

Spatial separation of endothelial small- and intermediate-conductance calcium-activated potassium channels (K_{Ca}) and connexins: possible relationship to vasodilator function?

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Abstract

Activation of endothelial cell small- (S) and intermediate- (I) conductance calcium-activated potassium channels (K_{Ca}) and current or molecular transfer via myoendothelial gap junctions underlies endothelium-derived hyperpolarization leading to vasodilation. The mechanism underlying the K_{Ca} component of vasodilator activity and the characteristics of gap junctions are targets for the selective control of vascular function. In the rat mesenteric artery, where myoendothelial gap junctions and connexin (Cx) 40 are critical for the transmission of the endothelial cell hyperpolarization to the smooth muscle, SK_{Ca} and IK_{Ca} provide different facets of the endothelium-derived hyperpolarization response, being critical for the hyperpolarization and repolarization phases, respectively. The present study addressed the question of whether this functional separation of responses may be related to the spatial localization of the associated channels? The distribution of endothelial SK_{Ca} and IK_{Ca} and Cx subtype(s) were examined in the rat mesenteric artery using conventional confocal and high-resolution ultrastructural immunohistochemistry. At the internal elastic lamina–smooth muscle cell interface at internal elastic lamina holes (as potential myoendothelial gap junction sites), strong punctate IK_{Ca} , Cx37 and Cx40 expression was present. SK_{Ca} , Cx37, Cx40 and Cx43 were localized to adjacent endothelial cell gap junctions. High-resolution immunohistochemistry demonstrated IK_{Ca} and Cx37-conjugated gold to myoendothelial gap junction-associated endothelial cell projections. Clear co-localization of K_{Ca} and Cxs suggests a causal relationship between their activity and the previously described differential functional activation of SK_{Ca} and IK_{Ca} . Such precise localizations may represent a selective target for control of vasodilator function and vascular tone.

Key words cell coupling; endothelium; gap junction; potassium channels; smooth muscle.

Introduction

Endothelium-derived hyperpolarization, independent of nitric oxide and prostaglandin activity, results in smooth muscle cell hyperpolarization and subsequent vessel dilation. However, the nature and specific mechanism

of endothelium-derived hyperpolarization activity is heterogeneous, in that the role of myoendothelial gap junctions, eicosanoids and K^+ (and perhaps C-type natriuretic peptide) contribute to endothelium-derived hyperpolarization to a variable degree within and between vascular beds, during development, ageing and disease, as well as between species, strains and sex (McGuire et al. 2001; Campbell & Gauthier, 2002; Sandow, 2004). The transfer of diffusible factors and/or contact-mediated electrical coupling accounts for endothelium-derived hyperpolarization activity, with K^+ and the direct myoendothelial gap junction-mediated transfer of current or small molecules fulfilling these roles in many vascular beds (Griffith, 2004; Sandow, 2004).

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Although the specific signalling mechanisms involved in endothelium-derived hyperpolarization activity are still under debate, it is accepted that agonist-induced release of endothelial cell intracellular calcium is critical for the response, and that this activates endothelial cell calcium-activated potassium channels (K_{Ca}) of small (S) and intermediate (I) conductance. The subsequent outflow of potassium may act directly on the adjacent smooth muscle as an endothelium-derived hyperpolarizing factor (EDHF), and/or generate a hyperpolarizing current that is transferred from the endothelium to the smooth muscle via myoendothelial gap junctions (McGuire et al. 2001; Crane et al. 2003; Sandow, 2004).

In the rat mesenteric bed, agonist-induced endothelium-derived hyperpolarization is separated into two differentially activated components: an SK_{Ca} apamin-sensitive hyperpolarization phase and an IK_{Ca} 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34)-sensitive repolarization phase (Crane et al. 2003). Integral to this functional relationship is the hypothesis that it may be related to the differential spatial arrangement of SK_{Ca} and IK_{Ca} (Crane et al. 2003) and the connexins (Cxs) responsible for endothelium-derived hyperpolarization activity (Sandow et al. 2002; Mather et al. 2005). Thus, we determined here the spatial arrangement of endothelial cell K_{Ca} and myoendothelial gap junction Cxs integral to endothelium-derived hyperpolarization vasodilator activity.

Methods

Experiments were carried out in accordance with University of New South Wales Animal Care & Ethics

Committee and UK Home Office animal legislation [UK Scientific Procedures (Animals) Act 1986] guidelines.

Tissue preparation

Segments of mesenteric artery, as detailed below, were taken from animals where the characteristics and density of myoendothelial gap junctions had previously been determined (Sandow & Hill, 2000; Sandow et al. 2002).

Conventional immunohistochemistry

Male Wistar rats, 10–12 weeks old, were anaesthetized [44/8 mg kg⁻¹ ketamine/xylazine or 3 g kg⁻¹ urethane, i.p.] and perfusion fixed (2% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS)) according to conventional procedures (Sandow et al. 2002). First-order branches of the superior mesenteric artery from the fourth to sixth arcade of the mesentery after the stomach (Sandow et al. 2002) were removed into PBS. Tubular vessel segments were cut along the lateral plane and pinned out as a flat sheet with the intima uppermost. Care was taken to avoid damage to the endothelium. Whole mount tissues were subsequently incubated in blocking buffer, as PBS containing 1% normal donkey serum (NDS)/bovine serum albumin (BSA), 0.2% Tween 20, for 1 h at room temperature (RT), rinsed in PBS (3 × 5 min) and incubated in primary antibody in blocking buffer (Table 1) for 18 h at RT or for 36 h at 4 °C. Tissue was subsequently rinsed in PBS (3 × 5 min) and incubated in secondary antibody diluted in 0.01% Tween 20, for 2 h. For double labelling,

Table 1 Antibody characteristics

Antibody (Ab)	Raised against/antigen (Ag; species/epitope site)	[Ab]	Ag peptide block	Supplier	Host
K _{Ca} 2.3 (SK3)	Human N' aa 2–21	1 : 100	yes	Alomone (APC-025)	rabbit
K _{Ca} 2.3 (SK3)	Human N' aa 2–17 (M74)	1 : 300	yes	GlaxoSmithKline R&D, UK (Chen)	rabbit
K _{Ca} 2.3 (SK3)	Human C' aa 559–574	1 : 100	no	Sigma (P4747)	rabbit
K _{Ca} 2.3 (SK3)	Human N' aa 2–21	1 : 100	yes	University of Queensland (Sah)	sheep
K _{Ca} 3.1 (IK1, SK4)	rat C' aa 350–363	1 : 100	yes	Alomone (APC-064)/Sigma (P4997)	rabbit
K _{Ca} 3.1 (IK1, SK4)	Human N' aa 2–17 (M20)	1 : 400	yes	GlaxoSmithKline R&D, UK (Chen)	rabbit
K _{Ca} 3.1 (IK1, SK4)	rat N' aa 2–17	1 : 1500	no	University of Melbourne (Neylon)	rabbit
Connexin 37	rat C' aa 318–333	1 : 100	yes	Alpha Diagnostics (Cx37B12-A)	rabbit
Connexin 37	rat C' aa 266–281	1 : 100	no	Australian National University (Hill)	sheep
Connexin 40	rat C' aa 254–270	1 : 100	no	Australian National University (Hill)	sheep
Connexin 43	rat C' aa 365–381	1 : 100	yes	Zymed (71-0700)	rabbit
Connexin 43	Mouse C' 19-aa peptide	1 : 100	yes	Chemicon (AB1727)	rabbit
Smooth muscle α-actin	Mouse N' IgG2a	1 : 200	yes	Sigma (A5288)	mouse
von Willebrand Factor	Human purified VWF	1 : 300	no	Sigma (F3520)	rabbit

after rinsing (3 × 5 min), tissues were again incubated in blocking buffer (as above), incubated in the second paired primary antibody (Table 1; rabbit and sheep paired hosts) diluted in blocking buffer, rinsed (3 × 5 min) and incubated in the matching secondary antibody [rabbit and goat (which is analogous to the sheep epitope), respectively]. The secondary antibodies used were Alexa Fluor® 633 goat anti-rabbit IgG (Molecular Probes/Invitrogen, Mt Waverly, Australia; A21070), Alexa Fluor® 633 donkey anti-goat IgG (A21082), Alexa Fluor® 546 goat anti-rabbit IgG (A11010) and Alexa Fluor® 546 donkey anti-goat IgG (A11056). Tissue was subsequently rinsed in PBS (3 × 5 min) and mounted with the intima uppermost in buffered glycerol and examined with a confocal microscope (Leica TCS SP/Olympus FV500-SU/Olympus FV300) with filter sets appropriate to the secondary antibody characteristics. The primary antibodies and their working dilutions are detailed in Table 1. For double labelling, pairs of antibodies were chosen that were raised in rabbit and sheep.

Tissue was examined and optically sectioned from the intimal surface through to the innermost layer of smooth muscle cells. Additionally, endothelial cell and smooth muscle cell layers were differentiated by their respective orientations in the vessel wall, with the long axis of endothelial cells being parallel to the longitudinal vessel axis and smooth muscle cells being at ~90° to this axis; the patency of the endothelium was further confirmed by von Willebrand factor staining. The internal elastic lamina was demonstrated via aldehyde-induced autofluorescence at ~488 nm. Data for each vessel were verified from 3–10 different animals. Note that further quantification of the confocal immunohistochemical data is not valid, as it is not possible to obtain consistently the very flat individual specimens required for comparable focus on the very small focal plane at the internal elastic lamina–smooth muscle cell interface. Furthermore, owing to the technical difficulty of following through serial sections for quantitative immunoelectron microscopy, it is not feasible to quantify such data.

Controls for antibody specificity were carried out using standard procedures and involved omission of the primary antibody and pre-incubation with a ten-fold excess of peptide, where available (Table 1). Additional characterization of IK1 (SK4) and SK3 antibody specificity was previously undertaken using Western blots and immunolabeling of transfected HEK and CHO cells (Furness et al. 2003; Chen et al. 2004). Additional characterization of Cx antibody specificity

was previously undertaken using Western blots and immunolabelling of transfected HeLa, COS and Rin cells (Yeh et al. 1998, 2000; Rummery et al. 2002, 2005; Sandow et al. 2003b).

High-resolution immunohistochemistry

Segments of mesenteric arteries from anaesthetized rats (as above) were frozen at high pressure (BAL TEC, HPM 010 or Leica EM HPF; ~2100 bar), freeze-substituted (Leica, AFS; -90 °C) in 0.1% uranyl acetate in acetone for 4 days, infiltrated and embedded in LR gold (ProSciTech, C028) at -25 °C, polymerized under UV light. Serial thin transverse sections, mounted on formvar (0.5% in chloroform) and carbon-coated (5–10 nm) slot grids, were incubated in blocking buffer (as above) for 30 min, followed by incubation in antibodies (Table 1) raised to IK1 (Neylon), IK1 (Alomone) and Cx37 (Hill) in blocking buffer (18 h at 37 °C or 4 °C), followed by 5- or 10-nm colloidal gold-conjugated secondary antibodies (1 : 40; British Biocell; EM.GAR5, EM.RAG10) in 0.01% Tween 20 for 2 h. Sections were subsequently incubated in 1% glutaraldehyde in PBS, rinsed and stained conventionally with saturated uranyl acetate and lead citrate, with the specificity of antibody staining confirmed as above. Sections were photographed in a transmission electron microscope at ×10 000 – ×60 000 magnification.

Results

Distribution of endothelial cell K_{Ca}, internal elastic lamina holes, myoendothelial gap junctions and connexins

To determine the distribution of IK1 (SK4), reflecting IK_{Ca} distribution, three IK1 antibodies produced from different sources, two targeting different epitopes, were used. IK1 labelling was the same using each of the antibodies, although they differed in their level of background staining. An overlay of autofluorescence of the internal elastic lamina with IK1 staining demonstrated a positive correlation between strong punctate IK1 expression and internal elastic lamina holes (Figs 1B,C, 2A,C,F, 3A,C,F and 4B–D). High-resolution immunohistochemistry using two IK1 antibodies targeting different epitopes (at the C' and N' terminus; Alomone and Neylon antibodies, respectively) conjugated to colloidal gold confirmed the presence of IK1 at endothelial cell projections abutting smooth muscle cells,

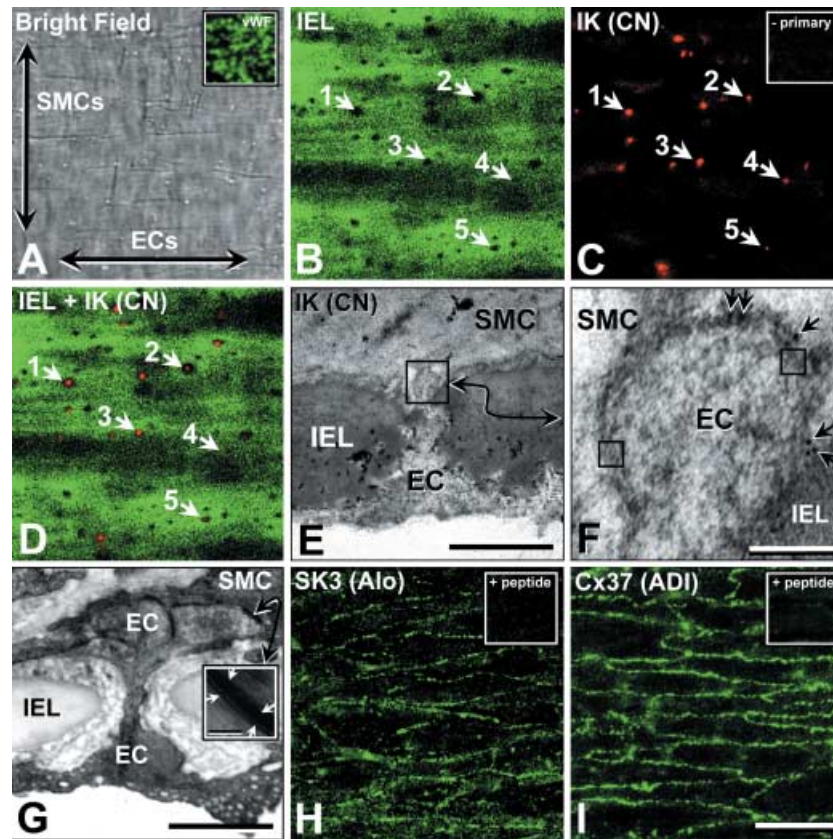


Fig. 1 Endothelial cell (EC) single label immunohistochemical K_{Ca} expression in rat mesenteric artery. Confocal images from the same region of a vessel (A–D), with the longitudinal vessel and EC axis running left to right and smooth muscle cells (SMCs) arranged at -90° to this axis (A), with von Willebrand factor (A, inset) demonstrating endothelial patency. Internal elastic lamina (IEL; B) autofluorescence demonstrates IEL holes (examples, arrowed, 1–5) as potential sites for myoendothelial gap junctions to pass through. Punctate IK1 (SK4; CN) staining, representative of IK_{Ca} expression (C; examples, arrowed, 1–5), occurs strongly at discrete points, and over the EC surface in a diffuse and non-uniform manner. An overlay of IK1 and IEL autofluorescence (D) demonstrates co-localization of punctate IK1 sites and IEL holes (examples, B–D arrowed, 1–5). Arrows (B–D, 1–5) correspond to the same region in each panel. Ultrastructural localization of IK1 (E,F; SK4; CN) antibody conjugated to 5-nm colloidal gold (F, examples at arrows) accumulated at EC projections, characteristic of pentalamellar myoendothelial gap junction sites (F, examples in boxes) in the mesenteric artery (G; modified from Mather et al. 2005, with permission, Lippincott, Williams & Wilkins; see also Figs 3J and 4E,F). SK3 (Alomone) staining, representative of SK_{Ca} expression (H), occurs strongly at EC–EC borders, as highlighted with Cx37 staining (I; ADI), indicative of EC–EC gap junction sites (Goto et al. 2004). Pre-incubation in the corresponding antigenic peptide (H,I inset, for SK3 and Cx37, respectively), and omission of the primary IK antibody (C, inset) reduced staining to background levels, with no punctate IK staining being present (C, inset). Scale bars: A–D,H,I, 20 μ m; E,G, 1 μ m; F, 100 nm; G, inset, 25 nm. Scale bars for insets A,C,H and I are the same as the associated respective main panels.

characteristic of myoendothelial gap junction sites (Figs 1E–G and 4E,F, respectively). The distribution of SK3, reflecting SK_{Ca} distribution, was determined using four SK3 antibodies produced from different sources, three targeting different epitopes. SK3 staining was the same using each of the antibodies, although they differed in their level of background staining. SK3 staining was localized at apparently adjacent endothelial cell gap junction sites, as indicated by Cx37 staining (cf. Fig. 1H and Fig. 1I), and was not observed at myoendothelial gap junction sites.

Double label immunohistochemistry of IK1 and Cx40 demonstrated a positive correlation of internal elastic lamina holes, IK1 and Cx40 expression at punctate sites (Fig. 2A–F). Confocal and high-resolution immunohistochemistry were recently used to confirm Cx40 expression at myoendothelial gap junctions in rat mesenteric artery (Mather et al. 2005). Double label immunohistochemistry of IK1 and Cx37 demonstrated a positive correlation of internal elastic lamina holes, IK1 and Cx37 expression at punctate sites (Fig. 3A–F), suggesting their presence at myoendothelial gap junctions.

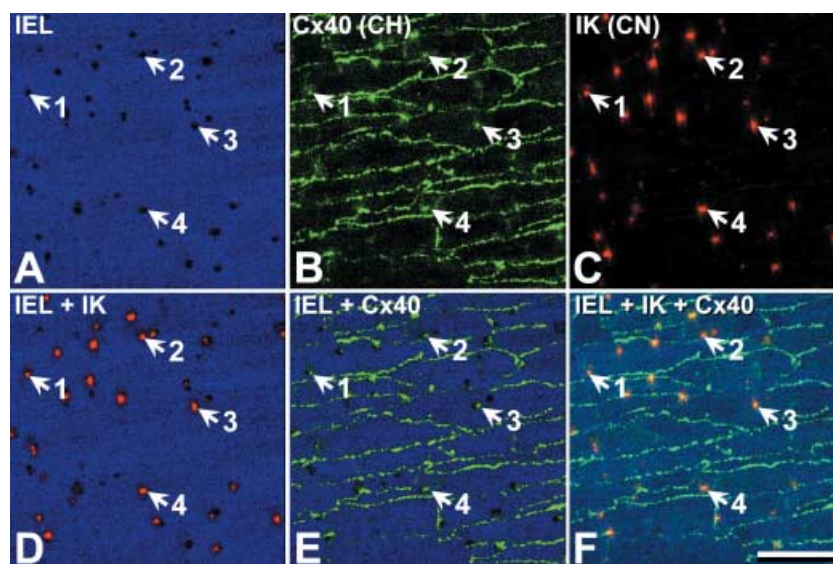


Fig. 2 Endothelial cell (EC) double label immunohistochemical IK_{Ca} and Cx40 expression in rat mesenteric artery. Confocal images from the same region of a vessel (A–F), with internal elastic lamina (IEL; A) autofluorescence demonstrating IEL holes (examples, arrowed, 1–4) as potential sites for myoendothelial gap junctions to pass through. Cx40 (CH) expression (B) occurs at adjacent EC–EC borders, characteristic of gap junction sites (Goto et al. 2004). Punctate IK1 (SK4; CN) staining, representative of IK_{Ca} expression (C), is strong at discrete points (arrowed, 1–4), and over the EC surface in a diffuse and non-uniform manner. An overlay of IK1 and IEL autofluorescence (D) demonstrates co-localization of punctate IK1 sites and IEL holes (arrowed, 1–4). An overlay of Cx40 near the IEL–SMC interface (previously shown at the confocal level to be punctate at the IEL–SMC interface and at the ultrastructural level to be at myoendothelial gap junctions; Mather et al. 2005), and IEL autofluorescence (E) suggests co-localization of Cx40 (CH) at IEL holes with myoendothelial gap junction sites (arrowed, 1–4; Mather et al. 2005). An overlay of IEL autofluorescence, IK1 and Cx40 (F) shows co-localization of IEL holes, punctate IK1 and Cx40 at discrete sites (arrowed, 1–4). Arrows (A–F, 1–4) correspond to the same region in each panel. Longitudinal vessel axis runs left to right. Scale bars: 20 μ m.

Confocal and high-resolution immunohistochemistry confirmed the presence of Cx37 at myoendothelial gap junctions (Fig. 3G–J). At the confocal and high-resolution immunohistochemical level, low-level Cx43 staining was found at adjacent endothelial cell gap junctions (see Hong & Hill, 1998; Gustafsson et al. 2003; Goto et al. 2004; Kansui et al. 2004), but not at myoendothelial gap junction sites (see Supplementary material online).

No immunolabelling was found when the primary antibodies were omitted or the staining blocked by pre-incubation in the respective antigenic peptide. Further confirmation of antibody specificity was demonstrated in previous studies using Western blots and immunolabelling of transfected cells (Yeh et al. 1998, 2000; Rummery et al. 2002, 2005; Furness et al. 2003; Sandow et al. 2003b; Chen et al. 2004).

Immunoelectron microscopy and myoendothelial gap junctions

The ultrastructural integrity of the vessel wall was well maintained in the tissue prepared for high-resolution

antigen studies, with actin staining localized to myosin filaments within smooth muscle cells (Fig. 5D–F), and pentalaminar membrane, characteristic of gap junctions, found between endothelial cells and smooth muscle cells (Figs 1F and 4F). No subendothelial or sub-smooth muscle space adjacent to the internal elastic lamina was present in tissue prepared for immunoelectron microscopy, in contrast to that in conventionally prepared tissue (Figs 1E, 3J and 4E, cf. Fig. 1G). Controls in which sections were exposed to primary antibody which had been pre-incubated in antigenic peptide, or to secondary antibody alone, showed no gold label (Fig. 5A–C).

Discussion

The present study shows that a high level of localized IK_{Ca}, Cx37 and Cx40 expression is present at myoendothelial gap junctions, and that SK_{Ca} are localized to adjacent endothelial cell gap junctions in the rat mesenteric artery, which is a model resistance bed for studies of vasodilator function and specifically endothelium-derived hyperpolarization. The spatial

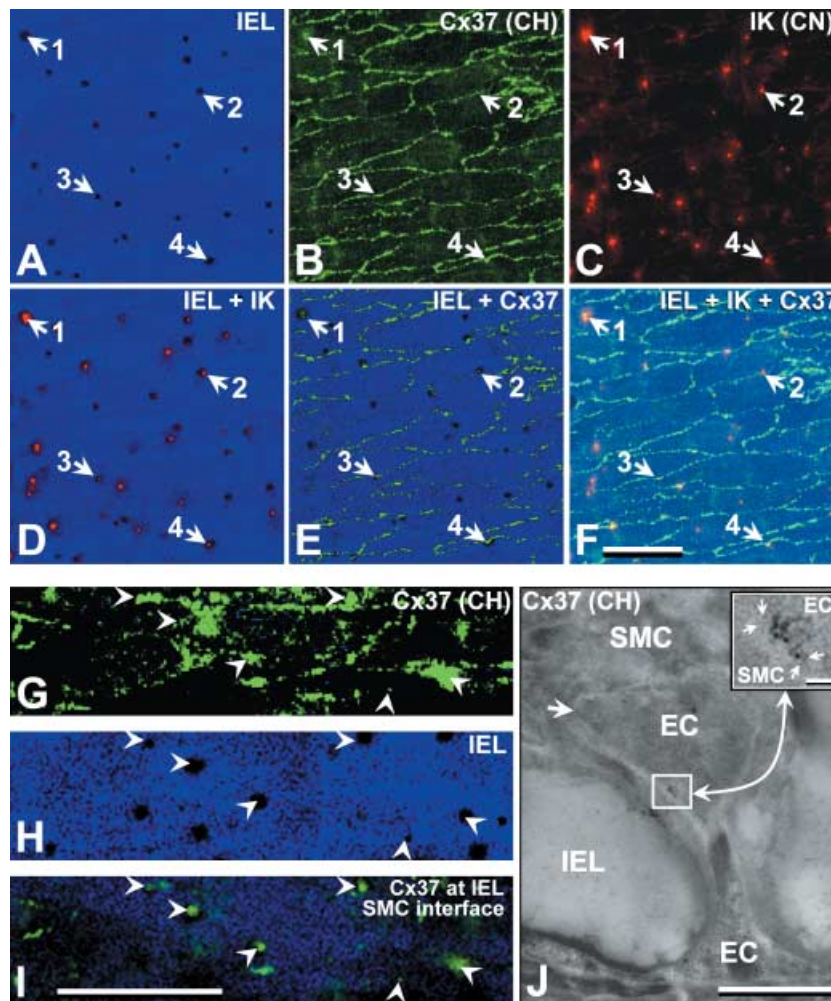


Fig. 3 Endothelial cell (EC) double label immunohistochemical IK_{Ca} and Cx37 expression (A–F) and EC single label immunohistochemical Cx37 expression (G–J) in rat mesenteric artery. Confocal images from the same region of a vessel (A–F), with internal elastic lamina (IEL) autofluorescence (A) demonstrating IEL holes (examples, arrowed, 1–4) as potential sites for myoendothelial gap junctions to pass through. Cx37 (CH) expression (B) occurs at adjacent EC–EC borders, characteristic of gap junction sites (Goto et al. 2004). Punctate IK1 (SK4; CN) staining, representative of IK_{Ca} expression (C), occurs strongly at discrete points (examples arrowed, 1–4), and over the EC surface in a diffuse and non-uniform manner. An overlay of IK1 and IEL autofluorescence (D) demonstrates co-localization of punctate IK1 sites and IEL holes (arrowed, 1–4). An overlay of Cx37 near the IEL–smooth muscle cell (SMC) interface and IEL autofluorescence (E) demonstrates potential co-localization of Cx37 (CH) and IEL holes (arrowed, 1–4). An overlay of IEL autofluorescence, IK1 and Cx37 (F) shows co-localization of IEL holes, punctate IK1 and Cx37 at discrete sites (arrowed, 1–4). Arrows (A–F, 1–4) correspond to the same regions in each panel. At high magnification, on small flat regions of the vessel, Cx37 (CH) is localized to adjacent EC–EC borders (G), characteristic of EC–EC gap junctions (Goto et al. 2004). Internal elastic lamina (IEL) autofluorescence at the same region shows IEL holes (H, arrowheads) as potential sites for myoendothelial gap junctions to pass through. An overlay of a single confocal section (at IEL–SMC interface) of the IEL at the same site reveals bright spots of fluorescence, indicating potential myoendothelial gap junction Cx37 labelling (I, arrows), where adjacent EC–EC border staining is limited (cf. G,I). Arrows (G–I) correspond to the same regions in each panel and highlight the potential close spatial relationship of myoendothelial gap junctions and EC–EC gap junctions. Localization of Cx37 (CH) antibody conjugated to 10-nm colloidal gold occurs at small regions on EC projections passing through the IEL and abutting the adjacent SMC (J, inset and arrow), with such sites being characteristic of myoendothelial gap junctions (see also Figs 1E–G and 4E,F). Longitudinal vessel axis runs left to right (A–I). Scale bars: A–I, 20 μm; J, 1 μm, inset, 50 nm.

separation and relationship of IK_{Ca}, SK_{Ca} and Cxs suggest a possible relationship to the TRAM-34-sensitive IK_{Ca}-dependent repolarization and the apamin-sensitive SK_{Ca}-dependent hyperpolarization phases of endothelium-

derived hyperpolarization (Crane et al. 2003), respectively. Indeed, the anatomical observations of the present study support the hypothesis advanced by Crane et al. (2003) that the two differentially activated components

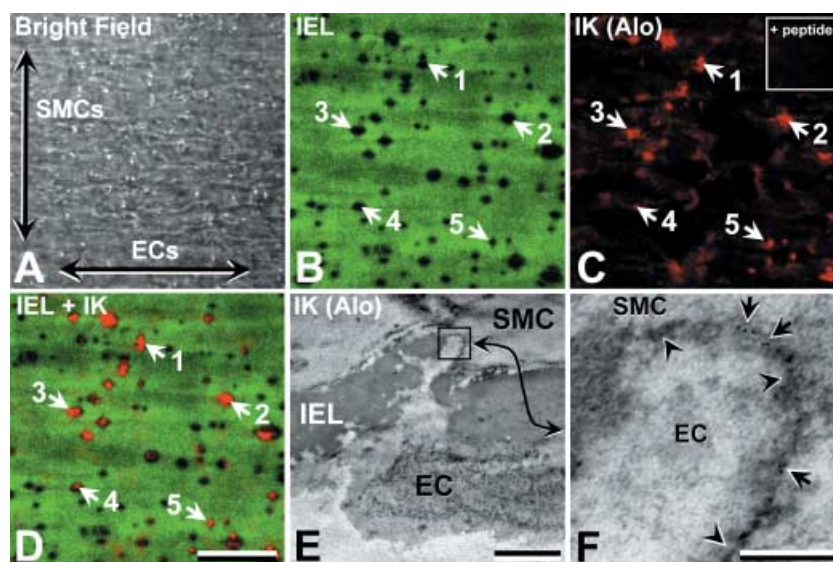


Fig. 4 Endothelial cell (EC) single label immunohistochemical IK_{Ca} expression in rat mesenteric artery. Confocal images from the same region of a vessel (A–D), with the longitudinal vessel and EC axis running left to right with the smooth muscle cells (SMC) being arranged at $\sim 90^\circ$ to this axis (A). Internal elastic lamina (IEL) autofluorescence (B) demonstrates holes in the IEL (examples, arrowed, 1–5) as potential sites for myoendothelial gap junctions to pass through. Punctate IK1 (SK4; Alomone) expression (C) occurs strongly at discrete points (examples arrowed, 1–5), and over the EC surface in a diffuse and non-uniform manner. This staining was blocked by pre-incubation in the corresponding antigenic peptide (C, inset). An overlay of IK1 and IEL autofluorescence (D) demonstrates co-localization of IK1 (SK4) and IEL holes (arrowed, 1–5), as potential myoendothelial gap junction sites (Sandow et al. 2002; Mather et al. 2005). Arrows (B–D, 1–5) correspond to the same region in each panel. Ultrastructural localization of IK1 (E,F; SK4; Alomone) antibody conjugated to 5-nm colloidal gold (F, arrows) at the EC projection, characteristic of pentalaminar myoendothelial gap junction sites (F, arrowheads) in the mesenteric artery (see also Figs 1E–G and 3J and Sandow et al. 2002; Mather et al. 2005). Scale bars: A–D, 20 μm ; E, 1 μm ; F, 100 nm. Scale bar for inset C is the same as the associated respective main panel.

of the endothelium-derived hyperpolarization response are related to the spatial separation of SK_{Ca} and IK_{Ca}.

The myoendothelial gap junctions that are typically found on endothelial cell projections in the rat mesenteric artery have previously been shown to be critical for endothelium-derived hyperpolarization activity (Sandow et al. 2002; Mather et al. 2005). Combined, the anatomical observations of the present study and the earlier functional data (Crane et al. 2003) suggest an elegant mechanism for the selective activation of multiple interactive, yet independent, second-messenger pathways, in a similar manner to that described in smooth muscle and neuronal systems (Delmas & Brown, 2002; Poburko et al. 2004; Raymond & Redman, 2006).

Heterogeneity in endothelial cell channel and receptor expression, and the associated post-receptor coupling and signal transduction mechanisms, has been suggested to reflect heterogeneity in the mechanisms controlling vessel tone (Hill et al. 2001; Aird, 2003). There is accumulating evidence for the cellular compartmentalization of ion channels and receptors (Shui & Jung,

2003; Poburko et al. 2004), or the microdomain-specific activation of channels and the region-specific site of activation of Ca²⁺ fluxes (Delmas & Brown, 2002; Poburko et al. 2004). Unlike arterial smooth muscle, where microdomain signalling has been well characterized (Poburko et al. 2004), little is known about the spatial arrangement of such domains in endothelial cells of intact vessels and nothing of the potential association of K_{Ca} and Cxs that are critical for endothelium-derived hyperpolarization. With the earlier Crane et al. (2003) data, this study is the first to describe this phenomenon in endothelial cells of intact vessels. These data support the existence of endothelial cell K_{Ca} and Cx microdomains in intact blood vessels, and thus support the potential for the focal activation of K_{Ca} and other components of the endothelium-derived hyperpolarization pathway, resulting from the focal release of calcium and potassium. Indeed, recent studies using co-cultured mouse aortic endothelial and smooth muscle cells, suggest that IP₃R1 and TRPC3 are preferentially localized to the endothelial projection associated

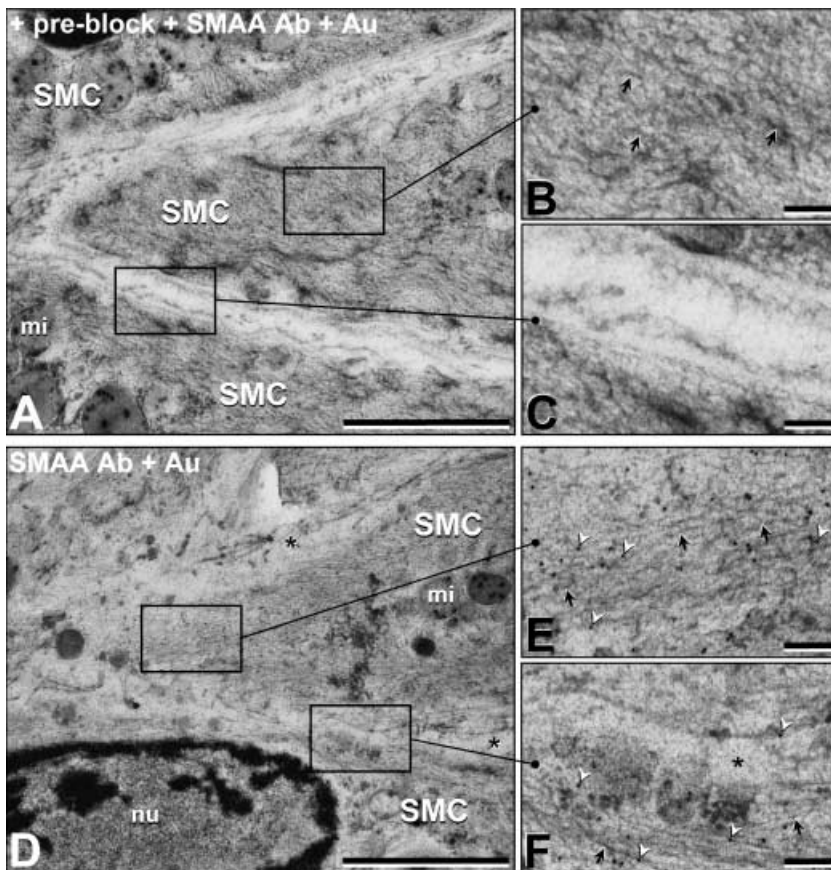


Fig. 5 Verification of high-resolution ultrastructural antigen localization methodology. In order to examine the patency of the high-pressure freezing, freeze substitution and low-temperature embedding method for optimizing antigenic and ultrastructural preservation, the distribution of a highly expressed antigen, smooth muscle alpha actin (SMAA), was examined in the media of the mesenteric artery. Smooth muscle cells (SMCs) were well preserved with myosin present as densely packed dark filaments aligned roughly parallel and slightly diagonal to the long axis of the SMCs (A,B and D,E,F, arrows) and associated with gold-labelled actin (E,F; arrowheads), with no labelling in areas between cells (*; D,F). Morphology of mitochondria (mi) and nuclei (nu) were also maintained (A,D). Exposure to gold-labelled secondary antibody alone or to actin antibody which had been pre-incubated with SMAA (A–C) resulted in no gold labelling. Scale bars: A,D, 1 μ m; B,C,E,F, 100 nm.

with myoendothelial gap junctions, supporting the proposition that the restriction of channels to distinct domains in endothelial cells is critical for myoendothelial signalling (Isakson & Duling, 2006).

The punctate IK_{Ca}–internal elastic lamina hole relationship described in the rat mesenteric artery is also present in the basilar artery of the rat and cremaster arteriole of the mouse where myoendothelial gap junctions are present and IK_{Ca} plays a significant role in endothelium-derived hyperpolarization (Haddock et al. 2006; Potocnik et al. 2006, respectively), although it does not appear to reflect myoendothelial gap junction presence in the middle cerebral artery of the rat (McNeish et al. 2006). In this latter vessel, where myoendothelial gap junctions are present, it is possible that a lack of an IK_{Ca}–internal elastic lamina hole relationship is not found owing to the IK_{Ca} localization being below the resolution of the confocal microscope and thus that further high-resolution immunohistochemical studies are required to clarify this. Of note in the present study, and as described in the caudal and femoral artery of the rat (Sandow et al. 2002, 2003a), is

that not all internal elastic lamina holes have myoendothelial projections passing through them, and thus not all internal elastic lamina holes are associated with myoendothelial gap junctions. Such sites may represent a low-resistance pathway for the diffusion of substances between endothelial cells and smooth muscle cells.

The high-resolution antigen localization method used in the present study resulted in tissue preservation comparable with that obtained with conventional methods. Indeed, the lack of a subendothelial/subsmooth muscle space near the internal elastic lamina in vessels prepared with this method suggests that significant shrinkage occurs in tissue prepared using routine ultrastructural methods and that a state closer to that *in vivo* may be achieved with this more complex preservation method (McDonald, 1999; Yuchi et al. 2002). Such shrinkage may result in appreciable artefacts at these sites and contribute to an underestimation of the density of myoendothelial gap junctions previously described.

Interestingly, consistent with observations in the present study on the primary mesenteric artery of the

rat, previous studies have described high levels of Cx37 and Cx40 expression at adjacent endothelial cell borders in arterioles and primary and secondary mesenteric arteries of the rat (Gustafsson et al. 2003; Goto et al. 2004; Kansui et al. 2004). By contrast, Cx43 expression at adjacent endothelial cell borders shows variability, being highly expressed in smaller diameter distal vessels and sparsely expressed in larger proximal vessels (compare data in Hong & Hill, 1998; Gustafsson et al. 2003; Goto et al. 2004; Kansui et al. 2004). Data from the present study of the rat primary mesenteric artery support this apparent proximo-distal trend in endothelial Cx43 expression, although the potential functional significance of this is unknown.

The localization of SK_{Ca} to adjacent endothelial cell borders, as typical sites of adjacent endothelial cell gap junctions (Goto et al. 2004), has previously been observed in porcine coronary artery, although no evidence to suggest a possible role for this relationship was presented (Burnham et al. 2002). Interestingly, decreased expression of SK3 is associated with an increase in vascular resistance and blood pressure (Taylor et al. 2003), perhaps suggesting that an altered association of SK_{Ca} and adjacent endothelial cell gap junctions may be a factor in vascular disease. Additionally, the close spatial association of myoendothelial gap junctions and adjacent endothelial cell gap junctions (Sandow et al. 2003a,b) may be related to the location of K_{Ca} channels and the dynamics of second messenger and calcium movement within and between vascular cells on which endothelium-derived hyperpolarization is dependent, as recently reported in a co-culture model of myoendothelial coupling (Isakson & Duling, 2006). Further studies will address these questions in the endothelium of intact vessels.

The localization of K_{Ca} and Cxs at the cellular and subcellular levels in the rat mesenteric artery suggests the potential for the dynamic regulation of these key components of endothelium-derived hyperpolarization activity to thus allow endothelial cells to respond in specific ways to diverse physiological and pathological stimuli. It further suggests that the spatial and related functional heterogeneity in the rat mesenteric artery may be important for the selective control of vasodilator function. The possibility that differential calcium storage and release pathways underlie differential activation of the SK_{Ca} and IK_{Ca} components of endothelium-derived hyperpolarization is the subject of ongoing investigations.

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Supplementary material

The following supplementary material is available for this article online at www.blackwell-synergy.com:

Figure S1. Endothelial cell (EC) single label immunohistochemical Cx43 expression in rat mesenteric artery.