β-Dystroglycan binds caveolin-1 in smooth muscle: a functional role in caveolae distribution and Ca^{2+} release

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Summary

The dystrophin–glycoprotein complex (DGC) links the extracellular matrix and actin cytoskeleton. Caveolae form membrane arrays on smooth muscle cells; we investigated the mechanism for this organization. Caveolin-1 and β-dystroglycan, the core transmembrane DGC subunit, colocalize in airway smooth muscle. Immunoprecipitation revealed the association of caveolin-1 with β-dystroglycan. Disruption of actin filaments disordered caveolae arrays, reduced association of β-dystroglycan and caveolin-1 to lipid rafts, and suppressed the sensitivity and responsiveness of methacholine-induced intracellular Ca^{2+} release. We generated novel human airway smooth muscle cell lines expressing shRNA to stably silence β-dystroglycan expression. In these myocytes, caveolae arrays were disorganized, caveolae structural proteins caveolin-1 and PTRF/cavin were displaced, the signaling proteins PLCβ1 and $G_{\alpha q}$, which are required for receptor-mediated Ca^{2+} release, were absent from caveolae, and the sensitivity and responsiveness of methacholine-induced intracellular Ca^{2+} release, was diminished. These data reveal an interaction between caveolin-1 and β-dystroglycan and demonstrate that this association, in concert with anchorage to the actin cytoskeleton, underpins the spatial organization and functional role of caveolae in receptor-mediated Ca^{2+} release, which is an essential initiator step in smooth muscle contraction.

Key words: Airway smooth muscle, Dystrophin, Actin, G-protein coupled receptors, Cytoskeleton, Contraction

Introduction

Smooth muscle in hollow organs is subject to cellular deformation and mechanical stress during contraction and changes in transmural pressure. Mechanisms have evolved to maintain integrity of the contractile apparatus and its association with the membrane and extracellular matrix, so that force can be transmitted between cells, thus preventing plasma membrane damage (Kuo and Seow, 2004; Lapidos et al., 2004). The dystrophin–glycoprotein complex (DGC) provides a strong mechanical link between the intracellular actin cytoskeleton and the extracellular matrix. The complex is composed of several transmembrane, cytoplasmic and extracellular protein subunits, with dystrophin, a large intracellular rod-like protein, serving as a tether between cytoskeletal actin and β-dystroglycan, the core transmembrane subunit of the DGC (Ervasti and Campbell, 1993; Lapidos et al., 2004). The extracellular domain of β-dystroglycan is linked to a peripheral protein subunit, α-dystroglycan, which serves as a receptor for laminin in the basal lamina (Montanaro et al., 1999).

Dystrophin exhibits a highly organized distribution in individual smooth muscle cells and co-segregates with caveolae-rich longitudinal sarcolemma arrays (North et al., 1993). Caveolae are cholesterol- and sphingolipid-enriched membrane invaginations that are abundant in mature smooth muscle cells (Cohen et al., 2004; Gosens et al., 2006a; Sharma et al., 2008). Caveolin-1 is the primary structural caveolae protein in smooth muscle, and forms a hetero-oligomeric network with caveolin-2 along the inner leaflet of the plasma membrane (Bauer and Pelkmans, 2006; Das et al., 1999). Caveolae and caveolin-1 have a key role in orchestrating activation of pathways that underpin cell proliferation, migration, and contraction (Cohen et al., 2004; Halayko and Stelmack, 2005). In contractile smooth muscle cells, caveolae are concentrated in close proximity to intracellular sarcoplasmic reticulum and mitochondria, forming nanospaces for Ca^{2+} homeostasis (Gabella, 1971; Gherghiceanu and Popescu, 2006; Kuo et al., 2003). Biochemical fractionation has revealed that caveolae are enriched in Ca^{2+}-handling and Ca^{2+}-binding proteins, and trimeric G-proteins are sequestered to these microdomains (Darby et al., 2000; de Weerd and Leeb-Lundberg, 1997; Gosens et al., 2007a; Li et al., 1995). Caveolae are functionally important, because they are needed to facilitate contraction and Ca^{2+} mobilization mediated by some G-protein-coupled receptors (GPCRs) in airway smooth muscle (Gosens et al., 2007b; Prakash et al., 2007). These observations suggest that the spatial organization of caveolae and the repertoire of proteins that localize to them are fundamental determinants of the contractile response of smooth muscle.

Mechanisms for the ordered distribution of caveolae in mature smooth muscle cells are not elucidated. In striated muscle, β-dystroglycan binds a putative WW-domain in caveolin-3 (Sotgia et al., 2000) and supports localization of T-tubules to costamers; however, there are no reports confirming that β-dystroglycan binds to endogenous caveolin-1 in smooth muscle. In smooth muscle, the expression of DGC subunits is associated with myocyte maturation, suggesting a specific role in functionally contractile myocytes (Anastasi et al., 2005; Ramirez-Sanchez et al., 2005;

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Sharma et al., 2008; Straub et al., 1999; Wheeler et al., 2002). In these cells, dystrophin is localized to caveolae-rich arrays that are associated with Ca^{2+}-handling proteins and organelles (Darby et al., 2000; Gherghiceanu and Popescu, 2006; Gosens et al., 2007a; North et al., 1993). There is evidence that the cytoplasmic tail of β-dystroglycan can serve as a scaffold for signaling proteins such as Grb2, nNOS and regulatory kinases (Cavaldesi et al., 1999; Grozdanovic and Baumgarten, 1999; Spence et al., 2004), but no direct link to excitation-contraction coupling and Ca^{2+} handling in smooth muscle has been made.

In the current study, using human and canine airway smooth muscle cells and tissue, we tested the hypothesis that spatial distribution of caveolae in contractile smooth muscle is determined by the association of caveolin-1 with β-dystroglycan and this interaction supports a functional role for caveolae in facilitating G-protein-coupled-receptor (GPCR)-mediated Ca^{2+} mobilization. Our data demonstrate, for the first time, the direct interaction between caveolin-1 and β-dystroglycan. In addition, we found that distribution of caveolae is determined by tethering to the actin cytoskeleton via caveolin-1 and the DGC, and the ordered distribution of caveolae in contractile smooth muscle cells is linked with the sequestration and function of proximal signaling proteins that mediate intracellular Ca^{2+} release.

Results

β-Dystroglycan co-fractionates and co-precipitates with caveolin-1

We reported that DGC subunits are abundant in human airway smooth muscle tissue and cells (Sharma et al., 2008). Moreover, fluorescent microscopy has shown dystrophin and caveolin-1 exhibit overlapping immunolabeling in guinea pig taenia coli smooth muscle (North et al., 1993). To determine whether DGC subunits and caveolae share cellular domains we used sucrose density gradient ultracentrifugation for subcellular fractionation of cells from human and canine airway smooth muscle tissue, and serum-deprived cultures of canine airway myocytes. DGC subunits (β-dystroglycan, β-, δ- and γ-sarcoglycan, and dystrophin) co-fractionate with high buoyant density caveolin-1-enriched microdomains (Fig. 1A,B). We next used double labeling of β-dystroglycan and caveolin-1 to assess their colocalization in individual elongate contractile smooth muscle cells in culture (Fig. 1C-E). The proteins were organized into markedly overlapping longitudinal arrays. However, in striking contrast, cells that lacked β-dystroglycan (a feature of a short spindle shaped non-contractile myocyte subpopulation) did not form discrete linear arrays of caveolin-1 (Fig. 1E). To determine whether β-dystroglycan and caveolin-1 proteins interacted directly we performed immunoprecipitation, and found they could be readily co-precipitated from human and canine smooth muscle (Fig. 1F). Collectively, these data demonstrate that caveolin-1 interacts with β-dystroglycan, and that this interaction might be crucial to orchestrate the ordered distribution of caveolae in contractile smooth muscle.

The actin cytoskeleton underpins the ordered distribution of caveolae

As the association of caveolin-1 with β-dystroglycan suggests that caveolae might be indirectly tethered to the intracellular actin cytoskeleton via dystrophin, we next assessed whether promoting loss of filamentous actin with latrunculin A affected the colocalization and distribution of caveolin-1 and β-dystroglycan. Phalloidin labeling confirmed that filamentous actin was depleted by exposure to latrunculin A (Fig. 2A,B). Moreover, this was associated with the disruption of discrete linear arrays of β-dystroglycan and caveolin-1, resulting in much less visible colocalization of these proteins (compare Fig. 1C-E with Fig. 2C-E). Notably, co-immunoprecipitation from canine smooth muscle revealed that the association of β-dystroglycan with caveolin-1 was not directly affected by latrunculin A, suggesting that the actin cytoskeleton underpins the sequestration of these proteins to specific

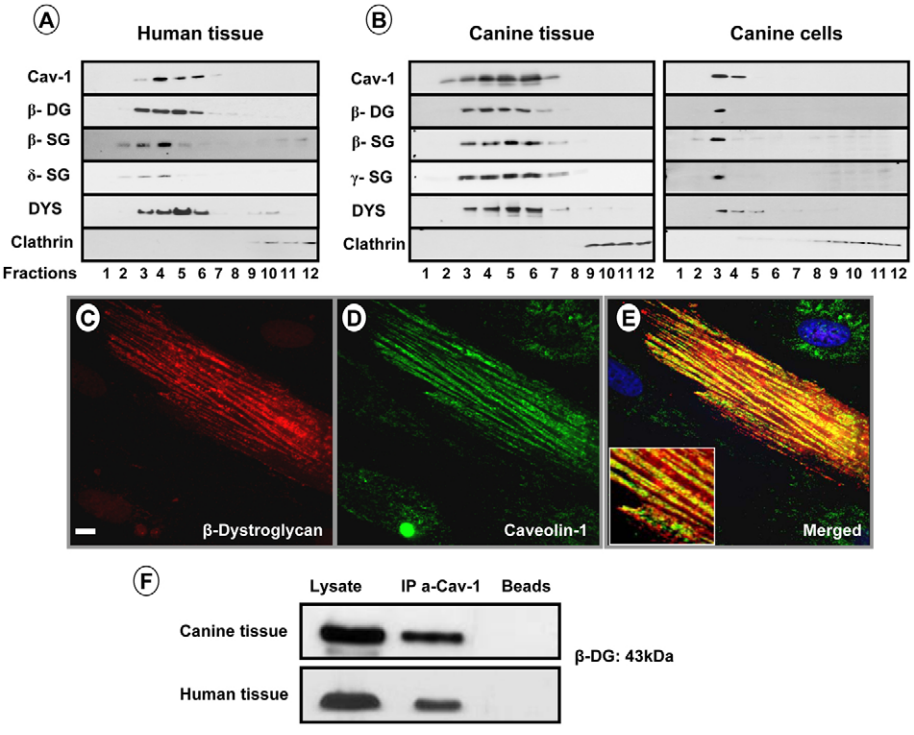

Fig. 1. Association of dystrophin–glycoprotein complex subunits with caveolin-1. Human bronchial smooth muscle tissue (**A**), canine airway smooth muscle tissue and serum-deprived canine airway smooth muscle cells (**B**) were lysed in carbonate buffer for isolation of caveolae-rich fractions using sucrose density gradient centrifugation. After centrifugation, 1 ml fractions were collected (buoyant density decreases with increasing fraction number). Equal amounts of protein from each fraction were subjected to immunoblot analysis for the indicated proteins. (**C-E**) Serum-deprived (contractile) canine airway smooth muscle cells were fixed and double labeled for β-dystroglycan (red) (C) and caveolin-1 (green) (D). The corresponding merged image (E) with a higher-magnification insert is also shown. Nuclei appear blue and are counterstained with Hoechst 33342 (10 μg/ml). Scale bar: 20 μm. (**F**) Lysates from canine and human airway smooth muscle tissue were used for immunoprecipitation (IP) with anti-caveolin-1 antibody (a-Cav-1) using protein-G-conjugated Sepharose beads. Lane labeled 'Beads' included sample but no antibody. Beads with immunoprecipitated proteins were used to perform protein blot analysis for β-dystroglycan.

membrane microdomains, but is not involved in their binding per se (Fig. 2F). To characterize the ultrastructural consequences associated with disruption of the actin cytoskeleton, we next used transmission electron microscopy. Individual myocytes in intact smooth muscle tissue exhibited characteristic arrays of membrane caveolae, whereas in latrunculin A-exposed specimens we observed greatly reduced numbers of caveolae invaginations, with the appearance of significant numbers of double membrane caveolae-like vesicles beneath the plasma membrane, suggesting that the loss of actin integrity promotes caveolae internalization (Fig. 2G-J). This was confirmed using sucrose density cell fractionation, which revealed that the abundance of both caveolin-1 and β-dystroglycan in cell fractions that typically harbor caveolae-enriched microdomains was dramatically reduced after actin disruption (Fig. 2K). Collectively, these results support a role for the actin cytoskeleton, through its tethering to a DGC–caveolin-1 complex, in the establishment and maintenance of discrete caveolae microdomains in smooth muscle cells.

Discrete organization of caveolae is required for receptor-mediated Ca^{2+} release

Through mechanisms involving the association of proximal signaling proteins with caveolin-1, caveolae modulate receptor-mediated Ca^{2+} release in the smooth muscle and other cell systems (Daniel et al., 2009; Darby et al., 2000; El-Yazbi et al., 2008; Gosens et al., 2007b; Prakash et al., 2007; Sengupta et al., 2008; Zhu et al., 2008). We assessed whether this functional role is linked to the unique ordered distribution of caveolae in contractile smooth muscle cells. Primary

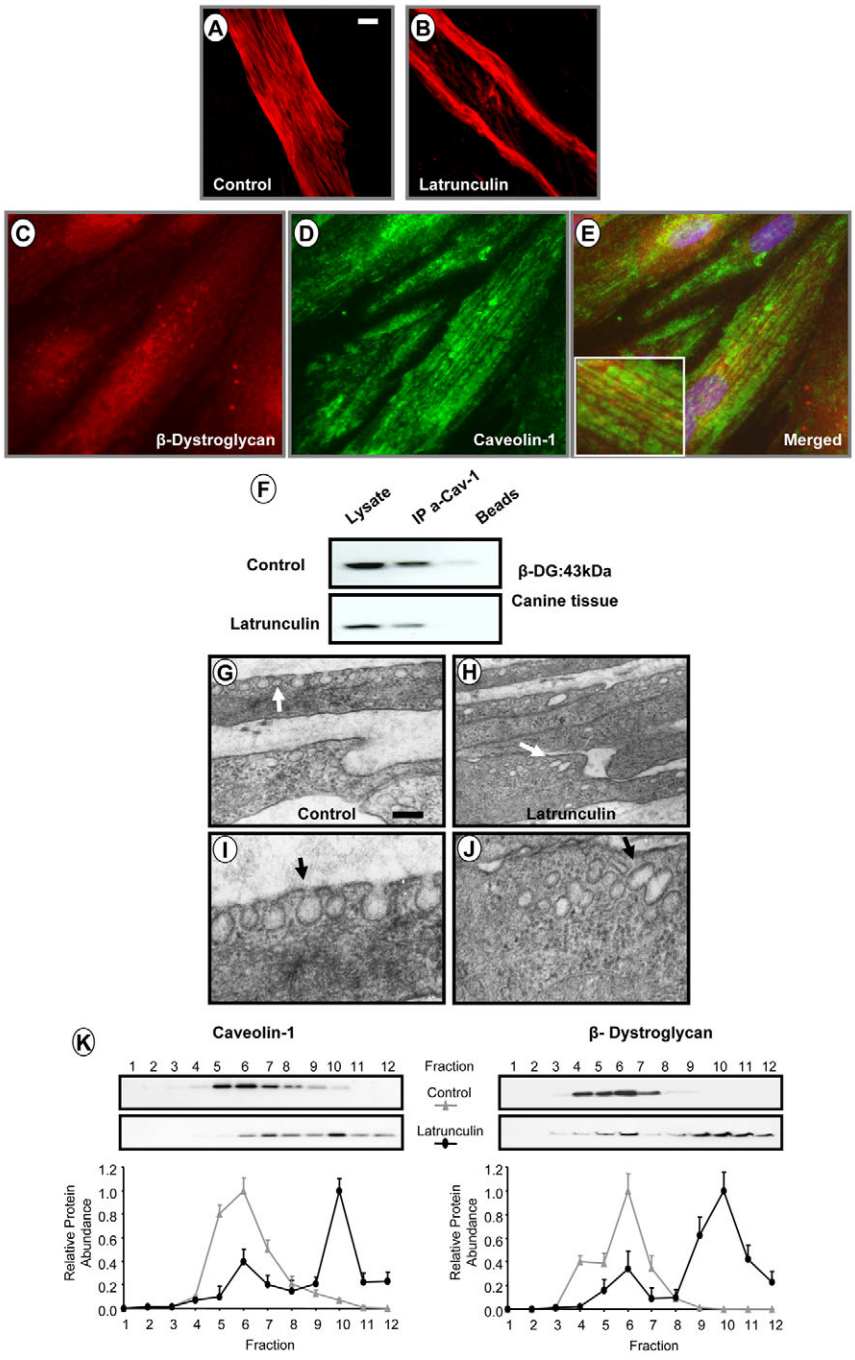

Fig. 2. Effect of actin disruption on localization and distribution of caveolin-1 with β-dystroglycan. (**A**,**B**) Serum-deprived (contractile) canine airway smooth muscle cells were either incubated in HBSS or latrunculin A (1 μM, 1 hour, 37°C), then labeled for filamentous actin using phalloidin-TxR (red). (**C-E**) Cells incubated with latrunculin A were fixed and double labeled for β-dystroglycan (red) and caveolin-1 (green). Nuclei were counterstained blue with Hoechst 33342 (10 μg/ml). The corresponding merged image with a higher-magnification insert is shown in E. Scale bar: 20 μm. (**F**) Lysates from canine airway smooth muscle tissue (Control) and those pre-treated with latrunculin A were immunoprecipitated with anti-Cav-1 antibody using protein-G-conjugated Sepharose beads. Lane labeled 'Beads' included sample but no antibody. Immunoprecipitated proteins were subjected to protein blot analysis for β-dystroglycan. (**G-J**) Canine airway smooth muscle tissue was incubated in either HBSS (G,I) or latrunculin A (1 μM, 1 hour, 37°C) (H,J), then fixed for transmission electron microscopy. Arrows in G and I indicate typical caveolae and linear arrays; whereas in panels H and J, arrows indicate internalized caveolae-like double-membrane structures that appear with latrunculin A treatment. Scale bar: 100 nm. (**K**) Control and latrunculin-A-treated canine airway smooth muscle tissue was homogenized and lysed in carbonate buffer for isolation of caveolae enriched by sucrose density gradient centrifugation. Equal amounts of protein from each fraction were subjected to protein blot analysis for caveolin-1 or β-dystroglycan. Results shown include three independent experiments performed on canine smooth muscle tissue.

cultured canine airway myocytes were loaded with the Ca^{2+}-sensitive dye, Fura-2, and we measured the effects of actin cytoskeleton disruption with latrunculin A on $G_{\alpha q}$-coupled muscarinic M3-receptor-mediated Ca^{2+} release, which is the initiating step for contraction. Airway myocytes exhibited a dose-dependent increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to the M3-receptor agonist methacholine (MCh), a profile that is reproducible in the same cells 1 hour later (Fig. 3A,B). By contrast, peak response elicited by all concentrations of MCh was abrogated or dramatically reduced after incubation with latrunculin A (Fig. 3A,B). Indeed, actin disruption significantly decreased sensitivity to MCh ($EC50_{Con}$=37±11nM; $EC50_{Lat}$=87±14nM; $P<0.05$), and actin disruption also reduced the maximum peak $[Ca^{2+}]_i$ induced with MCh (10^{-5}M) by 30% ($P<0.01$). These data demonstrate that the unique, highly ordered spatial distribution of caveolae underpinned by cytoskeletal actin has a central role in mediating GPCR-mediated mobilization of $[Ca^{2+}]_i$ in smooth muscle.

Association of β-dystroglycan with caveolin-1 is needed for modulation of $G_{\alpha q}$-coupled-receptor signaling by caveolae

As our data indicate that caveolae modulate intracellular $[Ca^{2+}]_i$ release and this capacity is linked to their actin-dependent spatial distribution on the sarcolemma, we next assessed whether direct interaction of caveolin-1 with β-dystroglycan was essential for caveolae integrity and regulation of GPCR excitation-contraction coupling. To address this, we prepared lines of primary human airway smooth muscle cells in which we stably silenced β-dystroglycan expression with lentivirus-delivered short-hairpin RNA (shRNA). shRNA interference using increasing lentiviral titers resulted in an initial 70–100% reduction of β-dystroglycan protein (Fig. 4A), and quantitative (real-time) PCR confirmed that the β-dystroglycan mRNA transcript was also reduced by 80–90% (Fig. 4B). Using puromycin selection, we generated human airway smooth muscle cell lines with stably silenced β-dystroglycan in which protein expression was abrogated even in cells subjected to prolonged serum deprivation: a condition that does promote the accumulation of β-dystroglycan in cell lines stably transduced with a non-coding β-dystroglycan refractory shRNA, and non-infected primary smooth muscle cells (Fig. 4C). To determine the impact of β-dystroglycan silencing on caveolae and caveolin-1 distribution, we performed subcellular fractionation using sucrose density gradient centrifugation. Both caveolin-1 and PTRF (polymerase I and transcript release factor, also known as cavin-1), the structural protein with unique affinity for caveolin-1-sequestered lipid rafts and required for the invagination of caveolae microdomains (Hill et al., 2008; Liu and Pilch, 2008), were displaced from high buoyant density caveolae-rich fractions in β-dystroglycan-silenced

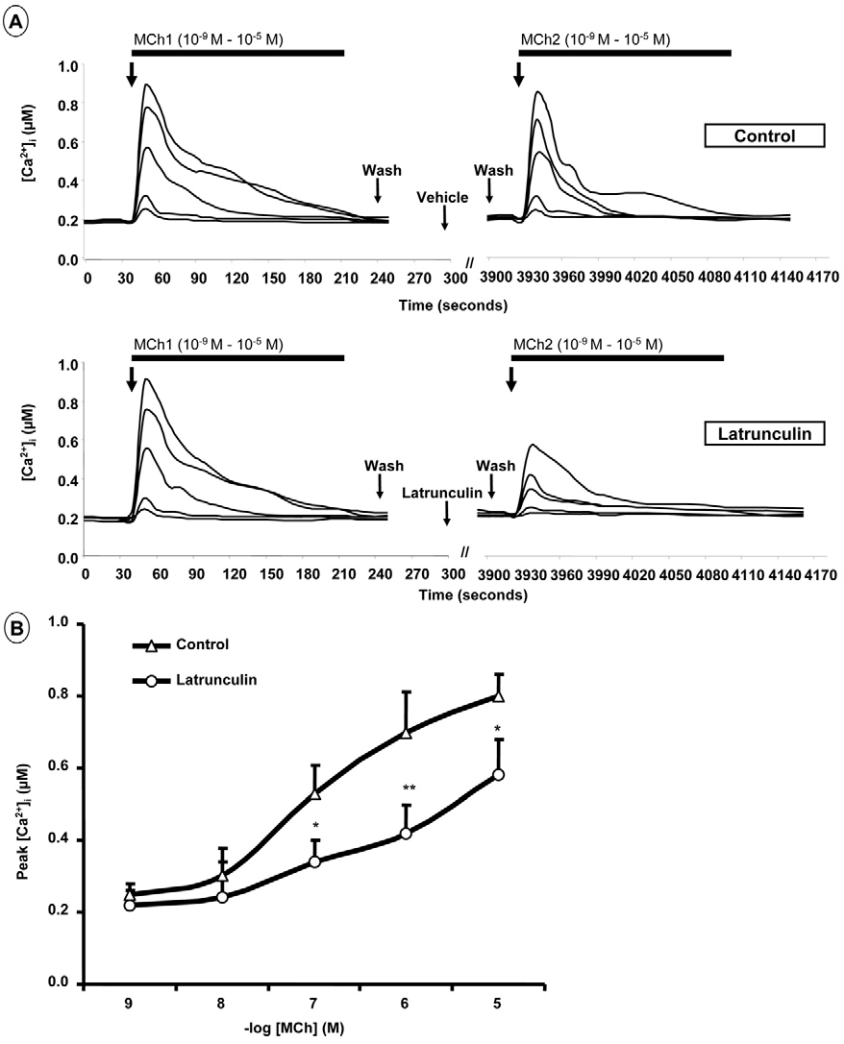

Fig. 3. Actin disruption markedly alters receptor-mediated Ca^{2+} mobilization. (**A**) Representative tracings from experiments using Fura-2-loaded canine airway smooth muscle cells grown to confluence then serum deprived in insulin-supplemented medium for 7 days. Each tracing is the mean of 8–12 elongate cells identified in a single microscopic field. Cells were first stimulated with methacholine (MCh1, left panels) (10^{-9} to 10^{-5} M) and changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) recorded. Thereafter, cells were incubated with either vehicle (top row) or latrunculin-A (1 μM, 37°C) (bottom row) for 1 hour, and were subsequently treated with methacholine (MCh2) at the same concentration used for MCh1. Changes in $[Ca^{2+}]_i$ in response to MCh2 were recorded for the same cells monitored after MCh1 treatment. (**B**) Concentration-response curves for MCh2 in control and latrunculin-A-treated cells plotted as peak $[Ca^{2+}]_i$. Curves are derived using individual data points that are the mean ± s.e.m. of at least 30 cells in total (assayed in at least three different experiments). *$P<0.05$, **$P<0.01$, for control versus latrunculin A at a given MCh concentration.

cells (Fig. 4D,E). These data indicate that β-dystroglycan is essential for the association of caveolin-1 with lipid rafts and for supporting caveolae formation and/or stability.

Muscarinic M3 receptors (M3Rs) are coupled to $G_{\alpha q}$, which mediates the activation of PLCβ1 to generate secondary messengers that trigger the release of Ca^{2+} from intracellular sarcoplasmic

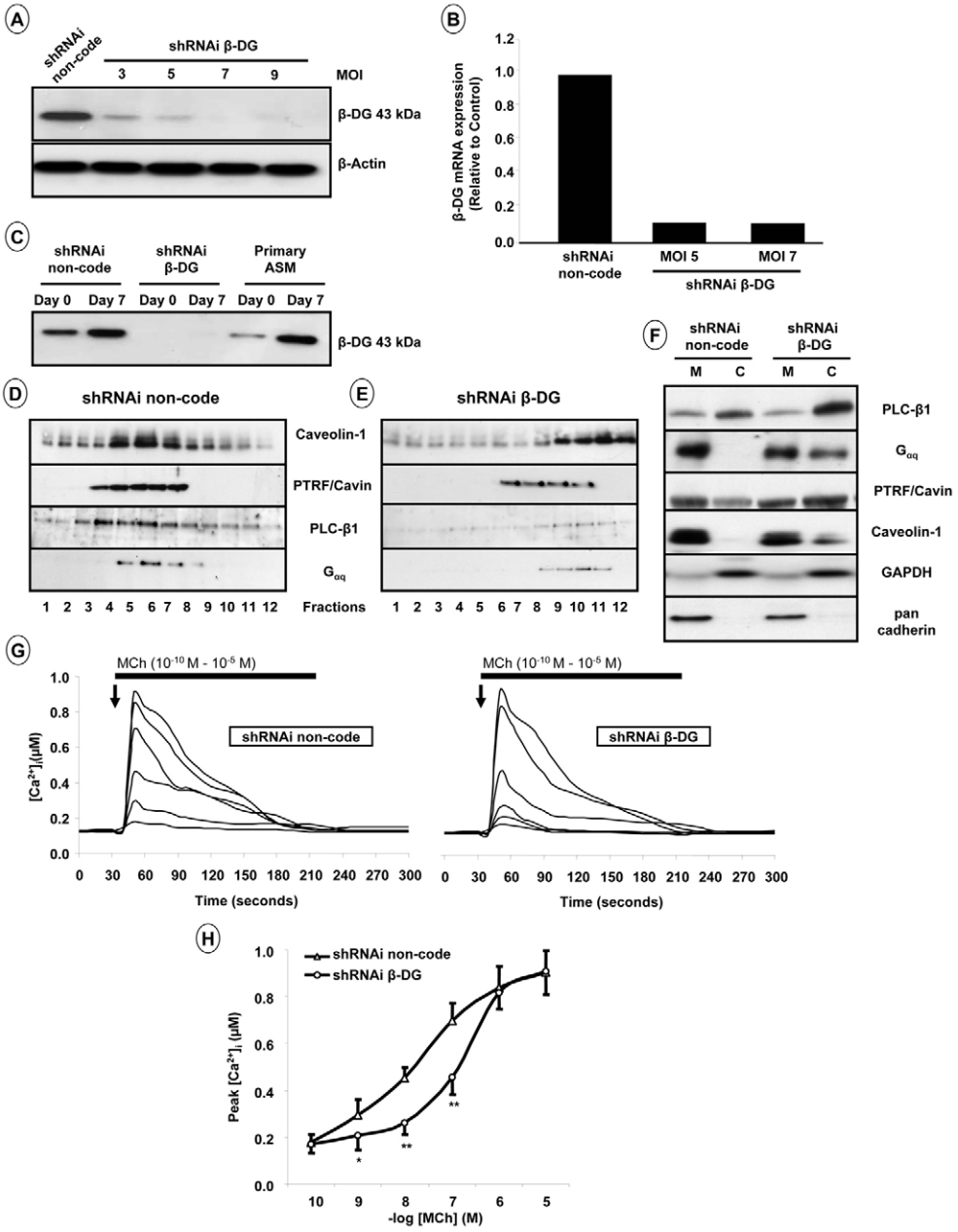

Fig. 4. shRNA interference of β-dystroglycan expression alters distribution of caveolae proteins and signaling molecules. (**A**) Representative protein immunoblot probed for β-dystroglycan (β-DG) 2 days after human airway smooth muscle (ASM) cells were transduced with lentivirus carrying β-dystroglycan-specific (shRNAi β-DG) or non-coding β-dystroglycan refractory shRNA (shRNAi non-code). The MOI of lentivirus used is indicated for each sample. (**B**) Quantitative (real-time) RT-PCR for mRNA encoding β-DG 2 days after human airway smooth muscle cells were transduced with lentivirus carrying shRNAi β-DG or a shRNAi non-code. Viral MOIs used are indicated. (**C**) Representative protein immunoblot for β-dystroglycan in puromycin-selected human ASM cell lines. After cells were transduced with lentivirus, they were subjected to 21 days of growth in the presence of puromycin, before passaging cells or experimentation. Before preparing lysates, shRNA-expressing cultures were grown to confluence (Day 0) and subjected to 7 days of serum deprivation (Day 7). Also shown are β-DG protein levels in extracts of non-infected primary human ASM cells (Primary ASM). (**D**,**E**) Human ASM cells expressing shRNAi non-code and shRNAi β-DG were grown to confluence, serum deprived for 7 days, then lysed in carbonate buffer for isolation of caveolae rich fractions by sucrose density gradient centrifugation. Buoyant density decreases with increasing fraction number. Equal amounts of protein from each fraction were subjected to protein blot analysis for the proteins indicated. (**F**) Isolated membrane (M) and cytosol (C) fractions from serum-deprived shRNAi non-code and shRNAi β-DG human ASM cell lines were assayed for the indicated proteins by immunoblotting. (**G**) Representative tracings showing changes in $[Ca^{2+}]_i$ in response to MCh (10^{-10} to 10^{-5} M) for Fura-2-loaded shRNAi non-code and shRNAi β-DG-expressing human ASM cells. (**H**) Concentration-response curve for peak $[Ca^{2+}]_i$ in response to MCh for shRNAi non-code and shRNAi β-DG expressing human ASM cell lines. Data at each concentration represent mean ± s.e.m. from 35 cells measured in at least three different experiments. **P*<0.05, ***P*<0.01.

reticulum stores (Gosens et al., 2006b). We have shown that M3Rs can be associated with caveolae in airway smooth muscle (Gosens et al., 2007a), therefore we used sucrose gradient fractionation to compare the subcellular distribution of $G_{\alpha q}$ and PLCβ1 in control and β-dystroglycan-silenced human airway myocytes. Both PLCβ1 and $G_{\alpha q}$ were enriched in high buoyant density caveolin-1-enriched fractions of control smooth muscle cells. By contrast, mimicking our observation for caveolin-1 and PTRF/cavin, the absence of β-dystroglycan resulted in both proteins being displaced to less-buoyant cell fractions (Fig. 4D,E). To further clarify the subcellular location of these signaling effectors, we compared their relative abundance in membrane and cytosolic fractions using protein blotting (Fig. 4F). In β-dystroglycan-deficient cells, the relative abundance of PLCβ1 and $G_{\alpha q}$ in the cytosol was increased; a pattern that mirrored caveolin-1 and PTRF/cavin distribution. These data indicate that $G_{\alpha q}$ and PLCβ1 colocalize with caveolin-1, but in the absence of β-dystroglycan, the stability of membrane caveolae is compromised, resulting in the loss of these proteins from the sarcolemma.

We next investigated the functional consequences of β-dystroglycan silencing on GPCR-mediated mobilization of $[Ca^{2+}]_i$ in smooth muscle cells (Fig. 4G,H). Cells expressing non-coding control shRNA exhibited typical concentration-response characteristics. However, cells lacking β-dystroglycan exhibited significant suppression in peak $[Ca^{2+}]_i$ induced with lower concentrations of MCh. Indeed, in β-dystroglycan-silenced myocytes, there was a significant reduction in the sensitivity to MCh ($EC50_{Control}$=50.3±14 nM versus $EC50_{shRNAi}$=220±24 nM; $P<0.01$). By contrast, and consistent with previous studies in which caveolin-1 expression was silenced (Gosens et al., 2007b), peak $[Ca^{2+}]_i$ induced with maximum concentrations of MCh were unaffected. Collectively, these data demonstrate that the requirement of β-dystroglycan for caveolae integrity is a crucial determinant of the spatial profile of proximal signaling effectors, which has important consequences on the initiation of contraction by physiologically relevant agonists.

Discussion

Expression of DGC subunits is coupled to airway myocyte phenotype maturation in vitro and they are abundant in smooth muscle tissues (Sharma et al., 2008). Since previous studies show that dystrophin colocalizes with caveolin-1 in specific membrane microdomains in contractile smooth muscle cells (North et al., 1993), we characterized the interaction of caveolin-1 with the DGC, which is tethered to the actin cytoskeleton, and investigated the functional significance of this interaction. Our data show that endogenous caveolin-1 binds to β-dystroglycan, an association that had only been predicted before from work using recombinant peptides (Sotgia et al., 2000). We also show that this interaction underpins a structural framework that involves filamentous actin and the DGC to support caveolae plasma membrane arrays. Importantly, our studies demonstrate that ordering of caveolae into stable cell membrane arrays is concomitant with sequestration of signaling effectors, including $G_{\alpha q}$ and PLCβ1, which transduce GPCR-mediated Ca^{2+} mobilization. Our study reveals a new level of regulation of receptor-mediated contraction of smooth muscle and is thus important for understanding the function of hollow organs in health and disease.

The plasma membrane of contractile smooth muscle cells is highly ordered, consisting of repeating longitudinal rib-like arrays of caveolae and adherens junctions (Gabella, 1984; Halayko and Stelmack, 2005; Montesano, 1979; Small, 1985; Small and Gimona, 1998). These domains are associated with disparate intracellular, membrane-associated and extracellular proteins; for example, adherens junctions are enriched in actin crosslinking proteins such as vinculin, transmembrane integrins and fibronectin in the extracellular basal lamina (Gabella, 1984; North et al., 1993; Small, 1985). North and colleagues (North et al., 1993) provided initial evidence that caveolae microdomains are marked by the presence of both caveolin-1 and dystrophin. This has contributed to models of the cytoskeletal organization in smooth muscle, but there has been no investigation that assesses the mechanisms of colocalization of caveolin-1 and dystrophin, or of the functional relevance of this association. Our new data show that several subunits of the DGC, including β-dystroglycan, colocalize with caveolin-1 in membrane arrays in contractile myocytes. In striking contrast, in non-contractile cultured cells lacking endogenous β-dystroglycan, caveolin-1 is expressed, but is not organized into linear arrays (see Fig. 1D). This suggests a need for the DGC in ordering of membrane caveolae domains, a conclusion supported by the fact that stable silencing of β-dystroglycan leads to disruption of caveolae linear arrays and loss of caveolin-1 and PTRF/cavin from lipid-raft-rich membrane fractions. PTRF/cavin is crucial for caveolae formation (Hill et al., 2008), and its presence on the inside surface of caveolae stabilizes these structures, probably through interaction with the cytoskeleton (Liu and Pilch, 2008). Thus, β-dystroglycan is required for the organization of caveolae arrays through its interaction with caveolin-1, and this might impact at a more fundamental level the association of PTRF/cavin and its role in forming and stabilizing caveolar structures. These observations are also important in light of our previous studies showing that DGC expression is a feature of contractile phenotype myocytes (Sharma et al., 2008), equipping them with the capacity to organize caveolae into arrays as a determinant of smooth muscle physiology.

We investigated the nature of the interaction between caveolin-1 and the DGC in smooth muscle. Caveolin-3 co-precipitates with β-dystroglycan in skeletal muscle, and a recombinant tagged peptide of a C-terminal WW domain from caveolin-3 (residues 34–129) appears to interact with a WW-binding motif from the cytoplasmic tail of β-dystroglycan (Sotgia et al., 2000). In the same study, a peptide harboring a putative WW domain in caveolin-1 (residues 61–156) was also used to co-precipitate a recombinant peptide encoding the β-dystroglycan WW-binding domain. To the best of our knowledge, our new studies confirm for the first time in any tissue that endogenous caveolin-1 associates with native β-dystroglycan. WW domains are widely distributed among structural, regulatory and signaling proteins, and are named after two highly conserved tryptophan (W) residues spaced 20–22 amino acids apart in a semi-conserved motif of 38–40 residues (Bork and Sudol, 1994; Sudol et al., 1995). WW domains mediate protein–protein interactions by binding to peptide sequences containing proline-rich motifs, such as PPXY, as is found in WW-binding domain in β-dystroglycan (residues 884–895) (Chen and Sudol, 1995; Kay et al., 2000; Sotgia et al., 2000). Although our studies did not directly address whether the putative WW domain in caveolin-1 is the effector binding site for β-dystroglycan in smooth muscle cells, they do suggest a protein–protein interaction because caveolin-1 co-fractionates and co-immunoprecipitates with β-dystroglycan. This association was refractory to actin disruption, suggesting their interaction is not reliant on indirect association. Moreover, it was retained during disruption of caveolar structures.

Thus we provide new evidence for an interaction between caveolin-1 and β-dystroglycan that appears to underpin the role of the DGC in orchestrating plasma membrane distribution of caveolae in contractile myocytes.

There is abundant evidence that the actin cytoskeleton has a key role in smooth muscle contraction, both as a principal component of a plastic contractile apparatus and cytoskeleton, and by effecting modulation of ion channels involved in Ca^{2+} mobilization (Tang and Gunst, 2004; Yamboliev et al., 2000; Yao et al., 2008; Zhang et al., 2005). The DGC interacts with and stabilizes actin filaments through a link involving dystrophin (Rybakova et al., 2006). This led us to hypothesize that actin tethering is important for stabilization and organization of the DGC and its role in orchestrating caveolae distribution via caveolin-1 on the sarcolemma. Latrunculin A is widely used to depolymerize actin because it binds to and prevents addition of G-actin monomers (Mehta and Gunst, 1999). Our study demonstrates that disruption of actin filaments drastically changes the ultrastructure and molecular organization of membrane caveolae, with significant changes in the localization of β-dystroglycan and caveolin-1 and loss of membrane caveolae arrays. Transmission electron microscopy also suggests that caveolar structures are internalized as a result of actin depolymerization. These observations are consistent with those of Mundy and colleagues (Mundy et al., 2002) who showed that actin dynamics are essential for location of caveolae to the cell membrane. A point that our study did not address is the role of microtubules in the organization of caveolin-1 in caveolae, and its interaction with the DGC. This might be an important issue deserving future work because disruption of microtubules appears to decrease inward cycling of caveolae and increases the abundance of plasma membrane caveolin-1 and caveolae (Mundy et al., 2002). Nonetheless, our findings extend understanding, because they reveal that the DGC holds an important role in linking caveolae to a dynamic intracellular actin network.

We and others have shown in smooth muscle cells that depletion of cholesterol or silencing of caveolin-1 expression disrupts the organization of membrane caveolae and alters functional responses, as measured by contractile agonist-induced intracellular Ca^{2+} release and force generation (Gosens et al., 2007b; Prakash et al., 2007). Our new data are consistent with these observations and those showing a role for the actin cytoskeleton in modulating Ca^{2+} responses in several other cell types (Calaghan et al., 2004; Rosado et al., 2000; Sabala et al., 2002), because induced changes in ultrastructure and the protein profile of caveolae were associated with a reduction both sensitivity to MCh and in maximum peak $[Ca^{2+}]_i$. Trimeric G-proteins are sequestered to caveolae through the interaction of α-subunits with caveolin-1, and the signaling machinery required to mobilize Ca^{2+} within smooth muscle cells is organized in caveolae (Darby et al., 2000; de Weerd and Leeb-Lundberg, 1997; Gosens et al., 2007b; Kifor et al., 1998; Li et al., 1995). Although PLCβ1 is concentrated at the cell membrane, it is also present throughout the cell, whereas $G_{\alpha q}$ is more-or-less membrane specific, where it stably associates with PLCβ1 to allow for rapid transmission of intracellular signals via GPCRs such as the M3 muscarinic receptor (Dowal et al., 2006). Recent evidence suggests that caveolin-1 can have a regulatory role, promoting dissociation of Gα subunits from βγ subunits to facilitate GPCR signal transduction (Sengupta et al., 2008). Interaction between caveolin-1 and β-dystroglycan appears to be necessary for such a functional role, because in human airway smooth muscle cells with stably silenced β-dystroglycan, PLCβ1 and $G_{\alpha q}$ were lost from caveolae and accumulated in fractions that are typically devoid of caveolae. As this change mirrored that for PTRF/cavin and caveolin-1, it suggests that interaction of caveolae with structural proteins and the actin cytoskeleton supports sequestration of signaling molecules for Ca^{2+} mobilization, and enable a regulator role for caveolin-1.

The role of caveolin-1 in GPCR-mediated Ca^{2+} flux is also linked with the existence of an organelle triad involving caveolae, the sarcoplasmic reticulum and mitochondria that form nanospaces for localized signal transduction leading to induction of contraction (Gabella, 1971; Gherghiceanu and Popescu, 2006; Kuo et al., 2003). Concomitant with loss of PLCβ1 and $G_{\alpha q}$ from caveolae membrane, silencing of β-dystroglycan disturbed the spatial distribution of caveolae. Studies using animal models that lack expression of DGC subunits indicate that this deficiency contributes to altered Ca^{2+} homeostasis in smooth muscle (Cohn et al., 2001; Lipskaia et al., 2007). In our present study, silencing of β-dystroglycan reduced sensitivity for muscarinic M3-receptor-mediated $[Ca^{2+}]_i$ mobilization but had no effect on maximum peak $[Ca^{2+}]_i$. This is consistent with previous work, which showed that siRNA silencing of caveolin-1 results in reduced sensitivity to MCh without impacting peak response (Gosens et al., 2007b). The lack of effect of caveolin-1 depletion on peak $[Ca^{2+}]_i$ is due to the existence of a significant M3 receptor reserve in airway smooth muscle. A similar mechanism is also likely to be the root of our observations linked to β-dystroglycan silencing in the present study. In contrast to the effect of silencing of caveolin-1 or β-dystroglycan, forced actin de-polymerization does suppress peak $[Ca^{2+}]_i$. This difference might be due to the fact that the actin cytoskeleton affects the activity of various ion channels, including some that localize to caveolae, which are involved in Ca^{2+} mobilization (Tang and Gunst, 2004; Yamboliev et al., 2000; Yao et al., 2008; Zhang et al., 2005).

In summary, our study demonstrates that the unique membrane distribution of caveolae in contractile smooth muscle cells is facilitated through the direct interaction of caveolin-1 with β-dystroglycan and tethering of the DGC to the intracellular actin cytoskeleton network. Interaction of β-dystroglycan with caveolin-1 is required to organize membrane caveolae enriched in key GPCR effectors and caveolae-stabilizing proteins. This appears to be crucial for effective spatial organization of caveolae arrays with respect to Ca^{2+}-mobilizing effectors and machinery, thus revealing a new role for the actin cytoskeleton as a determinant of smooth muscle function. Collectively, our data suggest that interaction of caveolin-1, β-dystroglycan and the actin cytoskeleton is important for the structural and spatial distribution of membrane caveolae, and has a key role in $G_{\alpha q}$- and PLCβ-1 mediated Ca^{2+} mobilization in the cell.

Materials and Methods

Reagents and antibodies

Horseradish-peroxidase (HRP)-conjugated goat anti-mouse IgG, HRP-conjugated goat anti-rabbit IgG and primary antibodies were obtained from the following sources: Caveolin-1 (BD Transduction Labs and Santa Cruz), γ-sarcoglycan, Clathrin HC (Santa Cruz), α-dystroglycan (provided by Kevin Campbell, Howard Hughes Medical Institute, Iowa City, IA), β-dystroglycan, β- and δ-sarcoglycan (Novocastra: NCL-b-DG, NCL-b-SARC, NCL-γ-SARC), Dystrophin (Chemicon, MAB1692), FITC-, Cy3- or Cy5-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories. Hoechst 33342 (H-3570), Latrunculin-A (L-12370), Texas-Red-X Phalloidin (T7471) were from Molecular Probes. Tissue-Tek OCT embedding medium was from Sakura Finetek. Cell culture media (DMEM and Ham's F12) and supplements (fetal bovine serum, ITS-A, penicillin and streptomycin) were obtained from Invitrogen. All other chemicals were of analytical grade.

Primary human and canine airway smooth muscle cell culture

Primary airway myocytes for cell culture were obtained from dissociated canine or human trachealis, as previously described (Naureckas et al., 1999). Cells were plated

onto 100 mm culture dishes or pre-cleaned sterile coverslips placed in six-well culture clusters and grown to confluence using Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum. At confluence, myocytes were serum deprived for a further 7–10 days using Ham's F12 medium supplemented with insulin, transferrin and selenium (ITS-A, 1%) to induce a contractile phenotype. Cultures were maintained in a humidified chamber at 37°C, 5% CO_2 and all media contained both 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate. For experiments involving actin disruption, cells were incubated for 60–90 minutes in HBSS [1.26 mM $CaCl_2$, 0.493 mM $MgCl_2{\cdot}6H_2O$, 0.407 mM $MgSO_4{\cdot}7H_2O$, 5.33 mM KCl, 0.441 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 137.93 mM NaCl, 0.338 mM Na_2HPO_4 (anhydrous) and 5.56 mM Dextrose] with or without 1 μM latrunculin-A. Myocytes were used at passage 0 or 2 in these studies.

Preparation of protein lysates from human and canine airway smooth muscle tissue and cells

Intact airway smooth muscle tissue was isolated from human bronchial or canine tracheal specimens by microdissection at 4°C. Smooth muscle tissues and primary cultured cells were homogenized in ice-cold RIPA buffer (40 mM Tris-HCl, 150 mM NaCl, 1% IgepalCA-630, 1% deoxycholic acid, 1 mM NaF, 5 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 7 μg/ml pepstatin A, 1 mM PMSF, pH 8.0) using a polytron. The lysate was transferred to 1.5 ml plastic tube, centrifuged (760 ***g***, 5 minutes) and the supernatant stored at –20°C for subsequent protein assay and immunoblot analyses.

Isolation of caveolae-enriched membranes

Membrane caveolae from cells or tissue were isolated by sucrose density gradient ultracentrifugation using a detergent-free protocol (Gosens et al., 2006a). Briefly, cells were lysed by sonication in 500 mM carbonate buffer (pH 11) containing 2 mM PMSF (after mincing, tissue was homogenised using a polytron and then sonicated in carbonate buffer). Sonicates were then combined with an equal volume of 90% sucrose in MES buffer (25 mM MES, 150 mM NaCl, pH 6.5) and the resulting 45% sucrose layer overlaid with a stepwise gradient of 30%, 20% and 5% sucrose buffers. The gradient was ultracentrifuged at 200,000 ***g*** for 16 hours at 4°C, and sequential 1 ml fractions were taken from the top of the gradient for later analysis by SDS-PAGE and western blotting. Caveolae membranes were isolated as opaque light-scattering bands at the 5%-20% and 20%-30% sucrose interfaces.

Protein blot analysis

Protein content in supernatant samples was determined using the Bio-Rad protein assay with bovine serum albumin as a reference. Immunoblotting was performed using standard techniques (Halayko et al., 1999; Sharma et al., 2008). Briefly, after reconstituting samples in denaturing buffer, equal protein was loaded per lane and size-separated electrophoretically under reducing conditions using SDS-polyacrylamide gels. Thereafter, proteins were transferred to nitrocellulose membranes by electric current. The membranes were subsequently blocked with 5% w/v skimmed milk in Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) with or without Tween-20 (0.2%). Blocked membranes were incubated with primary antibodies diluted in TBS containing 1% w/v skimmed milk with or without Tween-20. The membranes were incubated with HRP-conjugated secondary antibody, and then visualized on photographic film using enhanced chemiluminescence reagents (Amersham, UK). β-actin was used to correct for equal loading of samples.

Immunoprecipitation

For immunoprecipitation, protein-G-conjugated Sepharose beads (GE Healthcare) were mixed with 500 μl cell lysate. After 1 hour of incubation at 4°C, beads were washed four times with Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 and once with PBS. Beads with immunoprecipitated proteins were stored at –80°C until used for protein blot analysis.

Immunocytochemistry

Human or canine ASM cells were plated onto pre-cleared glass coverslips in six-well culture dishes as described previously (Sharma et al., 2008). Cells were fixed for 15 minutes at 4°C in CB buffer containing 3% paraformaldehyde (PFA). Cells were then permeabilized by incubation for 5 minutes at 4°C in CB buffer containing 3% PFA and 0.3% Triton X-100. For immunofluorescence microscopy, fixed cells were first blocked for 2 hours at room temperature in cyto-TBS buffer containing 1% BSA and 2% normal donkey serum. Incubation with primary antibodies occurred overnight at 4°C in cyto-TBST using anti-caveolin-1 (1:1000), anti-β-dystroglycan (1:50), anti-α-dystroglycan (1:50) or anti-dystrophin antibody (1:50). Incubation with FITC- or Texas-Red-conjugated secondary antibodies was for 2 hours at room temperature in cyto-TBST. Coverslips were mounted using ProLong antifade medium (Molecular Probes). Fluorescent imaging was performed by capturing a mid-cell section of 0.3 μm focal depth using an Olympus LX-70 FluoView Confocal Laser Scanning Microscope (Olympus) equipped with a 10×, 40× or 60× objective or Olympus IX-70 inverted microscope coupled to either a PerkinElmer UltraPix FSI CCD camera.

Transmission electron microscopy

The ultrastructure of intact canine trachealis was assessed as described previously with slight modification (Gosens et al., 2007b). Specimens consisting of two cartilage rings with intact trachealis were prepared from the cervical segments using a sharp scalpel. Specimens were incubated at 37°C in oxygenated Krebs-Henseleit solution (KH; 117.5 mM NaCl, 5.6 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.28 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 5.55 mM D-glucose, gassed with 5% CO_2 and 95% O_2, 37°C, pH 7.4) for 1 hour in the presence or absence of Latrunculin A (1 μM). Specimens were washed once with fresh KH buffer and fixed in 2.5% glutaraldehyde in PBS (pH 7.4) for 1 hour at 4°C, washed and fixed in 1% osmium tetroxide, before embedding in Epon. Thereafter, the smooth muscle layer was removed from each ring and subjected to postfixation with 1% osmium tetroxide and embedded in LX-112 acrylic medium. Ultra-thin cross-sections of the muscle tissue were then prepared, mounted onto coated grids, and stained with 1% uranyl acetate and lead citrate. Transmission electron microscopy was performed with a Philips CM10, at 80 kV, on ultra-thin sections (100 nm on 200 mesh grids) stained with uranyl acetate and counterstained with lead citrate.

β-dystroglycan RNAi

The β-dystroglycan lentiviral shRNA construct was purchased from Open-Biosystems (Huntsville, AL) distributed by the Biomedical Functionality Resource, at University of Manitoba as a bacterial culture (Clone Id: V2LHS_24095). Individual colonies were grown in 2 ml LB broth with 100 μg/ml ampicillin (bacterial selection) for 8 hours at 37°C, with shaking at 280 r.p.m. The cultures were then added to 200 ml LB broth with ampicillin (100 μg/ml) and incubated as above overnight. The culture was centrifuged and plasmid purified with a Hi Speed Plasmid Maxi Kit (Qiagen cat. no. 12663). The plasmid was transfected into HEK293T cells using a Ca^{2+}-phosphate three-plasmid transfection VSVG (envelope vector), 8.2Δvpr (packaging vector) and expression vector for β-dystroglycan to generate lentivirus by counting the puromycin-resistant colonies, as previously described (Ghavami et al., 2010; Kung et al., 2000). A non-coding β-dystroglycan refractory shRNA (shRNAi non-code) was used as a transduction control. The cells were incubated for 3 days at 37°C and supernatant containing virus was concentrated by ultracentrifugation. The virus was resuspended in DMEM 0.5% FBS for 24 hours at 4°C and aliquoted and stored at –80°C. For the transduction, human primary ASM cells were grown up to 70–80% confluence in 12-well plates and virus was added to the cells at a varied multiplicity of infection (MOI) of 3–9. The transduction was repeated twice and then the cells were allowed to grow in complete medium for 48 hours. The cells were then selected with puromycin 4 μg/ml. Viability of the transduced cells undergoing experiment was >98% as assessed by Trypan Blue dye after completion of the experiment.

RNA isolation and real-time RT-PCR analysis

Total RNA was extracted from human ASM cells using the RNeasy Plus Mini Kit in accordance with the manufacturer's recommendations (Qiagen, Mississauga, ON). The RNA concentration and purity were assessed with optical density measurements (Chirgwin et al., 1979). Total RNA (1 μg) was reverse transcribed using the Quantitect Reverse Transcription Kit as recommended by the supplier (Qiagen). Real-time PCR for cDNAs of interest was carried out with the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA), using primer pairs for β-dystroglycan as previously described (Sharma et al., 2008) and 18S ribosomal RNA (calibrator gene: NCBI X03205.1. Forward 5′-CGCCGCTAGAGGTGAAATTC-3′, Reverse 5′-TTGGCAAATGCTTTCGCTC-3′). Each reaction contained the following: 2× Power SYBR Green PCR Master Mix (Applied Biosystems), 0.4 μM each primer and cDNA template in a final volume of 20 μl. After initial denaturation for 10 minutes at 95°C, the reactions were cycled 40 times for 15 seconds at 95°C, 1 minute at the annealing temperature of 60°C and 30 seconds at 72°C for extension of both β-dystroglycan and 18S ribosomal RNA. Product specificity was determined by melting-curve analysis. Relative quantification of gene expression was performed using the 7500 Sequence Detection software v.1.4 (Applied Biosystems).

Subcellular fractionation

Cytosolic and membrane fractions were generated using a subcellular fractionation technique at 4°C as previously described (Ghavami et al., 2010). Cells were scraped in ice-cold buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and protease inhibitor cocktail), sonicated on ice three times for 5 seconds, and then the homogenate was separated into cytoplasmic and membrane fractions by ultra-centrifugation (100,000 ***g*** for 35 minutes). The membrane fraction was obtained by solubilization in dissociation buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM dithiothreitol, 1% SDS, 1 mM EDTA, 1 mM EGTA and protease inhibitor cocktail). For the detection of specific protein by immunoblotting, an equal amount of membrane and cytosolic protein fraction was subjected to standard SDS-PAGE and transferred to nitrocellulose membranes.

Intracellular $[Ca^{2+}]_i$ measurement

Real-time quantification of cytosolic Ca^{2+} in cultured ASM cells was performed using the Ca^{2+}-sensitive ratiometric fluorescent dye Fura-2 AM, as described previously (Gosens et al., 2007b; Mitchell et al., 2000). All measurements were carried out using myocytes grown on glass coverslips or chamber slides. Myocytes were washed briefly with HBSS/HEPES buffer containing 0.1% BSA and then incubated with 5 μg/ml Fura-2 AM (37°C, 1 hour) in buffer supplemented with 0.01% pluronic acid. Cells were then washed three times and incubated in buffer for

a further hour at room temperature to allow for Fura-2 AM de-esterification. Real-time changes in $[Ca^{2+}]_i$ were recorded using an Olympus LX-70 inverted epifluorescent microscope (20× objective) coupled to a Nikon CCD camera controlled by NIS imaging software. The system was further coupled to a Sutter Instruments Lambda 10-2 filter wheel and controller with repeated 100 msecond excitation at 340 and 380 nm; emission at 510 nm was recorded continually for up to 5 minutes after the addition of contractile agonists. Maximum change in $[Ca^{2+}]_i$ was calculated as the average baseline value subtracted from the peak $[Ca^{2+}]_i$ response to agonist. The ratio of emission at 510 nm excited by 340- and 380-nm light was converted to $[Ca^{2+}]_i$ values from a calibration curve generated using Ca^{2+} standards and calculated by the method of Grynkiewicz (Grynkiewicz et al., 1985). For studies examining the effects of actin disruption on $[Ca^{2+}]_i$ mobilization induced by contractile agonist, Fura-2-loaded cells were incubated at room temperature for 1 hour in buffer containing 1 μM latrunculin-A. Controls cultures were incubated for the same time period in buffer only.

Data analysis

Values reported for all data represent means ± s.e.m. For all studies, replicate data from three to four different cell lines were obtained. The statistical significance of the difference between two means was determined by an unpaired two-tailed Student's *t*-test or one-way ANOVA with Bonferroni's multiple comparison test when appropriate. Differences were considered to be statistically significant when $P<0.05$.

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