Incidence of protein on actin bridges between endothelium and smooth muscle in arterioles demonstrates heterogeneous connexin expression and phosphorylation

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Within Resistance Vessels, immunocytochemistry has demonstrated the presence of multiple connexin isoforms within endothelial cells (ECs; e.g., Refs. 15 and 17) and vascular smooth muscle cells (VSMCs; e.g., Refs. 15 and 23). However, an analysis of the incidence of protein expression between ECs and VSMCs from resistance vessels has not been done. This is vital to understanding EC and VSMC integration since it reveals the possible differences in how the two cell types may use connexins for heterocellular communication (e.g., Ref. 2). One of the most anatomically unique areas of resistance vessels is the location between ECs and VSMCs, where the two cell types make physical contact, termed the myoendothelial junction (MEJ). The MEJ is formed when ECs and/or VSMCs extend cellular extensions through the internal elastic lamina (IEL) and make contact. Since Rhodin (20) identified MEJs in transmission electron microscopy (TEM) sections of arterioles, it has been suggested that the MEJ acts as a possible “nexus” of signaling molecules and receptors between arteriolar ECs and VSMCs. However, the size of the MEJ (~0.5 μm by 0.5 μm width and length) and its location between ECs and VSMCs have made the assessment of protein expression such as connexin, dependent on TEM (23) or resorting to a cell culture-based model (6). Because of this, little is known of the MEJ in terms of protein expression; structurally, however, we do know that it can contain endoplasmic reticulum (7) and, because MEJs are cellular extensions, must be membranous and contain actin.

It has been hypothesized that gap junctions play a key role in the integration of VSMC and EC function (e.g., Ref. 18). With the use of TEM, previous work has confirmed that the typical gap junctional “pentalaminar” structure is present between ECs and VSMCs at the MEJ in resistance vessels (22, 29). Connexin proteins compose gap junctions by forming a dodecameric channel linking two adjacent cells. The gap junction channel permits cell-cell communication either on a rapid (e.g., second messengers and current flow) or a slower (e.g., control of mitosis) timescale, with recent work demonstrating roles in intracellular communication (32). Because the types of connexin isoforms determine solute permeability (e.g., Ref. 16), and thus cellular responses, providing information as to the degree and type of heterogeneous connexin expression between ECs and VSMCs would provide powerful insight into how ECs and VSMCs are functionally integrated.

Here we present a method whereby protein expression, as detected by antibodies, can be evaluated between ECs and VSMCs. This method uses phalloidin labeled with a fluorescent conjugate to track actin-based cellular extensions through the IEL, identified using Z-sections produced by confocal microscopy. By overlaying the “actin bridges” in the IEL and connexin antibodies as revealed with a fluorescent tag, we plot the incidence of connexin proteins found in the cellular extensions linking ECs and VSMCs in mouse arterioles and compare it with rat mesenteric arteries where connexin detection at the MEJ has used electron microscopy. We believe that this method will enable a rapid screening for protein between ECs and VSMCs and is an important first step toward the quantification of protein at the MEJ.

MATERIALS AND METHODS

Animals. All C57/B16 mice (Taconic) were males between 6–10 wk of age, and all animal protocols were approved by the University

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Once identified, the confocal microscope was activated, and a pinned down on Sylgard-coated polyester petri dishes, and the first secondary antibodies conjugated with Alexa 488 (Invitrogen) were previously tested. All primary antibodies were identified using (Zymed; Ref. 25) and monoclonal desmin (Sigma) antibodies have all EC adhesion molecule 1; Ref. 34), NG2 (Ref. 19), were obtained from Cell Signaling Technologies (1). CD31 (platelet antibodies against phosphorylated Cx43 at serine-368 (Cx43-S368) (kind gift from T. H. Steinberg, Washington University) (14). Primary mouse Cx45 and has previously been extensively tested and verified significany tested as previously described for mouse (6). Two Cx43 antibodies were used, a polyclonal and a monoclonal (Sigma) (6, 17).

Antibodies. Cx37 and Cx40 antibodies were from ADI with specificity tested as previously described for mouse (6). The rabbit Cx45 antibody was raised against the carboxyl terminus of mouse Cx45 and has previously been extensively tested and verified (kind gift from T. H. Steinberg, Washington University) (14). Primary antibodies against phosphorylated Cx43 at serine-368 (Cx43-S368) were obtained from Cell Signaling Technologies (1). CD31 (platelet EC adhesion molecule 1; Ref. 34), NG2 (Ref. 19), N-cadherin (Zymed; Ref. 25) and monoclonal desmin (Sigma) antibodies have all been previously tested. All primary antibodies were identified using secondary antibodies conjugated with Alexa 488 (Invitrogen).

Visualizing of proteins between ECs and VSMCs. Cremasters were pinned down on Sylgard-coated polyester petri dishes, and the first arteries supplied by feed arteries were identified as described (33). Once identified, the confocal microscope was activated, and a ×100 1.0 numerical aperture water immersion lens was used to identify the ECs and VSMCs within the arteries using phalloidin-Alexa 594. We were unable to detect any significant autofluorescence at the IEL (e.g., supplemental Fig. 1; note: all supplemental material can be found published with the online version of this article) in the 488 channel.

XY sections in the Z-direction at 0.2-μm steps brought a focal plane into focus containing VSMCs perpendicular to the ECs as determined by transverse views of VSMCs that appeared as circles due to the phalloidin staining of the cortical actin and lateral cortical actin lines of the ECs (Fig. 1, A and B, and supplemental Fig. 1). The Fluoview software was used to zoom in on short segments (the end result of the zoom was ~1 to 2 VSMCs across, or a ×2 zoom). Unstained (i.e., cell free) areas such as the IEL appeared black between the phalloidin-stained ECs and VSMCs, with actin bridges linking the two cell types (Fig. 1C). The actin bridges were membrane associated as demonstrated with the staining for the fixable membrane stain FM 1-43FX (Fig. 1D), indicating membranous and cytoskeletal extensions into the normally cell-free IEL. Although we cannot be at all certain that the actin bridges are MEJs, these observations in mouse cremaster arteries correlate with MEJs found in mouse cremaster arteries (Ref. 7; and S. Sandow, personal communication).

When fluorescence from an antibody was thought to be localized between ECs and VSMCs (Fig. 2A), the fluorescence emitted from phalloidin was used to plot horizontal and vertical intensity lines to define the approximate location of an actin bridge (Fig. 2B). With the use of the pixel intensity lines for phalloidin, a horizontal line running through the IEL would have little to no intensity unless it came across an area of fluorescence representing actin (Fig. 2B, horizontal histogram), whereas a vertical intensity line would have high intensity from ECs and VSMCs until it came across the cell-free IEL (Fig. 2B, vertical histogram). The horizontal intensity line was moved vertical until the intensity histogram was nearly flat with high intensity due to the phalloidin in ECs and VSMCs. The vertical intensity line was moved horizontal until the intensity histogram was nearly flat with high intensity due to the phalloidin in ECs, the actin bridge, and VSMCs. The intersections of the new vertical and horizontal intensity lines formed a box unique to each actin bridge. Because of irregular MEJ shapes and sizes, these intensity lines were moved 5% outward to encompass as much area as possible; this formed a region of interest (ROI) over the actin bridge unique to each particular actin bridge tested (Fig. 2B, blue box).

So as to compare the relative fluorescence from the antibodies in ECs, the actin bridge, and on the VSMCs, the ROI box created above was placed over the fluorescent image from the antibody being tested (Fig. 2C, box 2), as well as the ECs immediately above (Fig. 2C, box B).
**INCIDENCE OF PROTEIN IDENTIFICATION BETWEEN ECs AND VSMCs**

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**I. Innovative Methodology**

**A.** Representative for all other images in this figure.

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**B.** Representative intensity plots running horizontal and vertical defined the internal elastic lamina (IEL) and approximate localization of an actin bridge indicated by asterisk in A. Yellow lines are guides for localization of intensity on the histograms. The intersections of the new vertical and horizontal intensity lines formed a box unique to each actin bridge. Because of irregular myoendothelial junction shapes and sizes, these intensity lines were moved 5% outward to encompass as much area as possible; this formed a region of interest (ROI) over the actin bridge (blue box). C: blue boxes demonstrate the ROI for ECs (box 1), the actin bridge (box 2), and VSMCs (box 3). The mean pixel intensity from each of these boxes is in histogram format in C. The gray line is 25% below the value in ROI 3 and used as a benchmark for pixel intensity in ROI 2. In this incidence, the protein is found on the actin bridge. See MATERIALS AND METHODS for details. White bar in A is 3 μm and is representative for all other images in A; in B, white bar is 1 μm and is representative for all other images in B and C.

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**C.** Mean Intensity vs. ROI.

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**D.** Determination of protein expression on actin bridges be-

**Table:**

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<th>ROI</th>
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**RESULTS**

Heterogeneous protein expression on actin bridges between ECs and VSMCs. Although connexin expression in mouse cremasteric arterioles can be identified in cross section (supplemental Fig. 3), we sought to delineate connexin expression between ECs and VSMCs. Using actin bridges as a guide, we used the methodology described in Fig. 1 to examine connexin expression. Using this technique, we observed that both Cx37 and Cx45 had variable expression patterns (Fig. 3, B and E), whereas both Cx40 and Cx43 were routinely found on the actin bridges (Fig. 3, C and D). Intensity plots under each image for both actin and connexin demonstrate apparent colocalization.

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Connexin phosphorylation on actin bridges between ECs and VSMCs. Gap junctional coupling between ECs and VSMCs in mouse cremaster has recently been reported to be poor or nonexistent (26). For this reason, we tested the hypothesis that the Cx43 on the actin bridges was phosphorylated at serine-368, a PKC-mediated phosphorylation event that is correlated with closure of gap junctions (12). Extensive testing of the Cx43-S368 antibody demonstrated its specificity (supplemental Fig. 4, and Ref. 12). It is well accepted that Cx43 is expressed in VSMCs of the mouse aorta (27), as is evident in the aortic vessel wall. In contrast to the aorta, the arterioles from mouse cremaster muscle were positive for Cx43-S368 in both the ECs and VSMCs (Fig. 4B), as is evident in higher magnification images (Fig. 4C). Actin bridges in mouse cremaster arterioles were positive for the Cx43-S368 staining (Fig. 4D).

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**Determination of protein expression on actin bridges between ECs and VSMCs.** With the use of the methods described in Fig. 2, the frequency of detectable protein expression at the actin bridges is shown in Fig. 5. Neither NG2 (5.0%) nor CD31 (10.0%) was detectable at appreciable levels on actin bridges.
Desmin, a smooth muscle cell cytoskeletal component, and N-cadherin, an adhesion protein, were detected in 77.2% and 70.9%, respectively, of the bridges sampled (Fig. 5A). There was a significant difference in expression between the NG2 and CD31 antibodies and the N-cadherin and desmin antibodies (as demonstrated in raw images in supplemental Fig. 5). There was minimal expression of Cx45 (13.0%), and Cx37 expression appeared to be variable (31.8%; Fig. 5A). Cx40 (83.8%) had the highest percent expression on the actin bridges, whereas Cx43 (54.2%) was also expressed (Fig. 5A).

Of interest is the Cx43-S368 percent staining (52.7%; Fig. 5A) that was closely matched with that of Cx43, suggesting that all of the Cx43 was phosphorylated. When Cx40 was compared with any of the other connexins antibodies tested, there was a significant difference in expression. The incidence of these proteins lends increased confidence to the pattern of staining described in Fig. 3.

We sought to confirm our methodology with previously published results of immunohistochemistry of connexin proteins on electron microscopy images of the MEJ (23). Both...
VSMCs individually. Other drawbacks are discussed below.

Cytochemistry on TEM sections or the isolation of the ECs and specific cell types involved will continue to require immunocytochemistry (e.g., Ref. 20, and supplemental Fig. 7). Identification of types of cellular extensions composing MEJs found in vivo from which cell type the protein is derived due to the multiple VSMC integration. It cannot, however, be used to determine the incidence of protein expression between ECs and VSMCs. This method has the potential to be used as a screening technique to previously published TEM images of connexins at the MEJ (23).

**DISCUSSION**

The technique described has allowed for a determination of the incidence of protein expression between ECs and VSMCs. This method has the potential to be used as a screening mechanism for proteins that may be important for EC and VSMC integration. It cannot, however, be determined from which cell type the protein is derived due to the multiple types of cellular extensions composing MEJs found in vivo (e.g., Ref. 20, and supplemental Fig. 7). Identification of specific cell types involved will continue to require immunocytochemistry on TEM sections or the isolation of the ECs and VSMCs individually. Other drawbacks are discussed below.

It is clear the actin bridges are membranous (Fig. 1D), which indicates that they are cellular extensions from ECs, VSMCs, or both. Thus the identified proteins are at least uniquely found on cellular extensions protruding into the IEL. However, the identified proteins, using polyclonal antibodies, present a problem since it is not likely that all antibodies have the same affinity for the antigen or indeed the same accessibility of the antibodies due to protein-protein interactions (e.g., Cx43 and N-cadherin interactions; Refs. 24 and 31). Another potential problem is our use of a single plane of focus, and the MEJ arguably resembles more of a cylindrical structure and we therefore believe it is a possibility that we have not correctly identified some of the protein that is expressed. In addition, we expanded the guidelines for the ROI out by 5% when analyzing protein expression on the actin bridges. This was done due to the variability in MEJ structures (20); however, it is likely we have added contamination from the ECs and VSMCs as a whole into our analysis. Lastly, our method of collecting samples was to randomly choose actin bridges and then move three fields of view up or down the length of the arteriole. Although this technique was done in an attempt to determine the relationships between different proteins being expressed on actin bridges, this actually prevented us from determining whether shorter segments of the arterioles had differential expression of the connexins than other segments. Regardless of these problems, we are encouraged by the differences demonstrated in protein expression, which implicate selective protein segregation to the actin bridges, and the correlation of this technique to previously published TEM images of connexins at the MEJ (23).

**Incidence of connexin isoform within and between ECs and VSMCs.** With TEM, there are reports of connexin expressed at the MEJ in rat mesentery (23) and rat cerebral arteries (3). Based on the reported connexins from these studies and others (e.g., Refs. 17 and 27), we selected a panel of vascular connexins to examine at actin bridges, including Cx45, Cx37, Cx40, and Cx43. When Cx45 was examined using our model, the expression on the actin bridges was consistently at low levels (Fig. 5, A and B). The expression of Cx45 in mouse ECs and VSMCs of the microcirculation has been previously demonstrated in situ using lacZ (30) and by RT-PCR in primary culture of ECs (4). There is little known about Cx45 in the microcirculation, although Cx45/−/− mice had considerable vascular defects, indicating the importance of the particular connexin during vascular development (9).

In this study, Cx37 on actin bridges (31.8%) appeared to be variable in expression (Fig. 5A), a result consistent with previous reports of Cx37 variability in mouse kidney arterioles (N. H. Holstein-Rathlou, personal communication) and the lack of a pronounced Cx37/−/− vascular phenotype (27). It is interesting to note that murine cell culture models have also demonstrated limited or no Cx37 expression at points of heterocellular contact (6). Although the in vivo data here does demonstrate minor expression of Cx37 at the MEJ, in both the culture model and in vivo Cx40 and Cx43 predominated. We cannot, however, rule out that Cx37 may have an important role in discrete segments of the arteriole which may have

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**Fig. 5. Determination of antibody (Ab) threshold detection on the actin bridges.** With the use of the methods described in MATERIALS AND METHODS and in Fig 2, histograms of percentage of time that proteins could be detected on an actin bridge were plotted for mouse cremaster arterioles (A) and rat mesenteric arteries (B). Bars represent SD. #No significant difference. *Significant difference.
accounted for the variability observed, although, in rat mesentery, there was no detectable variability of Cx37 expression at the point of heterocellular contact (e.g., compare Fig. 5, A and B, as well as Ref. 23). It is clear that more work is required to understand these observations.

In our studies, Cx40 was the most consistent connexin tested to be found on actin bridges in mouse cremaster (Fig. 5A). Because this connexin is found in ECs and not VSMCs (27), it is likely that this connexin is selectively placed on the EC side of the MEJ, which corresponds with reports of Cx40 at the MEJ in rat (23). It is unclear how exactly Cx40 in mouse cremaster muscle integrates ECs and VSMCs, but previous experiments have demonstrated that Cx40 antibody could block endothelium-derived hyperpolarizing factor (EDHF) in rat mesentery (18).

Recently, Cx43 was identified as being important in the transmission of EDHF in humans (13), a result that coincides with our report of Cx43 on actin bridges in the majority of experiments (Fig. 5A). Because Cx43 was expressed in both ECs and VSMCs (supplemental Fig. 3), it was unclear whether both cell types contributed the connexins on the actin bridge. It is not clear why Cx43 was expressed over 50% of the time on actin bridges but not as often as Cx40.

There is a difference in phosphorylation states in rat Cx43 compared with mouse Cx43 (Fig. 5, A and B). In the mouse, the Cx43 present on the actin bridges is almost always phosphorylated by Cx43-S368; however, little Cx43 was detected on actin bridges in rat, and it was rarely found to be phosphorylated at serine-368. The Cx43 phosphorylation event at the MEJ may be a rationale for why EDHF responses are routinely noted in rat mesentery but not in mouse cremaster (26) (see next section).

Connexin phosphorylation on actin bridges. Gap junctions are not passive holes between cells but are highly regulated in terms of opening, closing, and solute movement. The regulation of the gap junction can be on timescales relating to connexin turnover (~1 to 4 h) or on timescales that are much shorter. The factors that can quickly regulate gap junction permeability include intracellular pH and Ca2+ concentrations (21), as well as nitrosylation (8) and phosphorylation (10).

Each of these factors has been shown to be able to occur in discrete domains within cells, making it possible that regulation of gap junctions could occur within localized cellular areas, e.g., the MEJ.

In the present study, we examined whether connexin phosphorylation was present on the actin bridges in mouse cremaster. The rationale for this arose from the knowledge that, anatomically, gap junctions are present at the MEJ (e.g., Refs. 6, 22, and 29); however, functional evidence in mouse cremaster has disputed their role at the MEJ (26). The descriptive evidence presented herein demonstrates that Cx43 is phosphorylated at S368. Phosphorylation of this site is generally considered to “close” gap junction-based communication (11, 12, 28), although it is not clear what effect, if any, there is on any other connexin proteins (i.e., they could still remain “open”).

We cannot exclude the possibility that factors inherited to the cremasteric removal (e.g., tissue damage and release of ATP) could also be the cause of the phosphorylation event. Taken together, we hypothesize that connexin phosphorylation observed on actin bridges in mouse cremaster may be the reason that poor EC and VSMC coupling has been previously reported. Future experiments that directly test the effects of connexin phosphorylation at the MEJ and their functional consequences are needed.

Conclusion. In summary, we have demonstrated a method using actin bridges between ECs and VSMCs to localize proteins at the interface between the two cell types. The incidence of the protein found on the actin bridges was dependent on the connexin isoform and the correlation with this method, and the TEM images of connexin expression at the MEJ in rat mesenteric arteries implicate that the actin bridges were possibly MEJs. We therefore believe that this method is an important first step toward the actual quantification of protein found between ECs and VSMCs, possibly at MEJs.

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