Homer Protein Increases Activation of Ca\(^{2+}\) Sparks in Permeabilized Skeletal Muscle*

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Members of the Homer family of proteins are known to form multimeric complexes capable of cross-linking plasma membrane channels (e.g. metabotropic glutamate receptor) and intracellular Ca\(^{2+}\) release channels (e.g. inositol trisphosphate receptor) in neurons, which potentiates Ca\(^{2+}\) release. Recent work has demonstrated direct interaction of Homer proteins with type 1 and type 2 ryanodine receptor (RyR) isoforms. Moreover, Homer proteins have been shown to modulate RyR-dependent Ca\(^{2+}\) release in isolated channels as well as in whole cell preparations. We now show that long and short forms of Homer H1 (H1c and H1-EVH1) are potent activators of Ca\(^{2+}\) release via RyR in skeletal muscle fibers (e.g. Ca\(^{2+}\) sparks) and potent modulators of ryanodine binding to membranes enriched with RyR, with H1c being significantly more potent than H1-EVH1. Homer did not significantly alter the spatio-temporal properties of the sparks, demonstrating that Homer increases the rate of opening of RyRs, with no change in the overall RyR channel open time and amount of Ca\(^{2+}\) released during a spark. No changes in Ca\(^{2+}\) spark frequency or properties were observed using a full-length H1c with mutation in the EVH1 binding domain (H1c-G89N). One novel finding with each Homer agonist (H1c and H1-EVH1) was that in combination their actions on [3H]ryanodine binding was additive, an effect also observed for these Homer agonists in the Ca\(^{2+}\) spark studies. Finally, in Ca\(^{2+}\) spark studies, excess H1c-G89N prevented the effects of H1c in a dominant negative manner. Taken together our results suggest that the EVH1 domain is critical for the agonist behavior on Ca\(^{2+}\) sparks and ryanodine binding, and that the coiled-coil domain, present in long but not short form Homer, confers an increase in agonist potential apparently through the multimeric association of Homer ligand.

Homer proteins constitute a family of proteins containing a highly conserved EVH1\(^1\) (Eva/Vasp homology 1) N-terminal domain (1, 2). This domain has been shown to bind specific proline-rich (PPXXF(R/S)) sequences on target proteins (2) that include metabotropic glutamate receptors (mGluRs) and inositol 1,4,5-trisphosphate receptors (InsP\(_3\)Rs), molecules involved with intracellular calcium signaling in neurons (3, 4). Constitutively expressed Homer proteins also contain a C-terminal coiled-coil domain and leucine zipper (3) that allows multimerization of the Homer molecules.

While the functional significance of Homer is yet to be elucidated, an attractive hypothesis is that Homer proteins functionally couple cell surface receptors and intracellular Ca\(^{2+}\) release channels into junctional signaling complexes in neurons (3). Recent results demonstrating that Homer-mediated coupling between mGluRs and InsP\(_3\)Rs is modulated by neuronal activity (1) are consistent with a functional coupling hypothesis. This modulation of Homer-mediated coupling is thought to occur via a competitive inhibition of the binding of Homer long form (Homer 1c; H1c) as a direct consequence of expression of the immediate early gene product Homer 1 “short form” (H1a). The H1a short form is composed of an identical EVH1 binding domain as H1c long form, but lacks the CC and leucine zipper region making self-multimerization unlikely (1, 3, 4).

Recently Homer proteins have been shown to be expressed in both skeletal and cardiac muscle (5, 6), where ryanodine receptors (RyRs), not InsP\(_3\)Rs, mediate the release of calcium from the sarcoplasmic reticulum during excitation-contraction (E-C) coupling (7, 8). Based on their sequence, RyRs are predicted to bind Homer proteins (2), and recent work has demonstrated (9) direct interaction of Homer proteins with skeletal and cardiac RyR isoforms (9–11)). Moreover, Homer proteins have been shown to modulate RyR-dependent Ca\(^{2+}\) release in isolated channels as well as in whole cell preparations (9–11) leading to the hypothesis that Homer may play a functional role in Ca\(^{2+}\) signaling in striated muscle.

Ca\(^{2+}\) sparks arise from the opening of small groups of RyRs in muscle (12). As such, sparks can provide insight into the function of native RyR Ca\(^{2+}\) release channels within an intact triadic structure and while associated with regulatory proteins, possibly distinct from isolated RyR in artificial membranes. In this investigation, we evaluate whether exogenously applied long and short forms of Homer 1, H1c and H1a, can exert a direct effect on RyR Ca\(^{2+}\) release channels within skeletal muscle fibers by monitoring Ca\(^{2+}\) sparks. We demonstrate that long and short forms of Homer H1 (H1c and H1-EVH1) are potent activators of initiation of Ca\(^{2+}\) release via RyR in skel-
Ryanodine Binding to Junctional Sarcoplasmic Reticulum—Junctional sarcoplasmic reticulum (JSR) membranes enriched in RyR1 were prepared from skeletal muscle of New Zealand White rabbits according to the method of Saito (18). The preparations were stored in 10% sucrose, 1 mM Hepes, pH 7.4 at −80°C until needed. Equilibrium measurement of specific high affinity [3H]ryanodine ([3H]Ry) binding was determined according to the method of Pessah et al. (19–21). SR vesicles (50 μg of protein/ml) were incubated with or without Homer protein in assay buffer containing HEPES (20 mM) pH 7.1, KCl (250 mM), NaCl (15 mM), varied CaCl2, and [3H]Ry for 3 h at 37°C. The reactions were quenched by filtration through GF/B glass-fiber filters and washed twice with ice-cold harvest buffer (20 mM Tris-HCl, 250 mM KCl, 15 mM NaCl, 50 μM CaCl2, pH 7.1). Nonspecific binding was determined by incubating SR vesicles with 1000-fold excess unlabeled ryanodine.

RESULTS

Long Form H1c Increases the Frequency of Occurrence of Spontaneous Ca2+ Sparks—The frequency of occurrence of Ca2+ sparks provides a measure of the rate of opening of the RyR Ca2+ release channel or channels that initiate the Ca2+ spark. Application of long form H1c to permeabilized frog skeletal muscle fibers induced a dramatic rise in the frequency of occurrence of spontaneous Ca2+ sparks, indicating an increased rate of RyR activation. Fig. 1A shows 4 successive 1-s line scan images, first in a control condition and subsequently (−5 min) following application of 6.25 nM of H1c. In a group of fibers (n = 4) application of 6.25 nM H1c increased Ca2+ spark frequency from 0.023 ± 0.01 to 0.078 ± 0.01 events·sarc−1·sec−1 (p < 0.05). This represents a 3.4-fold increase above the baseline frequency determined within the same fibers (Fig. 2A). An ensemble average of single identified sparks and pseudocolor surface projection for sparks recorded in control and 25 mM H1C is presented in Fig. 1B. Events were superimposed with alignment at the midpoint of the rising phase, and averaged to obtain an image representing the population average. The average sparks were essentially the same in the presence and absence of H1c. Because of the −14-fold increase in Ca2+ spark frequency in the presence of H1c compared with the control condition, the vast majority of events contributing to increased spark frequency observed are likely the direct result of H1c. Furthermore, H1c-modified fibers exhibit spark events that maintain similar spatio-temporal characteristics to those of control fibers (see Fig. 2C, below).

The concentration dependence of the H1c effect on Ca2+ spark occurrence is shown in Fig. 2A. Application of 5–50 nM H1c revealed a linear rise in the frequency of Ca2+ spark occurrence with concentration, indicating that 50 nM is well below the dissociation constant of the RyR for H1c and that the maximal effect of H1c on spark frequency is likely to be much greater than shown in Fig. 2A. Further increasing H1c concentration (≥100 nM) resulted in a higher level of Ca2+ spark activity and increase in non-spark fluorescence making the detection and analysis of Ca2+ spark properties unreliable at these high concentrations of H1c (data not shown). A “sham” condition (n = 3) consisting of a solution change to an identical internal solution (0 nM H1c), resulted in a small, non-significant increase in Ca2+ spark frequency thus demonstrating that any mechanical disturbance due to changing experimental conditions had a negligible effect.

The spatio-temporal properties of a spark reflect the overall duration and amount of Ca2+ release from the channels that generate Ca2+ sparks (22). The effect of H1c on the spatio-temporal parameters for Ca2+ sparks is evaluated between the control and 6.25 nM H1c conditions within the same fibers (n = 4) in Fig. 2C. Despite the robust increase in Ca2+ spark frequency, no significant differences in mean values for spark amplitude (0.99 ± 0.1 versus 1.14 ± 0.1 FL/F), rise-time (6.2 ± 0.1 versus 5.8 ± 0.2 ms), spatial width (FWHM; 2.0 ± 0.1 versus 1.14 ± 0.1 FL/F), and full-width at half-maximal amplitude (FWHM; μm) and amplitude (peak ΔF/F).

A sequence of 30–50 line scan images were collected 5 min after application of the control solution (internal solution) and subsequently 5 min after exchange of internal solution containing H1c, H1-EVH1, H1c point mutant G89N, or Mena (singly or in combination as described in the figure legends), or a solution change to another control internal solution (i.e., Sham condition). Spark morphometric parameters were compared using non-parametric analysis (Mann-Whitney Test on Ranks) with significance set at p < 0.05. Frequency responses between conditions were evaluated with analysis of variance with significance set at p < 0.05.
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1.9 ± 0.1 μm or temporal duration (FDHM: 12.4 ± 0.2 versus 12.3 ± 0.2 ms) was seen between the control and H1c, respectively. Thus, although H1c does markedly increase the rate of opening of the RyR channels that initiate the sparks (i.e., frequency of Ca\(^{2+}\) sparks), the overall duration and amount of Ca\(^{2+}\) released in a spark does not appear to be significantly altered by the Homer protein.

**Specificity and Effectiveness of Long versus Short Form Homer 1 on Eliciting Spontaneous Ca\(^{2+}\) Spark Frequency**—As reported above, the application of 5–50 nM H1c induced a robust increase in the frequency of occurrence of spontaneous Ca\(^{2+}\) sparks. To evaluate the specificity of H1c effects on spark activity, a point mutation of H1c (H1c-G89N) with impaired ability to bind Homer ligand, but with maintained ability to multimerize, was tested (1). This mutant exhibits negligible binding to the Homer ligand domains (1, 23). When applied at a 10-fold higher concentration than that of H1c, H1c-G89N failed to significantly alter Ca\(^{2+}\) spark frequency (Fig. 3A). These results suggest that enhanced Ca\(^{2+}\) spark activity is mediated by the interaction between the EVH1 domain of H1c and the Homer ligand domain of RyRs. The specificity of the H1c effect toward RyR shown above was further tested by preincubation of muscle fibers with the InsP\(_3\)R inhibitor heparin (50 μg/ml). The H1c effect in heparin-treated fibers (n = 2) was similar to that of control fibers (n = 2; data not shown). Application of heparin in the absence of Homer protein also had no effect on spark frequency or properties (n = 2; data not shown) indicating that InsP\(_3\)R's are not responsible for the Ca\(^{2+}\) sparks seen in frog skeletal muscle, either in the presence or absence of exogenously applied H1c.

Short form H1a is an alternatively spliced version of H1c protein composed only of the H1-EVH1 binding domain and lacking the CC domain necessary for self-multimerization. Permeabilized muscle fibers challenged either with 30 nM (n = 7), 50 nM (n = 2), or 150 nM (n = 2) recombinant H1-EVH1 exhibited a significant concentration-dependent increase in spontaneous Ca\(^{2+}\) spark frequency (Fig. 2B). However, 30–150 nM H1-EVH1 was less effective in inducing an increase in spontaneous spark activation than 5–50 nM H1c. H1-EVH1 required ~5-fold higher concentration than H1c to elicit similar increases in Ca\(^{2+}\) spark occurrence, and was unable to induce the highest level of activation seen with H1c in this preparation (Fig. 2B). The spatio-temporal properties of the events measured in the presence of H1-EVH1 were not significantly different from those seen in control and H1c-treated muscle fibers (Fig. 2C).

**FIG. 1.** Homer H1c increases spontaneous Ca\(^{2+}\) spark occurrence in permeabilized frog skeletal muscle fibers. A, line scan images are presented of a permeabilized skeletal muscle fiber bathed in a control internal solution (see "Materials and Methods") containing the Ca\(^{2+}\) indicator Fluo-3 (50 μm). Images report the ΔF/F fiber fluorescence resulting from line scan imaging a 138-μm length of fiber for 1024 ms. Following four successive images in the control condition (top), the addition of long form H1c (bottom) revealed a robust increase in the occurrence of spontaneous Ca\(^{2+}\) sparks. B, the average spark image of events from the above experiment (Control, n = 96 sparks; 25 nM H1c, n = 336 sparks) is displayed. Events have been superimposed with alignment at the midpoint of the rising phase and averaged to obtain a population average. In each condition the mean xy image (blue) is presented with spatial and temporal transients extracted through the peak ΔF/F of the image. The pseudocolor (green) surface plot of the mean event seen is presented below. The mean parameters for the sparks in Control versus 25 nM H1c from this fiber are ΔF/F (0.85 ± 0.24; 0.89 ± 0.26), RT (4.5 ± 0.21; 4.06 ± 0.19), FDHM (9.6 ± 0.4; 10.0 ± 0.3), FWHM (1.4 ± 0.1; 1.3 ± 0.1).
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**Fig. 2.** Homer H1c and H1-EVH1 increases the frequency of Ca\(^{2+}\) spark occurrence without altering the spatio-temporal properties of Ca\(^{2+}\) sparks. A, concentration dependence of long form H1c effect on Ca\(^{2+}\) spark frequency at 0, 6.25, 25, and 50 nM Homer H1c. B, concentration dependence of the short form H1-EVH1 effect on Ca\(^{2+}\) spark frequency at 0, 30, 50, and 150 nM. The dashed line represents the best fit line for the H1c condition for comparison. C, distributions of the spatio-temporal properties of the total population of Ca\(^{2+}\) sparks in control (white; n = 609), H1c (red; n = 1180), and H1-EVH1 (yellow; n = 453). Box plots indicate the 25, 50 (i.e. median), 75 percentiles, while the whiskers indicate the range between the 1st and 99th percentile values of the distribution (*, p < 0.05). Mean values (control, H1c, H1-EVH1) for the population were: Amplitude (ΔF/F): 1.06 ± 0.02, 1.03 ± 0.02, 1.05 ± 0.04, RiseTime (ms): 5.79 ± 0.11, 6.24 ± 0.10, 5.95 ± 0.24, FWHM (µm): 1.86 ± 0.02, 2.03 ± 0.02, 1.97 ± 0.04, FDHM (ms): 12.3 ± 0.2, 12.4 ± 0.13, 12.45 ± 0.37.

The protein Mena-EVH1 (MENA; murine Ena homologue) was used to test the specificity of H1a effects reported above. Mena-EVH1 lacks a CC domain and is structurally similar to Homer EVH1, yet it binds to a distinct proline-rich sequence independent of the Homer ligand site (1). This protein at 50 nM had no effect on the frequency of occurrence of Ca\(^{2+}\) sparks (Fig. 3).

**Fig. 3.** Specificity of Homer in eliciting Ca\(^{2+}\) spark in permeabilized frog skeletal muscle fibers. The spark frequency under experimental conditions is expressed as a fold-increase over the measured basal frequency in the fiber prior to application of the experimental condition. The H1c effect (6.25 nM) was significantly different (*, p < 0.05) from the control condition within the same fibers. H1C-G89N (50 nM) was no different from the control condition. H1-EVH1 (30 nM) elicited a significant increase in Ca\(^{2+}\) spark frequency (*, p < 0.05) to a level similar to that of 6.25 nM H1c. MENA (50 nM) was ineffective in modulating Ca\(^{2+}\) spark activity.

The Potential Role of the Homer Multimerization via the CC Domain in Ca\(^{2+}\) Spark Activation—The current results indicate that the activity of Homer 1 toward enhancing the frequency of RyR-mediated spark activity is specific to the EVH1 domain and does not require the CC domain. However the presence of the CC domain of H1c does increase effectiveness of a given concentration of Homer by ~5-fold (H1c versus H1-EVH1). One possible mechanism responsible for the greater activity of H1c compared with H1a could involve the multimeric nature of H1c via the CC domain. To evaluate this possibility, H1c (6.25 nM) was allowed to equilibrate (~30 min) in solution together with 10-fold excess of the non-binding point mutant H1c-G89N (above) before challenging the fiber. We predict that pre-equilibration of H1c with excess H1c-G89N should promote the formation of homodimers of H1c-G89N, heterodimers of H1c/H1c-G89N and an insignificant fraction of H1c homodimers based on the stoichiometry at equilibrium (~83, 16, and 1% respectively). This stoichiometry, we hypothesize, should result in a diminution in the relative efficacy of H1c if multimerization through the CC domain is essential for high efficacy. The results summarized in Fig. 4 show that 6.25 nM H1c, which forms homodimers, promoted the most robust elevation in Ca\(^{2+}\) spark frequency. By comparison, preincubated samples containing 6.25 nM H1c + excess H1c-G89N (in which H1c homodimers constitute ~1%) applied to fibers greatly blunted the response expected for 6.25 nM H1c alone to a level no different than the control condition. The suppression of the H1c response by excess H1c-G89N cannot be ascribed to simple competitive inhibition at the RyR Homer ligand site since H1c-G89N is a non-binding mutant (1, 23), indicating that heterodimers of H1c/H1c-G89N are ineffective at activating Ca\(^{2+}\) sparks. The subsequent addition of excess H1c (50 nM) to the fibers did promote a drastic increase in Ca\(^{2+}\) spark activation (data not shown), verifying that the spark activating potential remained.

To further evaluate this hypothesis, 30 nM H1-EVH1 was preincubated with excess (210 nM) H1c-G89N and subsequently applied to a permeabilized fiber. The activity of this mixture
toward enhancing Ca\(^{2+}\) spark activity was slightly but significantly lower than that seen with an equivalent concentration of H1a alone (Fig. 4). However, H1c-G89N is clearly not as effective in suppressing the enhanced Ca\(^{2+}\) spark frequency elicited by H1-EVH1 short-form when compared with the suppression observed with H1c-G89N + H1c. Taken together these results support a role for the CC domain in the enhanced effectiveness of a given concentration of H1c compared with H1-EVH1 toward activating Ca\(^{2+}\) sparks in this system.

In order to further test this hypothesis, 6.25 nM H1c (long form) was pre-equilibrated with 30 nM H1-EVH1 (short form), and the fiber was challenged with the mixture. This condition resulted in an additive effect between long and short forms toward enhancing Ca\(^{2+}\) spark frequency (−6-fold over control levels; Fig. 4). This level of activation is consistent with H1c (−3.5-fold) and H1-EVH1 (−2.5-fold) each exerting an independent effect on Ca\(^{2+}\) spark activation. This is consistent with each individual protein within the linear concentration range for spark activation.

**DISCUSSION**

**Long and Short Homer Proteins Promote RyR Activation**—It is well known that RyR-dependent SR Ca\(^{2+}\) release is modulated by endogenous ions such as Ca\(^{2+}\) and Mg\(^{2+}\) (16, 24, 25). In addition, RyR-dependent Ca\(^{2+}\) release is also modulated by regulatory proteins such as calmodulin (26, 27) and FKBP12 (28, 29), which associate at distinct binding sites on the RyR (30, 31). In permeabilized and thus continuously depolarized skeletal muscle fibers, DHPR voltage sensors are inactivated (32) and unable to initiate SR Ca\(^{2+}\) release. The appearance of Ca\(^{2+}\) sparks in this preparation thus presumably arises from ligand-dependent activation of RyRs; most likely through calcium-induced calcium release (CICR) mechanisms (8, 12).

Recent reports have demonstrated that skeletal type RyR contains the proline-rich (PPXXFS) Homer binding sequence, and recent studies have demonstrated a direct binding interaction of Homer long and short forms with RyR in GST pull-down assays (10). Recent investigations have also demonstrated that Homer increases the open probability of RyR in the bilayer and also increases the CICR sensitivity in intact skeletal muscle myotubes (10); with H1c being more effective than H1-EVH1 at the same concentration.

In contrast to these findings, a recent report by Hwang et al. (9) demonstrated that Homer short and long forms differentially regulated RyR1. In this study, long form Homer (e.g. H1c; V-1L) both bound and activated RyR1 whereas Homer short form (e.g. H1-EVH1; V-1s) bound to RyR1 yet did not activate

**FIG. 4.** The potential role of the Homer multimerization via the CC domain in Ca\(^{2+}\) spark activation. A, varying Homer conditions to investigate Homer interaction are shown. Spark frequency expresses as a percentage of control. All protein concentrations listed are in nanomolar concentrations. B, schematic representation of the predicted ability of the protein to bind a Homer ligand target, to dimerize into a multimeric complex, and to cross-link target proteins. A–B, H1c (6.25 nM; red bar) was allowed to equilibrate together with 10-fold excess H1c-G89N (70 nM; ~30 min) before application to the fiber. This condition (red-black checker) is predicted to result in homodimers of the H1c-G89N, heterodimers of H1c and H1c-G89N, and an insignificant amount of H1c homodimers based on the stoichiometry at equilibration (~83, 16, and 1%, respectively). This combination greatly blunted the increase in frequency seen with the H1c alone. 30 nM H1-EVH1 (yellow bar) with excess H1c-G89N (210 nM; yellow-black checker) resulted in a small but significant reduction in Ca\(^{2+}\) spark frequency when compared with the H1-EVH1 condition. However, H1c-G89N + H1-EVH1 was clearly not as effective in suppressing Ca\(^{2+}\) spark frequency as seen with H1c-G89N + H1c. H1c (6.25 nM) pre-equilibrated with H1-EVH1 (30 nM) resulted in an additive effect in Ca\(^{2+}\) spark activation to ~6-fold over control levels. This level of activation is consistent with H1c (~3.5-fold) and H1-EVH1 (~2.5-fold) each exerting an independent effect on Ca\(^{2+}\) spark activation. This is consistent with each individual protein within the linear concentration range for spark activation.
the channel. In addition, Homer short form dose-dependently decreased the effect of Homer long form, an effect that was not evident in our experiments. Furthermore, in contrast to the marked potentiation of spark frequency observed here for both H1c (6.25 nM resulted in a ~4-fold increase, see Fig 2A) and H1-EVH1 (30 nM resulted in ~5-fold increase, see Fig. 2B), Hwang et al. (9) found only a modest 1.8-fold increase in RyR channel P_{open} with 100 nM H1c. The disparity between this and our current data may be due to differences in the activity of recombinant Homer peptides used for the respective studies. Based on early experiments (12) our group did consider a differential regulation of Homer long and short form on Ca^{2+} spark behavior in the permeabilized frog fiber. In extending our experiments we realized significant variability of our results among different preparations of purified Homer protein. This variability led us to verify the sequence of all protein preparations (see “Materials and Methods”) prior to performing the experiments presented here. With sequence verified protein, we largely eliminated experimental variability between preparations, and have proceeded to demonstrate Homer-dependent RyR agonist behavior of both H1c and H1-EVH1 with three different methodologies: Ca^{2+} sparks in permeabilized frog muscle and ryanodine binding to JSR fractions in the current study, and single channel recording using reconstituted RyR1 in the BLM (10).

Our present functional studies now show a direct and additive effect of H1c and H1-EVH1 on activating Ca^{2+} sparks in permeabilized muscle fibers. In agreement with these findings we also report that H1c and H1-EVH1 modulate ryanodine binding to RyR1 in JR in a purely additive manner. Full-length Homer (H1c) was a more potent agonist of the RyR channel (~5-fold) than H1-EVH1 both in measurements of Ca^{2+} spark activity and in radioligand binding analysis. The similar relative effectiveness between the two different methodologies used here, as well as previous reports demonstrating differing efficacy of H1c and H1-EVH1 in the BLM (10), suggests a similar mode of action by which Homer long form and short form activate RyR, whether in the permeabilized muscle fiber or in the isolated membrane or single channel experiments.

Ca^{2+} Spark Properties Are Not Altered by Homer: Insight into the Homer Effect—Following RyR activation, the shape of the Ca^{2+} spark (i.e. spatio-temporal properties) is determined by the underlying RyR channel behavior (12, 22). Several reports have demonstrated that alterations in the spatial and/or temporal properties of spontaneous Ca^{2+} sparks correlate with alterations in RyR channel gating seen in planar lipid bilayer experiments (12-14, 33). In this investigation, the effect of both H1c and H1-EVH1 was specific to an increase in Ca^{2+} spark frequency, with minimal alteration in the shape of the individual Ca^{2+} sparks (i.e. spatio-temporal properties). It is widely accepted that Ca^{2+} sparks arise from a CRU containing a small number of RyR channels and that the stereotypic properties of Ca^{2+} sparks most likely are the result of some level of coordinated control. Based on this assumption, the present results support the conclusion that Homer long and short forms increase Ca^{2+} spark frequency by increasing the probability of opening of the RyR Ca^{2+} release channel(s) that initiate the Ca^{2+} sparks (trigger event within the CRU), without effecting the overall RyR channel opening underlying the Ca^{2+} spark. This finding is supported by recent work by Feng et al. (10) who demonstrated that in the BLM both Homer H1c and H1-EVH1 increases single channel gating activity without altering open dwell time.

Proposed Mechanisms for the Homer Effects Seen in this Investigation—Recent reports have hypothesized several mechanisms for Homer action on RyR ranging from a mechanical linking of related Ca^{2+} signaling proteins (34-36) to the tethering of Ca^{2+} signaling proteins to the skeletal muscle triad (9). Both of these mechanisms are thought possible due to the multimerization of Homer. Homer long form (H1c) differs from H1-EVH1 in that H1c contains a ~240 amino acid region on the C-terminal end of the EVH1 domain. This region is predicted to form a coiled-coil tertiary structure, which is thought to promote self-assembly of the Homer monomers into multimeric complexes (3, 37). The facts that an isolated single channel in BLM can be activated upon addition of H1c (9-11) and that H1-EVH1 activates both single channels in the BLM (10) and Ca^{2+} sparks (this investigation) suggest that the amplification of the Ca^{2+} signal stems from the interaction of the EVH1 domain with RyR, and that cross-linking of RyR with other proteins possessing Homer ligand is not necessary. However, based on the experiments presented here, it appears that the presence of the CC domain in conjunction with the EVH1 domain (i.e. H1c) augments this EVH1-dependent activation effect. Furthermore, the H1c/H1c-G89N heterodimer having one
wild type (binding) and one mutated (nonbinding) EUH1 domain appears to be ineffective in Ca\(^{2+}\) spark activation. A possible DHPR-RyR interaction secondary to cross-linking of DHPR and RyR by wild-type multimeric H1c would not be a likely explanation for our results. First, H1-EVH1 was an agonist of RyR activation and this protein is without a CC domain and cannot form complexes. Therefore, H1-EVH1 could act on either the DHPR or the RyR, but not by linking the two proteins in any manner. Second, in the present experiments, Homer was either added to JSR membrane or to a permeabilized muscle fiber. In ryadonine binding experiments, exogenous Homer promotes ryanodine binding to JSR independent of the DHPR, arising solely from the RyR (no InSP3 involvement). Therefore, any sparks that arise in control conditions are likely spontaneous in nature, independent of the DHPR, arising solely from the RyR (no InSP3 involvement).

Recent biochemical and in vitro evidence also supports the agonist behavior of Homer being primarily on RyRs. Taken together we can suppose that the effect of Homer on the RyR is to augment RyR sensitivity to CICR (10). While we did not expressly test this hypothesis, spontaneous Ca\(^{2+}\) sparks seen here are thought to occur via CICR mechanisms. In addition, limited experiments (\(n = \frac{2}{\text{mouse}}\); data not shown) in which a Homer challenge was performed in conditions with less CICR (or ryanodine binding) due to either a higher affinity of the wild type (binding) and one mutated (nonbinding) EUH1 do-