Ca\(^{2+}\) Signaling in Microdomains

Homer1 MEDIATES THE INTERACTION BETWEEN RyR2 AND Cav1.2 TO REGULATE EXCITATION-CONTRACTION COUPLING*

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Excitation-contraction (E-C) coupling and Ca\(^{2+}\)-induced Ca\(^{2+}\) release in smooth and cardiac muscles is mediated by the L-type Ca\(^{2+}\) channel isoform Ca\(_{1.2}\) and the ryanodine receptor isoform RyR2. Although physical coupling between Ca\(_{1.1}\) and RyR1 in skeletal muscle is well established, it is generally assumed that Ca\(_{1.2}\) and RyR2 do not directly communicate either passively or dynamically during E-C coupling. In the present work, we re-examined this assumption by studying E-C coupling in the detrusor muscle of wild type mice. In the present work, we re-examined this assumption by studying E-C coupling in the detrusor muscle of wild type and Homer1\(^{-/-}\) mice and by demonstrating a Homer1-mediated dynamic interaction between Ca\(_{1.2}\) and RyR2 using the split green fluorescent protein technique. Deletion of Homer1 in mice (but not of Homer2 or Homer3) resulted in impaired urinary bladder function, which was associated with higher sensitivity of the detrusor muscle to muscarinic stimulation and membrane depolarization. This was not due to an altered expression or function of RyR2 and Ca\(_{1.2}\). Most notably, expression of Ca\(_{1.2}\) and RyR2 tagged with the complementary C- and N-terminal halves of green fluorescent protein and in the presence and absence of Homer1 isoforms revealed that H1a and H1b/c reciprocally modulates a dynamic interaction between Ca\(_{1.2}\) and RyR2 to regulate the intensity of Ca\(^{2+}\)-induced Ca\(^{2+}\) release and its dependence on membrane depolarization. These findings define the molecular basis of a “two-state” model of E-C coupling by Ca\(_{1.2}\) and RyR2. In one state, Ca\(_{1.2}\) couples to RyR2 by H1b/c, which results in reduced responsiveness to membrane depolarization and in the other state H1a uncouples Ca\(_{1.2}\) and RyR2 to enhance responsiveness to membrane depolarization. These findings reveal an unexpected and novel mode of interaction and communication between Ca\(_{1.2}\) and RyR2 with important implications for the regulation of smooth and possibly cardiac muscle E-C coupling.

The Homer family of scaffolding proteins consists of three members, Homer1, Homer2, and Homer3, and several splice variants (1). The Homers are typified by an N-terminal Ena/VASP homology 1 (EVH) domain and C-terminal coiled coil and leucine zipper domains. The EVH domain is a protein-protein binding module that recognizes the proline-rich motifs PXPF, PXPF, and LPSSP (2), whereas the coiled coil domain and leucine zippers serve to multimerize the Homers into scaffolds that assemble signaling proteins into complexes (1). The Homer1 gene is unique in that it codes for the short Homer1a (H1a) and a long Homer1b/c (H1b/c) (3). H1a lacks the coiled coil domain and leucine zipper, and it antagonizes the functions of the multimerizing Homers by dissociating the complexes formed by them.

Many Ca\(^{2+}\) signaling proteins express Homer binding sequences, including G protein-coupled receptors, plasma membrane Ca\(^{2+}\) ATPase pumps, IP\(_{3}\) receptors, canonical transient receptor potential (TRPC) channels, and notably, the ryanodine receptors (RyRs) and the α1C subunit of Cav1.2 and α1D subunit of Cav1.3 L-type Ca\(^{2+}\) channels (1, 2, 4–7). The Homers recruit the Ca\(^{2+}\) signaling proteins to the plasma membrane (8) and cellular microdomains, such as dendritic spines (9), but are not essential for assembly or retention of the complexes, because deletion of the Homers does not affect localization of the complexes (10). On the other hand, the Homers modulate the efficiency (2) and intensity of Ca\(^{2+}\) signaling (10) by negatively regulating the activity of proteins within the Ca\(^{2+}\) signaling complex.

Another important function of Homer1 is the mediation of the conformational coupling between IP\(_{3}\)Rs and TRPC channels. Thus, Homer1 binds TRPC channels and the IP\(_{3}\)Rs to allow the gating of TRPC channels by IP\(_{3}\) (2, 11). This form of coupling is reminiscent of excitation-contraction (E-C) coupling in muscle, which is mediated by the sarcoplasmic voltage-activated L-type Ca\(^{2+}\) channels and the RyRs Ca\(^{2+}\) release channels in the sarcoplasmic reticulum (12–14). Homer1 may play a role in E-C coupling, because it was reported to activate

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The abbreviations used are: EVH, Ena/vasodilator-stimulated phosphoprotein homology 1; E-C, excitation-contraction; CICR, Ca\(^{2+}\)-induced Ca\(^{2+}\) release; RyR2, ryanodine receptor type 2; [Ca\(^{2+}\)]\(_e\), free intracellular Ca\(^{2+}\) concentration; HEK, human embryonic kidney; H1a, Homer1a; H1b/c, Homer1b/c; GFP, green fluorescent protein; TRPC, canonical transient receptor potential; IP\(_{3}\), inositol 1,4,5-trisphosphate; WT, wild type; DSM, detrusor smooth muscle; IP\(_{3}\)R, IP\(_{3}\) receptor.
the skeletal and cardiac muscle RyR isoforms RyR1 (15–17) and RyR2 (18), respectively. Furthermore, recently it was suggested that the short, dissociating H1a activates L-type Ca\(^{2+}\) current in neocortex pyramidal cells (19).

There are three forms of E-C coupling, depending on muscle type (12–14). Skeletal muscle displays mechanical coupling between the skeletal muscle L-type Ca\(^{2+}\) channel isoform Ca\(_{1.1}\) and RyR1, whereby the Ca\(^{2+}\) release units are organized in tetraads mediated by physical interaction between the cytosolic domains of RyR1 and Ca\(_{1.1}\) (12). The activating depolarization is sensed by Ca\(_{1.1}\) and is directly conveyed to RyR1 to initiate Ca\(^{2+}\) release that then propagates by a Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) mechanism. In cardiac muscle, RyR2 communicates with the cardiac muscle L-type Ca\(^{2+}\) channel Ca\(_{1.2}\), but the channels do not physically interact. CICR is initiated by Ca\(^{2+}\) influx through Ca\(_{1.2}\) that then activates RyR2 (13). However, at the cardiac Ca\(^{2+}\) release units, RyR2 and Ca\(_{1.2}\) are in close proximity, and Ca\(^{2+}\) entering through Ca\(_{1.2}\) is rapidly sensed by RyR2 (12, 13). Although all RyR isoforms were reported to be expressed in smooth muscle, RyR2 is the major isoform in this muscle type (20), including the urinary bladder detrusor muscle (21). Ca\(^{2+}\) influx during E-C coupling of smooth muscle is mediated by Ca\(_{1.2}\) (22). However, E-C coupling in smooth muscle was termed loose coupling (14) based on the relatively slow rate of activation of CICR (23).

The properties of E-C coupling and CICR in cardiac and smooth muscles led to the belief that Ca\(_{1.2}\) and RyR2 do not directly communicate either passively or dynamically during E-C coupling (12–14, 24). We re-examined this assumption by studying E-C coupling in the detrusor muscle of wild type (WT) and Homer1\(^{-/-}\) mice and by demonstrating a Homer1-mediated interaction between RyR2 and Ca\(_{1.2}\) using the split GFP technique. We report that Homer1 modulates a dynamic interaction between Ca\(_{1.2}\) and RyR2 to regulate the intensity of CICR and its dependence on membrane depolarization. These findings define the molecular basis of a “two-state” model of E-C coupling by Ca\(_{1.2}\) and RyR2. In one state, Ca\(_{1.2}\) couples to RyR2 by H1b/c, which results in reduced responsiveness to membrane depolarization, and in the other state, H1a uncouples Ca\(_{1.2}\) and RyR2 to enhance responsiveness to membrane depolarization. These novel forms of interaction between RyR2 and Ca\(_{1.2}\) have important implications for smooth and possibly cardiac muscle E-C coupling.

**EXPERIMENTAL PROCEDURES**

**Materials and Solutions**—Fura-2/AM and Fluo-3/AM were from Teff Laboratories, Inc., Indo1 was from Molecular Probes, anti-α1C subunit of Ca\(_{1.2}\) antibodies were from Alomone, and anti-RyR2 antibodies C3–33 were from Sigma. The standard bath solution contained (in mM) 140 NaCl, 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 10 HEPES (pH 7.4 with NaOH), and 10 glucose. The standard solution B used for experiments with muscle strips and cells contained (in mM) 120.5 NaCl, 4.8 KCl, 1.2 MgSO\(_4\), 1.2 NaH\(_2\)PO\(_4\), 20.4 NaHCO\(_3\), 1.6 CaCl\(_2\), and 10 glucose, pH 7.4. High K\(^{+}\) solutions were prepared by isosmotic replacement of NaCl with KCl. The α1C, β, and α,δ plasmids were generously provided by Dr. Ilya Bezprozvanny (Department of Physiology, University of Texas Southwestern Medical Center), and the RyR2 plasmid was generously provided by Dr. Andrew Marks (Department of Physiology, Columbia University).

**Transfection**—Human embryonic kidney (HEK)293 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were seeded in a 6-well plate at a density of 0.3 × 10\(^6\) cells/well on the day before transfection using the Lipofectamine and Plus reagents with 1 μg of DNA/well, in a ratio of 2:1:1.2:4 (α1C: βα1δ: RyR2:empty vector/H1a/H1b/c).

**Western Blot Analysis and Co-immunoprecipitation**—Microsomes were prepared by homogenization in a buffer containing (in mM) 250 sucrose, 10 HEPES, pH 7.4, 1 EDTA, 1 dithiothreitol, and 0.2 phenylmethysulfonyl fluoride. The homogenates were centrifuged at 1,000 × g for 10 min at 4 °C. The supernatants were collected and centrifuged at 40,000 × g for 20 min. The pellets were suspended in lysis buffer composed of (in mM) 50 Tris (pH 6.8 with HCl), 150 NaCl, 2 EDTA, 2 EGTA, and 1% Triton X-100 supplemented with protease inhibitors (0.2 phenylmethysulfonyl fluoride, 10 μg/μl leupeptin, 15 μg/μl aprotinin, 1 mM benzamidin) and extracted by a 1-h incubation on ice. The lysate was centrifuged to remove insoluble material and analyzed by SDS-PAGE.

For the co-immunoprecipitation experiments, 150-μl extracts prepared from transfected HEK cells, as described above, were incubated with 5 μl of anti-α1C antibodies overnight at 4 °C under gentle agitation. The immune complexes were collected with 30 μl of a 1:1 goat anti-IgY antibody-conjugated microbead slurry and were incubated for an additional 2 h at 4 °C. The beads were collected, washed three times with lysis buffer, and the precipitates were probed for H1b/c.

**Animals**—All experiments with mice have been approved by the Animal Care and Use Committee of the University of Texas Southwestern Medical Center and adhere to National Institutes of Health guidelines.

**Assay of Urinary Bladder Continence**—Bio-Rad filter paper sheets (Model 583) were placed over fresh cage bedding material, and the mice were placed over the filter papers while having free access to water and food. After 2 h, the filter papers were collected and the urine spots were counted under UV light.

**Preparation of Detrusor Smooth Muscle (DSM) Cells and Strips**—DSM cells and strips were prepared as described previously (25, 26). In brief, the bladder was excised into solution B, and the urothelium was dissected away. DSM cells were prepared by mincing the muscle and incubation for 20 min at 37 °C in 5 ml of solution B containing 1 mg/ml papain, 1 mg/ml dithiothreitol, and 1 mg/ml bovine serum albumin. The fragments were washed twice with solution B and incubated at 37 °C for 10 min with 5 ml of solution B containing 1 mg/ml collagenase type 2, 100 μM Ca\(^{2+}\), and 1 mg/ml bovine serum albumin. The tissue was washed, and cells were released by trituration. The cells were collected by passing through 125-μm nylon mesh and were concentrated by centrifugation at 100 × g.

DSM strips were prepared by cutting the bladder muscle into three strips of ~0.5 × 0.5 × 8.0 mm. The strips were stretched (×1.2 slack length), and incubated in the dark with solution B containing 10 μM Indo-1/AM or 10 μM Fura-2/AM, 0.01% plu-
ronic F-127, and 0.02% cremophor for 4 h at room temperature. The strips were then incubated in fresh solution B for 30 min at room temperature to allow completion of dye hydrolysis and used for measurements of $[Ca^{2+}]_i$ and force (Indo-1) or $[Ca^{2+}]_i$ only (Fura2).

**Measurement of Ca$_{1.2}$ Current in DSM Cells and HEK Cells—** The whole-cell current was measured with pipette solution containing (in mM) 140 CsCl, 10 EGTA, 1 MgATP, and 10 HEPES adjusted to pH 7.2 with CsOH. The bath solution contained (in mM) 140 NaCl, 10 BaCl$_2$, 4 KCl, and 10 HEPES adjusted to pH 7.4 with NaOH. When desired, recombinant H1a and H1b/c prepared in MgATP-free pipette solution were diluted into the pipette solution just before use. Ca$_{1.2}$ current was recorded using Ba$^{2+}$ as the current carrier to avoid the $[Ca^{2+}]_{i}$-mediated inhibition of the current. The current was sampled at 10 kHz and filtered at 1 kHz. Current/voltage relationships were obtained by holding the membrane potential at $-80$ mV and stepping at 10-mV intervals for 800 ms to $+60$ mV. Clampex and Clampfit software were used for data acquisition and analysis.

**Measurement of $[Ca^{2+}]_{i}$ and Force in Smooth Muscle Strips—** Ca$^{2+}$ and force of muscle strips were measured as described before (25). In brief, Indo-1-loaded strips were mounted on a force transducer and were illuminated at 365 nm. Emitted light at 405 and 485 nm was used to obtain the 405/485 ratio. When only $[Ca^{2+}]_{i}$ was measured, Fura2-loaded DSM strips were taped onto glass coverslips, and fluorescence was measured by illumination at 350 and 380 nm, and the emitted light was used to calculate the 350/380 ratio.

**Measurement of $[Ca^{2+}]_{i}$, in HEK Cells—** Thirty hours after transfection, the HEK cells were trypsinized and replated onto glass coverslips. After 18 h, the cells were incubated in solution A containing 10 μM Fura-2/AM for 30 min at room temperature. Fluorescence was recorded at an excitation wavelength of 350 and 380 nm, and the emitted light at 500 nm was used to calculate the 350/380 ratio.

**Preparation of Split GFP-tagged Ca$_{1.2}$ and RyR2—** The α1C was tagged with the C-terminal half of GFP as follows. First, the stop codon of α1C was removed by PCR amplification using the primers 5’-GGGGTACCATGGTCAATGAAAACACGAG-3’ (sense; the underlined region is a KpnI site) and 5’-ATGCCGGCGGTCAGTTGCGTAGAC-3’ (antisense; the underlined region is a NotI site). The bolded base is an insert sequence, and therefore deletion of Homer1 may affect the activity of Homer1 in E-C coupling. The PCR product of α1C was cloned into pcDNA3.1(+). Positive clones were confirmed by sequencing and referred as α1CΔS. Thereafter, the C-terminal half of GFP (amino acids 158–237) was amplified using the primers 5’-ATGCCGGCGGCGCGCAAGAATTGGAATCAGAAG-3’ (sense; underlined region is a NotI site, and bolded region is a linker) and 5’-ATGCCGGCCCTATTGGTATAGTTCAATCCA (antisense; underlined region is a NotI site) and inserted into the NotI site of the C-terminal of α1CΔS. Positive clones were confirmed by sequencing and by current measurement.

The second half GFP was fused to the N terminus of RyR2 as follows. The N-terminal half GFP (amino acids 1–157) was amplified by the sense primer 5’-ATAGCGGCCGCAATGAGTAGGGTTTGTCTGCCATGATG-3’ and antisense primer 5’-ATGCCGGCGGCGCGCTGCCCCTGCCCCTTTGTTTGTCTGCCAATGATG-TAGAC-3’ (the underlined region is a NotI site, and the bolded region is a linker) and inserted into the NotI site of the RyR2 plasmid, which is located upstream of the open reading frame of RyR2. Positive clones were confirmed by sequencing and caffeine-mediated Ca$^{2+}$ release.

**Measurement of GFP Fluorescence and $[Ca^{2+}]_{i}$, by Confocal Microscopy—** Images were acquired with a Bio-Rad 1024 laser scanning confocal microscope as single images at low rate/high resolution or by continuous monitoring at a resolution of two images/sec (see Fig. 7). For measurement of depolarization-triggered CICR, after monitoring the GFP fluorescence, the cells expressing split GFP-tagged Ca$_{1.2}$ and RyR2 and H1b/c were loaded with Fluo-3 by incubation with Fluo-3/AM for 20 min at room temperature or until Fluo-3 fluorescence was 20-fold higher than the GFP fluorescence. GFP and Fluo-3 fluorescence was measured using the 488 line of the microscope.

**RESULTS AND DISCUSSION**

**Deletion of Homer1 Increases Detrusor Muscle Activity—** The amino acid sequences 812–817 and 1964–1967 in RyR2 and 1526–1530 in the smooth and cardiac muscle α1C isoform of Ca$_{1.2}$ are proline-rich, Homer binding consensus sequences, and indeed, RyR2 (18) and Cav1.2 (see below) bind Homer. Therefore, it was of interest to determine whether the Homers participate in E-C coupling. We followed urinary bladder function, because the detrusor muscle expresses RyR2 (21) and Ca$_{1.2}$ (22), and detrusor muscle contraction is mediated by CICR. Fig. 1, A–C, shows measurement of bladder continence as reported by urination patterns of freely moving WT mice and mice from which the Homer1, Homer2, or Homer3 genes were deleted. WT, Homer2$^{-/-}$, and Homer3$^{-/-}$ mice produced an average of 2.7 ± 0.7, 2.6 ± 0.9, and 6.5 ± 2.6 (n = 3–15, trials) respectively, large and medium urine spots in 2 h. By contrast, the Homer1$^{-/-}$ mice produced 169 ± 42 (n = 18) small urine spots during the same period of time, indicating overactive bladder in the Homer1$^{-/-}$ mice.

Overactive bladder can result from altered detrusor muscle activity and/or altered neuronal control of muscle function, because the L-type Ca$^{2+}$ channels and the Ca$^{2+}$ release channels in both cell types can bind Homer. Moreover, deletion of the K$^+$ channel mSlo1 also results in overactive bladder (27) and therefore deletion of Homer1 may affect the activity of mSlo1 or the membrane potential. We consider this unlikely, because mSlo1 does not have a Homer binding ligand. In addition, although the membrane potential in the isolated cells is low because of the extensive collagenase digestion and the trituration needed to isolate the cells, the membrane potential is similar in WT (−17 ± 3.2 mV) and Homer1$^{-/-}$ cells (−16.8 ± 2.8 mV).

Because we are interested in muscle E-C coupling, we asked whether deletion of Homer1 affected muscle function independent of neuronal function. Therefore, to test for a specific role of Homer1 in muscle E-C coupling, we measured [Ca$^{2+}$]$_i$ and contraction in isolated, urethra-lumen-free, detrusor muscle strips from WT and Homer1$^{-/-}$ mice. Fig. 1D shows representative traces of Ca$^{2+}$ and contraction measurements in...
Homer1 in E-C Coupling

![Image](image_url)

**FIGURE 1. Detrusor muscle function in Homer1Δ/Δ mice.** Urinary bladder continence was assayed by counting urine spots after placing WT (A) or Homer1Δ/Δ (B) mice for 2 h in cages lined with filter paper. C summarizes the results obtained with WT (n = 15), Homer1Δ/Δ (n = 9), Homer2Δ/Δ (n = 4), and Homer3Δ/Δ mice (n = 3). D, sample traces of simultaneous measurement of peak [Ca2+], and force in response to the stimulation of the detrusor muscles from WT mice with 10 μM carbachol. The dose response for carbachol-stimulated [Ca2+], (E) and force (F) was measured in detrusor muscle strips from four WT and four Homer1Δ/Δ mice. Two strips were used from each bladder. The same number of mice and strips were used to measure the muscle response to stimulation with 10 μM carbachol of muscle strips (G and H). Results are presented as mean ± S.E.

**FIGURE 2. RyR2 activity in WT and Homer1Δ/Δ detrusor muscle.** Three detrusor muscle strips from each of three WT (A and C) and three Homer1Δ/Δ (B and C) mice were used to measure the [Ca2+]i increase in response to the indicated concentrations of caffeine. The amount of RyR2 was assayed by Western blot (D).

response to stimulation with 10 μM carbachol of muscle strips from WT mice. The summary in Fig. 1, E and F, shows that deletion of Homer1 results in increased sensitivity of the muscle to stimulation of the muscarinic receptors with carbachol, whereas not affecting the [Ca2+]i, or force response to maximal stimulation with 10 μM carbachol. Notably, Fig. 1, G and H, shows that the same effect was observed in response to depolarizing the muscle strips with high extracellular K+ (K+o), indicating that the effect of deletion of Homer1 does not involve specific biochemical pathways stimulated by carbachol but rather involves E-C coupling directly. Moreover, these results indicate that deletion of Homer1 specifically affected muscle function.

**Deletion of Homer1 Does Not Affect the Activity of RyR2—** Increased sensitivity to membrane depolarization in the Homer1Δ/Δ mice could result from increased activity of Ca,1.2, increased activity of RyR2, or altered coupling between Ca,1.2 and RyR2. Because previous studies reported activation of RyR1 (15–17) and inhibition of RyR2 (18) by Homers, we tested the activity of RyR2 in the intact muscle strips by measuring the [Ca2+]i, increase in response to caffeine, an established activator of all RyR isoforms (13, 14). Fig. 2, A and B, shows representative traces, and Fig. 2C shows the summary of [Ca2+]i increases evoked by different concentrations of caffeine between 2.5–40 mM. It is clear that deletion of Homer1 had no measurable effect on the activity of RyR2 in vivo. In addition, Fig. 2D shows that deletion of Homer1 had no effect on the expression of RyR2 (WT/Homer1Δ/Δ ratio, 1.1 ± 0.1, n = 4). Hence, the increased sensitivity of the muscle to stimulation with carbachol and membrane depolarization cannot be attributed to increased activity of RyR2.

**Homer1 Activates Ca,1.2—** An increased Ca,1.2 activity in the Homer1Δ/Δ mice could account for the increased sensitivity of the muscle to membrane depolarization. Measurement of Ca,1.2-mediated whole-cell Ba2+ current in detrusor muscle cells revealed an unexpected 39 ± 12% (n = 8) reduction in Ca,1.2 current (Fig. 3), opposite of what is expected from the increased sensitivity of the muscle to membrane depolarization. Ba2+ was used as a charge carrier to avoid Ca2+-mediated inactivation of the channel and exclude effect of Homer1 on regulation of the channel by Ca2+. In addition, Homer1 does not affect the regulation of Ca,1.2 by voltage (Fig. 3, B and C), suggesting that Homer1 increases the current amplitude of Ca,1.2. Fig. 3D shows that the higher Ca,1.2 current in WT cells was not due to reduced expression of Ca,1.2 in the Homer1Δ/Δ cells. In fact, deletion of Homer1 slightly increased expression of Ca,1.2 by 1.2 ± 0.07-fold (n = 4), which may be an adaptive response to the reduction in Ca,1.2 current. These findings are similar to a recent report showing that infusion of H1a enhances Ca2+ influx via the L-type Ca2+ channel in neocortex pyramidal cells (19).

Presence of a Homer binding sequence (amino acids 1526–1530) in the α1C subunit of Ca,1.2 raised the possibility that Homer1 binds to and directly activates Ca,1.2. To test this possibility, we first determined binding of H1b/c to α1C alone and also to α1C+β and α1C+β+α2δ subunits of Ca,1.2 by a coimmunoprecipitation assay. Fig. 4A shows that H1b/c does co-
immunoprecipitate with α1C, and expression of the β and α2δ subunits does not reduce or enhance the binding. Next, we tested whether infusion of the dissociating H1a and the multimerizing of H1b/c into WT cells affect Ca_{1.2} current. Unlike the finding in pyramidal cells (19), infusion of either Homer isoform had no effect on Ca_{1.2} current in WT cells (Fig. 3B). By contrast, infusion of either H1a or H1b/c activated Ca_{1.2} in Homer^{+/−} cells and restored the current amplitude to that measured in WT cells. Importantly, a point mutation in the EVH domain of Homer (W27A) that destroys Homer binding to proline-rich sequences in target proteins (2) completely prevented activation of Ca_{1.2} by H1a and H1b/c. To further verify activation of Ca_{1.2} by the two Homer isoforms, we measured the effect of the recombinant Homers on Ca_{1.2} expressed in HEK cells. Fig. 4D shows that H1a and H1b/c activated Ca_{1.2}, and H1b/c was slightly more potent than H1a.

The results in Figs. 3 and 4 suggest that Homer1 directly regulates the activity of Ca_{1.2}. The present findings are somewhat different from those in neocortex pyramidal cells (19), in that we found that the Homers had no effect in WT cells and that both H1a and H1b/c activate Ca_{1.2}. The reason for the different findings is not known at present. It is possible that regulation of Ca_{1.2} by Homer is different in neocortex pyramidal cells that express RyR3 or that Homer differentially regulates the neocortex pyramidal cells α1D and the bladder smooth muscle α1C subunits of Ca_{1.2}. Our findings indicate that the EVH domain of Homer1 activates the channel and that multimerization of Ca_{1.2} by Homer1 or Homer1-mediated interaction of Ca_{1.2} with other proteins, such as RyR2 (see below), is not required for activation of Ca_{1.2} by Homer1, because H1a and H1b/c similarly activate the channel.

Reconstitution of Homer1 Effect on E-C Coupling in HEK Cells—The results in Figs. 1–4 raised a conundrum. On the one hand, the deletion of Homer1 enhanced E-C coupling, indicating that Homer1 should inhibit Ca_{1.2} or RyR2 or both. On the other hand, deletion of Homer1 has no effect on the activity of RyR2, and Homer1 activates Ca_{1.2}. A potential solution for this conundrum is that Homer1 mediates a communication between Ca_{1.2} and RyR2 to regulate CICR and muscle contraction. To this end, we reconstituted the effect of Homer1 on E-C coupling in the expression system of HEK cells. Fig. 5A shows that expression of Ca_{1.2} and RyR2 resulted in a depolarization- and caffeine-activated [Ca^{2+}], increase, indicating that both Ca_{1.2} and RyR2 are active. Expression of the complexes dissociating H1a increased the sensitivity, whereas expression of the complexes forming H1b/c reduced the sensitivity of membrane depolarization to increase [Ca^{2+}]. Hence, although both H1a and H1b/c activate Ca_{1.2} (Fig. 4), they have the opposite effect on CICR initiated by activation of Ca_{1.2}. Notably, the effects of H1a and H1b on CICR reproduce the finding in vivo. Thus, it is possible to...
reconstitute the effect of Homer1 in E-C coupling by expression of the respective Homers together with RyR2 and Ca,1.2 in HEK cells.

Homer1-mediated Dynamic Interaction between Ca,1.2-RyR2—Dynamic interaction between Ca,1.2 and RyR2 in living cells was studied with the split GFP technique (28). In this technique, GFP is split into two halves (amino acids 1–157 and 158–237), and each half is attached to one of two proteins to detect their interaction. The GFP halves do not fluoresce, but when the proteins carrying them interact, the two halves are assembled into GFP and fluorescence is restored. Because the restored GFP fluorescence is transient, a leucine zipper is attached to each GFP half to stabilize the interacting halves, resulting in stable GFP fluorescence (28).

Because we are interested in the effect of Homer1 on the interaction between Cav1.2 and RyR2, we did not include the leucine zippers but rather used H1a and H1b/c as the regulators of the reassembled GFP. The N-terminal half on GFP was attached to the N terminus of the RyR2, because the N terminus of RyR2 contains its Homer binding motifs. For the same reason, the C-terminal half on GFP was attached to the C terminus of the Cav1.2, because the C terminus of Cav1.2 contains its Homer binding motif.

To determine the role of Homer1 in the interaction between Ca,1.2 and RyR2, the fusion proteins were expressed in HEK cells alone or with H1a or H1b/c. Panels A–C of Fig. 6 show representative images, and panel D is the summary. The superimposed bright field and fluorescent images show that, when Ca,1.2 and RyR2 were expressed alone, ~7 ± 2% of the cells showed fluorescence. Expression of the dissociating H1a, together with the channels, almost completely eliminated the fluorescent cells. Note that Ca,1.2, RyR2, and CICR are fully active and more sensitive to membrane depolarization in these cells (Fig. 5).
contrast, expression of the multimerizing H1b/c dramatically increased the number of fluorescent cells to 74 ± 13%.

As depicted in the model, the results in Fig. 6, A–D, indicate that H1a loosens and H1b/c tightens the interaction between CaV1.2 and RyR2. Together with the results in Fig. 5, loosening the interaction between the channels leads to enhanced efficiency of CICR, and tightening the interaction leads to reduced efficiency of CICR. Intuitively, we expected the opposite results. That is, that enhanced interaction between the channels would lead to better access of the Ca2+ entering through CaV1.2 to the RyR2 and activation of CICR. To further verify the findings in Fig. 6, A–D, we attempted to correlate between the intensity of the GFP fluorescence and response of the cells to depolarization. In each experiment, the cells with 40% or below the maximally recorded GFP fluorescence were considered as having low fluorescence and all others as having high fluorescence. Fig. 6E displays representative traces, and Fig. 6F is the summary. Only cells with relatively low fluorescence routinely responded to depolarization with 22.5 mM K+o, whereas stimulation with 80 mM K+o or 20 mM caffeine similarly increased [Ca2+]i, in all cells. Cells with high GFP fluorescence fell into two categories, those that modestly responded to depolarization with K+o and about 20% that did not respond to membrane depolarization. Most notably, caffeine increased [Ca2+]i in all fluorescent cells, indicating that the channels were active in the cells that did not respond to membrane depolarization. Therefore, the lack of response to depolarization must be due to impaired E-C coupling between CaV1.2 and RyR2.

To date, the split GFP technique has been used only to study passive protein-protein interaction (28–30). We reasoned that, because Homer1 is the natural ligand mediating the interaction between CaV1.2 and RyR2 to reconstitute the GFP fluorescence, we should be able to monitor conformational changes during E-C coupling by monitoring changes in GFP fluorescence. For these experiments, HEK cells were transfected with CaV1.2, RyR2, and H1b/c, and GFP fluorescence was monitored at a temporal resolution of two images/s. Fig. 7 shows that membrane depolarization and stimulation with carbachol caused a rapid increase in GFP fluorescence that reversed upon the removal of the stimulus. An increase in GFP fluorescence indicates altered interaction between CaV1.2 and RyR2 during initiation of E-C coupling. Interestingly, the GFP fluorescence did not change in cells with very high GFP fluorescence (marked by arrows). The same cells did not respond to membrane depolarization by a [Ca2+]i increase.

CONCLUSIONS

Unlike skeletal muscle E-C coupling that involves direct interaction between CaV1.1 and RyR1, smooth and cardiac muscle E-C coupling does not involve direct interaction between CaV1.2 and RyR2. Accordingly, all models of smooth and cardiac muscle E-C coupling assume that CaV1.2 and RyR2 do not interact but affect the activity of each other only through changes in Ca2+ at the Ca2+ release unit (12–14). The present work calls into question this assumption by reporting a novel mode of interaction between CaV1.2 and RyR2 that is mediated by Homer1. Both channels express Homer binding sequences and both bind Homer (Fig. 4) ((18)). The regulation of E-C coupling in vivo is specific to Homer1, because a change in E-C coupling and muscle contraction was observed only in Homer1−/− mice. This is not due to selective expression of Homer1, because cardiac and smooth muscle express all Homer isoforms (31). It is more likely that the action of the Homers is pathway-specific. In this respect, we showed earlier that Homer2 (but not Homer1) accelerates the GAP activity of RGS proteins and PLCβ to regulate IP3 production and Ca2+ signaling (10).

The Homer1-mediated interaction between CaV1.2 and RyR2 plays an important regulatory role in E-C coupling by reducing the responsiveness of the muscle to cell stimulation. This is depicted in the model in Fig. 8. This conclusion is based on the findings that the deletion of Homer1 in mice increases the responsiveness of the detrusor muscle to membrane depolarization. Similarly, expression of H1a increased, whereas expression of H1b/c decreased the CICR in response to mem-
brane depolarization in HEK cells expressing Ca$_{1.2}$ and RyR2. Because deletion of Homer1 does not affect the activity of the native RyR2 and Homer1 activates rather than inhibits the native Ca$_{1.2}$, Homer1 must affect E-C coupling by a mechanism downstream of Ca$_{1.2}$ and upstream of RyR2. The split GFP technique suggests that the most likely mechanism is a conformational mechanism in which Homer1-mediated interaction of Ca$_{1.2}$ and RyR2 solidifies the interaction between the channels to restrain the conformational change required for efficient CICR. Deletion of Homer1 or expression of H1a loosens the interaction between the channels to facilitate the conformational change that triggers CICR. This interpretation is supported by the findings that the ability of membrane depolarization to activate CICR inversely correlates with the intensity of GFP fluorescence (Fig. 6, E and F).

Homer1-mediated interaction of Ca$_{1.2}$ and RyR2 is dynamic and is enhanced during the activation of E-C coupling. The enhanced interaction between the channels further indicates that Homer1 acts to negatively regulate E-C coupling. In fact, the results in Fig. 7 are the first demonstration of a dynamic change in the interaction between L-type Ca$^{2+}$ channels and RyRs during E-C coupling. Further refinement of the split GFP technique should allow careful future analysis of this conformational change.

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