Essential control of an endothelial cell $I_{SOC}$ by the spectrin membrane skeleton

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Mechanism(s) underlying activation of store-operated Ca$^{2+}$ entry currents, $I_{SOC}$, remain incompletely understood. F-actin configuration is an important determinant of channel function, although the nature of interaction between the cytoskeleton and $I_{SOC}$ channels is unknown. We examined whether the spectrin membrane skeleton couples Ca$^{2+}$ store depletion to Ca$^{2+}$ entry. Thapsigargin activated an endothelial cell $I_{SOC}$ (−45 pA at −80 mV) that reversed at +40 mV, was inwardly rectifying when Ca$^{2+}$ was the charge carrier, and was inhibited by La$^{3+}$ (50 μM). Disruption of the spectrin–protein 4.1 interaction at residues A207-V445 of βSpIΣ1 decreased the thapsigargin-induced global cytosolic Ca$^{2+}$ response by 50% and selectively abolished the endothelial cell $I_{SOC}$ without altering activation of a nonselective current through cyclic nucleotide–gated channels. In contrast, disruption of the spectrin–actin interaction at residues A47-K186 of SpII 1 decreased the charge influx factor of unknown molecular identity (Randriamampita and Tsien, 1993; Csutora et al., 1999; Trepakova et al., 2000), cyclic GMP (Pandol and Schoeffield-Payne, 1990; Bahnson et al., 1993; Xu et al., 1994), small GTP binding proteins (Bird and Putney, 1993; Fasolato et al., 1993; Rosado and Sage, 2000[c]), cytochrome P450 products (Alvarez et al., 1992), and others have all been implicated as putative diffusible messengers, though none of these molecules has achieved uniform acceptance as the primary mechanism of channel activation. Conformational coupling between the endoplasmic reticulum and plasmalemma has also been advanced as a mechanism of channel activation. Direct interaction of Ca$^{2+}$ release channels with membrane Ca$^{2+}$ entry channels was proposed by Irvine (1990); coimmunoprecipitation of inositol 1,4,5-trisphosphate receptors (Ca$^{2+}$ release channel) with transient receptor potential 1 (Rosado and Sage, 2000[b]) and transient receptor potential 3 (Boulay et al., 1999; Kiselyov et al., 1999) channels provides recent support for this model. Finally, a secretion-like model has been proposed that suggests translocation of the endoplasmic reticulum to the plasmalemma is an important mechanism of coupling between membranes (Patterson et al., 1999; Yao et al., 1999). In this model, F-actin in the membrane skeleton or peripheral cortical rim may impaire membrane coupling. Indeed, reorganization of F-actin from a peripheral to centrally localized pattern appears important for activation of store-operated Ca$^{2+}$ entry, suggesting reorganization is important for protein coupling between the membranes (Moore et al., 1998; Patterson et al., 1999; Norwood et al., 2000; Rosado et al., 2000; Rosado and Sage, 2000[a,b]). Both conformational and secretion-like models implicate a physical coupling mechanism between Ca$^{2+}$ store depletion and activation of Ca$^{2+}$ entry.

The role of F-actin in regulation of store-operated Ca$^{2+}$ entry is controversial, however, and may be cell type–specific.

Introduction

Ca$^{2+}$ depletion from the endoplasmic reticulum activates a so-called store-operated Ca$^{2+}$ entry pathway that represents the principal mode of Ca$^{2+}$ entry in nonexcitable cells (Putney, 1986). However, many uncertainties exist regarding how the depletion of stored Ca$^{2+}$ activates Ca$^{2+}$ entry. Various models have been developed to address the coupling process. Evidence that a diffusible messenger either released or activated in response to store depletion has been advanced. A Ca$^{2+}$ influx factor of unknown molecular identity (Randriamampita and Tsien, 1993; Csutora et al., 1999; Trepakova et al., 2000), cyclic GMP (Pandol and Schoeffield-Payne, 1990; Bahnson et al., 1993; Xu et al., 1994), small GTP binding proteins (Bird and Putney, 1993; Fasolato et al., 1993; Rosado and Sage, 2000[c]), cytochrome P450 products (Alvarez et al., 1992), and others have all been implicated as putative diffusible messengers, though none of these molecules has achieved uniform acceptance as the primary mechanism of channel activation. Conformational coupling...
(Rosado and Sage, 2000a). F-actin appears to play a central role in mechanically sensitive cells, namely platelets (Rosado et al., 2000) and endothelial cells (Holda and Blatter, 1997; Norwood et al., 2000), but not in NIH 3T3 cells (Ribeiro et al., 1997) or DDTMF-2 and A7r5 muscle cell lines (Patterson et al., 1999). Though speculative, Rosado and Sage (2000a) suggested recently that the cell-specific distribution of F-actin, or alternatively its dynamic regulation, may account for these disparate findings. F-actin appears in a cortical membrane rim in platelets and endothelial cells. Particularly in endothelial cells, activation of store-operated Ca\(^{2+}\) entry is tightly coupled to reorganization of the F-actin membrane skeleton into stress fibers (Moore et al., 1998). Disruption of F-actin prevents activation of store-operated Ca\(^{2+}\) entry currents and stabilization of F-actin has similar effects (Norwood et al., 2000; Rosado et al., 2000), suggesting that in platelets and endothelial cells the dynamic activity of F-actin is required to link Ca\(^{2+}\) store depletion to Ca\(^{2+}\) entry. In contrast, F-actin is distributed throughout the cytosol of NIH 3T3 and smooth muscle cells and does not similarly reorganize in response to activation of store-operated Ca\(^{2+}\) entry (Ribeiro et al., 1997; Patterson et al., 1999).

It is unclear how the F-actin membrane skeleton regulates store-operated Ca\(^{2+}\) entry channel function. Spectrin is a principal component of the membrane skeleton that cross-links F-actin and provides structural support for the plasmalemma and intracellular organelles (Bennett and Gilligan, 1993; Hartwig, 1994, 1995; Goodman, 1999), including the endoplasmic reticulum (Devarajan et al., 1997). In its simplest form spectrin is a large heterodimer comprised of \(\alpha\) and \(\beta\) subunits oriented in an antiparallel fashion. Spectrin interacts with integral membrane proteins both directly and through its binding to ankyrin and protein 4.1 (Hartwig, 1994, 1995). The spectrin–protein 4.1 locus is functionally significant because it resides within the NH\(_2\) terminus of \(\beta\) spectrin (residues A207–V445), 21 amino acids downstream of the actin binding domain (residues A47–K186) (Ma et al., 1993; Zimmer et al., 2000). Although \(\alpha\) spectrin normally binds and crosslinks F-actin with a \(K_D = 10^{-4}\) M, its affinity for F-actin increases eight orders of magnitude in the presence of protein 4.1 (\(K_D = 10^{-12}\) M) (Goodman et al., 1988). Thus, protein 4.1 tethers spectrin to the membrane and controls F-actin crosslinking, providing a cytoskeletal connection between the endoplasmic reticulum and the plasmalemma. Prior studies have established that the spectrin-based membrane skeleton localizes ion channels to discrete cellular microdomains. In premyelinated axons spectrin localizes voltage-gated Na\(^{+}\) channels to nodes of Ranvier (Srinivasan et al., 1988; Bennett and Lambert, 1999), and in
MDCK cells it localizes the Na+/K+ ATPase to the basolateral plasma membrane with E-cadherin (Piepenhagen and Nelson, 1998). Therefore, we sought to explore whether coupling between store depletion and Ca2+ entry was dependent on the spectrin-based membrane skeleton.

**Results**

**Expression of nonerythroid spectrin**

Few studies have demonstrated expression of spectrin in endothelial cells (Pratt et al., 1984; Heltianu et al., 1986). Consequently, initial reverse transcriptase (RT)-PCR cloning experiments were performed to identify the expression of nonerythroid spectrin αSpIIΣ1 and βSpIIΣ1 subunits in pulmonary artery and microvascular endothelial cells. Single PCR products of predicted sizes were identified, and cloning experiments confirmed that the products were α and β subunits of nonerythroid spectrin (Fig. 1, A and B). Western analysis (Fig. 1 C) and immunocytochemistry (unpublished data) using an anti-α subunit antibody confirmed protein expression in pulmonary artery endothelial cells (PAECs)* and pulmonary microvascular endothelial cells (PMVECs). Since spectrin is a heterodimer comprised of α and β subunits, similar abundance of the α subunit in PAECs and PMVECs suggests these two cell types express comparable amounts of functional spectrin.

**Store-operated Ca2+ entry**

We have previously characterized peptide-specific antibodies SG43, SG48, and SG921 prepared against functional regions of nonerythroid β spectrin (βSpIIΣ1) (Sikorski et al., 2000; Zimmer et al., 2000). These antibodies have been used to characterize the role of spectrin in synaptic transmission (Sikorski et al., 2000). Data indicate they diffuse rapidly in the cytosol, with rates of diffusion resembling large molecules, including IgG (Alder et al., 1992), myoglobin (Arancio et al., 1996), and albumin (Popov and Poo, 1992). Antibody SG43 (targeting residues D8–R24) is directed against a region immediately adjacent to the actin binding domain (Karinch et al., 1990; Fig. 2). SG43 specifically disrupts spectrin–actin binding. (B) PAECs were grown to confluence on Celllocate coverslips and microinjected with either a PBS control or ~70 μg/ml of the SG43 antibody (see Materials and methods for details). Cells were loaded with fura2/AM in a Heps-buffered Kreb's solution containing 2 mM extracellular Ca2+ and [Ca2+]i responses to thapsigargin (1 μM; EC50) measured in the standard buffer. Baseline [Ca2+]i ratios were normal, indicating resting [Ca2+]i was ~100 nM. Similarly, the response to thapsigargin was within a normal range, indicating an increase in [Ca2+]i to near 600 nM. Average peak and plateau (measured 10 min after thapsigargin application) responses are depicted in C. Asterisk denotes different from Control responses. Means are ± SEM.

* Abbreviations used in this paper: PAEC, pulmonary artery endothelial cell; PMEC, pulmonary microvascular endothelial cell; RT, reverse transcriptase.
Figure 3. Disruption of the spectrin–protein 4.1 interaction decreases the [Ca\(^{2+}\)]\(_i\) response to activation of store-operated Ca\(^{2+}\) entry. (A) Antibodies SG921 and SG48 target different regions within the protein 4.1 binding domain, which possesses overlap with the synapsin binding domain, on β spectrin. Whereas SG921 targets a region located at the upstream region, SG48 targets a region located at the downstream region, of the protein 4.1 binding domain. Experiments were performed using PAECs grown to confluence on Cellocate coverslips and microinjected with either PBS control or 70 μg/ml of the SG921 (B) and SG48 (C) antibodies. Cells were loaded with fura2/AM in a Hepes-buffered Kreb’s solution containing 2 mM extracellular Ca\(^{2+}\), and [Ca\(^{2+}\)]\(_i\) responses to thapsigargin (1 μM; EC\(_{95}\)) measured in the standard buffer. Baseline [Ca\(^{2+}\)]\(_i\) ratios and the response to thapsigargin were within a normal range. Average peak and plateau (measured 10 min after thapsigargin application) responses are depicted in D. Asterisk denotes different from control responses. Means are ± SEM.

\(^{125}\)I-protein 4.1 to unlabeled lung spectrin immobilized on nitrocellulose paper, whereas SG43 has no effect (unpublished data). We examined whether the spectrin–protein 4.1 interaction contributes to linkage between Ca\(^{2+}\) stores and store-operated Ca\(^{2+}\) entry channels. In contrast to findings with the SG43 antibody, injection of both SG921 and SG48, which target the NH\(_2\) and COOH regions of the protein 4.1 binding domain, respectively, reduced the peak and plateau thapsigargin response by ~50% (Fig. 3, B–D). These data suggest that the spectrin–protein 4.1 interaction critically regulates at least a subset of membrane channels activated by depletion of Ca\(^{2+}\) stores.

Figure 4. Disruption of the spectrin–protein 4.1 interaction does not decrease Ca\(^{2+}\) release. PAECs were grown to confluence on Cellocate coverslips and microinjected with either PBS control or ~70 μg/ml of the SG921 (A) and SG48 (B) antibodies. Cells were loaded with fura2/AM, washed, and [Ca\(^{2+}\)]\(_i\) responses to thapsigargin (1 μM; EC\(_{95}\)) measured in the buffer containing 100 nM extracellular Ca\(^{2+}\). Baseline [Ca\(^{2+}\)]\(_i\) ratios and the response to thapsigargin were within a normal range for low extracellular Ca\(^{2+}\) experiments. Neither SG921- (A) nor SG48- (B) injected cells exhibited evidence of a decrease in Ca\(^{2+}\) release. Average peak [Ca\(^{2+}\)]\(_i\) responses are depicted in C. Means are ± SEM.
Since spectrin forms a membrane skeleton stabilizing both the plasmalemma and endoplasmic reticulum, SG921 and SG48 could have disrupted the spectrin–protein 4.1 association with either membrane. This possibility raises the concern that these antibodies prevented or reduced Ca\(^{2+}\) release, consequently limiting the magnitude of store depletion. To address this concern, cells were microinjected with SG921, loaded with fura2/AM, and thapsigargin was applied in low extracellular Ca\(^{2+}\). This protocol is commonly used to examine the magnitude of Ca\(^{2+}\) release, and to discriminate between Ca\(^{2+}\) release and Ca\(^{2+}\) entry components of the global [Ca\(^{2+}\)] response. Fig. 4 indicates that disruption of the spectrin–protein 4.1 interaction did not reduce Ca\(^{2+}\) release, suggesting suppression of Ca\(^{2+}\) entry observed in Fig. 3 was due...
to an effect on plasmalemma ion channel function. To confirm these findings, SG48 was injected and thapsigargin was applied in low extracellular Ca\(^{2+}\). Similar to the findings with SG921, disruption of the spectrin–protein 4.1 interaction using SG48 did not decrease Ca\(^{2+}\) release. Thus, the spectrin–protein 4.1 interaction regulates store-operated Ca\(^{2+}\) entry.

**\(I_{SOC}\) requires the spectrin–protein 4.1 interaction**

Data from Figs. 3 and 4 indicate that disruption of the spectrin–protein 4.1 interaction reduces the global [Ca\(^{2+}\)] response to thapsigargin. However, this reduction in [Ca\(^{2+}\)] could be due to either a decrease in Ca\(^{2+}\) entry or an increase in Ca\(^{2+}\) extrusion. Decreased Ca\(^{2+}\) entry could also be secondary to membrane depolarization that is not controlled in cell physiology experiments. Thus, we sought to examine whether endothelial cell \(I_{SOC}\) was regulated through a physical coupling mechanism. We (Moore et al., 1998; Norwood et al., 2000) and others (Schilling et al., 1992; Vaca and Kunze, 1994, 1995; Fasolato and Nilius, 1998) have demonstrated previously that thapsigargin activates an endothelial cell \(I_{SOC}\) that is relatively small (approximately −60 to −80 pA at −80 mV), reverses near +40 mV, and is inwardly rectifying. This current requires the presence of intracellular ATP, an intact cytoskeleton, myosin light chain kinase activity, and is inhibited by low concentrations of La\(^{3+}\).

\[\text{SG48}\]

Fig. 5 illustrates the typical thapsigargin-activated Ca\(^{2+}\) current in endothelial cells. As seen in Fig. 5A, application of La\(^{3+}\) immediately (within 2 min) shifts the reversal potential to 0 mV, and eliminates the thapsigargin-activated current altogether. These data are taken as support for the idea that thapsigargin activates a Ca\(^{2+}\)-selective \(I_{SOC}\). These findings are consistent with the Ca\(^{2+}\)-selective \(I_{GRAC}\) found in mast cells and T lymphocytes (Hoth and Penner, 1992; Zweifach and Lewis, 1993), suggesting thapsigargin activates a Ca\(^{2+}\)-selective \(I_{SOC}\) in endothelial cells (Fasolato and Nilius, 1998).

To examine whether the spectrin membrane skeleton regulates Ca\(^{2+}\) selective \(I_{SOC}\), we performed studies to selectively disrupt spectrin from actin and protein 4.1. Injection of SG43 neither reduced the \(I_{SOC}\) current (Fig. 5, B and C) nor left-shifted the reversal potential. These data are consistent with those in Fig. 3 which demonstrate that the specific interaction of spectrin with F-actin at residues A47–K186 of \(\beta\)-Sp\(\Pi\Sigma\)1, e.g., the F-actin binding domain of \(\beta\) spectrin, does not significantly affect the store-operated Ca\(^{2+}\) entry response. In contrast to SG43, injection of SG48 (and SG921; unpublished data) nearly abolished the \(I_{SOC}\) current normally activated by thapsigargin (Fig. 5, D and E). The remaining current was left-shifted to approximately +20 mV, consistent with inhibition of a Ca\(^{2+}\) current. In these studies, the antibodies were injected ∼1 h before electrophysiology recordings. Therefore, we sought to determine whether acute application of the antibodies would similarly ameliorate \(I_{SOC}\). Fig. 6 illustrates that application of SG48 through the patch pipette along with thapsigargin resulted in an ∼40% reduction in current (from control = −47 pA to SG48 = −28 pA at −80 mV), whereas SG43 was without effect. These data indicate a functional interaction between spectrin and protein 4.1 at residues A207–Y445 of \(\beta\)-Sp\(\Pi\Sigma\)1, e.g., the protein 4.1 binding domain of \(\beta\) spectrin, is essential for \(I_{SOC}\).

![Figure 6. Disruption of the spectrin–protein 4.1 interaction acutely inhibits, but does not abolish, \(I_{SOC}\). SG48 or SG43 were applied in the patch pipette with thapsigargin (1 \(\mu M\)) and the current measured 3–5 min later. Thapsigargin activated a typical \(I_{SOC}\) that was not affected by disruption of the spectrin–actin interaction with SG43. However, disruption of the spectrin–protein 4.1 interaction using SG48 in the patch pipette inhibited \(I_{SOC}\) by ∼40%. Means are ±SEM.](https://jcb.rupress.org/article-pdf/154/6/1230/15305295j00154-06.pdf)

We have recently identified a cyclic nucleotide–gated nonselective cation channel that is activated by thapsigargin in endothelial cells (Wu et al., 2000). cGMP stimulated this nonselective current, though it was not clear whether thapsigargin increased GMP through Ca\(^{2+}\)–dependent activation of nitric oxide and soluble guanylyl cyclase or by direct activation of membrane guanylyl cyclase (Gukovskaya et al., 2000). To investigate whether the spectrin–protein 4.1 interaction selectively regulates \(I_{SOC}\), we evaluated the effect of SG48 on cyclic nucleotide–gated channel activation. However, unlike \(I_{SOC}\), disruption of spectrin from protein 4.1 did not alter the ability of thapsigargin to stimulate a nonselective cation current (Fig. 5). Therefore, these data support the specific regulation by protein 4.1 of channels that constitute a Ca\(^{2+}\)-selective \(I_{SOC}\). Since disruption of the spectrin–protein 4.1 interaction inhibits \(I_{SOC}\), these data also indicate that activation of \(I_{SOC}\), per se, is not the Ca\(^{2+}\) source needed to increase cGMP and stimulate cyclic nucleotide–gated cation channel activity.

**Discussion**

Mechanism(s) linking Ca\(^{2+}\) store depletion to Ca\(^{2+}\) entry remain elusive. In certain cell types, particularly platelets and endothelium, the trigger appears to involve dynamic activity of F-actin. F-actin does not interact directly with ion channels, and to this point the manner in which F-actin regulates store-operated Ca\(^{2+}\) entry is unknown. Spectrin crosslinks F-actin in the membrane skeleton and tethers directly and indirectly to ion channels and other transmembrane proteins. Therefore, our present studies were undertaken to examine whether spectrin fulfills a central role in physical-coupling models, regulating store-operated Ca\(^{2+}\) entry.

Two general approaches have been used previously to illustrate the role of F-actin in regulation of store-operated Ca\(^{2+}\) entry (Rosado and Sage, 2000a). First, dissolution of
**Materials and methods**

**Isolation and culture of pulmonary endothelial cells**

Rat pulmonary artery and microvascular endothelial cells were isolated and cultured using a method described by Stevens et al. (1999). Cells were routinely passaged by scraping. Cultures were characterized using SEM, uptake of 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine-labeled low-density lipoprotein (DiI-acetylated LDL), and a lectin binding panel.

**Molecular biology**

Standard techniques for RT-PCR subcloning were followed. All chemical reagents used were molecular biology grade. In brief, total RNA was extracted with RNA Stat-60 (Tel-Test “B”) from cells grown to 100% confluence. cDNA was synthesized from 1 μg of DNaseI-treated total RNA. PCR was then performed with the following sets of primers: α-spectrin, 5'-ATG GCA ACC TCC CGA AGA G-3' (sense) and 5'-CTT GAA TGG CTG -3' (antisense); β-spectrin, 5'-CAT CCA GAA GCC TGA GAA TG-3' (sense) and 5'-CTT GAG AAC TGA TGG ACC TC-3' (antisense). PCR products were ligated into TA cloning vector pCR2.1 (Invitrogen) and transformed into chemically competent Escherichia coli. Positive clones (verified by sequencing) were selected and grown in Lauria-Bertani broth with kanamycin (50 μg/ml) for 18–20 h at 37°C. Plasmids were isolated by the QIAprep® spinprep system (QIAGEN) and submitted to the Biopolymer Laboratory at the University of South Alabama for automated fluorescence sequence analysis (AB373XL DNA stretch sequencer). Sequencing of both strands using double-stranded plasmids as templates and universal primers confirmed the product accuracy. Nucleotide and amino acid alignments were achieved with BLAST (NCBI) and DNASIS v2.0 (Hitachi Software) programs.

The spectrin–actin interaction is stabilized in a ternary complex by protein 4.1, which also tethers the spectrin membrane skeleton to transmembrane proteins. Consequently, reorganization of the membrane skeleton could alter protein 4.1’s interaction with transmembrane proteins dependent or independent of the spectrin–actin association. Though prior studies have not specifically demonstrated that protein 4.1 binds directly to cation channels, our data indicate store-operated Ca^{2+} entry is regulated by a spectrin–protein 4.1–dependent interaction. Disruption of the spectrin–protein 4.1 interaction using antibodies targeting either the NH₂ or COOH region of β spectrin’s 4.1 binding domain reduced store-operated Ca^{2+} entry by ∼50%. Most importantly, disruption of the spectrin–protein 4.1 interaction abolished I_{SOC} and had no effect on cyclic nucleotide–gated cation channel activity, indicating a specific subset of thapsigargin-stimulated channels are selectively regulated through this component of the cytoskeleton.

In summary, our studies have addressed the role of the spectrin membrane skeleton in regulation of store-operated Ca^{2+} entry. Our findings support the idea that a highly localized region of β spectrin (residues A207–V445), through its interaction with protein 4.1, contributes to the linkage between Ca^{2+} store depletion and I_{SOC} consistent with physical-coupling models. The physiological significance of these findings remain speculative. However, these data suggest the possibility that a restricted locus on β spectrin (e.g., protein 4.1 binding domain on β-spectrin; Fig. 3A) functionally links calcium store depletion with calcium entry through specific I_{SOC} channels. Molecular identity of endogenous I_{SOC} channels will be required to ultimately define the protein–protein interactions responsible for regulation of calcium entry through this pathway.

F-actin using cytochalasin D immediately accentuates store-operated Ca^{2+} entry or directly activates a cationic conductance. The dynamic activity of the F-actin cytoskeleton is required for channel function, consistent with physical-coupling models. This requisite for dynamic activity of F-actin supports the possibility that channel activation involves an actomyosin-based molecular motor (Gregory et al., 1999; Norwood et al., 2000), a mechanism of channel regulation also consistent with physical-coupling models.

**Materials and methods**

**Isolation and culture of pulmonary endothelial cells**

Rat pulmonary artery and microvascular endothelial cells were isolated and cultured using a method described by Stevens et al. (1999). Cells were routinely passaged by scraping. Cultures were characterized using SEM, uptake of 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine-labeled low-density lipoprotein (DiI-acetylated LDL), and a lectin binding panel.

**Molecular biology**

Standard techniques for RT-PCR subcloning were followed. All chemical reagents used were molecular biology grade. In brief, total RNA was extracted with RNA Stat-60 (Tel-Test “B”) from cells grown to 100% confluence (~10^6 cells) in 75-cm² tissue culture flasks. First strand synthesis was performed with reverse transcriptase and oligo(dT) primer (Life Technologies). cDNA was isolated as described (Piepenhagen and Nelson, 1998) to generate microdomains in other cell types. Thus, we initially examined whether the spectrin–actin association is required for Ca^{2+} store depletion to promote Ca^{2+} entry, thinking that specific disruption of this association might mimic the effects of other experimental strategies to disrupt F-actin. However, the results did not support the idea that F-actin regulates store-operated Ca^{2+} entry channels, particularly I_{SOC} through its direct interaction with spectrin, because disruption of this interaction did not prevent thapsigargin from activating I_{SOC}.  

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**Figure 7.** Disruption of the spectrin–protein 4.1 interaction does not prevent thapsigargin from activating cyclic nucleotide–gated cation channels. Single cells were microinjected with either PBS control or 7–70 μM of the SG48 antibody. After 1 h, cells were isolated in a whole cell voltage clamp mode and thapsigargin (1 μM) was applied through the patch pipette. Solutions were used to isolate a cyclic nucleotide–gated channel as described (Wu et al., 2000). Data indicate that disruption of the spectrin–protein 4.1 interaction does not decrease the thapsigargin-activated nonselective cationic conductance. Means are ± SEM.
Western blots

Cells were rinsed and then scraped into ice-cold detergent extraction buffer (40 μl per 60-mm dish; detergent extraction buffer, 10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 10 mM MgCl₂, 2 mM EDTA, 0.25 mM DTT, 1 mM PMSF, 1% [vol/vol] Triton-X, 4 mM DFP, 100 μg/ml antipain, 100 μg/ml leupeptin, 100 μg/ml E64 [Li-trans-3-Carboxyoxiran-2-carbonyl-L-ty-rcylagmatine], 0.4 mM benzamidine, and 10 mM iodocacetamide) (all chemicals from Sigma-Aldrich). The mixtures were cleared by centrifugation and subjected to SDS-PAGE for analysis.

Electrophoresis of α and β spectrin was through standard 5% SDS-PAGE gels at 100 V for 1.5 h. Proteins were transferred to nitrocellulose membrane in buffer containing 150 mM glycine, 20 mM Tris-base, and 20% (vol/vol) methanol. Transfer of proteins was performed overnight at 30°C. Membranes were then washed with TBS containing 0.1% Tween-20 to remove Tween-20 and subjected to blocking buffer. Incubations were at 4°C overnight with constant, gentle agitation. Blots were washed with TBS-Tween-20 (0.1% times for 30 min each,HRP-conjugated anti-rabbit IgG (1:20,000) was added to the blots in blocking buffer (room temperature) for 1 h then washed off as described above. Detection of secondary antibody was achieved using the SuperSignal™ West Pico Chemiluminescent System (Pierce Chemical Co.).

Isolation of lung spectrin, human erythrocyte 4.1, and binding analysis

Rat lung spectrin (α-SpⅡ1/β-SpⅡ1) was isolated by low ionic strength extraction (37°C) of crude membranes as described previously for brain spectrin (Sikorski et al., 1991). Modifications from our previously published procedure included a reduction in buffer volumes and size of the PAGE was performed followed by autoradiography. The spectrin–actin complexes were separated from free spectrin by sedimentation at 50,000 g/ml by centrifugation at 4°C. Spectrin (Sikorski et al., 1991). Modifications from our previously published procedure included a reduction in buffer volumes and size of the PAGE was performed followed by autoradiography. The spectrin–actin complexes were separated from free spectrin by sedimentation at 50,000 g/ml by centrifugation at 4°C.

Activation-activated Ca²⁺ entry by microinjected guanine nucleotide analogues. Evidence for the involvement of a small G-protein in capacitative calcium entry.

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Cytosolic Ca²⁺ measurements

Patch clamp electrophysiology

Conventional whole-cell voltage clamp configuration was performed to measure transmembrane currents in single rat PAECs by the standard gigaseal patch clamp technique, as described by Moore et al. (1998). Confluent rat PAECs were enzyme dispersed, seeded onto 35-mm plastic culture dishes, and then allowed to reattach for at least 24 h before patch clamp experiments were performed. Patch clamp recordings were obtained from single electrically isolated rat PAECs exhibiting a flat, polygonal morphology. These cells were chosen for study because their morphology was consistent with rat PAECs from a confluent monolayer. Recording pipettes were heat polished to produce a tip resistance in the range of 3–5 megahms in the internal solution. To examine Ca²⁺ currents, the pipette solution contained (in mM): 130 N-methyl-D-glucamine, 10 Hepes, 15 EGTA, 1 CaCl₂, 2 MgCl₂-ATP, 1 N-phenylanthranilic acid, 0.1 5-Nitro-2-(3-phenylprcpylamino benzoic acid (pH 7.2, adjusted with methane sulfonic acid). The external (bath) solution contained (in mM) 120 aspartic acid, 5 Ca(OH)₂, 5 CaCl₂, 10 Hepes, 0.5 3,4-diaminopyridine (pH 7.4, adjusted with tetraethylammonium hydroxide). To examine nonselective currents the pipette solution contained (in mM) 140 KOH, 5 NaOH, 145 glutamic acid, 10 EGTA, 10 Hepes, 1 N-phenylanthranilic acid, pH 7.2. The external (bath) solution contained (in mM) 140 NaOH, 5 KOH, 145 glutamic acid, 15 Hepes, 1 N-phenylanthranilic acid, pH 7.4. All solutions were adjusted to 290–300 mosM with sucrose. Currents were recorded with a computer-controlled EPC9 patch clamp amplifier (HEKA). Cell capacitance and series resistance were calculated with the software-supported internal routines of the EPC9 and compensated before each experiment. Voltage pulses were applied from –100 to +60 mV in 20 mV increments after the whole-cell configuration was achieved, with 200 ms duration during each voltage step and a 2 s interval between steps. The holding potential between each step was 0 mV. Data acquisition and analysis were performed with Pulse/Pulsefit software (HEKA) and filtered at 2.9 kHz.

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