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Gating of Store-Operated Channels by Conformational Coupling to Ryanodine Receptors

Kirill I. Kiselyov,* Dong Min Shin,*
Yaming Wang,† Isaac N. Pessah,‡
Paul D. Allen,† and Shmuel Muallem*§
*Department of Physiology
University of Texas Southwestern Medical Center
Dallas, Texas 75235
†Department of Anesthesia
Brigham and Women's Hospital
Harvard Medical School
Boston, Massachusetts 02115
‡Department of Molecular Biophysics
School of Veterinary Medicine
University of California, Davis
Davis, California 95616

Summary

We report here that RyRs interact with and gate the store-operated hTrp3 and I_{crac} channels. This gating contributes to activation of hTrp3 and I_{crac} by agonists. Coupling of hTrp3 to IP_3Rs or RyRs in the same cells was found to be mutually exclusive. Biochemical and functional evidence suggest that mutually exclusive coupling reflects clustering and segregation of hTrp3-IP_3R and hTrp3-RyR complexes in plasma membrane microdomains. Gating of CCE by RyRs indicates that gating by conformational coupling is not unique to skeletal muscle but is a general mechanism for communication between events in the plasma and endoplasmic reticulum membranes.

Introduction

Receptor-evoked Ca^{2+} signals include Ca^{2+} release from internal stores and subsequent Ca^{2+} influx across the plasma membrane (PM) (Putney and McKay, 1999). The two activities are linked in that Ca^{2+} release from the stores is obligatory for activation of Ca^{2+} influx. This gating behavior led to the formulation of the capacitative Ca^{2+} entry (CCE) hypothesis (Putney, 1990). The hypothesis was established as a gating behavior with the identification of the Ca^{2+} release activated Ca^{2+} current (I_{CRAC}) in the plasma membrane of nonexcitable and excitable cells (Parekh and Penner, 1997).

In the last year, experimental evidence supported two mechanisms to account for the CCE phenomenon (Putney, 1999). The first is a secretion mechanism, which proposes that store depletion triggers a delivery of conducting units containing CCE channels to the plasma membrane. Fusion of the conducting units inserts the channels in the PM to activate CCE (Patterson et al., 1999; Yao et al., 1999). The second mechanism is based on the conformational-coupling hypothesis (Irvine, 1991; Berridge, 1995) in which the IP_3-activated Ca^{2+} release channels (IP_3 receptors [IP_3Rs]) in the store membrane couple to and gate Ca^{2+} influx channels in the PM. Conformational changes in IP_3Rs due to IP_3 binding and store depletion result in opening of CCE channels in the plasma membrane. Indeed, store-operated hTrp3 channels stably expressed in HEK293 cells are gated by interaction with IP_3Rs (Kiselyov et al., 1998). The interaction is limited to the N-terminal domain of the IP_3Rs (Kiselyov et al., 1999a), which is sufficient for interaction and gating of hTrp3 channels. The domains in IP_3R3 and hTrp3 and other store-operated hTrp channels that interact with each other to modulate hTrp3 activity and CCE were recently identified (Boulay et al., 1999). Gating by IP_3Rs was also observed with the native I_{CRAC}-like store-operated I_{min} channels (Zubov et al., 1999).

Conformation coupling (C-C) between IP_3Rs and CCE channels is analogous to excitation-contraction (E-C) coupling between the L-type Ca^{2+} channels and the ryanodine receptors (RyRs) in skeletal muscle (Beam and Franzini-Armstrong, 1997). In these cells, the L-type Ca^{2+} channels sense the membrane depolarization to activate the RyRs. RyRs in turn regulate the gating of the L-type Ca^{2+} channels (Nakai et al., 1996). Of the three known RyR isoforms, RyR1 is expressed in skeletal muscle and couples to L-type Ca^{2+} channels. RyR2 is expressed in cardiac myocytes and is not capable of coupling to L-type Ca^{2+} channels (Nakai et al., 1997). RyR3 is widely expressed in both excitable and nonexcitable cells (Sorrentino and Reggiani, 1999). In neonatal but not adult skeletal muscle (Bertocchini et al., 1997), and in neuronal cells (Balschun et al., 1999), it appears to participate in E-C coupling. Although studies in RyR3 null mice suggest that RyR3 may mediate Ca^{2+} release secondary to activation of Ca^{2+} entry (Sorrentino and Reggiani, 1999), the ability of RyR3 to couple to the L-type Ca^{2+} channel was not tested directly. The role of RyR3 in nonmuscle cells is not known.

Growing evidence suggests that Ca^{2+} stores of many nonmuscle cells, including neurons (Balschun et al., 1999), neuroendocrine (Bennett at al., 1998), epithelial (Lee et al., 1997b), and HEK293 cells (Querfurth et al., 1998) express RyRs. Thus, pharmacological agents such as caffeine and the second messenger cADPR (Lee, 1997) can release Ca^{2+} from internal stores of these cells. Furthermore, Ca^{2+} release by activation of RyRs in nonmuscle cells activates CCE in a manner similar to Ca^{2+} release from the IP_3-sensitive stores (Bennett et al., 1998; Usachev and Thayer, 1999). Notably, expression of RyR1 in an HEK293 clone lacking RyRs strongly increased Ca^{2+} entry due to Ca^{2+} release evoked by caffeine (Figure 6 in Tong et al., 1999). Considering the coupling and gating of CCE channels by IP_3Rs (Kiselyov et al., 1998,1999a), the question that arises is whether RyRs expressed in nonmuscle cells can also couple to and gate CCE channels such as hTrp3 and I_{crac}. The answer to this question should have important implications to the generality and origin of C-C as a mean of communication between events in the plasma membrane and the endoplasmic reticulum.

We report here that Ca^{2+} release due to activation of RyRs in T_3 cells results in activation of hTrp3 channels in a manner similar to Ca^{2+} release initiated by activation of IP_3Rs. Maximal activation of hTrp3 in T_3 cells and I_{crac} in HSG cells by agonist stimulation requires activation of the CCE channels regulated by RyR. Biochemical and functional evidence indicate that in intact cells coupling

§To whom correspondence should be addressed (e-mail: smuall@mednet.swmed.edu).

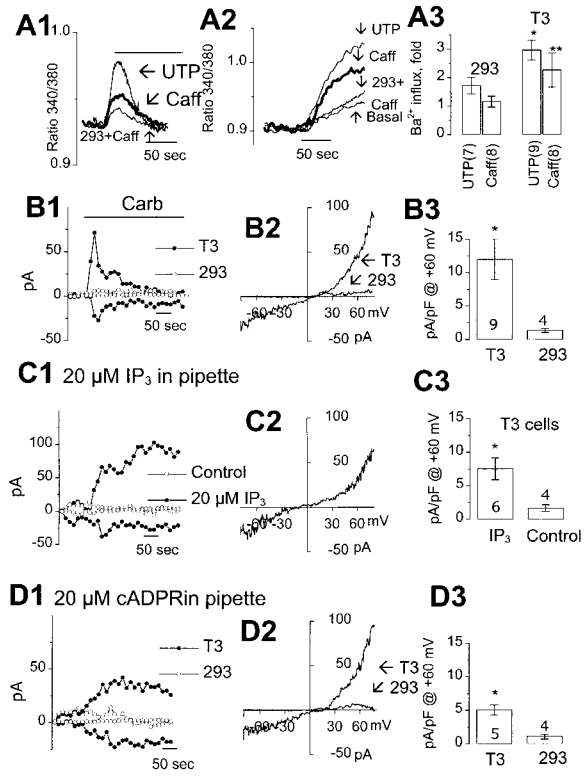

Figure 1. Ca^{2+} Release from a RyRs-Sensitive Pool Activates hTrp3

(A) The effect of agonist and caffeine on Ca^{2+} release (A1) and Ba^{2+} influx (A2) in HEK293 and T_3 cells was measured in Ca^{2+}-free solution containing 100 μM UTP or 20 mM caffeine (A1) and then 10 mM Ba^{2+} (A2). (A3) summarizes results from the multiple experiments. (B–D) Closed symbols in (B1)–(D1) show examples of hTrp3 activation by agonist (B1), IP_3 (C1), or cADPR (D1). Open symbols show similar experiments with control cells. (B2)–(D2) show macroscopic I/V plots from experiments with control or T_3 cells. (B3)–(D3) show summary of the maximal currents recorded in multiple experiments. Currents were recorded at −40 mV. Repetitive trains of five consecutive 250 ms RAMPs from −80 to +80 mV were delivered every 15 s. Data were superimposed and averaged within each train. Currents measured at +60 and −60 mV are plotted as a function of time. The currents present at the time of patch breakdown were subtracted.

of hTrp3 channels to IP_3Rs and RyRs is mutually exclusive, and mutually exclusive coupling reflects clustering of hTrp3-RyRs or hTrp3-IP_3Rs complexes in plasma membrane microdomains. These findings indicate that E-C coupling in skeletal muscle may be viewed as a specialized case of the general phenomenon of gating of plasma membrane Ca^{2+} influx channels by conformational coupling to Ca^{2+} release channels.

Results

Ca^{2+} Release by Activation of RyRs Activates hTrp3 and I_{crac}

The parental HEK293 cells and the HEK 293 cells stably expressing the hTrp3 channels (T_3 cells) used in the present study showed a similar Ca^{2+} release in response to stimulation with the RyRs ligand caffeine (Figure 1, A1), which averaged 38.7 ± 7.7% (n = 5) and 37.2 ± 3.8% (n = 7) of that caused by stimulation of the Gq-coupled P2Y receptor with UTP. By contrast, caffeine-induced Ba^{2+} influx was significantly larger ($P < 0.05$) in T_3 than in control cells (Figure 1, A2 and A3). In eight experiments, caffeine increased Ba^{2+} influx by 1.15 ± 0.19-fold in control cells and by 2.26 ± 0.6-fold (n = 8) in T_3 cells. Hence, as was found with agonist, similar Ca^{2+} release from internal stores caused larger Ca^{2+} influx in T_3 cells.

To verify that activation of RyRs activates hTrp3 channels, we measured hTrp3 current directly using the whole-cell configuration of the patch clamp technique. Figure 1B shows that the Ca^{2+} mobilizing agonist carbachol activated a nonselective, outward rectifying current in T_3 but not in control cells. The outward rectification is due to the regulation of hTrp3 channel open probability by the membrane potential (Kiselyov et al., 1998). Ca^{2+} release from internal stores due to infusion of IP_3 into the cytosol activated the hTrp3 channels (Figure 1C). Notably, infusion of the RyRs activator cADPR (Lee, 1997) through the patch pipette also activated a current typical of hTrp3 (Figure 1D). Interestingly, IP_3, and in particular cADPR alone, were less effective than agonist in activating hTrp3. Indeed, when infused together cADPR and IP_3 activated hTrp3 by 1.44 ± 0.21 (n = 4)-fold higher than IP_3 alone in the same set of experiments. None of the Ca^{2+} mobilizing agents activated a hTrp3-like current (Figure 1, B2, D2, and B3–D3).

It was important to establish the physiological significance of the regulation of hTrp3 and Ca^{2+} influx by RyRs. Since IP_3Rs and RyRs are positively and negatively regulated by Ca^{2+} and can influence each other's activity, the best protocol to isolate the contribution of RyRs to Ca^{2+} influx is to determine the effect of RyR inhibitors on Ca^{2+} influx activated by agonist stimulation. The results of such experiments with T_3 cells and the human submandibular gland (HSG) cell line, in which I_{crac} can be reliably measured (Liu et al., 1998), are shown in Figure 2. Figure 2A shows that inhibition of RyRs with either the cADPR antagonist N_2-8-cADPR or with Ruthenium Red (RuR) reduced agonist-dependent activation of hTrp3 by about 40%. Importantly, these inhibitors reduced agonist-dependent activation of I_{crac} in HSG cells by about 35% (Figure 2B). Furthermore, the two inhibitors appear to facilitate the spontaneous, time-dependent inactivation of I_{crac} current in these cells. Hence, we conclude that Ca^{2+} release from RyR-expressing stores is needed for maximal activation of store-operated Ca^{2+} influx by agonists.

Having established the physiological importance of Ca^{2+} release by RyR to agonist-stimulated Ca^{2+} influx, we proceeded to further study the relationship between hTrp3 and the RyRs channels by recording the activity of hTrp3 in cell-attached patches and in the same patches after excision into different media. Figure 3A shows that application of 20 mM caffeine to T_3 cells activated single channels with the properties of hTrp3 described before (Kiselyov et al., 1998). Excising the patches to a caffeine-free medium resulted in a complete loss of channel activity (Figure 3A, left panel). The same behavior was observed in more than 15 experiments. However, when patches from caffeine-responding cells were excised into a medium containing 20 mM caffeine, hTrp3 channel activity was observed in 11/15 experiments (Figure 3A, right panel). Several of the kinetic properties of the channel activated by caffeine in excised patches are shown in Figures 3B and 3C. The channel had an averaged conductance of either 16.0 ± 0.3 or 64.5 ± 0.3 (n = 6) pS (panel B) and a mean open time of 0.76 ± 0.06 (n =

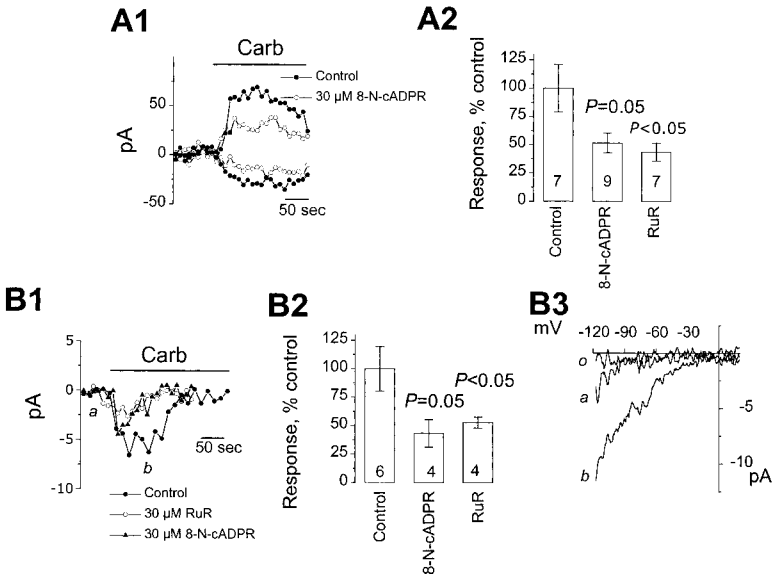

Figure 2. Regulation of hTrp3 and I_{crac} by RyRs Is Required for Maximal Activation of the Channels by Agonists

hTrp3 (A) or I_{crac} currents were measured in T_3 or HSG cells, respectively, as detailed in the Experimental Procedures. Pipette solution contained either 30 μM cADPR or 30 μM RuR. About 3 min after establishing the whole-cell configuration, the cells were stimulated with 100 μM carbachol. (A1) and (B1) show individual examples, and (A2) and (B2) show summary of the indicated number of experiments. (B3) shows the I/V plot of the macroscopic I_{crac} current at the times indicated in (B1).

5) ms (panel C). The current was nonselective with respect to K^+, Na^+, and Ca^{2+}, and showed voltage dependence of NP_o with steep outward rectification (data not shown). All these parameters are the same as those described for hTrp3 channels activated by Ca^{2+}-mobilizing agonists in intact cells and by IP_3 in excised patches (Kiselyov et al., 1998). Equally important, application of cADPR to the cytoplasmic face of patches excised from caffeine-responsive cells into caffeine-free solutions activated hTrp3 in 4/18 attempts. An example is given in Figure 3D.

Interaction of RyRs and hTrp3 Channels

The maintained regulation of hTrp3 channels by activators of RyRs in excised patches suggests that the hTrp3 and RyRs channels directly or indirectly interact with each other. Two experimental protocols were used to test this prediction. In the first protocol, we determined whether the two channels can be coimmunoprecipitated (Co-IP). Figure 4 shows that the antibody recognizing the HA-tagged hTrp3 protein Co-IP hTrp3 and RyRs from T_3 cells but not control cells. Further, antibodies recognizing RyRs IP RyRs from either control or T_3 cells but Co-IP the RyRs and hTrp3 only from T_3 cells.

In the second protocol, the ability of all known RyRs isoforms to recouple and gate hTrp3 channels was studied in detail. The experimental protocol used for these studies and the results are summarized in Figure 5. hTrp3 channel activity was measured using the cell-attached mode in T_3 cells stimulated with caffeine. Patches containing responding hTrp3 channels were excised into a control solution and were washed to strip the patches from attached RyRs. Stripping of RyRs was considered achieved when the hTrp3 channels no longer responded to application of caffeine (the first portion in each trace). The patches were then exposed to the specified native (n) or recombinant (r) RyR isoforms and in the presence or absence of RyR ligands. The results are summarized in the table in terms of the number of successful attempts in which gating of hTrp3 channels by RyRs was restored. The source of RyR1 was either microsomes containing recombinant RyR1 expressed in dyspedic myoblasts isolated from mice lacking RyR1 (see Experimental Procedures), or sarcoplasmic reticulum microsomes prepared from skeletal muscle and containing native RyR1. Recombinant or native RyR1 alone or together with cADPR restored hTrp3 channel activity in only 1/10 attempts. Incubation of RyR1 with caffeine resulted in 4/21 successful attempts. Similar findings were observed with RyR3. In the absence of ligands, RyR3 activated hTrp3 in 1/11 experiments. In the presence of cADPR or caffeine, successful reconstitution of RyR3 with hTrp3 was observed in 5/21 and 6/21 attempts, respectively.

The statistical significance of the findings in Figure 5 was analyzed by a Chi-square test. For this, we combined the experiments with ligands but no receptors into control group A (0/76 attempts). Experiments with RyR1 with and without cADPR and with RyR3 but without ligands were combined into group B (4/41 attempts). Experiments with RyRs and ligands (RyR1 + Caffeine and RyR3 + Caffeine or cADPR) were combined into group C (15/63 attempts). The difference between group A and B and A and C was statistically significant ($P < 0.01$). The difference between groups B and C (requirement for occupation or RyRs with ligands) had a $P = 0.069$. Hence, it seems that ligands tended to improve the coupling of RyRs to hTrp3. In all 20 experiments, attempt to reconstitute gating of hTrp3 with RyR2 prepared from cardiac muscle failed. This may be anticipated from the mode of E-C coupling in cardiac muscle. Unlike skeletal muscle RyRs, RyR2 expressed in cardiac cells cannot couple to the L-type Ca^{2+} channels (Nakai et al., 1997).

To extend the physiological significance of gating of CCE by RyR to I_{CRAC}-like channels, we used the protocol of Figure 5 to examine the interaction of RyRs with I_{min}. I_{min} shares many kinetic and pharmacological properties with I_{CRAC} (Kiselyov et al., 1999b), including gating by IP_3R (Zubov el al, 1999). Since regulation of I_{min} was not studied before in HGS cells, we determined first whether I_{min} can be activated by IP_3 and caffeine in these cells. Figure 6A shows that excising patches from UTP-responding cells in the cell-attached mode to a control solution resulted in disappearance of I_{min} activity. When

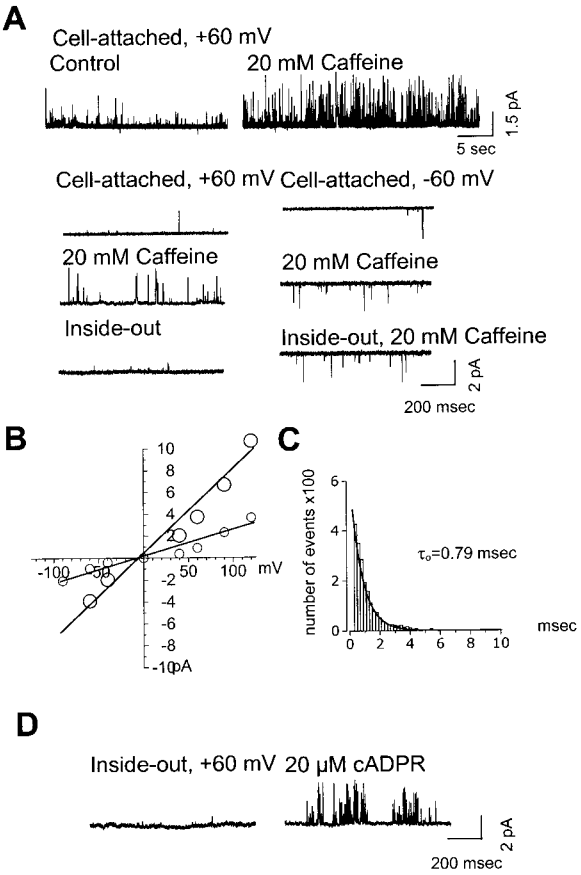

Figure 3. Single-Channel Properties of RyRs-Dependent hTrp3 Channels

(A) Single-channel recordings of caffeine-induced hTrp3 channel activity in intact cells (cell-attached) and excised (inside-out) patches.

(B) Single-channel I/V relationship of caffeine-activated hTrp3 channel. The large symbols represent the 66 pS conductance and small symbols the 17 pS substate. Errors (SEM) are smaller than the symbol sizes. Each point is the average of three to seven experiments.

(C) Dwell-time distribution of the large, 66 pS openings induced by caffeine. The curve is a single exponential fit of the data.

(D) Activation of hTrp3 channels in excised patches by cADPR. Caffeine responsive patches were excised into control medium to ensure no channel activity in the absence of cofactor and were then exposed to 20 μM cADPR. The experiment in (D) represents 4/18 successful attempts to activate hTrp3 by cADPR.

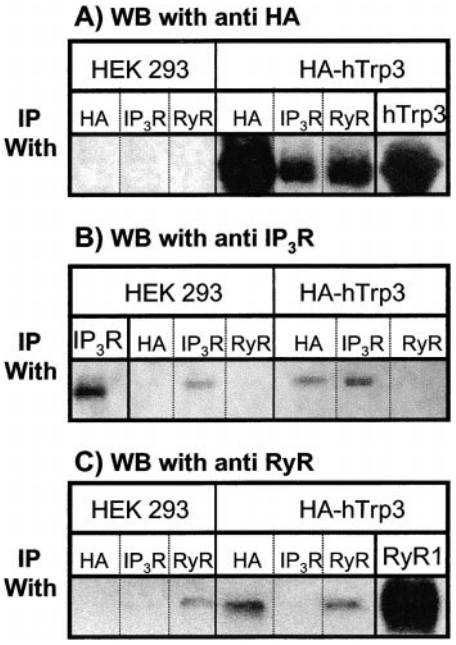

Figure 4. Immunoprecipitation of hTrp3-RyRs and hTrp3-IP_3Rs Complexes

Extracts from HEK293 or T_3 cells were used to IP hTrp3, RyRs, or IP_3Rs with the respective antibodies. Proteins were blotted by anti-HA (A), anti-IP_3Rs (B), or anti-RyRs (C) antibodies. Extracts from T_3 cells were used for hTrp3 (last lane in [A], -Con) and IP_3R3 (first lane in [B], -Con) standards and from skeletal muscle microsomes for RyRs standards (last lane in [C], -Con).

seven of these patches were exposed to IP_3, I_{min} was observed in three patches (Figure 6B). Similarly, exposing seven of the patches to caffeine resulted in reactivation of I_{min} in three patches (Figure 6C). After washing the patches 3–4 times with the bath solution, caffeine could no longer activate I_{min} in 16 patches (Figure 6D). Exposing these patches to caffeine and microsomes containing recombinant RyR3 reconstituted I_{min} activity in 4/16 patches (Figure 6E). This rate is similar to that found for reconstitution of hTrp3 gating by RyR3 (Figure 5). Hence, similar to hTrp3, I_{min} is gated by both IP_3Rs and RyRs.

Coupling of hTrp3 Channels to IP_3Rs and RyRs Is Mutually Exclusive

Gating of hTrp3 channels by RyRs and IP_3Rs in the same cells raised the question of whether the same hTrp3 channels are coupled simultaneously to the two types of Ca^{2+} release channel or whether coupling of hTrp3 channels to IP_3Rs and RyRs is mutually exclusive. To distinguish between these possibilities, we embarked on the series of experiments illustrated in Figure 7. The reasoning behind these experiments is as follows. Mutually exclusive coupling requires that RyRs-coupled hTrp3 channels will be excised with RyRs and should be sensitive to and only to activators of RyRs. That is, hTrp3 channels activated by caffeine in the cell-attached mode should retain their activity after patch excision into caffeine-containing medium and lose their activity when excised into control or IP_3-containing medium. Conversely, hTrp3 channels not responsive to caffeine in the cell-attached mode should be coupled to IP_3Rs and should retain their activity when excised into IP_3-containing but not caffeine-containing medium. If hTrp3 channels are simultaneously coupled to IP_3Rs and RyRs or hTrp3 channels coupled to either receptor are present in the same patch, then they should respond to IP_3 or caffeine after patch excision. The results in Figure 7 demonstrate that in intact cells coupling of hTrp3 to RyRs and IP_3Rs is mutually exclusive. The type of coupling present in the patches was evaluated by the ability of caffeine to activate the channels in the cell-attached mode. hTrp3 channels activated by caffeine were considered coupled to RyR. For example, in Figure 7A 15/24 and in Figure 7C, 12/27 of the channels were considered coupled to RyRs, whereas the remaining are assumed to be coupled to IP_3R. Figure 7A shows that excision of 15 patches containing RyRs-coupled hTrp3 channels into control medium resulted in loss of hTrp3 channel activity. Figure 7B shows that in another set of experiments

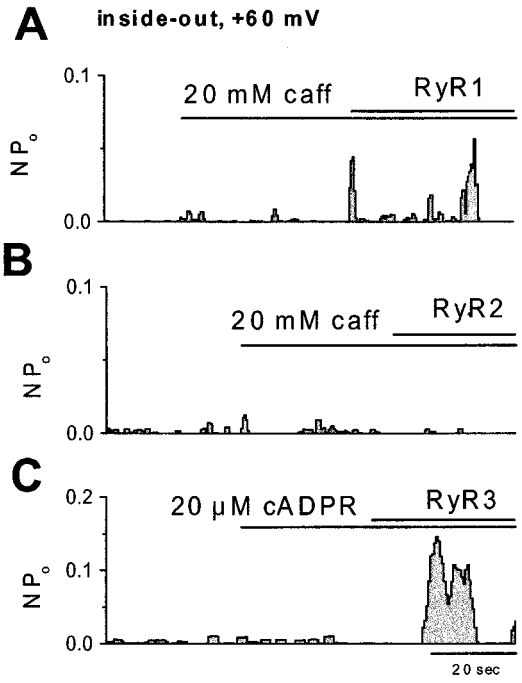

Figure 5. Specificity of RyRs Isoforms in Reconstituting hTrp3 Gating in Excised Patches

(A)–(C) These panels illustrate typical experiments to test reconstitution of gating of hTrp3 channels by the indicated RyR in the presence of 20 mM caffeine. Results are shown in the form of increased NP_o. Patches containing caffeine-responsive hTrp3 channels were washed (three to four times with 1.5 ml while keeping bath solution at 100 μl to reduce patch rupture) before application of 20 mM caffeine and then caffeine and control microsomes (microsomes from nontransfected cells) or caffeine and the indicated RyR isoforms. The table in (D) summarizes the results of all experiments. The source of RyRs was either native (n) (skeletal [RyR1] or cardiac [RyR2] SR) or recombinant (r) (RyR1 and RyR3).

D

	alone	+cADPR	+CAFF
no receptor	0/33	0/21	0/22
RyR1	1/10r + 1/10n	1/10r	4/21n
RyR2	0/10n	-	0/10n
RyR3	1/11r	5/21r	6/21r

11 of 15 patches containing RyRs-coupled hTrp3 channels retained hTrp3 channel activity when excised into medium containing caffeine. Figure 7C shows that none of the 12 RyRs-coupled hTrp3 channels responded to IP_3 after patch excision into IP_3-containing medium. By contrast, of the remaining 15 patches that did not respond to caffeine in the cell-attached mode, 10 responded to IP_3 after patch excision (Figure 7D). As an additional control, we observed that excision of seven patches containing caffeine-insensitive hTrp3 channels into medium containing caffeine did not result in any channel activity (Figure 7D). Thus, the patches contained hTrp3 channels coupled to either IP_3Rs or RyRs.

Spatial Segregation of hTrp3-IP_3Rs and hTrp3-RyRs Complexes

Possible explanations for the mutually exclusive coupling of hTrp3 channels to IP_3Rs and RyRs are (1) the density of coupled hTrp3 channels is low and each patch contains a single hTrp3 channel coupled to IP_3Rs or RyRs; and (2) hTrp3 channels coupled to IP_3Rs or RyRs are spatially segregated in plasma membrane microdomains. Biochemical and functional evidence supports the second alternative. To determine the relationship between hTrp3 channels coupled to RyRs and IP_3Rs, we tested the ability of anti-RyRs antibodies to Co-IP IP_3Rs and the ability of anti-IP_3R3 antibodies to Co-IP hTrp3 and RyRs. Figure 4A shows that anti-IP_3Rs and anti-RyRs antibody Co-IP similar amount of hTrp3 protein. However, anti-IP_3Rs antibodies IP IP_3Rs, but not RyRs, and conversely, anti-RyRs antibodies IP RyRs but not IP_3Rs. Similar results were observed in seven experiments. Thus, hTrp3 channels form separate complexes with RyRs and IP_3Rs.

Functional segregation of the complexes is demonstrated in Figure 8. In these experiments, we used large diameter pipettes ($\geq$ 5 μm) to increase the probability of finding patches containing at least two hTrp3 channels. In 20 of the large cell-attached patches, the presence of two caffeine-activated RyRs-coupled hTrp3 channels could be clearly resolved. The very short dwell time of hTrp3 (0.8 ms, Figure 3) dictates that simultaneous opening of more than one hTrp3 channels present in a patch must be a rare event. This indicates that the number of hTrp3 channels per patch is underestimated in all experiments. Thus, simultaneous opening of two

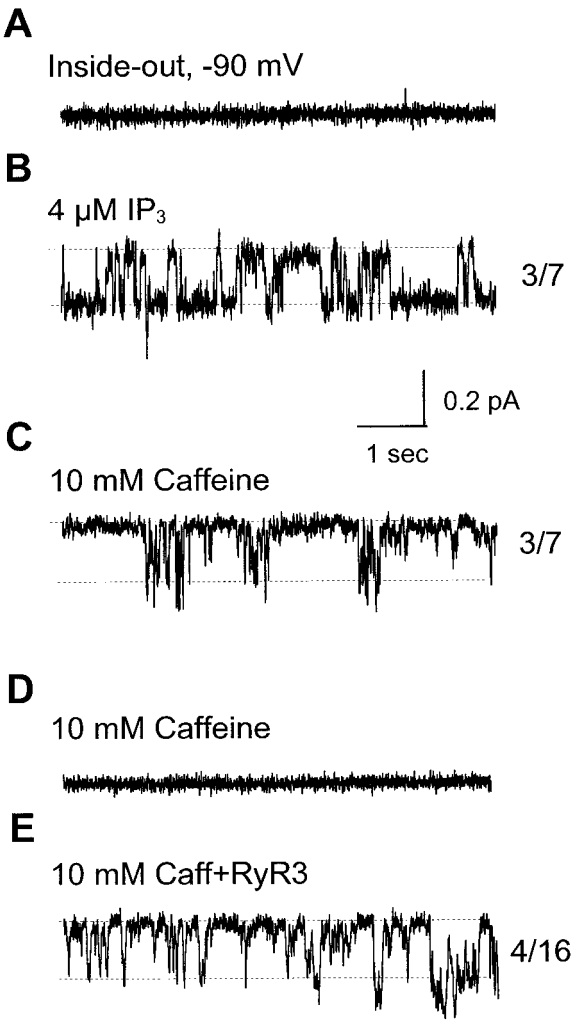

Figure 6. Interaction of the I_{crac}-like Channel I_{min} with RyRs in HSG Cells

The presence of I_{min} in each patch was verified in the cell-attached mode. The patches were then excised into a control solution (A) that also contained 4 μM IP_3 (B) or 10 mM caffeine (C). In another set of experiments, patches were excised into a solution containing either IP_3 or caffeine. If I_{min} activity was present, the patches were washed until they failed to respond to caffeine ([D], 16 patches). The patches were incubated with 10 mM caffeine and microsomes expressing recombinant RyR3 (E). Successful reconstitution of I_{min} was observed in 4/16 experiments.

hTrp3 channels in a patch likely reflects clustering of several hTrp3 channels in the membrane patch. Examples of patches containing two resolvable hTrp3 channels are given in Figures 8A and 8B. In 9/9 of these patches, hTrp3 activity was retained after excision into a medium containing caffeine. The high rate of hTrp3 channel activity conservation (100%) probably reflects the presence of more than one active channel in the patches. By contrast, excision of 11 patches containing two resolvable RyRs-coupled hTrp3 channels into medium containing IP_3 failed to preserve hTrp3 channel activity. Since IP_3 activated hTrp3 channels in 10/15 patches that contained caffeine nonresponsive channels (Figure 7D), the results in Figure 8 indicates that the hTrp3-RyRs and hTrp3-IP_3Rs complexes are segregated in plasma membrane microdomains.

In the next set of experiments, an attempt was made to determine whether the mutually exclusive coupling is due to a structural modification(s) of subsets of hTrp3 channels. For this, we tested whether hTrp3 channels coupled to RyRs can be stripped of RyRs and made to recouple to IP_3Rs. The experimental protocol is shown in Figure 9. Of the 27 patches studied, 11 responded to caffeine in the cell-attached mode. The 11 patches were excised into medium containing IP_3 to ascertain the absence of IP_3Rs-coupled channels in the patches. Indeed, 11/11 patches failed to respond to IP_3. The patches were then further washed to strip them of RyRs and treated with microsomes rich in IP_3R1. hTrp3 channel activity was restored in 4/11 patches. For controls, eleven of the caffeine-insensitive patches in the cell-attached mode were excised into a medium containing IP_3 and washed to strip them from IP_3Rs in the same manner as patches obtained from caffeine-responsive cells. Microsomes containing IP_3R1 reactivated hTrp3 in 5/11 of these patches. Hence, it appears that IP_3Rs can couple to hTrp3 channels previously coupled to IP_3Rs or RyRs with similar efficiency.

Discussion

Ca^{2+} release from internal stores by activation of either IP_3Rs or RyRs leads to activation of CCE. In the present work, we extended these findings to show that Ca^{2+} release from RyR-sensitive stores activates I_{CRAC} and RyRs participate in activation of I_{CRAC} during agonist stimulation. Furthermore, I_{min}, which is probably a form of I_{CRAC} (Kiselyov et al., 1999b; Zubov et al., 1999), is gated by RyRs. Thus, our findings indicate that I_{CRAC} is gated by both IP_3Rs and RyRs. This conclusion appears in contradiction with a recent study in which an inhibitor of IP_3Rs, 2-Aminoethoxydiphenyl Borate (2ABP), completely inhibited CCE in a T_3 clone in which all stores were depleted of Ca^{2+} (Ma et al., 2000). However, it is possible that unlike our clone, the T_3 clone used in these studies did not express RyRs. HEK293 clones vary considerably with respect to expression of RyRs. In addition, at the concentration used, 2ABP was reported to have nonspecific effects in some cell types (Maruyama et al., 1997). It is thus possible that 2ABP at least partially inhibited RyRs (if present) in the cells used in Ma et al. (2000) studies. Based on the findings of the present studies, we therefore conclude that, when expressed, RyRs participate in gating of CCE channel during agonist stimulation.

In recent years, studies focused on the mechanism by which I_{CRAC} can sense and respond to Ca^{2+} content in the stores. Experimental evidence supported two major mechanisms, regulation of Ca^{2+} influx by exocytosis (Patterson et al., 1999; Yao et al., 1999; Ma et al., 2000) or by conformational coupling (C-C) between the Ca^{2+} release channels and CCE (Kiselyov et al., 1998, 1999a; Boulay et al., 1999). Regulation by exocytosis was suggested based on findings in *Xenopus* oocytes in which inhibitors of SNARE proteins inhibited CCE (Yao et al., 1999) and on findings in several cell lines in which solidification of the cytoskeleton inhibited CCE (Patterson et al., 1999; Ma et al., 2000). Regulation by C-C was concluded from the findings that IP_3Rs and the store operated hTrp3 channels interact with each other (Boulay et al., 1999; Kiselyov et al., 1999a). Partially purified

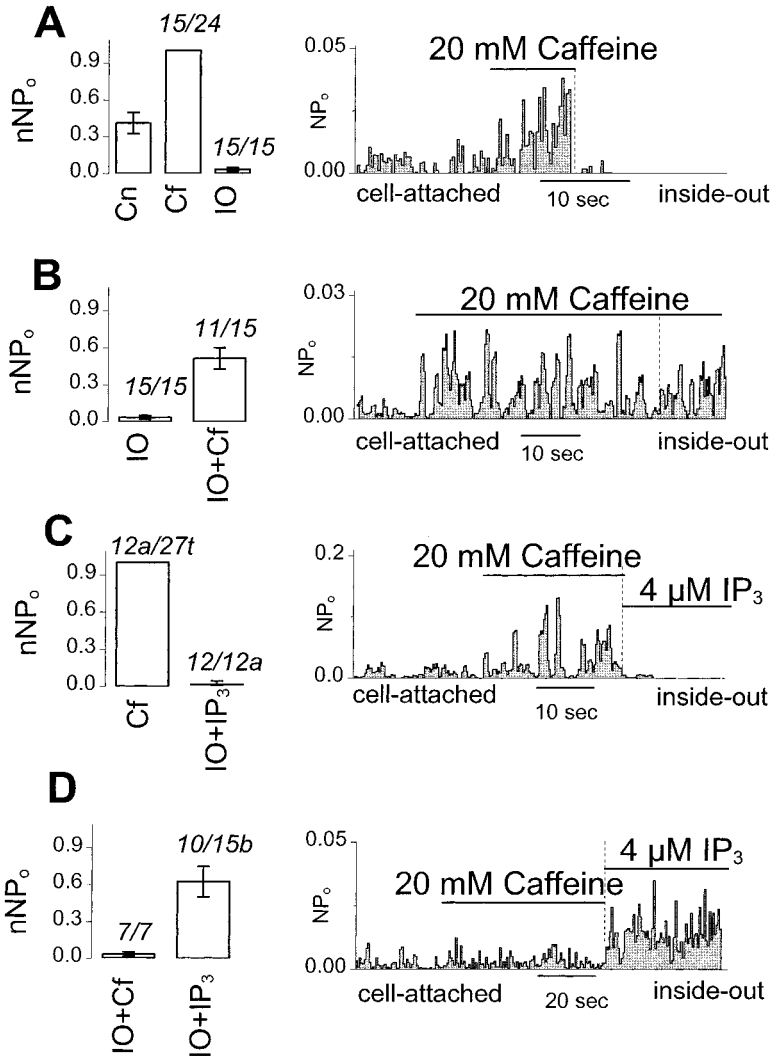

Figure 7. Mutually Exclusive Coupling of hTrp3 to RyRs or IP_3Rs

The experimental protocol was as follows. Cells in the cell-attached configuration were stimulated with caffeine, and patches were excised into a control solution or solutions containing caffeine or IP_3. To facilitate comparison of the data, NP_o is calculated relative to the activity measured in caffeine-responsive cell-attached patches, which was taken as 1.0. Numbers above the bars show the number of experiments performed under each condition. In (A), 15/24 cells responded to caffeine in cell-attached mode, and hTrp3 activity was absent in all 15 patches when excised (IO-inside out) into control solution. In (B), the 15 cells in (A) are compared to another set of 15 caffeine-responding cells. In 11/15 patches, hTrp3 activity was retained after excision into a caffeine-containing solution. In (C), of the 12 cells that responded to caffeine, all lost hTrp3 channel activity when excised into IP_3-containing medium. The remaining 15 cells that did not respond to caffeine in the cell-attached mode were excised into IP_3-containing medium. (D) shows that 10 of these 15 patches retained hTrp3 channel activity. As a control for the latter, (D) also shows that excising patches from caffeine-nonresponding cells into medium containing caffeine did not result in hTrp3 activity in 7/7 patches. Right panels, examples of histograms of NP_o plots.

IP_3R1 reconstituted into liposomes (Kiselyov et al., 1998) or the soluble N-terminal domain of the IP_3R1 (Kiselyov et al., 1999a) can couple to and gate the activity of hTrp3 channels. Domains in hTrp3 and IP_3R3 directly interact with each other, and the interaction affects CCE (Boulay et al., 1999). Furthermore, IP_3R1 gates the miniature, I_{CRAC}-like and highly Ca^{2+} selective channel I_{min} (Zubov et al., 1999). Since disassembly of the cytoskeleton does not interfere with CCE either in oocytes or in cell lines (Patterson et al., 1999; Yao et al., 1999), prior cell stimulation is not needed to insert I_{min} (Zubov et al., 1999) or hTrp3 (K. I. K. and S. M., unpublished data) into the PM, and since the findings in cell lines (Patterson et al., 1999; Ma et al., 2000) can be explained well by a C-C mechanism, we tend to favor regulation of CCE by C-C.

In the present work, we were able to develop evidence that indicates that RyRs, like IP_3Rs, can gate store-operated channels by C-C. Thus, Ca^{2+} release from RyRs-sensitive stores activated hTrp3 and I_{CRAC}, activation of RyRs in cell-attached and the same excised patches activated the same hTrp3 channels and the RyRs and hTrp3 channels can be Co-IP with antibodies recognizing either RyRs or hTrp3. Moreover, in excised patches, activation of hTrp3 and I_{min} channels by activators of RyRs can be removed by washing the cytoplasmic face of the patch and reconstituted with native or recombinant RyRs. Several findings indicate that the reconstitution was specific: (a) Microsomes prepared from cells not expressing RyRs were without effect; (b) Notably, microsomes containing RyR2 prepared from cardiac muscle did not couple to gate hTrp3. The RyR2 does not couple to the L-type Ca^{2+} channel in cardiac muscle or in dyspedic cells expressing recombinant RyR2 (Nakai et al., 1997); (c) The ability of RyRs to gate hTrp3 channels required RyRs ligands and the ligands tended to enhance the reconstitution of RyRs and hTrp3 complexes. Interestingly, RyRs in the absence of caffeine and cADPR reconstituted gating of hTrp3 at low frequency (Figure 5). This may be explained by the effect of Ca^{2+} on the RyRs.

Based on the findings listed above, we conclude that RyRs can interact with and gate store-operated channels. This conclusion has several important implications. First, it shows that regulation of Ca^{2+} influx channels by RyRs is not unique to skeletal muscle. Thus, gating of PM Ca^{2+} channels by intracellular Ca^{2+} release channels may be viewed as a general and widespread paradigm in Ca^{2+} signaling. Second, it explains how Ca^{2+} release

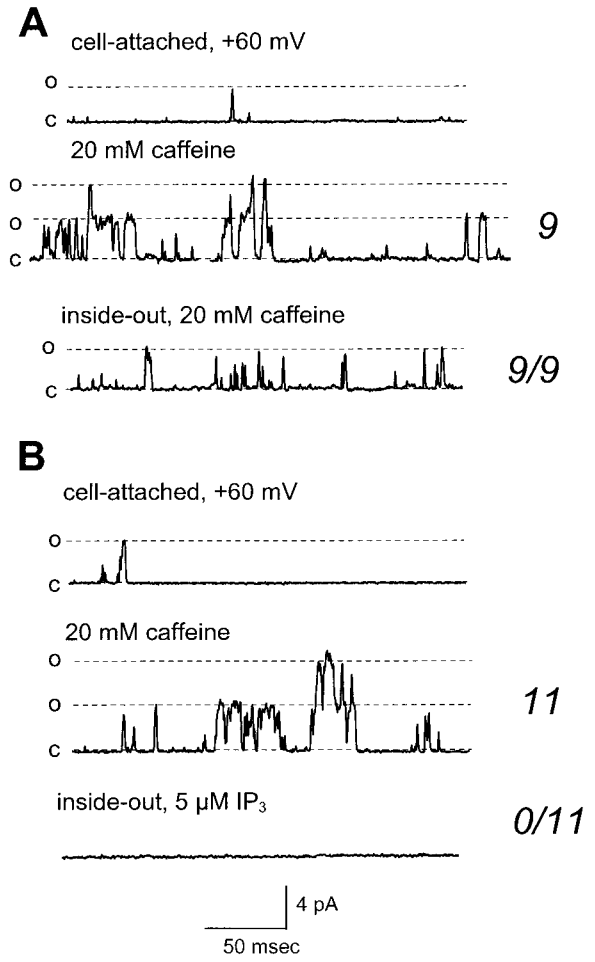

Figure 8. Multiple hTrp3-RyRs Channels in Excised Patches

The middle traces in (A) and (B) show examples of two channel openings in large cell-attached patches. hTrp3 channel activity was retained in 9/9 patches containing two resolvable hTrp3 channels when excised into 20 mM caffeine (A). hTrp3 channel activity was not observed in 11/11 similar patches when excised into medium containing 5 μM IP_3.

from RyRs-sensitive stores activates CCE and I_{crac}. Third, it can explain why truncation of IP_3Rs of B lymphocytes did not prevent CCE (Sugawara et al., 1997). Fourth, this conclusion implies similarities between E-C coupling in skeletal muscle and C-C in nonmuscle cells. In skeletal muscle, the L-type Ca^{2+} channel activates the RyRs but also the RyRs modulate the activity of the L-type Ca^{2+} channel (Nakai et al., 1996). Regulation of L-type Ca^{2+} channels by RyRs was also reported in neuronal cells (Chavis et al., 1997). In neuronal cells, the coupling between the L-type Ca^{2+} channels and RyRs survived patch excision and required activation of RyRs by caffeine (Chavis et al., 1997), similar to the findings reported here for the interaction between hTrp3 channels and RyRs. In this respect, it is interesting to note that amino acids 728–735 in the C-terminal tail of hTrp3 have the motif KCRRRRLQ, which consists of a stretch of five positively changed amino acids followed by a neutral and a negatively charged amino acid. A very similar motif, EKKRRKMS, was identified in the II-III loop of the skeletal muscle L-type Ca^{2+} channel as mediating the coupling of L type Ca^{2+} channels to RyR1 (Gurrola et al., 1999). In cardiac L-type Ca^{2+} channel, the second lysine is replaced with glutamate, and the methionine and serine are replaced with leucine and alanine, respectively (Gurrola et al., 1999). These amino acids substitutions prevented binding of the cardiac L-type Ca^{2+} channel to RyR1 (Gurrola et al., 1999). Hence, it will be of interest to determine whether the motif in hTrp3 homologous to that in the II-III loop of the skeletal muscle L-type Ca^{2+} channels is needed for gating CCE channels by RyRs and possibly IP_3Rs.

An interesting observation with implications to organization of Ca^{2+} signaling complexes in cellular microdomains is the mutually exclusive coupling of hTrp3 to RyRs or IP_3Rs. Mutually exclusive coupling can arise from the generation of two structurally distinct populations of hTrp3 channels by posttranslational modification. This is unlikely the case, since hTrp3 channels coupled to RyRs can be stripped of RyRs and recouples to IP_3Rs in a similar frequency as hTrp3 channels previously coupled to IP_3Rs.

Another mechanism to achieve mutually exclusive coupling is by targeting hTrp3 channels and subsets of RyRs-expressing or IP_3Rs-expressing internal stores to cellular microdomains. Support for such a mechanism comes from the ability of cells to target Ca^{2+} transport proteins such as IP_3Rs, SERCA pumps, and PMCA pumps to cellular microdomains (Sasaki et al., 1994; Lee et al., 1997a, 1997b). In addition, the Ca^{2+} pool responsible for gating of CCE consists of only a very small fraction of the mobilizable intracellular pool and is probably located just underneath the PM (Broad et al., 1999). Accordingly, anti-RyRs antibodies IP hTrp3-RyRs, but not hTrp3-IP_3Rs complexes and anti-IP_3Rs antibodies IP hTrp3-IP_3Rs, but not hTrp3-RyRs complexes. Furthermore, in large patches containing multiple hTrp3 channels, the coupling of hTrp3 to RyRs or

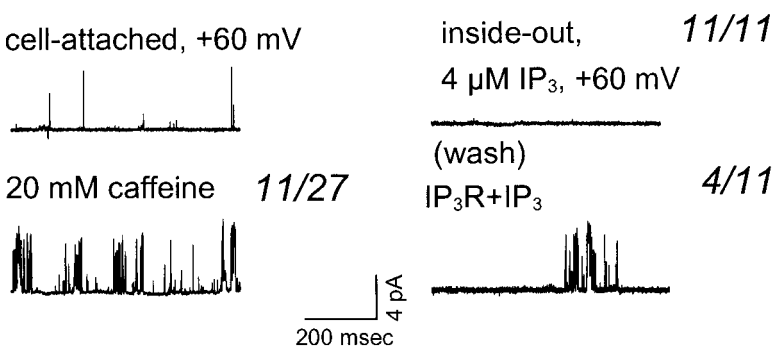

Figure 9. Recoupling of the RyRs-Coupled hTrp3 Channels to IP_3Rs

Caffeine-responsive patches in the cell-attached mode (left panels) were excised into IP_3-containing medium to verify lack of response to IP_3 (top right). After several washes, the patches were incubated with microsomes containing a high level of IP_3Rs (bottom right). Recoupling of hTrp3-RyRs to IP_3Rs was observed in 4/11 experiments.

IP_3Rs was mutually exclusive. It should have not been possible to observe mutually exclusive coupling in all patches containing more than one hTrp3 channel if the patches contained mixed populations of hTrp3 channels. Finally, estimation of the number of channels/cell and the rate of observing two hTrp3 in a 5–6 μm diameter patch also support channel clustering. In previous work, we estimated that T_3 cells contain 20 channels/cell (Kiselyov et al., 1998). The cell diameter is between 15–20 μm. A simple calculation shows that a 5–6 μm patch comprises ~1/30-1/40 of the cell surface area. Selection of patches containing hTrp3 channels for these experiments ensures that each patch will have at least one active hTrp3 channel. Assuming that 50% of channels are coupled to either IP_3Rs or RyRs and that the channels are evenly distributed in the plasma membrane, the probability of seeing one RyRs-hTrp3 channel in a patch is about 1/2. The probability of finding two RyRs-hTrp3 channels in a single patch is very low due to the low number of hTrp3 channels in a cell. The short dwell time of hTrp3 channels reduces this probability even further. Experimentally, two RyRs-hTrp3 channels in a patch were found in 20/42 large, caffeine-responsive patches. This can occur only if the hTrp3 channels are clustered in plasma membrane microdomains.

Spatial segregation of hTrp3-RyRs and hTrp3-IP_3Rs complexes in plasma membrane microdomains can be achieved by the aid of scaffolding proteins. dTrp channels are localized in the rhabdomere by binding to the PDZ domain of INAD (Tsunoda et al., 1997). In the postsynaptic density, IP_3R1 is localized close to L-type Ca^{2+} channels by binding to the scaffold protein Homer (Tu et al., 1998). The concept of targeting of signaling proteins into cellular microdomains is emerging as an important means for controlling cellular activity (Hunter, 2000). It is thus possible that binding to scaffold proteins segregates hTrp3 channels to signaling complexes in cellular microdomains. These structures may be in close proximity to population of internal stores containing IP_3Rs or RyRs that gate CCE channels.

Experimental Procedures

Cells

HEK293 cells stably expressing hTrp3 were kept in culture as described elsewhere (Kiselyov et al., 1998). For patch clamp experiments and fluorescent measurement, cells were seeded onto coverslips and maintained in culture for 1–3 days before use. HSG cells were handled essentially the same way as HEK293 cells and were used for the experiments within a day of reseeding.

Preparation of Skeletal and Cardiac SR

Membranes enriched in markers of junctional SR were prepared from rabbit fast skeletal muscle (Saito et al., 1984) in the presence of 100 μM PMSF and 10 μg/ml leupeptin. Purified heavy SR was pelleted, resuspended at a protein concentration of 2–6 mg/ml, frozen in liquid nitrogen, and stored at -80°C until needed. Membranes enriched in cardiac junctional SR were prepared from rabbit heart using according to Feher and Davis (1991).

Preparation of Recombinant RyR1 and RyR3

Primary dyspedic myoblasts were isolated from neonates homozygous for an insertion in the RyR1 gene that disrupted translation of the protein (Nakai et al., 1996). Myoblasts were grown and differentiated as before (Nakai et al., 1997), and the myotubes were infected at MOI 1 with helper virus-free HSV-1 amplicon virions containing cDNA encoding for either RyR1 or RyR3 (Wang et al., 1999). Twenty-four hours postinfection, cells from 10 plates were harvested into a buffer and centrifuged for 5 min at 100 × g. The pellets were suspended in an ice-cold hypotonic buffer containing a cocktail of protease inhibitors and homogenized. This homogenate was centrifuged at 110,000 g for 1 hr at 4°C, and the pellet was suspended in 4 ml of a buffer containing 10% sucrose and 10 mM HEPES (pH 7.4). The dispersed pellet was loaded on top of a 27%/45% sucrose gradient and centrifuged at 40,000 × g 1 hr. The fraction at the 27%/45% interface was collected, washed once, and resuspended in the 10% sucrose buffer. Aliquots were frozen in liquid nitrogen and stored at -80°C. Before use, aliquots were thawed on ice and diluted (1:10) into a solution containing 600 mM KCl and 10 mM HEPES (pH 7.4). After 30 min incubation at 0°C, the microsomes were collected by 30 min centrifugation at 100,000 × g and resuspended with the intracellular-like pipette solution for reconstitution experiments.

Electrophysiology

The whole-cell configuration of the patch clamp technique was used to follow hTrp3 and I_{crac} channel activities in intact cells. Currents were recorded using an Axopatch 200A patch-clamp amplifier and digitized at 1 kHz. Membrane potential of T_3 cells was held at –40 mV and that of HSG cells at +40 mV to minimize Ca^{2+}-dependent inactivation of I_{crac}. Membrane conductance of T_3 cells was probed with five consecutive 250 ms RAMPs from -80 to +80 mV, and that of HSG cells with RAMPs from -120 to +60 mV, delivered every 15 s. Pipette resistance for the whole-cell mode was between 4–6 MΩ, and seal resistance was always more the 2 GΩ. Single hTrp3 channels were recorded in cell-attached and inside-out patches. Currents were digitized at 5 kHz. Two types of pipettes were used for the cell-attached and inside-out patch recording. For most experiments, pipettes of 8–12 MΩ resistance were used. To obtain large patches containing more than one resolvable hTrp3 channel, pipettes of 4–6 MΩ resistance were used. To increase the probability of useable experiments, we attempted to select patches containing hTrp3 channels. This was accomplished by up to 3 min recording in the cell-attached mode and before cell stimulation to follow spontaneous activity of hTrp3. Only patches showing at least one opening of hTrp3 (i.e., positive patches) were used. This is particularly significant for the analysis of the experiments in Figures 5 and 7–9. Indeed, as expected, only about 40% of all patches using the small diameter pipettes contained hTrp3 channels. Current recording and analysis was performed with the use of pClamp 6.0.3 software suite. Experiments were carried out at room temperature (22°C–24°C). Results are given as mean ± SEM. Error bars denoting SEM are shown where they exceed the symbol size.

Fluorescence Measurements

Cells grown on glass coverslips were loaded with 2 μM FURA/2AM. FURA 2 loaded cells were placed in a perfusion chamber and washed 2–3 times with 1.5 ml of standard solution while maintaining a constant chamber volume of 300 μl. Application of agonist and Ba^{2+} was made by solution changes so that fluorescence is recorded under the same conditions as ionic currents. Loaded cells were illuminated by an alternating 340/380 nm light delivered every 0.5 s and fluorescence intensity at 510 nm was measured with a DeltaRAM fluorimetric system. The extent of divalent cation influx is derived from the change in the ratio of fluorescence intensity at 340 and 380 nm.

Solutions

The standard bath solution for $[Ca^{2+}]_i$ measurements contained (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES/KOH, (pH 7.5 with NaOH), 10 Glucose, and either 1 mM $CaCl_2$ or, 10 mM $BaCl_2$ or 0.1 mM EGTA. The extracellular solution for whole-cell current measurements (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 mM $CaCl_2$, 10 HEPES/KOH, (pH 7.5 with NaOH), and 10 Glucose. The standard intracellular-like, pipette solution used during whole-cell current recording contained (in mM): 140 K-Glutamate, 5 NaCl, 1 $MgCl_2$, 10 HEPES, 1.13 $CaCl_2$, and 2 EGTA/KOH (pCa 7) (pH 7.5). The pipette solution used in single channel patch clamp measurements contained (in mM): 140 Na-HEPES, 2 $CaCl_2$ and 5 NaCl (pH 7.5). In cell-attached experiments, the bath solution contained (in mM): 140 KCl, 5 NaCl, 10 HEPES/KOH, 1 $MgCl_2$, and 2 $CaCl_2$ (pH 7.5). After current recording

in cell-attached mode, the bath solution was changed to that specified for the pipette solution in whole-cell recording before patch excision.

I_{crac} measurements in HSG cells were performed using a bath solution containing (in mM): 140 Na Gluconate, 5 NaCl, 10 HEPES/KOH, (pH 7.4 with NaOH), 10 Glucose and 1 or 10 mM $CaCl_2$. The pipette solution contained (in mM): 140 Cs-Gluconate, 5 NaCl, 1 $MgCl_2$, 1 ATP, 10 HEPES, 10 EGTA/CsOH pH 7.4. Under these conditions I_{crac} in HSG cells is activated very slowly in the absence of agonist. Agonist stimulation or inclusion of IP_3 in the pipette solution resulted in a rapid activation of I_{crac} (Figure 2 and Liu et al., 1998)

Immunoprecipitation

Cells were washed with PBS, collected by centrifugation, and lysed with 200 μl of a buffer containing (in mM) 20 HEPES, 150 NaCl, 1 EDTA, 3 DTT, and 1% CHAPS (pH 8.0) supplemented with a cocktail of protease inhibitors. The cleared lysate was incubated with 4 μl anti HA antibodies, 5 μl anti IP_3R3 antibodies, or 30 μl anti RyRs antibodies for 1 hr before addition of 25 μl sepharose A beads and an overnight incubation at 4°C under gentle agitation. The immunoprecipitated proteins were analyzed by SDS-PAGE. Proteins were detected by Western blotting with the desired antibodies. Anti-HA monoclonal antibodies (5 mg/ml) were obtained from BABCO (Richmond, CA) and anti-IP_3Rs monoclonal antibodies (250 μl/ml) were obtained from Transduction Laboratories (Lexington, KY). The anti-RyRs monoclonal antibodies were prepared by Sutko et al. (Airey et al., 1990) and purchased from the Hybridoma Bank, University of Iowa.

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