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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 βSpIIΣ1 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 βSpIIΣ1 did not decrease the thapsigargin-induced global cytosolic Ca^{2+} response or inhibit I_{SOC}. Results indicate that the spectrin–protein 4.1 interaction selectively controls I_{SOC}, indicating that physical coupling between calcium release and calcium entry is reliant upon the spectrin membrane skeleton.

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, 2000c), 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, 2000b) 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 impair 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, 2000a,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

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Key words: store-operated Ca^{2+} entry; capacitative Ca^{2+} entry; I_{CRAC}; protein 4.1; F-actin

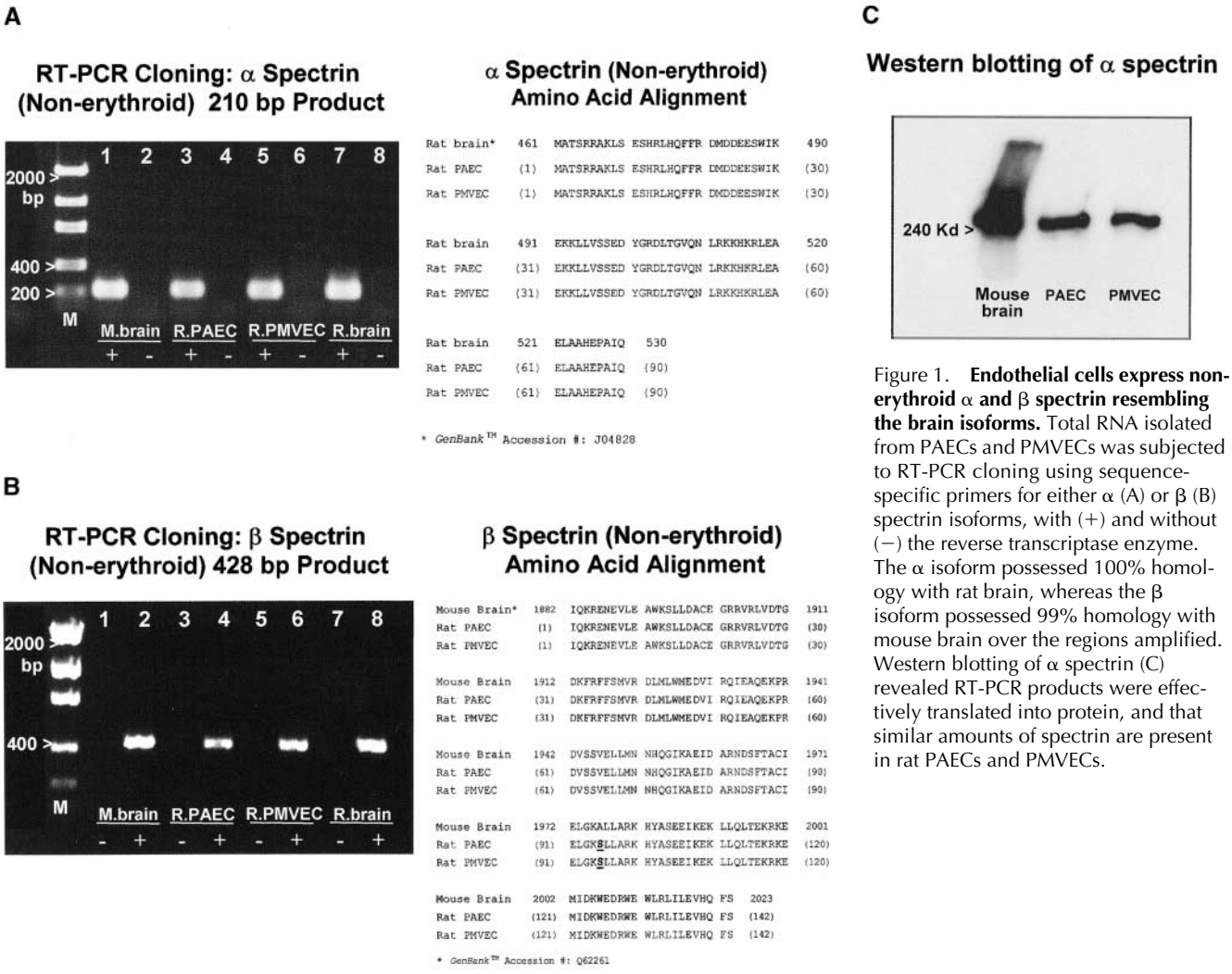

Figure 1. **Endothelial cells express non-erythroid α and β spectrin resembling the brain isoforms.** Total RNA isolated from PAECs and PMVECs was subjected to RT-PCR cloning using sequence-specific primers for either α (A) or β (B) spectrin isoforms, with (+) and without (−) the reverse transcriptase enzyme. The α isoform possessed 100% homology with rat brain, whereas the β isoform possessed 99% homology with mouse brain over the regions amplified. Western blotting of α spectrin (C) revealed RT-PCR products were effectively translated into protein, and that similar amounts of spectrin are present in rat PAECs and PMVECs.

(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 DDT_1MF-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 α and β 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 β 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 β spectrin normally binds and crosslinks F-actin with a $K_D = 2 \times 10^{-4}$ 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

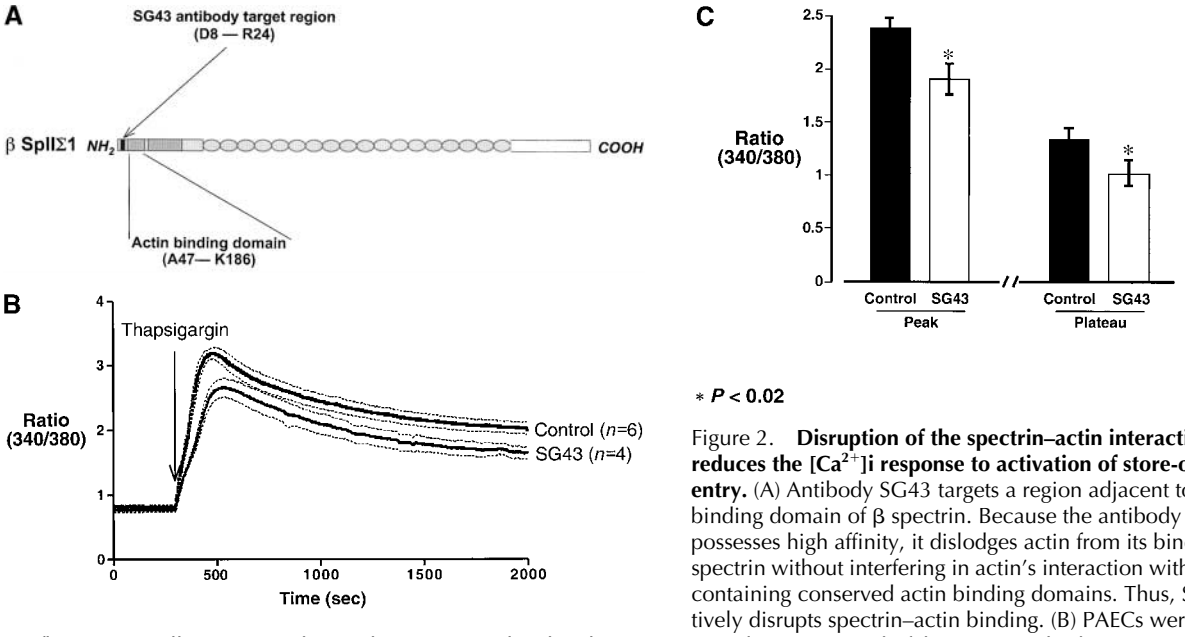

Figure 2. **Disruption of the spectrin–actin interaction modestly reduces the $[Ca^{2+}]i$ response to activation of store-operated Ca^{2+} entry.** (A) Antibody SG43 targets a region adjacent to the actin binding domain of β spectrin. Because the antibody is large and possesses high affinity, it dislodges actin from its binding site on β spectrin without interfering in actin's interaction with other proteins containing conserved actin binding domains. Thus, SG43 selectively disrupts spectrin–actin binding. (B) PAECs were grown to confluence on Cellocate 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 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 were normal, indicating resting $[Ca^{2+}]i$ was ~100 nM. Similarly, the response to thapsigargin was within a normal range, indicating an increase in $[Ca^{2+}]i$ 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.

MDCK cells it localizes the Na^+/K^+ ATPase to the basal-lateral plasma membrane with E-cadherin (Piepenhagen and Nelson, 1998). Therefore, we sought to explore whether coupling between store depletion and Ca^{2+} 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 αSpIIΣI and βSpIIΣI 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 Ca^{2+} 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 blocks the binding of ^{125}I-lung spectrin to F-actin in cosedimentation assays, whereas SG48 and SG921 have little effect (unpublished data; see Materials and methods for detail). To examine whether the spectrin–actin interaction physically couples Ca^{2+} store depletion to Ca^{2+} entry, either PBS control or SG43 were microinjected into confluent PAEC cultures. After microinjection, cells were loaded with fura2/AM and $[Ca^{2+}]i$ was measured. The application of thapsigargin to PBS-injected cells produced a slowly developing and sustained rise in $[Ca^{2+}]i$ (Fig. 2, B and C). The thapsigargin $[Ca^{2+}]i$ response was only modestly reduced (~15%) in SG43-injected cells, suggesting that the spectrin–actin interaction does not link Ca^{2+} store depletion to store-operated Ca^{2+} entry channels.

Antibodies SG921 (targeting residues M206–D221) and SG48 (targeting residues Q417–D428) are directed against the synapsin–protein 4.1 binding domain (Ma et al., 1993; Sikorski et al., 2000; Zimmer et al., 2000; Fig. 3). Both SG921 and SG48 block synaptic transmission by interfering with the spectrin–synapsin interaction, whereas SG43 has no effect (Karinch et al., 1990; Zimmer et al., 2000). Not surprisingly, SG921 and SG48 also block the binding of

*Abbreviations used in this paper: PAEC, pulmonary artery endothelial cell; PMEC; pulmonary microvascular endothelial cell; RT, reverse transcriptase.

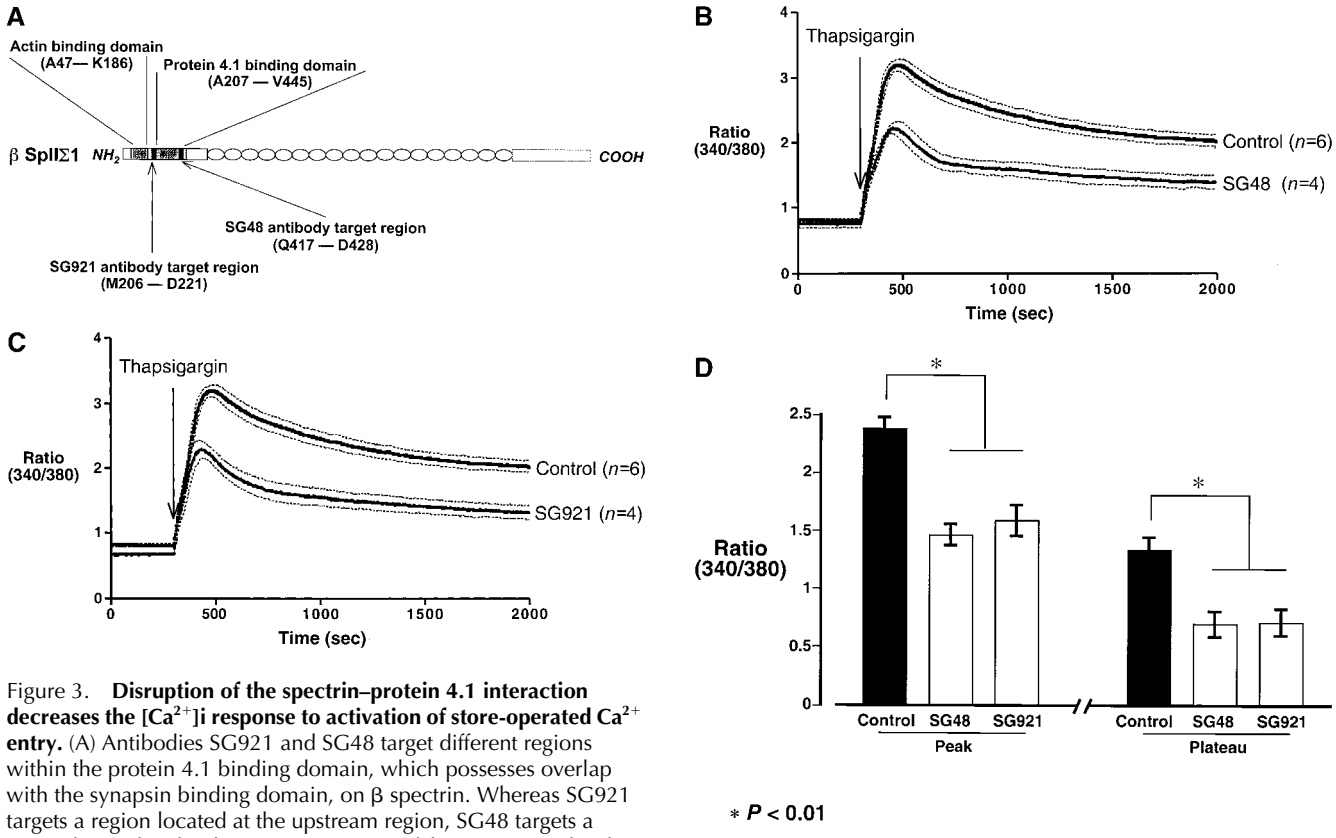

Figure 3. **Disruption of the spectrin–protein 4.1 interaction decreases the $[Ca^{2+}]i$ response to activation of store-operated Ca^{2+} entry.** (A) Antibodies SG921 and SG48 target different regions within the protein 4.1 binding domain, which possesses overlap with the synapsin binding domain, on 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.

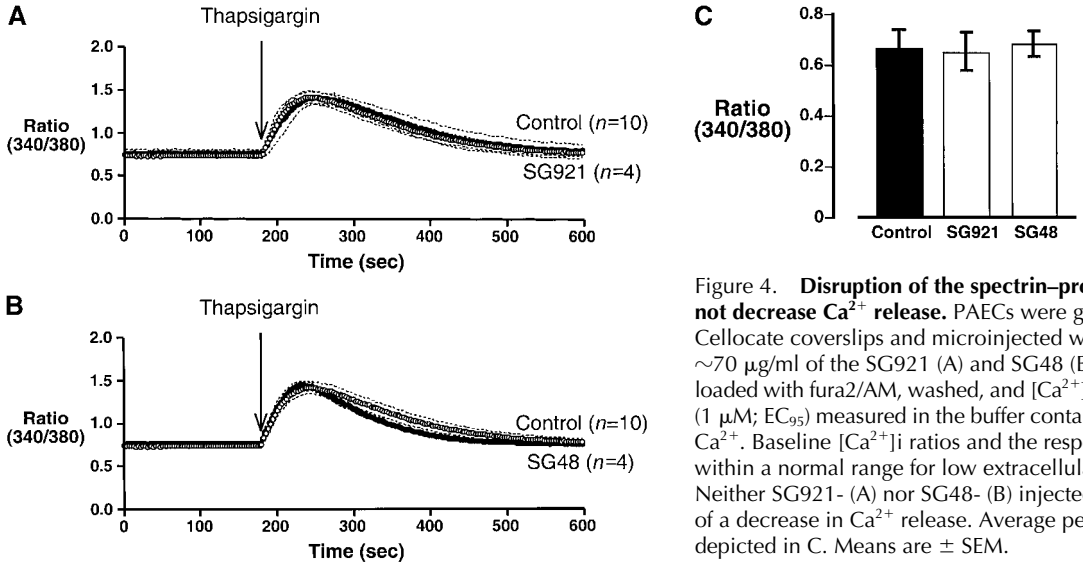

Figure 4. **Disruption of the spectrin–protein 4.1 interaction does not decrease Ca^{2+} release.** PAECs were grown to confluence on Cellocate coverslips and microinjected with either PBS control or ~70 μg/ml of the SG921 (A) and SG48 (B) antibodies. Cells were loaded with fura2/AM, washed, and $[Ca^{2+}]i$ 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.

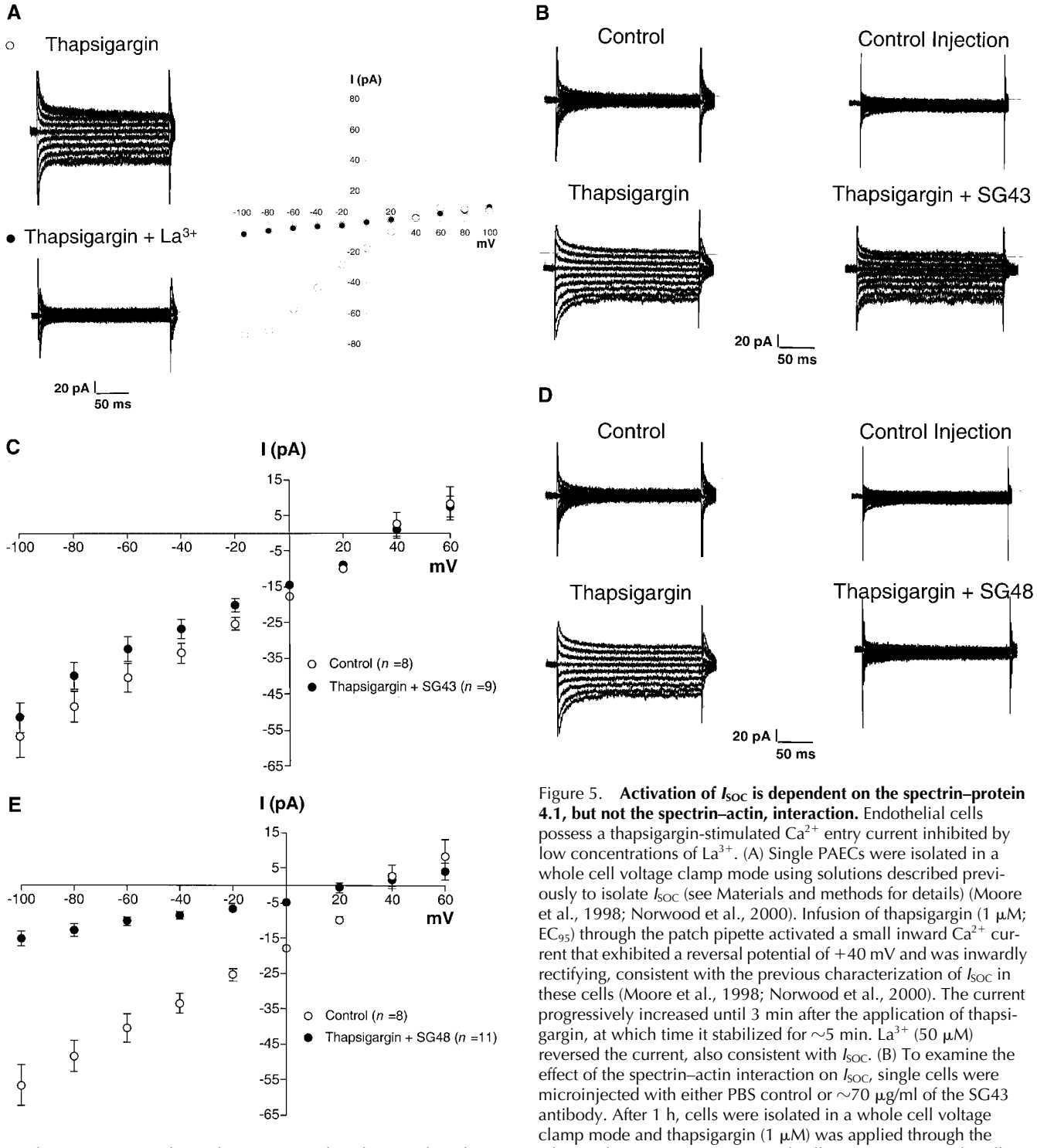

Figure 5. **Activation of I_{SOC} is dependent on the spectrin–protein 4.1, but not the spectrin–actin, interaction.** Endothelial cells possess a thapsigargin-stimulated Ca^{2+} entry current inhibited by low concentrations of La^{3+}. (A) Single PAECs were isolated in a whole cell voltage clamp mode using solutions described previously to isolate I_{SOC} (see Materials and methods for details) (Moore et al., 1998; Norwood et al., 2000). Infusion of thapsigargin (1 μM; EC_{95}) through the patch pipette activated a small inward Ca^{2+} current that exhibited a reversal potential of +40 mV and was inwardly rectifying, consistent with the previous characterization of I_{SOC} in these cells (Moore et al., 1998; Norwood et al., 2000). The current progressively increased until 3 min after the application of thapsigargin, at which time it stabilized for ~5 min. La^{3+} (50 μM) reversed the current, also consistent with I_{SOC}. (B) To examine the effect of the spectrin–actin interaction on I_{SOC}, single cells were microinjected with either PBS control or ~70 μg/ml of the SG43 antibody. After 1 h, cells were isolated in a whole cell voltage clamp mode and thapsigargin (1 μM) was applied through the patch pipette. Data indicate thapsigargin induced a I_{SOC} of similar magnitude in either PBS or SG43-injected cells (C). To examine the effect of the spectrin–protein 4.1 interaction on I_{SOC}, single cells were microinjected with either PBS control or ~70 μg/ml of the SG48 antibody and evaluated as described using the SG43 antibody. Data indicate that disruption of the spectrin–protein 4.1 interaction abolished I_{SOC} activated using thapsigargin (D and E). 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 $[Ca^{2+}]i$ 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 $[Ca^{2+}]i$ response to thapsigargin. However, this reduction in $[Ca^{2+}]i$ 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+}. Fig. 5 illustrates the typical thapsigargin-activated Ca^{2+} current in endothelial cells. As seen in Fig. 5 A, 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_{CRAC} 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 β-SpIIΣ1, e.g., the F-actin binding domain of β spectrin, does not significantly effect 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–V445 of β-SpIIΣ1, e.g., the protein 4.1 binding domain of β spectrin, is essential for I_{SOC}.

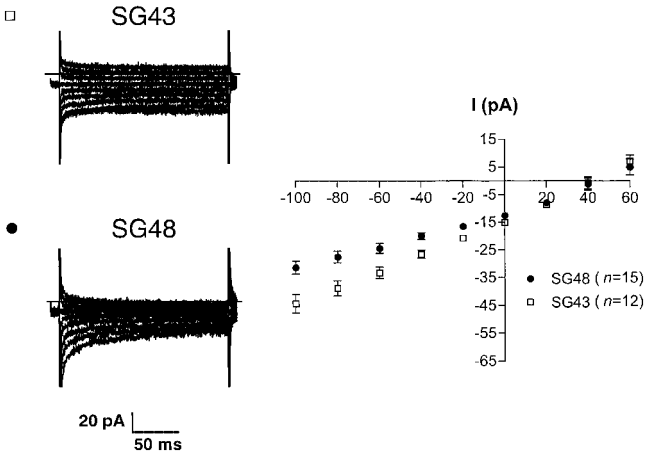

Figure 6. **Disruption of the spectrin–protein 4.1 interaction acutely inhibits, but does not abolish, I_{SOC}.** SG48 or SG43 were applied in the patch pipette with thapsigargin (1 μM) and the current measured 3–5 min later. Thaspigargin activated a typical I_{SOC} that was not effected by disruption 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.

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 cGMP 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. 7). 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

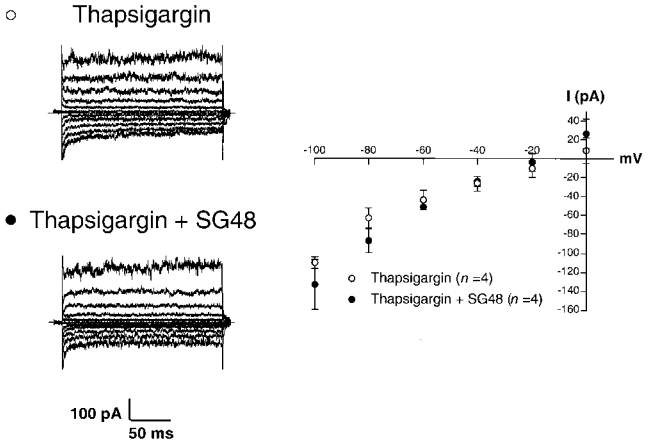

Figure 7. **Disruption of the spectrin–protein 4.1 interaction does not prevent thapsigargin from activating cyclic nucleotide–gated cation channels.** Single cells were microinjected with either PBS control or ~70 μg/ml of the SG48 antibody. After 1 h, cells were isolated in a whole cell voltage clamp mode and thapsigargin (1 μM) was applied through the patch pipette. Solutions were used to isolate a cyclic nucleotide–gated cation 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.

F-actin using cytochalasin D immediately accentuates store-operated Ca^{2+} entry or directly activates a cationic conductance, and over time reduces store-operated Ca^{2+} entry or prevents I_{SOC} (Holda and Blatter, 1997; Norwood et al., 2000; Rosado et al., 2000; Rosado and Sage, 2000a). Second, stabilization of F-actin using jasplakinolide prevents activation of store-operated Ca^{2+} entry and I_{SOC} (Holda and Blatter, 1997; Norwood et al., 2000; Rosado et al., 2000; Rosado and Sage, 2000a). These apparently disparate findings are rectified by the idea that 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.

Several features of these models implicate a role for spectrin in regulation of Ca^{2+} entry, particularly the β spectrin locus residing across the actin and protein 4.1 binding domains. Spectrin crosslinks F-actin, contributes to formation of the cortical actin rim, and regulates Mg^{2+}–myosin ATPase activity (Wang et al., 1987). Spectrin also localizes both voltage-gated Na^+ channels (Srinivasan et al., 1988; Bennett and Lambert, 1999) and the Na^+/K^+ ATPase (Piepenhagen and Nelson, 1998) to discrete 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}.

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- and protein 4.1–dependent interaction. Disruption of the spectrin–protein 4.1 interaction using antibodies targeting either the NH_2 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. 3 A) 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.

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′,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 (~10^7 cells) in 75-cm^2 tissue culture flasks. First strand synthesis was performed with reverse transcriptase and oligo(dT) primer (Life Technologies) on ~ 1 μg of DNaseI-treated total RNA. PCR was then performed with the following sets of primers: α-spectrin, 5′-CCT GAA TGG CTG GTT CGT GTG -3′ (sense) and 5′-ATG GCA ACC TCC CGA AGA G-3′ (antisense); β-spectrin, 5′-CAT CCA GAA GCG 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 PCR analysis) 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.

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 $MgCl_2$, 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 E-64 [L-trans-3-Carboxyoxiran-2-carbonyl-L-leucylagmatine], 0.4 mM benzamidine, and 10 mM iodoacetamide) (all chemicals from Sigma-Aldrich). The mixtures were cleared by centrifugation and subjected to SDS-PAGE for analysis.

Electrophoresis of α and β spectrins 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 V, at 4°C. Blots were stained with Ponceau S to visualize marker proteins, destained with TBS (50 mM Tris-HCl, 120 mM NaCl), and blocked with TBS plus 0.05% Tween-20 and 5% nonfat dry milk for 1 h. Spectrin antibodies (provided by Dr. Steven R. Goodman) were diluted 1:1,000 in blocking buffer. Incubations were at 4°C overnight with constant, gentle agitation. Blots were washed with TBS–Tween-20 (0.1%) three 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-SpIIΣ1/β-SpIIΣ1) 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 Sephacryl S-500 column (1.8 × 20 cm) because we started the isolation with only 10 g of frozen rat lungs and we eliminated steps that demyelinate brain homogenates. The final yield of lung spectrin was 100 μg from 10 g of tissue. Erythrocyte protein 4.1 was isolated by the method of Tyler et al. (1979). Rabbit muscle actin was purchased from Sigma-Aldrich and then further purified on a Sephacryl S-100 column to remove contaminants from the commercial preparation.

We tested the ability of β-SpIIΣ1 peptide–specific antibodies SG43 (residues 8–24, adjacent to the actin binding domain), SG921 (residues 206–221 at a synapsin attachment site), and SG48 (residues 417–428 within the protein 4.1–synapsin binding domain) (Ma et al., 1993; Sikorski et al., 2000; Zimmer et al., 2000) to block the spectrin–protein 4.1 or spectrin–actin interaction. To test the effect of the peptide-specific antibodies on the spectrin–protein 4.1 interaction, we loaded 1 μg/lane of pure lung spectrin on a 7% polyacrylamide mini gel and performed SDS-PAGE followed by transfer to nitrocellulose paper. The nitrocellulose paper was then blocked with 5% dry milk in PBS plus 0.05% Tween, and then dried strips were incubated with PBS or antibodies SG43, SG48, and SG921 diluted to 1:100 in PBS. The strips were then incubated with ^{125}I-protein 4.1 (10 ng/ml; 1,073,742 cpm/μg) and autoradiography was performed. After autoradiography, the antibodies were detected by immunoperoxidase staining and the spectrin bands were excised and counted in a Packard 500 gamma counter.

To observe the effect of the peptide-specific antibodies on the spectrin–actin interaction, we conducted spectrin–actin cosedimentation assays as described previously (Karinch et al., 1990). In brief, we preincubated 10 μg/ml lung spectrin (12,849 cpm/μg) with (1:10 dilution) peptide-specific antibodies SG43, SG48, SG921, or buffer for 30 min at 4°C. We then added an equal volume of actin (500 μg/ml) in polymerization buffer and incubated for 1 h at 4°C. The spectrin–actin complexes were separated from free spectrin by sedimentation at 50,000 *g* for 30 min at 4°C. Supernatants and pellets were loaded on 7% polyacrylamide minigels and SDS-PAGE was performed followed by autoradiography.

Antibody microinjection

Rat PAECs were seeded onto 25-mm circle microscope glass coverslips or Cellocate coverslips (Eppendorf) and grown for 24–48 h. Microinjection was performed as described in detail elsewhere (Norwood et al., 2000).

Cytosolic Ca^{2+} measurements

Rat PAECs were seeded onto 25-mm circle microscope glass coverslips (Fisher Scientific) and grown to confluence. Cytosolic Ca^{2+} $[Ca^{2+}]i$ was estimated with the Ca^{2+}-sensitive fluorophore fura 2/acetoxymethylester (Molecular Probes) according to methods described previously (Norwood et al., 2000). Calculations of free $[Ca^{2+}]i$ are routinely made using modifications of the formula described by Grynkiewicz et al. (1985) (Stevens et al., 1994).

Patch clamp electrophysiology

Conventional whole-cell voltage clamp configuration was performed to measure transmembrane currents in single rat PAECs by the standard giga-seal 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, polyhedral 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 megaohms in the internal solution. To examine Ca^{2+} currents, the pipette solution contained (in mM) 130 *N*-methyl-D-glucamine, 10 Hepes, 1.15 EGTA, 1 Ca^{2+}, 2 Mg^{2+}-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 $CaCl_2$, 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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