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Connexin Isoform Expression in Smooth Muscle Cells and Endothelial Cells of Hamster Cheek Pouch Arterioles and Retractor Feed Arteries

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Abstract

Objective—Gap junction channels formed by connexin (Cx) protein subunits enable cell to-cell conduction of vasoactive signals. Given the lack of quantitative measurements of Cx expression in microvascular endothelial cells (EC) and smooth muscle cells (SMC), the authors' objective was to determine whether Cx expression differed between EC and SMC of resistance microvessels for which conduction is well-characterized.

Methods—Cheek pouch arterioles (CPA) and retractor feed arteries (RFA) were hand-dissected and dissociated to obtain SMC or endothelial tubes. In complementary experiments, small intestine was dissociated to obtain SMC. Following reverse transcription, quantitative Real-Time PCR (qRT-PCR) was performed using specific primers and fluorescent probes for Cx37, Cx40 and Cx43. Smooth muscle α -actin (SMAA) and platelet endothelial cell adhesion molecule-1 (PECAM-1) served as respective reference genes.

Results—Transcript copy numbers were similar for each Cx isoform in EC from CPA and RFA (~ 0.5 Cx/PECAM-1). For SMC, Cx43 transcript in CPA and RFA (< 0.1 Cx/SMAA) was less ($p < 0.05$) than that in small intestine (~ 0.4 Cx/SMAA). Transcripts for Cx37 and Cx40 were also detected in SMC. Punctate immunolabeling for each Cx isoform was pronounced at EC borders and that for Cx43 was pronounced in SMC of small intestine. In contrast, Cx immunolabeling was not detected in SMC of CPA or RFA.

Conclusions—Connexin expression occurs primarily within the endothelium of arterioles and feed arteries, supporting a highly effective pathway for conducting vasoactive signals along resistance networks. The apparent paucity of Cx expression within SMC underscores discrete homocellular coupling and focal localization of myoendothelial gap junctions.

Keywords

Gap junctions; immunolabeling; microcirculation; quantitative Real-Time PCR; resistance arteries

Introduction

Intercellular communication is integral to the control of blood flow in vascular resistance networks. Within tissues, arteriolar networks govern the distribution and magnitude of local blood flow to parenchymal cells while proximal feed arteries control the total volume of flow entering the tissue. For network branches arranged in parallel as well as in series, the control of tissue blood flow requires coordinated interactions among daughter and parent segments (34). Such coordination is enabled by the conduction of vasomotor responses from cell to cell along the vessel wall through gap junction channels (12,27,35). Gap junction channels are unique among proteins expressed in the plasma membrane because the docking of respective hemichannel (connexon) subunits between adjacent cells forms a continuous aqueous pore (40) that enables the passage of ions and small (< 1 kDa) molecules from cell to cell. Each hemichannel is comprised of six connexin (Cx) subunits. Of more than 20 Cx isoforms that have been characterized, those most consistently associated with the vasculature are Cx43, Cx40 and Cx37 (18).

Localization of Cx expression in the vessel wall has relied primarily on immunolabeling and electron microscopy to locate gap junction plaques. A consistent observation in intact arterioles and feed arteries is that Cx proteins are highly expressed at the borders of EC (29,33,44). Further, integrity of the endothelium has proven essential to conducted vasodilation in these microvessels (12,29) with signals apparently able to travel into (and from) smooth muscle via myoendothelial gap junctions (8,11,46,48). Whereas functional studies (2,3,42) and mathematical modeling (7) have implicated a complementary role for gap junctional coupling between consecutive SMC along the vessel wall, their presence has been more difficult to confirm (3,33). Thus, a role for smooth muscle in providing an alternative conduction pathway (2,4,42) remains controversial (16,44). Contributing to this controversy is a distinct lack of quantitative measurements of Cx expression in respective cell types.

When conduction is absent (25,36) or impaired (6,14,24,30,44), the control of tissue blood flow is compromised. Thus a fundamental question of clinical significance concerns the relative contribution of endothelium vs. smooth muscle in providing the cellular pathway for signal transmission along the vessel wall (2,17,42,46). A direct approach to answering this question entails obtaining pure samples of SMC and of EC followed by analyses of cell-type specific gene expression. Because such analyses have not yet been performed in the microcirculation, a primary goal of the present study was to develop methods for determining cell-type specific expression of Cx isoforms (Cx43, Cx40 and Cx37) in EC and SMC of resistance microvessels. For this purpose, we used hamster cheek pouch arterioles (CPA) and retractor muscle feed arteries (RFA), two microvessels that have been well-characterized with respect to the conduction of vasomotor responses (11,12,35,37,42,45,46). We tested the null hypothesis that Cx expression is not different between microvascular EC and SMC. We report that EC from RFA and CPA consistently expressed robust transcript levels and protein immunolabeling for Cx37, Cx40 and Cx43. In contrast, SMC from respective microvessels expressed only marginal transcript levels without discernable immunolabeling. Nevertheless, SMC from small intestine exhibited robust transcript expression and clear immunolabeling for Cx43.

Methods

Animal care and tissue dissection

All procedures were approved by the Institutional Animal Care and Use Committee and were performed in accord with the *NIH Guide for the Care and Use of Laboratory Animals*. Male Syrian Golden hamsters (n=56; 80–125 g; Charles River Laboratories, Wilmington, MA) were anesthetized with an intraperitoneal injection of either pentobarbital sodium (60 mg/kg) or α -

chloralose (80mg/kg) + urethane (500mg/kg). The entire cheek pouch and retractor muscle with intact feed arteries (15) were excised bilaterally. A segment (~2 cm) of the small intestine (jejunum) was removed via a midline incision in the abdomen. Tissues were placed immediately into a 35-mm Petri dish containing ice-cold (4 °C, pH 7.4, 295 ± 5 mOsm) dissection buffer composed of (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 10 N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 10 Glucose, 0.01 sodium nitroprusside (SNP) and 0.1% Bovine Serum Albumin (USB Corp. #10856, Cleveland, OH). Reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Tissue samples were kept on ice for at least 10 min to relax SMC and then transferred to a dissection chamber containing ice-cold buffer and pinned onto a transparent Sylgard block. While viewing through a stereomicroscope, individual CPA and RFA were dissected by hand and pooled samples of each vessel were collected into respective 12 mm × 75 mm borosilicate glass tubes kept on ice and containing 4 ml dissociation buffer (as above with 0.1 mM CaCl₂ replacing SNP). The sample of jejunum was opened and pinned in the same ice-cold tissue chamber to remove serosal fat and connective tissue. The circular layer of smooth muscle (*muscularis externa*) was carefully peeled away using fine forceps, cut into ~ 6 mm × 6 mm pieces and then treated identically to CPA and RFA. All tissue samples were kept on ice before further processing. One hamster was used for each preparation from CPA and small intestine. Because there are fewer RFA than CPA in each hamster, two hamsters were used for each preparation of RFA in order to obtain enough cells for respective analyses.

Isolation of cells

For each sample of CPA and RFA, the dissection buffer was replaced with 1 ml of preheated (37 °C) dissociation buffer [as above containing 26 U/ml papain (Sigma # P-4762), 1 mg/ml dithioerythritol (Sigma # D-8255), 1.95 FALGPA U/ml collagenase (Sigma # C-8051)] and incubated for 30-40 min at 37 °C. This solution was replaced by 4 ml of enzyme-free dissociation buffer, incubated for 5 min at room temperature and supernatant was aspirated to leave samples suspended in ~200 µl. Trituration was performed slowly through a glass micropipette [internal diameter (ID), ~250 µm] to gently separate cells. Cell suspensions were transferred to a 35-mm Petri dish on the stage of a microscope (GFL, Zeiss). Using Koehler illumination (total magnification, 125×), individual SMC or endothelial tubes were identified (22) and aspirated into borosilicate glass capillary tubes [1.0 mm OD/ 0.58 mm ID; # 1B100-3, World Precision Instruments (WPI), Sarasota, FL] with heat-polished tips (ID, 40-50 µm) using a Nanoliter Injector (Cat # A203XVY; WPI) with a MICRO1 controller (WPI). Each sample of cells was ejected into a 0.5 ml nuclease-free microfuge tube kept on ice. In each preparation of CPA or RFA, as many cells as possible were collected within 20 min, resulting in 15-20 µl containing 1500-2000 SMC or 5-10 µl containing 6-10 endothelial tubes that were each ~500 µm long. For preparations of small intestine, similar numbers of SMC were sampled within the same period.

Viability of cell preparations

Freshly-dissociated SMC and endothelial tubes maintain functional integrity as demonstrated by robust calcium and electrophysiological responses to agonists (5,22). In light of the paucity of gene expression for Cx isoforms found in microvascular SMC (*see RESULTS*), a Live/Dead viability assay (Invitrogen # L3224; Carlsbad, CA) confirmed that 85-90 % of SMC labeled brightly with calcein dye, excluded ethidium homodimer (confirming the integrity of plasma membranes) and contracted in response to the α_1 -adrenoreceptor agonist phenylephrine (confirming the integrity of receptor-mediated signaling leading to contractile protein interaction). The morphological integrity of cell preparations for immunolabeling (*see below*) was evaluated using differential interference contrast (DIC) imaging.

Immunolabeling

Cell suspensions were placed in incubation wells on a slide (Grace Bio-Labs #CWCS-2R; Bend, OR), allowed to attach for 10-15 min at 37 °C, then washed and permeabilized with Tris-Buffered Saline Tween-20 (TBST; 136 mM NaCl, 24 mM Tris, 0.1% Tween-20; pH 7.4) for 5 min, fixed with cold (-20 °C) methanol for 2 min, washed twice with TBST and blocked with 5% nonfat milk in TBST for 1 h at room temperature. Following an additional wash with TBST, cells were incubated with one of the following rabbit primary antibodies: anti-Cx43 (Sigma C-9619, 1:200), anti-Cx40 (Chemicon AB1726, 10 µg/ml), or anti-Cx37 (Alpha Diagnostic CX37A11-A, 10 µg/ml) along with mouse anti-SMAA (Sigma A-2547, 1:400) for 1 h at room temperature. In each case, negative controls were performed by eliminating the primary antibody. Cells were washed with TBST, incubated with secondary antibodies (1:500; Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 546 goat anti-mouse; Invitrogen #A11034 and A11030, respectively) for 1 h at room temperature and washed with TBST. Incubation wells were replaced by a coverslip and slides were viewed on a Nikon E800 microscope equipped for DIC and fluorescence image acquisition with filter sets appropriate for respective secondary antibodies (Chroma Technology; Rockingham, VT). Whole-mount preparations of small intestine [and of intact microvessels to confirm previous observations (28,33): data not shown] were pinned on Sylgard® (#184; Dow-Corning; Midland, MI) and treated using the same procedures. Images were acquired using Nikon objectives: 20× [numerical aperture (NA) =0.5] or 60× and 100× oil-immersion (NA=1.4). At each magnification, digital images were acquired at identical settings for illumination and sensitivity using a SPOT cooled CCD Camera (Diagnostic Instruments; Sterling Heights, MI). Immunolabeling experiments were replicated in at least 4 independent preparations.

Quantitative Real-Time PCR

A “Cells to cDNA II” kit (Ambion #1722; Austin, TX) was used to extract total RNA, eliminate genomic DNA and generate cDNA from cell samples as follows. Ice-cold cell lysis buffer (15 µl) was added to each microfuge tube containing cells, incubated for 8-10 min at 75 °C and cooled 5 min at room temperature. One µl of DNase-I (40 U) was added to eliminate genomic DNA and incubated for 15 min at 37 °C followed by 5 min at 75 °C to inactivate DNase. Reverse transcription was performed by adding 8 µl dNTPs and 4 µl Oligo (dT) primers and heating for 3 min at 70 °C. Each microfuge tube was placed on ice for 1 min and then 4 µl of the 10× reverse transcriptase buffer, 2 µl of M-MLV reverse transcriptase enzyme, and 2 µl of RNase inhibitor were added. This mixture was incubated at 42 °C for 60 min followed by incubation at 95 °C for 10 min to inactivate the reverse transcriptase.

After generating cDNA, equal aliquots of the sample were used to evaluate each respective gene: Cx43 (accession #AF508037), Cx40 (accession #AF508036), and Cx37 (accession #AF508035). For housekeeping genes, an aliquot of each SMC sample was used to evaluate smooth muscle α -actin (SMAA; accession #AF508039) and an aliquot of each EC sample was used to evaluate platelet endothelial cell adhesion molecule-1 (PECAM-1; accession #AF508040). Using TaqMan chemistry (Applied Biosystems; Foster City, CA), specific primers and fluorescent probes for Real-Time PCR were custom-designed and synthesized for each gene based upon the sequence determined by PCR cloning of respective hamster genes (28) and detected by the 7900HT Real-Time PCR System (Applied Biosystems). The size of respective PCR products (base pairs, bp) for each primer and probe set were: Cx43, 64 bp; Cx40, 59 bp; Cx37, 68 bp; SMAA, 67 bp; PECAM-1, 68 bp.

For qRT-PCR, the reaction mixture (final volume, 25 µl) in each aliquot contained: 500-900 nM primers, 200-250 nM probe, 1× TaqMan Universal PCR Mastermix (Applied Biosystems #4304437), and 6-12 µl cDNA. For each cell sample, an equal amount of total cDNA was used to evaluate each gene. To generate standard curves, the reaction mixture contained 1 µl of

plasmid copies (range, 300 to 50,000) containing the cloned DNA sequence from respective genes. Plasmid copies were quantified using the Pico Green Assay (Invitrogen #P-7589). Thermocycler conditions for PCR were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Threshold cycle (Ct) values for standard curves were consistently between 24 and 34. A sample was excluded if there was no apparent expression of the respective housekeeping gene. Results were analyzed using Sequence Detection System Software (version 2.3; Applied Biosystems). The r^2 values for standard curves averaged 0.95 while slopes averaged -3.97. In accord with previous studies on intact microvessels (28,33) and to express Cx data relative to cell-type specific reference genes, respective Cx genes expressed in EC were normalized to platelet endothelial cell adhesion molecule-1 (PECAM-1) (9,13,23) while those in SMC were normalized to smooth muscle α -actin (SMAA) (20,31).

RNA Stability

Total RNA should increase in direct proportion to the number of cells sampled over time if RNA remains stable. To evaluate the stability of RNA in cell samples, from 50 to 400 SMC were collected in random order from preparations of the small intestine during sampling periods of up to 40 min (i.e., twice the duration used for obtaining cells from microvessels) and placed in microfuge tubes containing 5 μ l lysis buffer (*as described above*). Final volume was adjusted to 12 μ l using dissociation buffer. Cells were incubated for 10 min at 75 °C then 1 μ l of DNase was added to each tube after cooling room temperature for 5 min. Each microfuge tube was incubated at 37 °C for 15 min followed by 95 °C for 5 min to inactivate the DNase. Total RNA was quantified using Quant-it RNA Assay kit (Invitrogen) and the Qubit fluorometer.

Statistics

Data from qRT-PCR performed on SMC and EC samples were analyzed using Two-Way (gene $\times$ cell type) Analysis of Variance (SigmaStat version 3.5; Systat Software; Richmond, CA). When significant F-ratios were found, post-hoc comparisons were performed using Holm-Sidak tests with the overall level of significance set at 0.05. Standard curves for PCR reactions and the stability of total RNA were evaluated using linear regression.

Results

The morphological integrity of dissociated cell preparations was confirmed routinely by eye and documented using DIC imaging (Figures 1-4).

Immunolabeling

Smooth muscle cells—Smooth muscle cells isolated from CPA (Figure 1A-C) and RFA (Figure 1D-F) were identified by their elongated slender profile indicative of a relaxed state, often in a “C” shape reflecting their circumferential orientation in the vessel wall. The SMC from both vessels had robust labeling of SMAA but not for any of the Cx isoforms.

Whole-mount preparations of the *muscularis externa* exhibited punctate labeling for Cx43 at cell borders with more uniform distribution of SMAA (Figure 2A-C). Smooth muscle cells isolated from the small intestine were typically cigar-shaped with robust labeling for Cx43 along with SMAA (Figure 2D). These positive controls for Cx43 (26, 38) demonstrate our ability to detect Cx protein in freshly-dissociated SMC under conditions identical to those used for evaluating SMC from CPA and RFA. As seen for microvascular SMC, there was no immunoreactivity apparent for Cx40 or Cx37 protein in SMC from the small intestine (data not shown).

Endothelial cells—Gentle trituration produced intact endothelial ‘tubes’ after dissociating SMC from both CPA (Figure 3A-D) and RFA (Figure 3E-H). Tubes of contiguous EC are most clearly seen under lower magnification in 3A and 3E and their relationship to surrounding SMC is illustrated in Figure 4. Folds in endothelial tubes (e.g., Figure 3A) often resulted in acquiring higher-magnification images along an upper edge or ridge of these preparations. Figure 3 illustrates the consistent punctate staining for Cx37, Cx40, and Cx43 at EC borders, reminiscent of the pattern seen for intact vessels (33) (and confirmed in the present experiments; data not shown).

Partial dissociation—In 4 experiments, CPA and RFA segments were obtained in which SMC were partially dissociated from endothelial tubes. As shown for a representative RFA (Figure 4), punctate Cx43 labeling was manifest at the borders of EC while absent from SMC with immunoreactivity for SMAA only in SMC.

Quantitative Real-Time PCR

For each Cx isoform, copy numbers were normalized to SMAA for SMC and to PECAM-1 for endothelial tubes. These reference genes were evaluated in each preparation of respective cell types, enabling normalization within each experiment.

Smooth muscle cells—In both CPA and RFA, SMC expressed significantly greater levels of Cx43 transcript than for Cx40 or Cx37 (Figure 5A). For SMC of the small intestine (Figure 5B), the level of Cx43 transcript was several-fold greater ($p < 0.001$) than observed in SMC from microvessels while transcripts for Cx37 or Cx40 were marginally detected. There was no evidence of PECAM-1 transcript in SMC samples, indicating that the detection of Cx43 in SMC samples was not from contamination by EC (data not shown).

Additional control experiments were performed to test whether the copy number of SMAA was different between SMC of different origin. In ~30 ng of total DNA (which provided sufficient material for reference to standard curves), we found no significant difference in SMAA copy number (mean $\pm$ S.D.) between RFA (348 ± 28 ; $n = 3$) vs. CPA (240 ± 52 ; $n = 3$). However, both values were significantly less ($P < 0.05$) than found in small intestine (1343 ± 773 ; $n = 4$), further highlighting the relatively greater Cx43 expression in SMC of small intestine (Figure 5).

Endothelial cells—Transcripts for each Cx isoform were consistently expressed in endothelial tubes from CPA and RFA with no significant difference between Cx37, Cx40 or Cx43 for respective microvessels (Figure 6). Variable detection of SMAA transcript in endothelial tubes (data not shown) may be attributed to contamination from undetected SMC. However given the low level of Cx transcripts in SMC (above), contamination of endothelial preparations should have negligible effects on the data in Figure 6.

RNA Stability

As shown in Figure 7, total RNA increased in direct proportion to the number of SMC collected through 40 min. With randomized order in which samples of different cell numbers were obtained across SMC preparations, the strong correlation ($r^2 = 0.99$) indicates that RNA remained stable during the ~20 min periods in which cell samples were collected for determining gene expression in Figures 5 and 6.

Discussion

The conduction of hyperpolarization and depolarization from cell to cell along the wall of resistance microvessels is mediated by current flow through gap junctions (11,19,47).

However, the respective cell-type-specific expression of the Cx subunits that comprise gap junctions has not been established. To address this limitation, we evaluated Cx expression in the endothelium and smooth muscle of two microvessels in which conduction of underlying electrical signals has been well-characterized: Arterioles of the hamster cheek pouch (42,45, 46) and feed arteries of the adjacent retractor muscle (11,12). Intact CPA and RFA were individually dissected by hand, gently dissociated (22) and respective cell types were sampled to quantify copy numbers of Cx37, CX40 and Cx43 transcripts using qRT-PCR with reference to housekeeping genes for endothelium (PECAM-1) (9,13,23) and smooth muscle (SMAA) (20,31). In complementary experiments, immunolabeling was performed to resolve whether EC and/or SMC contained respective Cx proteins. The present findings are the first to demonstrate expression of transcript and protein for Cx37, Cx40, and Cx43 in isolated microvascular endothelial tubes. In contrast, although transcripts for each Cx isoform were detected in microvascular SMC, respective proteins were not present at levels that could be resolved with immunolabeling. Nevertheless, positive controls illustrate the expression of Cx43 transcript with immunolabeling for Cx43 protein in SMC isolated from the small intestine (26,38) and thereby confirm the effectiveness of our experimental procedures in resolving Cx expression for SMC that have been freshly dissociated from intact tissue samples.

The robust Cx expression in endothelial tubes (Figure 3) is consistent with an integral role for the endothelium in providing a pathway for conduction from cell to cell along the vessel through gap junctions (33,12,17,29,42,44). Within each preparation of endothelial tubes, the copy number of respective Cx isoforms was normalized to that of PECAM-1. Thus the data in Figure 6 indicate similar levels of expression for each Cx gene in EC of both CPA and RFA. Our results from immunolabeling (Figure 3) support this interpretation as Cx37, Cx40 and Cx43 protein were each consistently located at EC borders from both microvessels. Although the present data do not resolve the subunit composition of respective connexon hemichannels, our findings support immunolabeling profiles of intact CPA and RFA (33) while demonstrating that the integrity and localization of Cx proteins are maintained throughout our dissociation and sampling protocol.

In contrast to the endothelium, a role for smooth muscle in providing a cellular pathway for conduction has been controversial (4,12,16,42,44), as has the presence of effective myoendothelial coupling (12,39,42,46). It is therefore critical to establish whether (and if so, which) respective Cx isoforms are expressed in microvascular SMC. Connexin transcripts were detected across SMC yet respective proteins were not apparent with immunolabeling, even when EC were positive in the same preparation (e.g., Figure 4). Given the stability of Cx message and protein found in endothelial preparations (above), apparently low levels of Cx transcripts in SMC from both CPA and RFA (Figure 5A) led us to question whether these molecules were being degraded during SMC isolation and sampling. Therefore, based upon reports that Cx43 expression is manifest in smooth muscle of the small intestine in dog (26) and rat (38), we performed parallel experiments to evaluate Cx43 expression in the *muscularis externa* of hamster jejunum. The robust Cx43 immunolabeling (Figure 2) and transcript level (Figure 5B) found in these intestinal SMC imply that the paucity of Cx transcripts within SMC of CPA and RFA (Figure 5A) reflect their constitutive levels of expression. In control experiments for which different numbers of freshly-dissociated SMC were sampled randomly over 40 min (i.e., twice the duration for sampling cells to evaluate Cx expression) total RNA remained stable (Figure 7). Collectively, these observations indicate that Cx transcript and protein in freshly-dissociated SMC did not undergo substantive degradation. In turn, a paucity of Cx expression in SMC may reflect the discrete nature of homocellular and myoendothelial gap junctions, as found in electron micrographs of both CPA and RFA (33).

The use of different normalizing genes prevents direct comparisons between Cx expression in microvascular smooth muscle vs. endothelium. Nevertheless, SMC from small intestine had

several-fold higher levels of Cx43 transcript (Figure 5) and definitive immunolabeling for protein (Figure 2) as compared to SMC isolated from RFA or CPA (Figure 1). It is recognized that the expression of Cx transcripts can vary independently from corresponding proteins in SMC as well as EC (1,23). For example, the present data indicate that Cx37 transcript but not protein was detectable in SMC of CPA and RFA (Figures 1 and 5). Nevertheless, immunolabeling for Cx37 (but not for Cx40 or Cx43) was observed on rare occasion for SMC of these intact microvessels (33). Of greater physiological importance, only those Cx proteins that contribute to functional gap junction channels enable cell-to-cell coupling and discrete channels may be present even when their ensembles (i.e., characteristic 'plaques') cannot be detected. For example, despite the inability to confirm gap junctions with electron microscopy, dye transfer between SMC of coronary arteries (3) and electrical continuity between cardiac myocytes *in vitro* (43) implicated the presence of effective cell-to-cell coupling. Given that the functional coupling between cells can be regulated acutely [e.g., through phosphorylation of Cx43 (10,32)], we suggest that the controversy surrounding whether microvascular SMC are effectively coupled to each other or to the endothelium may be explained by acute regulation of discrete gap junction channels (or associated proteins) in response to experimental perturbations.

The focus on Cx37, Cx40 and Cx43 in the present experiments derives not only from earlier studies of intact hamster microvessels, but reflects complementary studies of regional variation in Cx expression throughout the vasculature of other mammals (16,18,29,41,44). The similarity of Cx isoform expression observed between arterioles and feed arteries found here contrasts with the regional heterogeneity reported among successive branches of arteriolar networks and arteries supplying different vascular beds (16,18,29). Studies of mice in which the expression of individual Cx has been genetically manipulated are providing critical insight into the role that specific Cx isoforms serve in the conduction of vasomotor responses. For example, Cx40 has found to be integral to conducted vasodilation in arterioles of the cremaster muscle (6, 14) and this role cannot be replaced by Cx45 (44). Although Cx45 transcript was detected previously in RNA isolated from intact CPA and RFA, there was no evidence of immunoreactivity for the corresponding protein (33). Whether specific vascular beds have the same profile of Cx expression across species remains to be established.

In summary, through dissociating hamster microvessels into their constitutive cells and selectively sampling endothelium and smooth muscle, this study presents the first quantification of cell-type specific gene expression in the microcirculation. This methodology should be applicable to determining the expression of other genes relevant to the function of smooth muscle and endothelial cells in microvessels; e.g., particular ion channels contributing to the electrical signaling underlying vasomotor control (21). The present focus on Cx isoforms in CPA and RFA of the hamster derives from these microvessels being well-characterized with respect to the conduction of vasomotor responses through cell-to-cell coupling enabled by gap junction channels. Consistent expression of Cx message and protein in endothelial tubes of RFA and CPA supports the effectiveness of the endothelium as a cellular pathway for conduction (4,12,17,29,42,44). In contrast, the apparent paucity of Cx expression in microvascular SMC underscores the difficulty of resolving gap junction structures between adjacent SMC and highlights the discrete localization of myoendothelial gap junctions (33).

Acknowledgments

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Figure 1. Co-Immunolabeling for SMAA and Cx isoforms in SMC from CPA and RFA
Immunolabeling for SMAA (red) and Cx isoforms (green) in single dissociated SMC from CPA (rows **A-C**) and RFA (rows **D-F**). Connexin isoforms were not detected in SMC from either vessel while SMAA was present in all SMC. DIC images of each cell are shown at left. **Insets:** negative controls in the absence of primary antibodies. Scale bar = 20 μ m and applies to all panels.

Figure 2. Co-Immunolabeling for SMAA and Cx isoforms in SMC of small intestine

Immunolabeling for SMAA (red) and Cx isoforms (green) in whole-mount preparations of *muscularis externa* from the jejunum (rows A-C) and from single dissociated SMC (row D). Each row contains the same observed field. Arrowheads point to punctuate Cx43 staining between SMC in the intact tissue (A) and at the cell border of a single SMC (D). Neither Cx40 nor Cx37 were detected. DIC images for each preparation are shown at left. **Insets:** Negative controls in the absence of respective primary antibodies. Scale bar in last panel of row C = 20 μ m and applies to all panels in rows A-C. Scale bar in last panel of row D = 50 μ m and applies to all panels in row D.

Figure 3. Immunolabeling for Cx isoforms in endothelial tubes

Immunolabeling for Cx isoforms within regions of endothelial tubes isolated from CPA (panels **A-D**) and RFA (panels **E-H**). Arrowheads point to labeling around borders of individual EC. The DIC images taken at lower magnification serve to illustrate the shape of representative intact endothelial tubes from CPA (**A**) and RFA (**E**) but do not correspond to the same fields of view as immunofluorescence. Note prominence of EC nuclei. *Inset*: negative controls in the absence of primary antibodies. Scale bar in panel **D** = 20 μm and applies to panels **B** and **C**; Scale bar in panel **H** = 20 μm and applies to **F** and **G**. Scale bars in panels **A** and **E** = 50 μm . See Figure 4 for orientation of endothelial tube tube relative to SMC.

Figure 4. Co-Immunolabeling for SMAA and Cx43 following partial dissociation of RFA

(A) DIC image showing endothelial tube in which SMC have been dissociated from half of the vessel segment while the remaining SMC have been “loosened” but still surround the endothelial tube. Panels B–D are higher magnification of area enclosed by upper box in panel A. (B) DIC image showing SMC remaining in register along the vessel wall. (C) Immunolabeling for SMAA (red) is restricted to SMC. (D) Immunoreactivity for Cx43 occurs only at borders of EC along endothelial tube surrounded by SMC in panel C. Panels E–G are higher magnification of area enclosed by lower box in panel A. (E) All SMC have been dissociated from this region of the isolated vessel segment, which consists entirely of endothelial tube. (F) No labeling for SMAA is apparent in endothelial tube. (G) Cx43 immunoreactivity at EC borders recapitulates that shown in panel D. Scale bar in A = 150 μm . Scale bar in panel G = 20 μm and applies to panels B–G.

Figure 5. Quantification of Cx isoform expression in smooth muscle of CPA, RFA and small intestine

A. Transcript copy numbers for Cx isoforms in SMC from CPA and RFA normalized to SMAA. For SMC of both microvessels, levels of Cx43 were significantly greater (* $p < 0.05$) than for Cx40 or Cx37 ($n = 5-9$ independent determinations for each gene).

B. Transcript copy numbers for Cx isoforms in SMC from small intestine normalized to SMAA. The level of Cx43 transcript was significantly greater (* $p < 0.025$) than that for Cx40 or Cx37. Summary data are means $\pm$ S.E. from 6 independent determinations.

Figure 6. Quantification of Cx isoform expression in endothelium of CPA and RFA

Normalized to PECAM-1, there were no significant differences in respective levels of Cx37, Cx40, or Cx43 transcript in either CPA or RFA, nor was there a difference between respective vessels. Summary data are means $\pm$ S.E. from 4-5 independent determinations.

Figure 7. Stability of total RNA

Total RNA increased in direct proportion to the number of cells sampled. Summary data are means $\pm$ S.E. from four independent experiments.