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Osmotic Stress Alters the Intracellular Distribution of Non-erythroidal Spectrin (Fodrin) in Bovine Aortic Endothelial Cells

A.C. Sun, I. Levitan
Institute for Medicine and Engineering, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

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Abstract. Cell swelling is known to result in unfolding of membrane invaginations and restructuring of F-actin. The effect of cell swelling on the intracellular distributions of other cytoskeletal proteins that constitute the submembrane cortical cytoskeleton is virtually unknown. This study focuses on the effects of cell swelling on non-erythroidal spectrin (fodrin, also known as spectrin II), a predominant component of the membrane cytoskeleton. The intracellular distribution of spectrin in vascular endothelial cells was studied by optical sectioning using a 3-D deconvolution microscopy system. Our results show that once bovine aortic endothelial cells (BAECs) reach confluency, the non-erythroidal spectrin is localized in the submembrane regions of the cells. Analysis of the intensity profiles of the non-erythroidal spectrin under isotonic and hypotonic conditions show that: (a) the width of the submembrane spectrin staining increases gradually with time within the first 5 minutes after the osmotic shock; (b) significant recovery is observed after 10 minutes even if the cells are maintained in hypotonic medium, and (c) spectrin distribution is altered by disrupting F-actin with latrunculin A but not by stabilizing F-actin with jasplakinolide. We suggest that cell swelling results in partial translocation of the submembrane spectrin to the cytosol and that it may play a major role in initiation of swelling-induced cellular events.

Key words: Cell volume regulation — Spectrin — Fodrin — Actin

Introduction

Restructuring of the actin cytoskeleton is a hallmark of the cellular response to hypotonic osmotic stress (Cornet, Lambert & Hoffmann, 1993; Lang et al., 1998; Papakonstanti, Vardaki & Stournaras, 2000). However, in spite of an extensive literature on osmotically-induced reorganization of F-actin (e.g., Cornet, Delpire & Gilles, 1987; Ziyadeh, Mills & Kleinzeller, 1992; Bibby & McCulloch, 1994; Levitan et al., 1995; Hallows et al., 1996), very little is known about the effects of hypotonic stress on other cytoskeletal proteins that constitute the actin-cytoskeleton complex. This study focuses on swelling-induced changes in the 3-D intracellular distribution of non-erythroidal spectrin (spectrin II or fodrin), one of the major components of the submembrane actin-cytoskeleton complex in non-erythroidal cells.

Spectrins constitute a family of heterotetrameric proteins $(\alpha\beta)_2$ that are ubiquitously expressed in metazoan cells where they interact with a variety of other cytoskeletal and integral membrane proteins (reviewed by Bennett, 1989; Morrow, 1993; De Matteis & Morrow, 2000; Bennett & Baines, 2001). Spectrin was first discovered in human erythrocytes where it interacts with actin, forming a two-dimensional submembrane actin-spectrin lattice (Yu, Fischman & Steck, 1973; Byers & Branton, 1985; Liu, Derick & Palek, 1987). Deficiencies in the eythroidal αI- and βI-spectrin isoforms (erythroidal spectrin isoforms) result in mechanical fragility of erythrocytes, indicating that a major function of the spectrin cytoskeleton is bestowing mechanical stability to the membrane lipid bilayer (e.g., Greenquist, Shohet & Bernstein, 1978; Bodine, Birkenmeier & Barker, 1984). Another member of the spectrin family, spectrin $(\alpha II\beta II)_2$ constitutes a major component of the submembrane cytoskeleton in a variety of non-erythroidal cells (e.g., Levine & Willard, 1983; Takemura et al., 1993; Piepenhagen & Nelson, 1998; Pradhan et al., 2001).

Since spectrin plays a central role in providing a mechanical scaffold for plasma membranes, it has

Correspondence to: I. Levitan; email: ilevitan@mail.med.upenn.edu

been suggested to participate in the coordination of surface and volume changes (Herring et al., 2000). The first step in addressing the hypothesis that spectrin is involved in sensing changes in cell volume is to determine whether osmotic stress alters the intracellular distribution of this protein. Using 3-D deconvolution microscopy, we show that, indeed, in vascular endothelial cells, exposure to a hypotonic shock results in a fast transient redistribution of non-erythroidal spectrin, supporting the hypothesis that it may constitute one of the cellular mechanotransducers. We also show that while intact F-actin is necessary for the normal distribution of spectrin, the sensitivity of spectrin to osmotic shock is not dependent on swelling-induced F-actin depolymerization.

Materials and Methods

Cell Culture

Bovine aortic endothelial cells were isolated by scraping them from the aortas and frozen in liquid nitrogen at passage 7. The cells were thawed and maintained between passages 7–15 by growing them in Dulbecco's Modified Eagle's Medium (DMEM; Cell Grow, Washington DC) supplemented with 10% bovine serum, 0.5% PennStrep and 1% L-glutamine (Gibco BRL, Grand Island, NY). Cell cultures were maintained in a humidified incubator at 37°C with 5% CO_2. The cells were fed and split every 3–4 days. For the experiments, cells were plated onto glass slides at a density of 5–7×10^5 cells/ml and grown to confluency (5–7 days). For immunostaining of single cells, the cells were plated at a density of 1×10^5 cells/ml and maintained in culture for 2–3 days.

Osmotic Challenge

Prior to an experiment, the culture medium was removed by washing the cells with PBS (300 mOsm). They were then challenged osmotically by exposing them to a diluted PBS solution (200 mOsm) for a time period ranging from 1 to 10 minutes. The osmolarity of the PBS was determined immediately before the staining with a vapor pressure osmometer (Wescor, Logan, UT) and was adjusted by the addition of water as required.

Immunofluorescence

Non-erythroidal spectrin was visualized with specific antibodies against the αII subunit. Antibodies against the β subunits of spectrin were not used because anti-β-spectrin antibodies that are commercially available do not discriminate between βI or βII isoforms (Chemicon).

Experimental Protocol

Immediately after the osmotic shock, BAECs were fixed in methanol at −20°C for 5 minutes, washed again in PBS (300 mOsm) 3 times and blocked with 4% goat serum for 3 hr at room temperature (RT). After blocking, cells were incubated with the primary antibodies (diluted in PBS) for 2 hr at RT. The primary antibodies for αII-spectrin (Mab 1622, Chemicon, Temecula, CA) were used at 1:100 dilution. After incubation with primary antibodies, cells were washed 3 times and incubated with rhodamine- or fluorescein-conjugated secondary antibodies. The protocol was performed at room temperature (RT). To test for possible methanol-induced dehydration effects, cells were fixed in 4% paraformaldehyde (PFA) at 22°C for 10 minutes. There were no apparent differences between spectrin distribution observed using the two fixation protocols, but the intensity of spectrin-specific staining in PFA-fixed cells was dimmer. In addition, the solution of 4% PFA dissolved in normal PBS is strongly hypertonic and to exclude the possibility of introducing artifacts during fixation, a series of control experiments was performed, in which PFA was prepared in diluted PBS, so that its osmolarity matched the osmolarity of the medium. Neither of the fixation protocols affected the height of the cells. Since spectrin-specific fluorescence was brighter after the methanol fixation, it was the protocol of choice in this study.

Deconvolution Microscopy

A deconvolution microscopy system (DeltaVision, Applied Precision, Issaquah, WA) was used to generate three-dimensional (3-D) images of endothelial cells. The system consists of a Zeiss Axiovert 100TV microscope (Germany) with a 63× Plan-Apochromat lens (NA 1.4), a precisely controlled XYZ stage (Applied Precision) and a scientific-grade cooled CCD camera (MicroMax, Princeton Instruments, Trenton, NJ). In order to maximize resolution along the optical axis, illumination from a mercury lamp was directed through a fiber optic scrambler to provide high-intensity, homogeneous illumination to the back aperture plane of the objective lens. Optical sectioning was performed by acquiring stacks of images 200 nm apart along the Z-axis. Constrained iterative deconvolution and 3-D rendering were performed using Delta Vision software, SoftWorx (Applied Precision) on an 02 R10000 RISC workstation (Silicon Graphics, Mountain View, CA), as described by Helmke, Goldman & Davies (2000). The optical resolution of the system, estimated by restoring the images of microbeads, was approximately 300 nm in the XY-plane and 800 nm along the Z-axis (Helmke et al., 2000).

Volume Measurements

Changes in the cell volume of BAECs were measured as described in our recent paper (Romanenko, Davies & Levitan, 2002). Briefly, the cells were loaded with 20 mM 6-methoxy-*N*-(3-sulfopropyl)-quinolinium (SPQ) in serum-free medium overnight. Cells were then washed three times with Cl^--free perfusion medium (in mM: 111 $NaNO_3$, 4 KNO_3, 1.6 $Ca(NO_3)_2$, 0.6 $Mg(NO_3)_2$, 50 HEPES, 5 Glucose, 28.5 Gluconic Acid, pH 7.2) and incubated in the same medium for 2 hr at 25°C to deplete Cl^-. Fluorescence was measured with a microplate fluorometer (Fluoroscan Ascent FL, Labsystems, Finland) for 2 hr at 30 minute increments to establish a baseline fluorescence. A 50% osmotic shock was administered using the fluorometer's built-in dispenser, after which readings were taken every 30 seconds for 20 minutes. Excitation and emission filters were 355 nm and 460 nm, respectively.

Analysis

To quantitatively analyze the spatial redistribution of spectrin induced by an osmotic shock, fluorescence intensity profiles of spectrin were obtained from optical sections of cells exposed to different experimental conditions. The intensity profiles were measured for every pair of adjacent cells in the visual field. The lines were drawn from a point in a center of a cell (chosen by eye) to a point in a center of an adjacent cell. This procedure was chosen to avoid a bias in choosing the width of the cell-cell borders. As ex-

pected, we have observed significant variation in the width of the intensity profiles between individual cells, but repeating the measurements for a large quantity of cells in several independent experiments yielded quantitatively consistent results. The quantification procedure: The width of the peak of the intensity profile represents the degree of spectrin dispersion: the tighter the spectrin distribution near the membrane, the narrower is the peak of the intensity profile. The width of the peak was calculated at the half-height of the profile peak, defined as follows: To detect the edges of the peak of the profile plot, we calculated the slopes of every 10 points using an algorithm similar to a "running average", where every new slope is calculated by shifting the data by one point. Figure 1 shows an example of an intensity profile (Fig. 1*B*) and of its "running slope" (Fig. 1*C*). In the cytosol, except for near the cell borders, the slopes were in a zero range, as expected, but near the cell border, fluorescence increased sharply. The maximal slope corresponded to the left edge of the profile peak (point *a*, Fig. 1*C*), whereas the point where the slope became zero again corresponded to the right edge of the profile peak (point *b*, Fig. 1*C*). The average of these two points was defined as the baseline fluorescence from which we calculated the half-height of the peak (point *c*, Fig. 1*B*).

The analysis was repeated for >300 individual profiles for every experimental condition and the data represents at least 3 independent experiments. Statistical analysis was performed using a standard two-sample Student's *t*-test assuming unequal variances of the two data sets. Statistical significance was determined using a two-tail distribution assumption and was set at the 5% level.

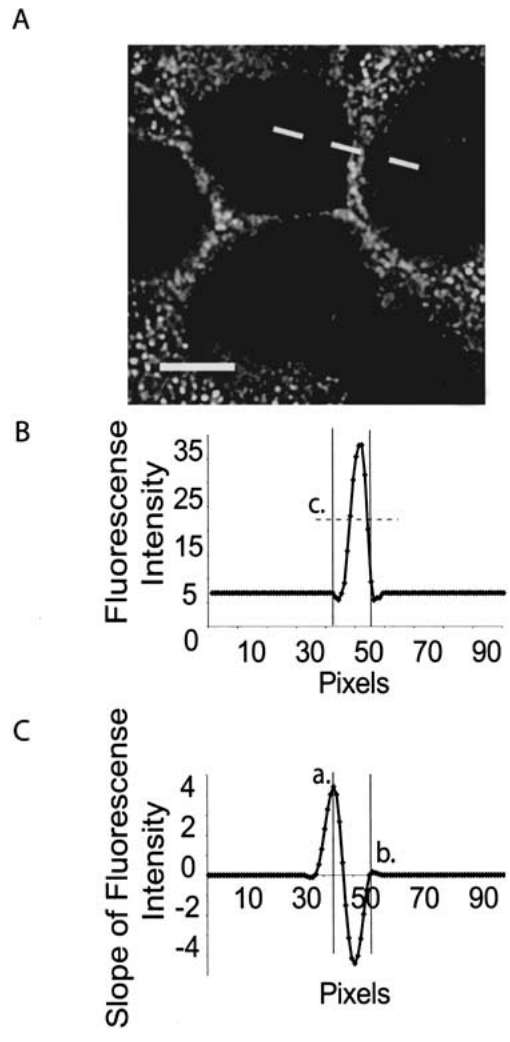

Fig. 1. Spectrin ring width measurement methodology. (*A*) A typical central optical section of spectrin in confluent BAECs. The dashed white line from the center of one cell to the center of an adjacent cell specifies the X-axis of the intensity profile shown in *B*. (*B*) The plot was generated using the profile plot function of Scion Image. Width is inferred from pixel values, where 1 pixel = 0.106 μm. (*C*) A "running slope" of spectrin width. The edge of the spectrin ring of the first cell corresponds to the maximum slope of the slope graph as indicated by *a*. The edge of the adjacent cell is where the slope approaches a zero slope again as indicated by *b*. A baseline fluorescence is derived by averaging the fluorescence intensity at points *a* and *b*. The width at the half height *c* is then calculated. Scale bar in *A* = 5 μm, corresponding to 47 pixels.

Results

3-D Intracellular Distributions of Spectrin in Single and Confluent BAECs

Optical sectioning shows that the intracellular distributions of non-erythroidal spectrin in single and confluent BAECs are significantly different (Fig. 2). While the resolution of the optical system in the Z-direction is not as high as in the X- and Y-directions, the Z-resolution was sufficient to clearly discriminate between optical sections 2 μm apart. In single cells (Fig. 2, left column), spectrin is dispersed throughout the cytoplasm in a random punctate pattern and appears both above and below the nucleus (sections *Aa* and *Ad*, respectively), with an enhanced staining in the perinuclear region (sections *Ab* and *Ac*). When the cells grow to confluency (Fig. 2, right column), however, spectrin is no longer scattered throughout the cytoplasm but localizes mostly in the submembrane regions. Panel *Ba* of Fig. 2 shows an apical section of a cell in a confluent monolayer (section *Ba*, arrow). Since individual cells in a monolayer have different heights, as has been shown earlier by the AFM-measurements (Barbee et al., 1995), only one cell was observed in the field that had its apical membrane in the focal plane of the optical section; for all other cells the section focuses below the apical membranes. As shown in the lower sections, spectrin colocalizes with the cortical cytoplasm and sites of cell-cell contacts with virtually no staining observed in the central regions of the cells or below the nuclei (sections *Bb*, *Bc*). Interestingly, the spectrin-specific fluorescence that was observed in the basal plane of the cells was centralized along the edges of the cells with no fluorescence in the cell center (section *Bd*). The inset compares the cells treated with the anti-spectrin antibodies and with non-immune antibodies from the same species, demonstrating that the level of nonspecific background fluorescence is negligible compared with the spectrin-specific fluorescence.

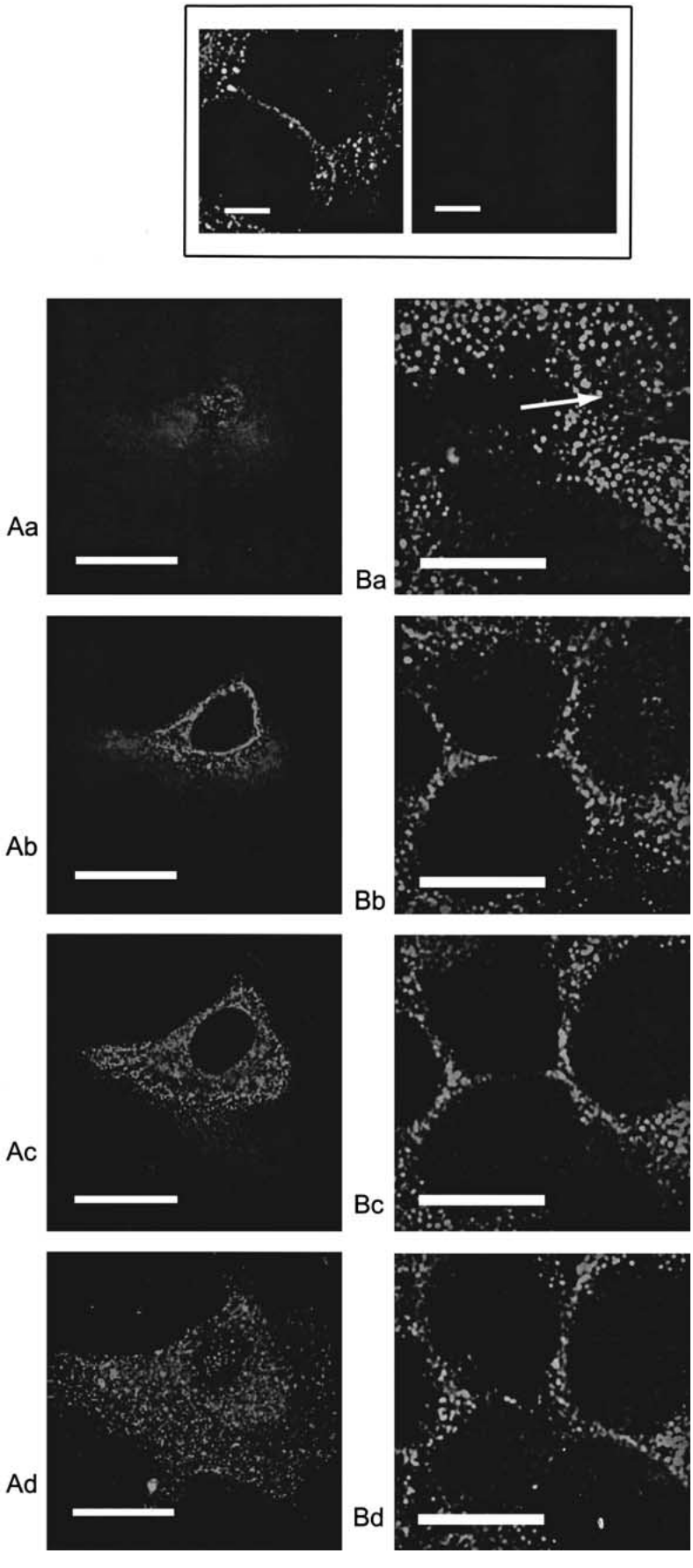

Fig. 2. 3-D distribution of non-erythroidal spectrin in single and confluent BAECs. Deconvolved optical sections for the planes of the apical membrane (*Aa*) and sections at 2-μm increments below (*Ab–Ad*) show that in single cells (left column), αII-spectrin is distributed throughout the cytoplasm (*A*), whereas in confluent cells it colocalizes with the plasma membrane (*B*). The inset shows the nonspecific fluorescence of cells exposed to nonimmune mouse antibodies. Scale bar = 10 μm.

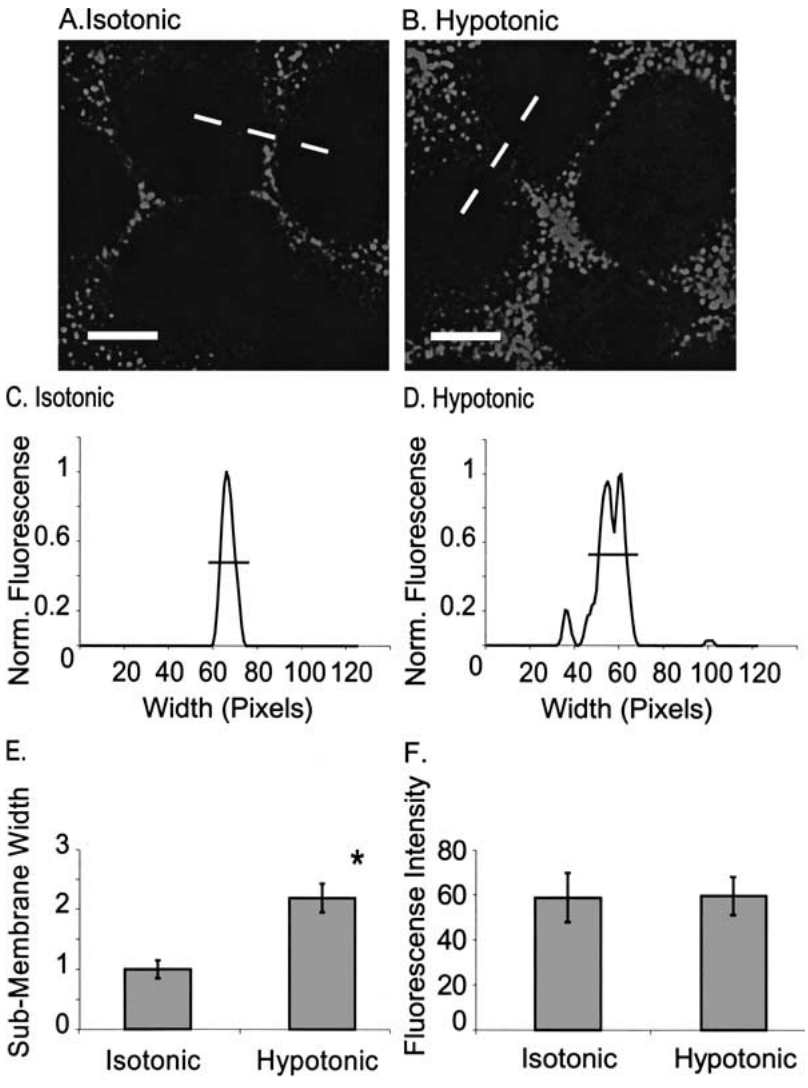

Fig. 3. Effect of osmotic stress on the distribution of spectrin. (*A*) Typical optical sections of cells maintained in isotonic environment or (*B*) exposed to a 33% osmotic gradient for 5 minutes. (*C*) Examples of intensity profiles measured along the dashed straight lines connecting the centers of two adjacent cells for isotonic and for (*D*) hypotonic conditions. (*E*) The bar graph shows the difference in the average width of the intensity peaks of αII-spectrin distribution in isotonic and hypotonic conditions ($p < 0.05$). The width of the peaks was measured at half height as described in Methods. At least 20 intensity profiles were analyzed for every experimental condition. The experiment was repeated in 5 independent trials and in each trial, the average width of the intensity profiles in isotonic conditions was significantly ($p < 0.05$) lower than that in hypotonic conditions. Scale bar = 5 μm.

Effect of Hypotonic Stress on the Intracellular Distribution of Spectrin

To test whether hypotonic stress results in redistribution of spectrin in BAECs, we have analyzed the degree of spectrin localization to the regions of cell-cell contacts (Fig. 3). Panels 3*A* and *B* show typical examples of spectrin distribution observed in optical sections through the cell center in cells maintained in isotonic conditions (300 mosM) or challenged with a 66.7% osmotic gradient (200 mosM) for 5 minutes respectively. The distribution consistently appeared to be more dispersed in cells challenged with an osmotic gradient (Panel 3*B*). We have quantified this effect by measuring the fluorescence intensity as a function of a position inside the cell, generating line intensity profiles for every pair of adjacent cells in the visual field, as described in detail in the Analysis section. Typical examples of the intensity profiles for isotonic and hypotonic conditions are shown in Panels 3*C* and *D*, respectively. The peaks of the profiles represent the localization of spectrin-specific fluorescence in the cell-cell contact region. Analysis of more than 300 cells for each experimental condition showed that challenging the cells osmotically resulted in about a twofold increase in the width of the spectrin area at the cell-cell boundary (Panel 3*E*). The intensity of the spectrin staining, however, remained the same (Panel 3*F*), suggesting that hypotonic stress results in the redistribution of spectrin in BAECs.

Figure 4 shows the time course of hypotonically induced reorganization of spectrin and that of cell volume changes. Significant dispersion of spectrin was observed within 1 minute of the osmotic challenge and was maintained at 3 and 5 minutes. Longer exposures, however, led to a recovery of the original spectrin distribution. After maintaining the cells in hypotonic environment for 10 minutes, the distribution of spectrin was not significantly different from that in cells maintained in isotonic conditions. To measure cell volume, the cells were loaded with SPQ, a fluorescent dye that is sensitive to cell volume

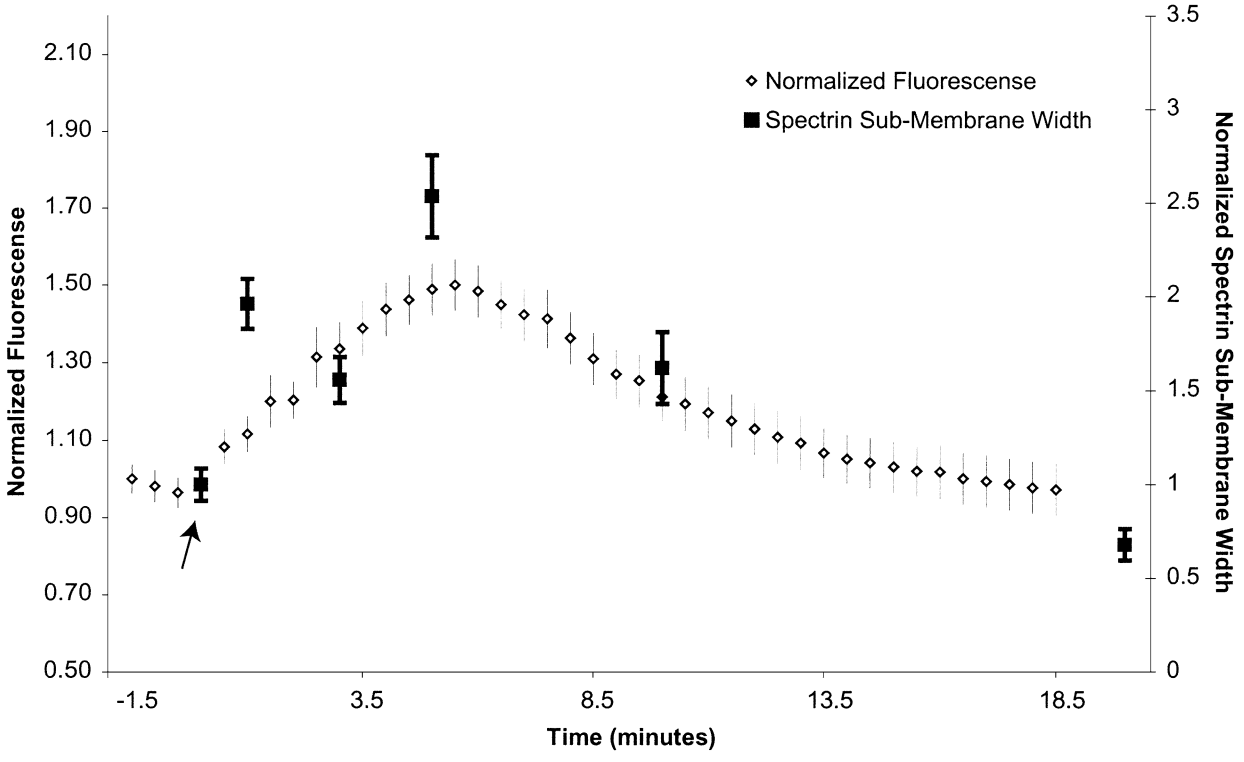

Fig. 4. The time courses of changes in spectrin distribution and of the recovery of cell volume. *Squares:* The widths of the intensity profiles measured in cells exposed to an osmotic gradient of 50% for 0 (isotonic), 1, 5, or 10 minutes and normalized to the value measured in control cells (at least 50 cells were analyzed for each time point). *Diamonds:* The time course of cell volume changes, as estimated by the fluorescence of the volume-sensitive dye SPQ (6-methoxy-1-(3-sulfopropyl) quinolinium monohydrate). The arrow represents the time of the osmotic shock. As described in Methods, a greater fluorescence reading relates to a larger volume.

(Srinivas and Bonanno, 1997; Srinivas, Guan & Bonanno, 1999). The principle of measuring cell volume with SPQ is that the dye is quenched by intracellular osmolytes, presumably by the intracellular anions. When cells swell, the intracellular concentrations of both the SPQ and the quencher decreases, lowering the probability of interaction between the two, and the fluorescence intensity increases. The experiment is performed in cells depleted of the intracellular Cl^- because SPQ is quenched by Cl^- ions. Intracellular Cl^- is substituted with the NO_3^- ion that has little effect on SPQ fluorescence (Orosz & Garlid, 1993). Indeed, after the anion substitution, SPQ fluorescence stabilized, producing a stable baseline with no detectable variation for the duration of 30 minutes. As can be noted from the graph, there is a direct correlation between the changes in the spectrin area and cell volume.

Dependence of the Intracellular Distribution of Spectrin on the Integrity of F-Actin

To test the relationship between swelling-induced reorganization of spectrin and reorganization and depolymerization of F-actin, the intracellular distribution of spectrin was analyzed in cells exposed to jasplakinolide, a toxin that stabilizes F-actin by preventing its depolymerization (Bubb et al., 1994). As expected, exposing the cells to jasplakinolide (1, 3, or 5 μM) significantly decreased RITC-phalloidin fluorescence, indicating that jasplakinolide was successfully loaded into the cells, where it competes with phalloidin for binding to F-actin (*not shown*). Since 3 μM of jasplakinolide was sufficient to decrease RITC-phalloidin fluorescence to the background level, this concentration was used to test the effect of F-actin stabilization on spectrin distribution. Figure 5 shows that loading the cells with jasplakinolide did not have an apparent effect either on spectrin distribution in cells maintained under isotonic conditions (*compare* Panels *A* and *C*). Furthermore, exposure to jasplakinolide did not prevent swelling-induced redistribution of spectrin (Panels *B* and *D*). Statistical analysis of the data confirms these observations (Fig. 6), indicating that stabilization of F-actin appears to have no effect on swelling-induced changes in the intracellular distribution of spectrin in BAECs.

In contrast, exposing the cells to latrunculin A, a toxin that disrupts F-actin (Spector et al., 1989) had significant effect on both the distribution of spectrin under isotonic conditions and on its sensitivity to cell

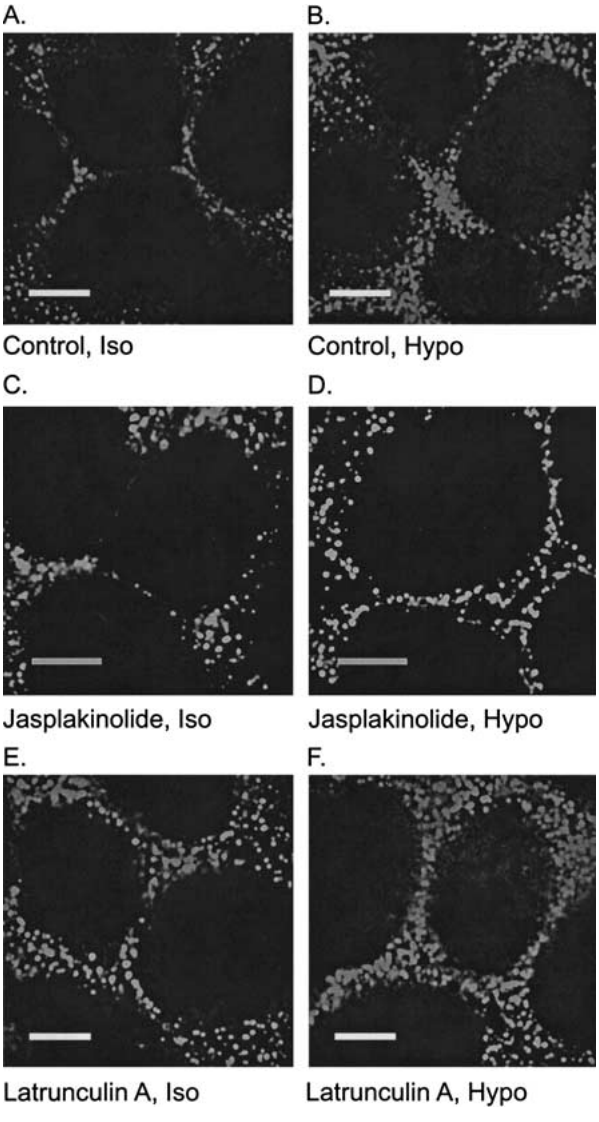

Fig. 5. The effects of jasplakinolide and latrunculin on spectrin distribution. Typical optical sections of spectrin of control cells maintained in isosmotic conditions (*A*) or challenged with a 33% osmotic gradient for 5 minutes (*B*). Optical sections in cells exposed to 3 μM jasplakinolide for 10 minutes and maintained in isotonic environment (*C*) or challenged with the same osmotic gradient (*D*). Optical sections in cells exposed to 1 μM latrunculin for 10 minutes in isotonic (*E*) or in hypotonic environment (*F*). Scale bars = 5 μm.

swelling (Fig. 5*E*, *F*). As expected, exposing the cells to 1–5 μM latrunculin A resulted in massive disruption of the F-actin network (*not shown*). The effect of latrunculin on the spectrin distribution was similar to that of the hypotonic challenge (*compare* Panels *A*, *B* and *E*). No further dispersion of spectrin was observed when latrunculin A-treated cells were challenged with an osmotic gradient (*compare* Panels *B* and *F*). Analysis of the spectrin intensity profiles for the six experimental conditions is summarized in Fig. 6.

Discussion

Cell swelling is known to have profound effects on the intracellular structure and signaling, including rearrangement of F-actin, gating of ion channels, activation of intracellular signaling pathways, and changes in gene expression (*reviewed* by Hoffmann & Simonsen, 1989; Nilius et al., 1997; Okada, 1997; Lang et al., 1998; Hoffmann, 2000; Pasantes-Morales, Cardin & Tuz, 2000; Kim, Stein & Chari, 2001). In several cell types an increase in cell volume is not accompanied by an increase in cell surface area, indicating that swelling has to be accompanied by unfolding of membrane invaginations, rather than by addition of membranes from intracellular vesicles (Levitan & Garber, 1997; Miwa, Ueda & Okada, 1997; Okada, 1997). Therefore, disruption and/or detachment of the submembrane cytoskeleton may play a major role in coupling cell swelling to a variety of swelling-induced cellular events (Okada, 1997). Indeed, Pedersen, Mills & Hoffmann (1999) showed that in Ehrlich ascites tumor cells, challenging the cells osmotically affects mainly the cortical (submembrane) F-actin with no visible changes in F-actin in other cell regions. Non-erythroidal spectrin, a cytoskeletal protein that underlies the plasma membrane in a variety of cells and exhibits unique extensibility and strength, was suggested to be a cell volume sensor candidate (Herring et al., 2000). This study analyzes the 3-D intracellular distribution of non-erythroidal spectrin in BAECs and demonstrates that hypotonic shock has a significant effect on its intracellular distribution in these cells. These data support the hypothesis that spectrin may play an important role in transducing the changes in cell volume to the intracellular responses. Submembrane localization of spectrin in BAECs observed in this study is consistent with spectrin localization and assembly at sites of cell-cell contacts in other cell types (Levine & Willard, 1983; Takemura et al., 1993; Piepenhagen & Nelson, 1998; Pradhan et al., 2001). A significant amount of spectrin in the apical membranes of BAECs may account for an earlier observation that in 2-D images spectrin appears to be scattered throughout the cytoplasm (Pratt et al., 1984; Heltianu et al., 1986). Interestingly, in all our experiments, spectrin staining at the borders of confluent BAECs is not continuous but punctuate. This is consistent with the observation of Pratt et al. (1984) who showed punctate spectrin staining in BAECs grown on fibronectin substrate, but the nature of the spectrin-containing structures is not identified yet. Further studies are needed to identify the nature of the spectrin-containing cellular structures in vascular endothelial cells.

While cell swelling does not affect the total cellular content of spectrin, it results in a significant redistribution of spectrin-specific fluorescence at sites

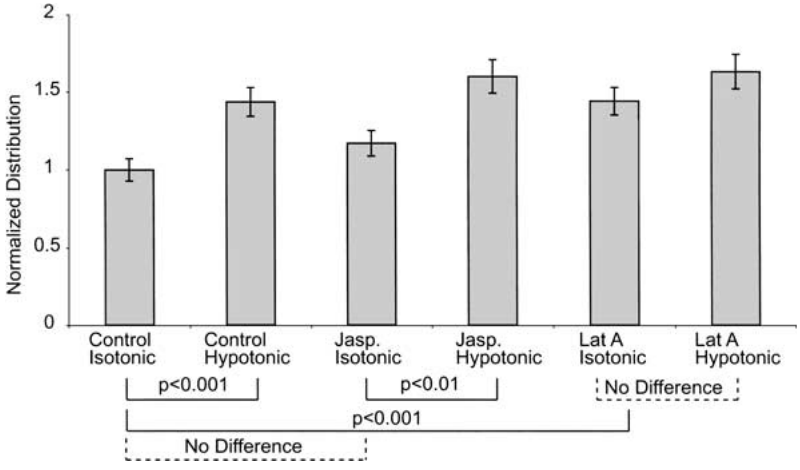

Fig. 6. Quantification of the effect of jasplakinolide and latrunculin A on submembrane spectrin distribution. The data were quantified by analyzing the line intensity profiles of the spectrin distribution as described in the Analysis section. At least 50 cells were measured for each condition in every experimental trial. The findings were confirmed by repeating the experiments in several (2–5) independent trials.

of cell-cell junctions. Since the total spectrin fluorescence does not increase, we suggest that the observed spectrin redistribution is due to spectrin dispersion rather than an increase in the number of spectrin-containing structures. Swelling-induced dispersion of spectrin appears to be one of the earliest swelling-induced events. Similar to the reorganization of F-actin (Ziyadeh et al., 1992; Pedersen et al., 1999), dispersion of spectrin can be detected within one minute after the osmotic challenge and develops in parallel to an increase of cell volume. The fast onset of spectrin reorganization suggests that it may act as a mechanical transducer by initiating a chain of events leading to other cellular responses. It is also significant that the effect of osmotic stress on the spectrin distribution is reversible. Similar to F-actin (Ziyadeh et al., 1992), the distribution of spectrin recovers when the cells are exposed to longer periods of osmotic stress, presumably due to the regulatory volume decrease, which leads to the recovery of cell volume. Indeed, significant cell volume recovery in BAECs is observed 10 minutes after the exposure to an osmotic shock. Fast reversibility of the effect is important because it is essential for sensing and transducing changes in cell volume.

To get an insight into the mechanism responsible for the swelling-induced reorganization of spectrin in BAECs, we tested the hypothesis that depolymerization and/or reorganization of F-actin may be the trigger that induces redistribution of spectrin. Our results show that the sensitivity of spectrin to the osmotic challenge is not blocked by stabilizing F-actin, indicating that F-actin depolymerization is not the trigger for the redistribution of the spectrin. Exposing the cells to latrunculin A, however, results in significant changes in spectrin distribution, indicating that the integrity of F-actin is critically important for the normal distribution of spectrin. These observations underscore the fact that pharmacological agents that disrupt F-actin may have significant effects on other cytoskeletal proteins. Thus, the interplay between F-actin and other cytoskeletal proteins has to be taken into account in analyzing the role of membrane cytoskeleton in cellular signaling.

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