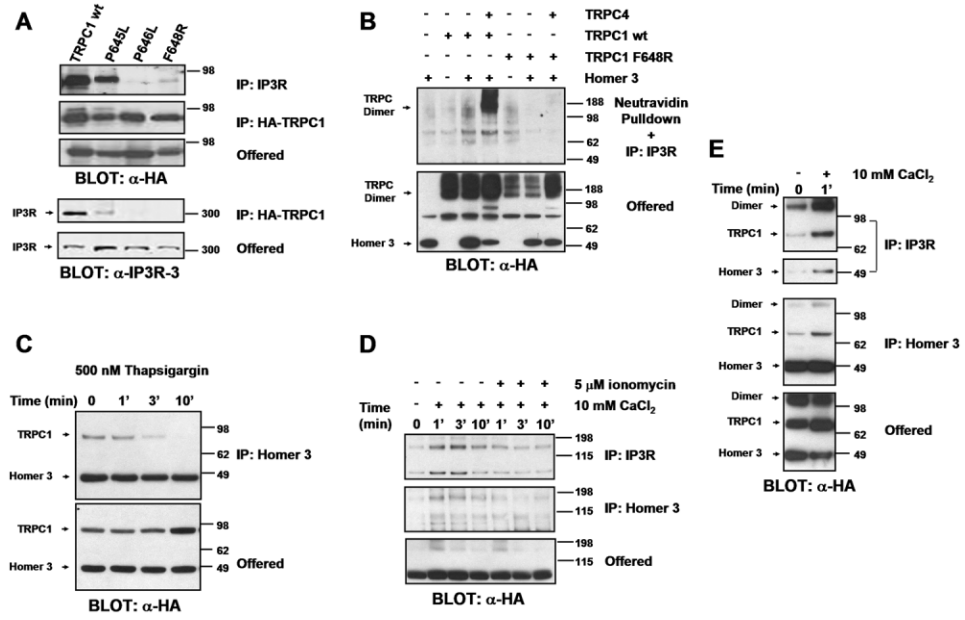

Figure 6. IP_3R Binding to TRPC1; Role of Homer and Store Ca^{2+}

(A) Mutations that disrupt Homer binding reduce TRPC1 association with the IP_3R. HEK293 cells were transfected with wt TRPC1 and C-terminal Homer site point mutants. Endogenous IP_3R3 co-IPs both wt TRPC1 and TRPC1(P645L) and vice versa. TRPC1(P646L) and TRPC1(F648R) do not co-IP with IP_3R3.

(B) IP3R coimmunoprecipitates surface-expressed (biotinylated) wt TRPC1 and wt TRPC1/TRPC4 dimers, but not TRPC1(F648R) or TRPC1(F648R)/TRPC4. HEK293 cells were transfected with the combinations as shown.

(C) Homer binding to TRPC1 is rapidly reduced by store depletion. Homer 3 and TRPC1 were coexpressed in HEK293 cells. Homer 3 co-IP of TRPC1 was assayed at 1, 3, or 10 min after addition of 500 nM thapsigargin to media (DMEM). Note Homer co-IP of TRPC1 is reduced within 3 min. Homer was consistently IPed at each time point.

(D) IP_3R and Homer association with TRPC1 increases upon Ca^{2+} store filling. HEK293 cells were transfected with TRPC1. TRPC1 co-IP with IP_3R Ab and Homer 3 Ab increased within 1 min of addition of 10 mM $CaCl_2$ and remained elevated to 10 min. This effect of added Ca^{2+} was blocked by simultaneous addition of ionomycin.

(E) TRPC1, Homer and IP3R are part of complex regulated by store Ca^{2+}. HEK293 cells were transfected with TRPC1 and Homer. Addition of 10 mM $CaCl_2$ induced parallel increases in TRPC1 co-IP with IP_3R Ab and with Homer 3 Ab. All Homer 3 and TRPC1 constructs were HA-tagged and detected with α-HA antibody.

the TRPC1-IP_3R interaction. Loss of this interaction is correlated with a spontaneously active TRPC1 channel.

TRPC4 and TRPC5 form functional heterodimers with TRPC1, and dimer TRPC channels are present in native tissues (Hofmann et al., 2002; Strubing et al., 2001). To examine the role of Homer in coupling of these channels with IP_3R, we coexpressed TRPC4 with TRPC1 or TRPC1(F648R), and assayed for co-IP with IP_3R Ab. To selectively assay dimers at the plasma membrane, expressing cells were first treated with sulfo-NHS-SS-biotin (Pierce) and biotinylated proteins were precipitated on neutravidin beads. Protein complexes were then dissociated from the beads with reducing agent (DTT) and IPed with anti-IP_3R Ab. TRPC1-TRPC4 heterodimer showed marked enrichment in this assay compared to TRPC1(F648R)-TRPC4, indicating that Homer interaction with TRPC1 is essential for establishment of the complex with IP3R at the membrane (Figure 6B). TRPC1 homodimers could also be detected in this assay, but were at the limit of detection, consistent with their lower expression at the membrane compared to TRPC1-TRPC4 (see also Figure 1). Similarly, Homer protein was detected with long exposures in the TRPC1-TRPC4 precipitate (data not shown). Homer proteins are enriched in neutravidin pull-downs of membrane TRPC1 homodimers and TRPC1-TRPC4 heterodimers indicating their prominent association with TRPC channels at the plasma membrane (Figure 1). Accordingly, the present result is consistent with the possibility that Homer dissociates from the complex during the more complicated IP procedure of the present assay. TRPC1 and IP_3R could remain in association by interactions that are Homer-independent or that are catalyzed by Homer (see Discussion). Nevertheless, these studies demonstrate the essential role for Homer binding to TRPC1 in forming TRPC channel complexes with IP_3R.

Homer Binding to TRPC1 Is Rapidly Regulated by Changes in Store Ca^{2+}

The present study suggests a model in which a physically coupled complex of TRPC1-Homer-IP_3R forms prior to its regulated gating by subsequent store depletion or agonist stimulation. To examine the dynamic features of this model, we monitored the TRPC1-Homer-IP_3R interaction under conditions that either deplete the Ca^{2+} stores or keep them at a filled state. HEK cells expressing wt HA-tagged TRPC1 and HA-tagged Homer 3 were treated with 500 nM thapsigargin, and Homer was IPed from the lysates. Thapsigargin inhibits the SERCA pumps in the endoplasmic reticulum and results in a time-dependent depletion of the ER Ca^{2+} store and opening of TRPC1 channels (Liu et al., 2000). TRPC1 co-IP with Homer was reduced within 3 min of addition of thapsigargin to the media and was further reduced by 10 min (Figure 6C). The time course is slightly slower than thapsigargin effects in physiological recordings, where media change is also more rapid. This result suggests that the Homer-TRPC1 interaction is interrupted upon depletion of the ER Ca^{2+} store. In this experiment, thapsigargin is anticipated to also result in a modest rise in cytosolic Ca since it was added to cells in DMEM without depletion of extracellular Ca^{2+}. Deletion of Ca^{2+} from the medium by addition of EGTA produced variable effects that appeared time and [EGTA] dependent. Accordingly, additional experiments were performed to assess the role of store Ca^{2+}.

To examine the effect of increased store Ca^{2+}, TRPC1 expressing HEK cells were treated by addition of 10 mM Ca^{2+} to the medium and assayed for TRPC1-Homer (native) and TRPC1-IP_3R associations by co-IP. Incubation in 10 mM Ca^{2+} for 15 min increased store Ca^{2+} content by 19 $\pm$ 5% ($n = 6$, $p < 0.05$), as assayed by the subsequent discharge of stored Ca^{2+} with ionomycin in a Ca^{2+}-free media. The amount of TRPC1 that co-IPed with Homer increased within 1 min of the addition of Ca^{2+} and remained elevated at 3 min and 10 min (Figure 6D). The amount of TRPC1 that co-IPed with IP_3R Ab showed a similar time course of increase. The dimer species of TRPC1 was strikingly enriched in the immunoprecipitates with both Homer and IP_3R relative to the total lysates. Since dimer species are enriched at the plasma membrane (Figure 1B), their enrichment in these immunoprecipitates is consistent with changes of the TRP-Homer-IP_3R complex at the membrane. It is notable that addition of extracellular Ca^{2+} is expected to produce a modest increase in cytosolic Ca^{2+}, much like addition thapsigargin in 6C. Since these manipulations produce opposite effects on TRP-Homer associations, they together support the notion that store Ca^{2+} is the critical regulator of the TRP-Homer-IP3R assembly. Moreover, the effect of extracellular Ca^{2+} was blocked if added in combination with ionomycin, which depletes store Ca^{2+}. These experiments indicate that ER Ca^{2+} load dynamically controls TRPC1-Homer and TRPC1-IP_3R associations. Depletion of the stores is followed by dissociation of TRPC1-Homer complex, which coincides with activation of TRPC1 and SOCs. Reloading of the stores induces assembly of the TRPC1-Homer-IP_3R complex and coincides with cessation of SOCs activation and Ca^{2+} influx.

The dynamic assembly induced by addition of extracellular Ca^{2+} offered an experimental paradigm to examine the TRPC1 molecular complex. In experiments that rely on native Homer, TRPC1 is detected in immunoprecipitates with either Homer Ab or IP_3R Ab, but it proved technically difficult to detect Homer in the IP_3R immunoprecipitates. To confirm that Homer is present in the IP_3R immunoprecipitates we examined HEK293 cells expressing HA-tagged TRPC1 and HA-Homer. As anticipated, addition of 10 mM Ca^{2+} resulted in a marked increase in IP_3R Ab pull-down of TRPC1 (Figure 6E). In the same immunoprecipitates, Homer showed similar increased association with the IP_3R. Moreover, immunoprecipitation of transgene Homer showed an induced increase in association with TRPC1. These studies confirm the presence of a molecular complex that includes TRPC1, Homer, and IP_3R, and is regulated by Ca^{2+} store.

Homer Proteins Alter Native Store-Operated Channel Activity

The effect of H1a to induce spontaneous TRPC1 channel activity provided a tool to examine the role of Homer in regulating native store-operated channels. The human salivary gland cell line (HSG) possesses a prominent SOC and expresses TRPC1 (Liu et al., 2000). To examine the activity of Homer proteins on native ion channels,

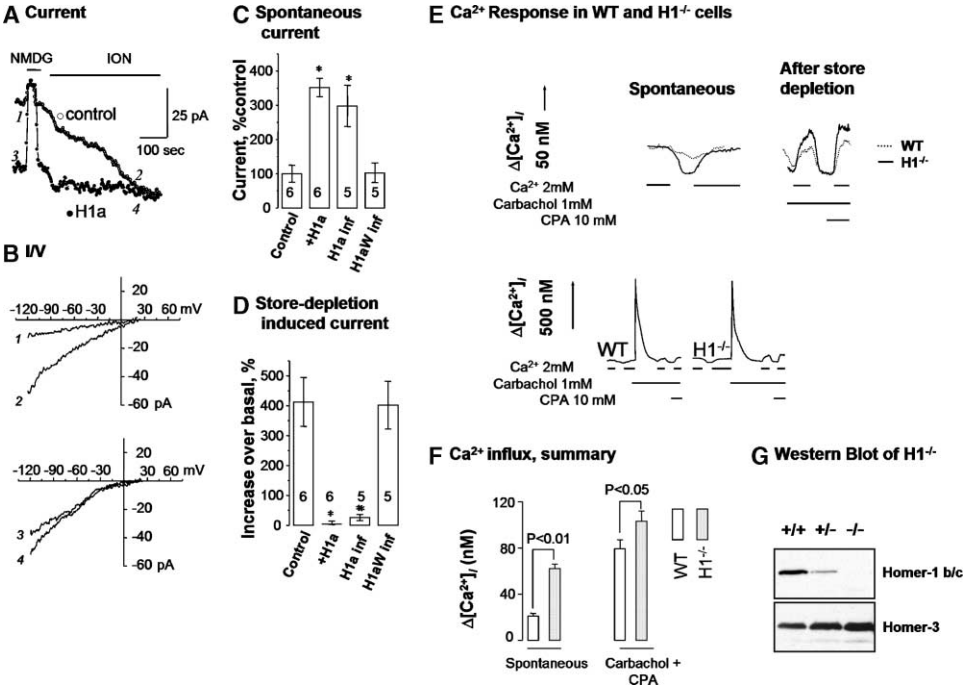

Figure 7. Homer 1a Induces Spontaneous Activity of Native SOCs and Deletion of Homer 1 Increases Spontaneous Ca^{2+} Influx

(A–D) Store-operated channels (SOCs) of the submandibular HSG cell line were activated by including 10 mM BAPTA in the pipette solution and depleting the stores with 2 μM ionomycin. The open circles in (A) show the time course of activation of SOC current in these cells after exposure to ionomycin. The same cell was infused with Homer 1a by including 5 μg/ml of recombinant Homer 1a in the pipette solution, and current was measured before and after treatment with ionomycin (close circles in A). Note that ionomycin produces a progressive increase in the inward current that is occluded by including H1a in the pipette. The traces in (B) show the I/V relations recorded at the times marked in (A). Traces (1 and 2) are from the control cell and traces (3 and 4) are from the cell infused with Homer 1a.

(C) depicts the spontaneous current and (D) the current activated by ionomycin in control cells and cells transfected with Homer 1a (+H1a) or cells infused with 5 μg/ml recombinant Homer 1a (H1a inf) or recombinant Homer 1a(W27A) mutant (H1aW inf). Note that transfection and infusion of Homer 1a similarly activate native SOCs. * denotes $P < 0.05$.

(E–G): Pancreatic acinar cells isolated from WT and Homer $1^{-/-}$ mice were loaded with Fura 2 to record spontaneous and store-depletion induced $[Ca^{2+}]_i$. As indicated by the bars [E, lower trace WT (left) and Homer $1^{-/-}$ (right)], the cells were perfused with standard bath solution containing 2 mM Ca^{2+} or 0.2 mM EGTA (Ca^{2+} free conditions) and stimulated with 1 mM carbachol with 100 μM of the SERCA pump inhibitor CPA to insure maximal store depletion. The upper traces show the superimposed influx measured in cells from WT (dashed lines) and Homer $1^{-/-}$ mice (solid traces) before ("Spontaneous") and after ("After store depletion") carbachol/CPA treatment. Spontaneous Ca^{2+} influx was increased 2.5-fold in H$1^{-/-}$ compared to WT mice. Depletion of Ca^{2+} stores in H$1^{-/-}$further increased Ca^{2+} influx by only 1.7-fold.

(F) shows the summary of the results obtained from multiple experiments (n = 3 mice, 13 acini). Acini were stimulated with a maximal concentration of carbachol in Ca^{2+} free medium (E; middle traces). Note that the agonist-mobilized Ca^{2+} stores are similar in both cell types. Spontaneous Ca^{2+} influx is increased in H$1^{-/-}$ cells compared to wt cells ($P < 0.01$). Generation of Homer $1^{-/-}$ mice is described in methods.

(G) demonstrates absence of Homer 1, and preservation of Homer 3, immunoreactivity in hippocampus of Homer $1^{-/-}$ mice.

we either transfected Homer expression constructs or added recombinant Homer proteins to the pipette solution (Figure 7). SOC was activated by including 10 mM BAPTA in the pipette solution and depleting the stores with 2 μM ionomycin. Transfection of H1a resulted in spontaneous SOC activation and no further current was activated by exposing the cells to ionomycin (Figure 7A). Infusing the cells with recombinant H1a was similarly effective in channel activation, whereas the mutant H1a-W27A was without effect (Figures 7A, 7C, and 7D). Image 7B shows the current voltage relations at the indicated times. These findings are consistent with those made with expressed TRPC1 and suggest that Homer proteins participate in regulation of the native SOC channels.

To further demonstrate the role of Homer proteins in native cells, we analyzed mice from which Homer 1 was deleted and measured Ca^{2+} influx in acini prepared from pancreas. SOC was monitored using a Ca^{2+} removal-Ca^{2+} readdition protocol, as illustrated in Figures 7E–7F. The lower traces in Figure 7E show that stimulation of cells with maximal concentration of carbachol increased $[Ca^{2+}]_i$ to similar levels in cells from WT and Homer $1^{-/-}$ mice, indicating that deletion of Homer 1 did not interfere with signaling from the receptor to Ca^{2+} release by IP_3. Additional experiments comparing wt and Homer $1^{-/-}$ mice verified intact carbachol-induced activation of PLCβ and IP_3 production, IP_3-mediated Ca^{2+} release, Ca^{2+} pumping into the ER by SERCA pumps and Ca^{2+} efflux across the plasma membrane (Shin et al., 2003). Exposing the cells to Ca^{2+}-free media prior to stimulation assessed spontaneous influx and revealed increased spontaneous Ca^{2+} influx in cells from Homer $1^{-/-}$ cells (Figures 7E and 7F). Deletion of Homer 1 increased the spontaneous Ca^{2+} influx by approximately 2.5-fold compared to cells from wt mice (left upper trace). Depletion of Ca^{2+} stores of these cells further increased Ca^{2+} influx by only 1.7-fold. By contrast, similar depletion of Ca^{2+} stores in cells from WT mice in-

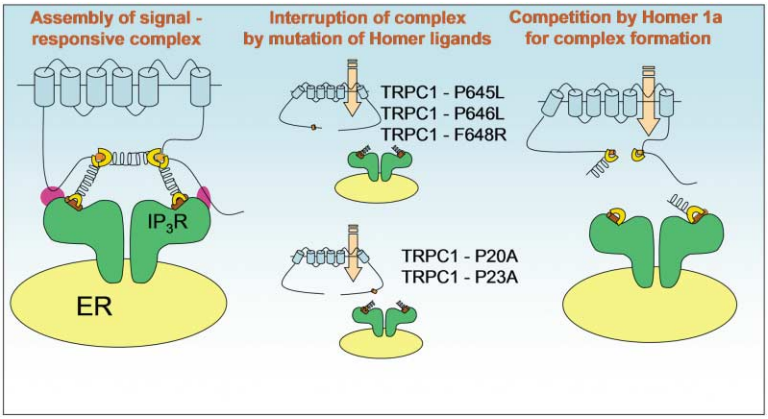

Figure 8. Homer-Assisted Formation of the TRPC-IP_3R Complex

In the signal responsive complex, Homer binding to TRPC1 couples the channel to the IP_3R. Cooperative Homer binding to the N- and C termini of the channel is indicated. Additional interactions (magenta) may contribute to complex formation and localize the TRPC channel at signaling microdomains. Interruption of Homer binding to either site of TRPC1 results in a spontaneously active channel (arrow) that is not further activated by store depletion or agonist stimulation. Expression of H1a, which lacks the coiled-coil domain, interrupts coupling and results in a more active channel.

creased Ca^{2+} influx 3.2-fold (right upper trace). Thus, Homer $1^{-/-}$ results in an increase in the ratio of spontaneous to store-depletion current [26% for wt and 59% for Homer $1^{-/-}$ $p > 0.01$]. Deletion of Homer 1 also resulted in about 1.3-fold upregulation of Ca^{2+} influx. Hence, deletion of Homer 1 was sufficient to increase spontaneous Ca^{2+} influx in native cells. The residual coupled influx may indicate adaptation of the cells to the deletion of Homer 1 or residual coupling of the Ca^{2+} influx channels to Homer 2 and 3. We suggest that the more complete effect of H1a addition in the above experiments is consistent with its ability to inhibit crosslinking by all Homer isoforms.

Discussion

The present study supports a model in which Homer proteins regulate a physical interaction between TRPC1 and IP_3R that is critical for gating of TRPC1 (Figure 8). Homer proteins that are capable of crosslinking facilitate the formation of a complex in which the TRPC1 channel is inactive, but is available for activation by agonists and/or store depletion. The immediate early gene form of Homer (H1a) does not facilitate assembly. The assembly and disassembly process is dynamically responsive to physiological manipulations that close or open the TRPC channel, respectively. Thus, TRPC1 channel activation appears be mechanistically linked to interruption of the Homer-dependent interaction.

TRPC1 mutants that do not associate with Homer form spontaneously active channels. Several observations indicate that this property of the TRPC1 channel is indicative of a natural regulatory mechanism and are not simply an artifact of mutation. In a gain-of-function experiment, gating of a TRPC1 mutant with reduced, but not absent, Homer binding [TRPC1(P645L)] is restored by overexpressing Homer 1b. This indicates the TRPC1 mutant channel can function normally if sufficient Homer 1b is present and demonstrates the subtle and specific impact of the mutation. Moreover, a spontaneously active state is induced in wt TRPC1 by H1a. Effects of Homer appear to be relevant to native SOC as H1a induces spontaneous SOC activity in salivary gland cells. Furthermore, Homer $1^{-/-}$ mice show increased spontaneous Ca^{2+} influx, consistent with a role for Homer in assembling the native SOC.

The Homer interaction with TRPC1 is also essential for formation of complexes between the TRPC1-TRPC4 heterodimer and IP_3R. TRPC1 is known to form functional heterodimers with other TRPC family members that possess unique channel properties (Hofmann et al., 2002; Strubing et al., 2001). Because TRPC1-TRPC4 heterocomplexes are enriched at the plasma membrane relative to TRPC1 homodimers, they facilitate biochemical studies of the complex as it assembles at the membrane. While Homer Ab efficiently IPs surface TRPC1-TRPC4 complexes (Figure 1), Homer is not equivalently abundant in precipitates with IP_3R Ab that pulldown TRPC1-TRPC4. Since Homer binding to TRPC1 is essential to form the membrane complex of TRPC1-TRPC4, these data suggest that Homer may function in a role other than a simple physical tether. Recent studies of ryanodine receptor function indicate a potent effect of Homer to increase the open channel probability, and this effect that does not require Homer's crosslinking activity (Feng et al., 2002). Together with the observation that Homer binds to a specific conformation of its proline rich ligand (Beneken et al., 2000), these data suggest that Homer could function as a catalyst for conformational assembly (Fagni et al., 2002).

The physical association between TRPC1 and IP_3R is likely to be complex and involve interactions in addition to those regulated by Homer. For example, peptides derived from the N terminus of the IP_3Rs are able to activate TRPC1, TRPC3, and TRPC4 (Boulay et al., 1999; Tang et al., 2001). Interestingly, IP_3R peptides compete with calmodulin for binding to TRPC channels (Tang et al., 2001), and addition of calmodulin prolongs the interval between Ca^{2+} release from intracellular stores and activation of Ca^{2+} influx (Vaca and Sampieri, 2002). These findings suggest a model in which calmodulin blocks IP_3R-dependent activation of the channel. However, we note that deletion of the calmodulin binding sequences in TRPC1 [TRPC1(1–650)] did not result in spontaneously active channels as might be expected if calmodulin binding effectively inhibits the channel. Since calmodulin binding affinity increases with elevated Ca^{2+}, this mechanism may be important for certain adaptations and be influenced by cytosolic Ca^{2+}. By contrast, the interaction of TRPC1 with Homer is essential for channel gating in normal HEK293 cells, and effects of Homer that support our model are evident in

the human salivary gland cell line and pancreatic acinar cells of the Homer $1^{-/-}$ mouse.

Analysis of Homer binding to TRPC1 provided several observations that may be important for understanding how Homer binding is regulated. First, TRPC family members selectively associate with different Homer family proteins. Such Homer specificity has not been noted previously and is striking in view of the high degree of conservation of the Homer EVH1 domains (Xiao et al., 1998). Second, the Homer binding site in the N terminus of TRPC1 (LPSSP) does not include a phenylalanine, unlike previous Homer binding sites (PXXF) (Beneken et al., 2000). We refer to the TRPC1 N-terminal site as a type 2 Homer ligand to distinguish it from ligands with conventional PPXXF sequence (type 1). Interestingly, a recent crystal structure of Homer 1 identifies a type 2-like proline motif (P-motif) that is present in Homer itself, C-terminal to the EVH1 domain (Irie et al., 2002). The P-motif contacts surfaces that partially overlap the type 1 ligand site. A similar P-motif is present in Homer 3, but not Homer 2. Third, binding studies of TRPC1 mutants indicate complex interactions between sites in the N and C termini of TRPC1. This suggests that intramolecular interactions of the target protein can also influence Homer binding.

The present study identifies the TRPC channel as a target for regulation by a cellular immediate early gene. While H1a was originally cloned from seizure-stimulated brain (Brakeman et al., 1997), the transcript can be detected in multiple tissues and cell lines (Soloviev et al., 2000a and P.F.W., unpublished data). In a normal brain, H1a induction is so closely associated with neuronal activity that its transcription can be used to detect neural networks involved in spatial learning (Vazdarjanova et al., 2002). TRPC1 is abundantly expressed in brain and in recent studies, we find that it mediates the mGluR1-dependent delayed inward current (Kim et al., submitted). If H1a plays a role in the dynamic assembly of TRP-IP_3R complexes, it would be expected to increase the duration of channel opening by competing with cross-linking Homer as the complex reassembles. It is therefore compelling to suggest that H1a represents a transcription and protein synthesis-dependent mechanism that regulates the communication between membrane TRPC channels and intracellular stores as a function of the activity history of the cells.

Experimental Procedures

In Vitro Binding and Protein Expression

GST fusion constructs were prepared by polymerase chain reaction with specific primers that included SalI NotI sequences and subcloned into pGEX4T-2 vector (Pharmacia Biotech, Uppsala, Sweden). Constructs were confirmed by sequencing. GST-fusion proteins were expressed in BL21 bacterial strains. Bacteria were harvested and lysed in PBS, 1% Triton X100, 2 mM phenylmethylsulfonyl fluoride (PMSF), and pelleted at 13,000 rpm (Sorvall SS-34) at 4°C for 5 min. Proteins were purified by incubating 1.5 ml bed volume glutathione-Sepharose (GST) beads (Sigma) with bacterial supernatant at 4°C for 30 min and washing twice with PBS and PBS plus 1% Triton X-100.

HEK293 cells were transfected with 10 μg of cDNA per 10 cm dish using calcium phosphate method and incubated at 37°C, 5% CO_2 for 2 overnights. Cells were lysed using 1 ml of binding buffer: 1× PBS buffer + 5 mM EDTA, 5 mM EGTA, 1 mM NaVO3, 10 mM NaPyrophosphate, 50 mM NaF [pH 7.4] containing 1% Triton X-100, and 100 μl of lysate were used for GST-pull-down assays, rocking for 2 hr at 4°C. GST beads were washed 3 times 10 min with 1× PBS, and SDS loading buffer was added.

Mutant Construction and Expression

TRPC cDNAs used in the experiments include: TRPC1: NM_003304; TRPC2: NM_011644; TRPC3: NM_003305; TRPC4: NM_016984; TRPC5: NM_009428; and TRPC6: NM_004621. The N-terminal 948 nucleotide (316 aa) fragment of human TRPC1 was amplified by polymerase chain reaction. Deletion mutants of TRPC1(Δ644–760 and Δ650–760) were made by PCR. Full-length N- and C-terminal point mutants and point mutants, in the N-terminal fragment were made using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The sequences of the primers used to generate each mutant will be supplied on request. All constructs were cloned into mammalian expression vector pRK5 (Genentech) with an N-terminal HA-tag.

Immunoprecipitation Assays

Adult rats were sacrificed by decapitation, and brain regions dissected, sonicated in 3 ml of binding buffer containing 1% CHAPS or 1% Triton X-100 and protease inhibitor cocktail (~100 mg wet weight/ml). The homogenate was centrifuged at 37,000 × g for 20 min at 4°C. 100 μl of the cerebellar or hippocampal extract were used for each immunoprecipitation assay with the following antibodies: 2 μl of crude Homer 1 or Homer 3 antibodies (Xiao et al., 1998). For HEK293 cell lysates, 100 μl of 1 ml were used for each immunoprecipitation assay with 2 μl of crude Homer 1, 2, or 3 antibodies; or 2 μl of crude IP_3R antibody (gift from Alan Sharp; Nucifora et al., 1996). For experiments that examined effects of thapsigargin or Ca^{2+} addition to IP3R-Homer-TRPC1 interaction, HEK293 cells were transfected as described. Before harvesting, cells were treated with 500 nM of thapsigargin or 10 mM of $CaCl_2$ for 1, 3, or 10 min. Cells were lysed in 1% TX-100, and co-IP assays were performed.

Antibodies and extracts were incubated for 30 min at 4°C, then 50 μl of 1:1 protein A or protein G (for goat Ab) Sepharose slurry was added. The antibody/extract/beads were incubated for an additional 90 min at 4°C. After washing three times for 10 min each in 1% TX-100 + PBS buffer, the proteins were eluted from the beads with 40 μl of 4% SDS loading buffer, and 20 μl were analyzed by SDS-PAGE and immunoblot.

Biotinylation Assays

Tissue culture plates were coated with poly-L-ornithine. HEK293 cells were transfected with cDNA and after a 2 overnights were washed 3 times 1× PBS, biotinylated with 0.5 mg/ml of sulfo-NHS-SS-biotin (Pierce) for 30 min, and quenched 3 × 5 min with 50 mM glycine. All of the above steps were done on ice. Cells were lysed in 1 ml of 1% Triton X-100, and 50 μl of 1:1 slurry of immobilized neutravidin beads (Pierce) was incubated with 100 μl of lysate for 2 hr at 4°C. Beads were washed 3 times 1× PBS, and SDS loading buffer was added. IP_3R coimmunoprecipitation of biotinylated TRPC1 and/or TRPC4 was performed using the method from Klingelhofer et al. (2003).

Measurement of $[Ca^{2+}]_i$ and Divalent Ions Permeability

$[Ca^{2+}]_i$ and divalent ions permeability were measured with the aid of Fura 2. The standard bath solution for $[Ca^{2+}]_i$ measurement contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES-KOH (adjusted to pH 7.4 with NaOH), 10 glucose, and either 1–2 mM $CaCl_2$ or 0.1–0.2 mM EGTA (Ca^{2+}-free). Agonists and other additives were included in the bath perfusate. HEK 293 Cells were grown on glass coverslips and transfected with the desired construct using lipofectamine reagent. Twenty-four to forty-eight hr posttransfection, the cells were loaded with 2 μM Fura 2 acetoxymethyl ester by 30 min incubation at 37°C. Pancreatic acinar cells were prepared by standard collagenase digestion procedure, as described before (Shin et al, 2000) and loaded with 5 μM Fura 2 acetoxymethyl ester by 20 min incubation at room temperature. After loading, cells were washed and incubated in extracellular medium for 20 min to allow completion of Fura 2/AM hydrolysis. Fura 2 fluorescence of HEK293 cells was measured by illuminating the cells with an alternating 340/380 nm light delivered every 0.5 s. Fluorescence intensity at 510 nm was measured with a DeltaRAM fluorimetric system (PTI Inc.,

Monmouth Junction, NJ). Changes in $[Ca^{2+}]_i$ are presented as the change in the ratio of fluorescence intensity at 340 and 380 nm. Fura 2 fluorescence of pancreatic acini was measured by an image acquisition system from PTI. For both cell types $[Ca^{2+}]_i$ was calculated using calibration curve obtained by exposing cells permeabilized to Ca^{2+} with 10 μM ionomycin to solutions containing 2 mM Ca^{2+} and 10 mM EGTA. Membrane permeability to divalent ions was estimated by replacing Ca^{2+} in the bath solution with Sr^{2+} or Ba^{2+} and measuring the rate and extent of fluorescence increase.

Electrophysiology

TRPC1 activity was evaluated from measurement of whole-cell current. The extracellular solution for whole-cell current measurement was the bath solution specified above that was used for measurement of $[Ca^{2+}]_i$. The pipette solution contained (in mM): 140 cesium-aspartate, 5 NaCl, 1 $MgCl_2$, 1 ATP, 10 HEPES, 0.1, 0.5, 2, or 10 EGTA-CsOH (or 10 BAPTA) (adjusted to pH 7.3 with CsOH). Current was recorded using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA) and digitized at 2 kHz. Throughout the experiment, the membrane potential was held at 0 mV to minimize Ca^{2+}-dependent inactivation of currents. The membrane conductance was probed with consecutive rapid (250 ms) alteration of membrane potentials ("RAMPs") from −120 to +60 mV delivered every 2 s. Pipettes had resistance between 5 and 7 MΩ when filled with an intracellular solution, and seal resistance was always more the 4 GΩ. Current recording and analysis was performed with the use of pClamp 6.0.3 (Axon Instruments) software suite. Experiments were carried out at room temperature (22–24°C). Results are given as mean ± SEM.

Generation of Homer 1 Mutant Mice

The Homer 1 targeting construct was generated from 14 kb SV129 mouse genomic DNA insert from a Stratagene lambda Fix2 library containing exons 2, 3, and 4 of the Homer 1 gene (MKS and PHS). A SV40-NEO cassette containing a single loxP site and two Frt sites from vector pSVKeoX-FRT2 was inserted into the unique BamHI site ~700 bp downstream of exon 2. The single EcoRI site located ~200 bp upstream of exon 2 was utilized to insert an ApoI-PacI-LoxP-EcoRI linker containing the second LoxP element. In order to check the transcription level of the targeted allele, five silent mutations were introduced into exon 2 (TGACCCGAACACAAA to CGAT CCAAATACCAA). The resulting targeting construct was linearized by SpeI digestion and electroporated into R1 ES cells. Cells were selected by G418. Positive clones were picked and homologous recombination was confirmed by Southern blotting. Clones were chosen and injected into blastocysts by standard methods and chimeras were mated to C57BL/6 mice to produce Homer 1 heterozygotes that were then mated sequentially to Flpe (Rodriguez et al., 2000) and CMV-Cre (Schwenk et al., 1995) transgenic mice to remove Neo and Exon 2 respectively, thus creating the functional knockout. Recombination events were confirmed by Southern blot and routinely monitored by PCR genotyping.

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