Metabolic organization in vascular smooth muscle: distribution and localization of caveolin-1 and phosphofructokinase

Johana Vallejo and Christopher D. Hardin

Department of Medical Pharmacology and Physiology, University of Missouri, Columbia, Missouri 65212

Submitted 16 October 2002; accepted in final form 25 August 2003

Vallejo, Johana, and Christopher D. Hardin. Metabolic organization in vascular smooth muscle: distribution and localization of caveolin-1 and phosphofructokinase. Am J Physiol Cell Physiol 286: C43–C54, 2004. First published August 27, 2003; 10.1152/ajpcell.00483.2002.—We have shown that a compartmentation of glycolysis and gluconeogenesis exists in vascular smooth muscle (VSM) and that an intact plasma membrane is essential for compartmentation. Previously, we observed that disruption of the caveolae inhibited glycolysis but stimulated gluconeogenesis, suggesting a link between caveolae and glycolysis. We hypothesized that glycolytic enzymes specifically localize to caveolae. We used confocal microscopy to determine the localization of caveolin-1 (CAV-1) and phosphofructokinase (PFK) in freshly isolated VSM cells and cultured A7r5 cells. Freshly isolated cells exhibited a peripheral (membrane) localization of CAV-1 with 85.3% overlap with PFK. However, only 59.9% of PFK was localized with CAV-1, indicating a wider distribution of PFK than CAV-1. A7r5 cells exhibited compartmentalization of glycolysis and gluconeogenesis and displayed two apparent phenotypes distinguishable by shape (spindle and ovoid shaped). In both phenotypes, CAV-1 fluorescence overlapped with PFK fluorescence (83.1 and 81.5%, respectively). However, the overlap of PFK with CAV-1 was lower in the ovoid-shaped (35.9%) than the spindle-shaped cells (53.7%). There was also a progressive shift in pattern of colocalization from primarily the membrane in spindle-shaped cells (both freshly isolated and cultured cells) to primarily the cytoplasm in ovoid-shaped cells. Overall, cellular colocalization of PFK with CAV-1 was significant in all cell types (0.68 ≤ R² ≤ 0.77). Common precipitation of PFK with CAV-1 further validated the possible interaction between the proteins. We conclude that a similar distribution of one pool of PFK with CAV-1 contributes to the compartmentalization of glycolysis from gluconeogenesis.

caveolae; glycolysis; compartmentation; phenotype

Glycolysis has traditionally been considered to behave as a homogeneous cascade within the cytoplasm with the intermediate metabolites able to mix freely. A growing body of evidence suggests that glycolytic enzymes are localized to many elements of the cytoarchitecture in a variety of cell types and thus are not freely diffusible. For example, evidence indicates that glycolytic enzymes bind to F-actin (3, 25, 27) and the microtubules (16, 26, 28, 29, 53, 62, 63), and they have been found in association with the plasma membrane with functional coupling to membrane ion channels (11, 18, 21, 35, 44, 45). Localized glycolytic enzymes may be important to provide metabolic support to nearby ATP-consuming processes. Extensive evidence indicates a coupling of metabolic cascades to specific processes in different types of cells, and this concept is now emerging as a more general feature of cellular organization. For example, it has been shown that the gating properties of the ATP-sensitive potassium channel are dependent on ATP derived from glycolysis (65, 66). Similarly, results of several studies suggest that there is also a close relationship between Na-K-ATPase activity and glycolysis (8, 43, 44, 49). One recent study demonstrated that the control of intracellular sodium in myocardial cells also relies on glycolytically derived ATP (11). Compartmentation of lactate and glucose metabolism has been shown in C6 glioma cells through 13C and 1H NMR spectroscopy studies (2). The same conclusion was drawn from previous studies on the metabolism of 13C-labeled pyruvate in rabbit heart (9, 30), indicating a metabolic heterogeneity of pyruvate pools within the myocytes. Recently, pyruvate compartmentation was also proposed in astrocytes (67). Thus glycolytic enzyme localization may also underlie the coupling of glycolytic ATP with a wide variety of ATP-dependent processes.

Phosphofructokinase (PFK) is a key enzyme in the control of glycolysis. It catalyzes the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate, a unidirectional and rate-limiting step in glycolysis. A number of reports describe the association of PFK with various cytoskeletal elements and signal transduction-related kinases, and thus this localization might underlie the observed compartmentation. In cardiac muscle, a fraction of PFK is associated with phospholipase A2 (23). In skeletal muscle, PFK associates with tubulin under certain conditions (29). Also, insulin stimulates binding of both PFK and aldolase to the muscle cytoskeleton (4).

Previous work by our laboratory has shown that glycolysis and gluconeogenesis occur in separate compartments within the cell and that their intermediates do not mix freely in the cytoplasm of vascular smooth muscle (VSM) cells (19, 20, 22, 33, 34). We have also found that an intact plasma membrane is essential for compartmentation of glycolysis from gluconeogenesis to exist, suggesting that glycolysis and gluconeogenesis may be associated with distinct plasma membrane microdomains (34, 35), and that disruption of the specific caveolae plasma membrane microdomains by treatment with cyclodextrin disrupts metabolic flux and compartmentation in VSM cells (35). Thus we proposed that caveolae might provide scaffolding for localization of glycolytic enzymes in VSM.

Caveolae are small invaginations of 50–100 nm in size in the plasma membrane with key functions in signal transduction and lipid transport (1, 36, 37, 47, 60). They are the major surface feature of many highly differentiated mammalian cells such as adipocytes, endothelial cells, and muscle cells (1, 56, 58). Biochemically, caveolae represent a subdomain of the plasma membrane enriched in cholesterol, glycophospholipids, and the protein caveolin (1, 56). Three mammalian isoforms of...
caveolin have been characterized and identified as caveolin-1, caveolin-2, and caveolin-3 (1, 12, 51, 56). Expression of each isoform of caveolin has been found in every smooth muscle so far tested, suggesting that each isoform may be ubiquitously expressed in smooth muscle and that each caveolin isoform assumes a predominantly plasma membrane distribution (58). Caveolae are organized as oligomers of caveolin proteins in the membrane and create a scaffold on which a variety of signaling molecules are brought together in preassembled signaling complexes (1, 6, 41, 51). Many signal transduction proteins are localized in caveolae, including G protein subunits (31, 32, 39), G protein-coupled receptors (5, 10), receptor tyrosine kinases (7, 31), the insulin receptor (42), the m3 acetylcholine receptor (14), small GTPases (31, 57), cellular adhesion proteins (46), protein kinase C (40), nitric oxide synthase (13, 17, 24, 55), the insulin receptor substrate 2 (IRS-2), the m2 nicotinic acetylcholine receptor (49), and the 

**C44 CAVEOLAE AND GLYCOLYTIC ENZYME LOCALIZATION**

**MATERIALS AND METHODS**

**Tissue handling.** Hog carotid arteries obtained from the abattoir were dissected free of loose fat, connective tissue, and adventitia and placed into a physiological saline solution (PSS) at pH 7.4 and 4°C, equilibrated by bubbling with a gas mixture of 95% O2 and 5% CO2. PSS was composed of 116 mM NaCl, 4.6 mM KCl, 1.16 mM KH2PO4, 25.3 mM NaHCO3, 2.5 mM CaCl2, 1.16 mM MgSO4, 0.85 mM penicillin, 0.069 mM streptomycin sulfate, and 5 mM glucose. Arteries were stored in PSS at 4°C overnight until needed.

**Arteries were stored in PSS at 4 mMPSS and incubated at 37°C for 90 min. After incubation, the enzyme solution was drained from the arteries, and the arteries were reinserted with suture. A needle and syringe were used to suspend the arteries in and out of the artery.**

The cell suspension was collected at the edge of each coverslip. A 650-nm excitation laser (Cantech, Cambridge, MA) was used to illuminate the cells and a Speed-Vac (Savant Instruments, Farmingdale, NY), and stored at −20°C until needed. Lyophilized samples were resuspended in 2 ml of 2H2O (Cambridge Isotope Laboratories, Andover, MA) containing 25 mM 3-(trimethylsilyl)-1-propanesulfonic acid (TMSPS) as a chemical shift reference. A 650-μl aliquot of this suspension was transferred to a 5-mm NMR tube, and 13C NMR spectroscopy was performed by using a Bruker DRX 500 spectrometer with the following acquisition parameters: 300 scans with 16 dummy scans, 30° pulse angle at 125.77 MHz, 33,333 Hz sweep width, and 1-s presaturation (33–35). A total of 32K points were acquired and processed with 1-Hz line broadening prior to Fourier transform. All spectra were broadband proton decoupled and referenced with TMS by 7.26 ppm. Peak intensities were determined using Bruker Software and expressed relative to the TMS peak. No corrections for nuclear Overhauser effects were made. Intensities of the peaks of interest were normalized to the intensity of the TMS peak.

**Immunofluorescence labeling of freshly isolated VSM cells.** Freshly isolated VSM cells were fixed in suspension with a paraformaldehyde solution (2% paraformaldehyde, 350 mM NaCl, 160 mM HEPES, and 10 mM CaCl2). Fixed cells were initially incubated in a permeabilization solution (200 μM β-escin, 150 mM NaCl, and 15 mM Na-citrate) containing 1% normal donkey serum (Sigma). After the initial permeabilization, cells were incubated overnight in permeabilization solution with 1% normal donkey serum, goat anti-rabbit PFK IgG (1:100) (Rockland Immunochemicals for Research, Gilbertsville, PA), and mouse monoclonal anti-CAV-1 IgG (1:100) (Research Diagnostics, Flanders, NJ). After incubation with both primary antibodies, cells were incubated for 3 h in permeabilization solution containing donkey anti-goat IgG conjugated to Alexa 488 (18) (1:100) (Molecular Probes, Eugene, OR) and donkey anti-mouse IgG conjugated to Alexa 594 (red, 1:100) (Molecular Probes). After incubation with both secondary antibodies, cells were rinsed with a citrate solution containing 150 mM NaCl, 15 mM Na-citrate, and 2% BSA. Finally, cells were transferred to glass slides (25 μl of suspension per slide) and covered with coverslips. Coverslips were mounted with Mowiol 4-88 mounting medium by addition of the medium around the edge of each coverslip.

**Immunofluorescence labeling of cultured A7r5 cells.** Cultured A7r5 cells were fixed in the 13-mm petri dishes in which they were grown with a paraformaldehyde solution (2% paraformaldehyde, 350 mM NaCl, 160 mM HEPES, and 10 mM CaCl2). Fixed cells were initially incubated in a permeabilization solution (50 μM β-escin, 150 mM NaCl, and 15 mM Na-citrate) containing 1% normal donkey serum.
ter

transmitted to a personal computer with the software programs La-
microscopy was performed by using the Bio-Rad Radiance 2000
with a coat of nail enamel to prevent sample from overdrying.
containing immunolabeled cultured cells were placed over glass slides
M NaCl, 15 mM Na-citrate, and 2% BSA. Finally, coverslips
Diagnostics). After incubation with both primary antibodies, cells
night in permeabilization solution with 1% normal donkey serum,
were incubated over glass slides (Sigma). After the initial permeabilization, cells were incubated over-
hyposmotic sucrose (850 

Image acquisition was done in the
argon laser at 488 nm (for green) and
emission filter cube that allowed for band-pass excitation and band-
40 nm (for red). All transmitted light

C. R. 2 ) and the slope

C45CAVEOLAE AND GLYCOLYTIC ENZYME LOCALIZATION

C. R. 80

C. R. 80

C. R. 1.0 g each and stored at

389 by 10.220.32.246 on November 6, 2017 http://ajpcell.physiology.org/ Downloaded from
The immune protein complexes from the immunoprecipitation experiments were separated by SDS-PAGE using 10% acrylamide gels and then electrotransferred to 0.2-μm nitrocellulose sheets for Western blot analysis. After transfer, nitrocellulose sheets were immunolabeled with rabbit polyclonal caveolin-1 antibody (1:1,000) (BD Transduction Laboratories) or goat anti-rabbit PFK IgG (1:1,000) (RockLand Immunochemicals for Research), followed by alkaline phosphatase-conjugated donkey anti-rabbit IgG (1:1,000) (both from RockLand Immunochemicals for Research). Nitrocellulose was developed in alkaline phosphatase developing buffer to visualize protein bands.

**Statistical analysis.** Results are expressed as means ± SE of n fields. Statistical significance was determined using a two-tailed paired Student’s t-test, assuming unequal variances. P values ≤0.05 were considered significant. All statistical calculations were performed using Microsoft Excel 2001 software.

**RESULTS**

Cultured A7r5 VSM cells exhibit compartmentalization of glycolysis from gluconeogenesis. Using 13C NMR, we previously measured compartmentation of glycolysis and gluconeogenesis in freshly isolated smooth muscle preparations (22, 33–35). Here we used 13C NMR to demonstrate that compartmentation of glycolysis from gluconeogenesis also exists in cultured A7r5 VSM cells (Fig. 1). We found that cultured A7r5 cells metabolized [2-13C]glucose to [2-13C]lactate (55.3% of total product) via glycolysis, whereas [1-13C]FBP, a glycolytic intermediate, was metabolized mainly to [1-13C]glucose (30.6% of total product) via gluconeogenesis rather than to [3-13C]lactate (14.1% of total product) via glycolysis (Fig. 1). Overall, 55.3% of metabolism came from [2-13C]glucose, whereas 44.7% of metabolism came from [1-13C]FBP. Glycolysis represented 69.4% of total product, whereas gluconeogenesis represented 30.6% of total product. These results confirmed the highly glycolytic capacity and the gluconeogenic ability of VSM as demonstrated previously (22, 33–35). Furthermore, these results confirmed the existence of compartmentalization of glycolysis from gluconeogenesis in VSM (freshly isolated preparations and cultured cells).

CAV-1 and PFK localization in VSM cells of different phenotypes studied by confocal immunofluorescence. The first passage of cultured A7r5 cells contained two apparent phenotypes of smooth muscle cells distinguishable by shape. One population tended to be more spindle shaped, whereas the other tended to be more round (Fig. 2). When individual cells were categorized visually into one of the two groups, the cells were analyzed for aspect ratio (length along longest axis/greatest width at a right angle with length axis). Cells with a length-to-width ratio >2.0 (average = 2.5 ± 0.19) were identified as spindle-shaped A7r5 cells. Cells with a length-to-width ratio <2.0 (average = 1.4 ± 0.13) were identified as ovoid-shaped A7r5 cells.

Confocal microscopy was used to determine the distribution and colocalization of PFK and CAV-1 in the VSM freshly isolated cell model (Fig. 3) and the VSM cultured cell model (Fig. 2) while considering the two A7r5 cell populations as separate groups in the analysis. We observed that CAV-1 largely exhibited a peripheral (membrane) localization in freshly isolated cells, consistent with the known locations of caveolae formation; however, CAV-1 fluorescence was also observed at intracellular loci (Fig. 3). In spindle-shaped A7r5 cells, CAV-1 exhibited some degree of peripheral (membrane) localization with a widespread localization at intracellular loci (Fig. 2). However, in ovoid-shaped A7r5 cells, CAV-1 localization was observed mainly at intracellular loci with a significantly less peripheral localization (Fig. 2). Caveolae have been reported as invaginations about 50–100 nm in size that usually cluster into groups of caveolae interspersed by membrane areas containing no caveolae (47). We assume that the punctate staining observed on confocal images represents clusters of caveolae and not individual ones.

In freshly isolated cells, PFK also exhibited a large degree of peripheral localization but exhibited a wider distribution throughout the cell than did CAV-1 (Fig. 3). PFK exhibited a distribution similar to CAV-1 in spindle-shaped A7r5 cells, with some degree of peripheral (membrane) localization and a widespread localization throughout most regions of the cell (Fig. 2). However, in ovoid-shaped A7r5 cells, PFK localization was significantly reduced at the periphery yet more distributed throughout the cell (Fig. 2).

Specific quantification of the overlap in between PFK and CAV-1 was performed by overlay of the PFK and CAV-1 pixels from images of each cell. In freshly isolated VSM cells, we found that 85.3 ± 2.8% of CAV-1 fluorescence overlapped with PFK fluorescence, indicating that most CAV-1 is localized near PFK, perhaps due to direct or indirect association of the proteins. However, only 59.9 ± 4.4% of the total PFK fluorescence overlapped with CAV-1 fluorescence (Fig. 4). These results indicate a wider distribution of PFK than CAV-1 within the freshly isolated VSM cell. We found that in both phenotypes of cultured A7r5 cells, CAV-1 fluorescence overlapped with PFK fluorescence (spindle-shaped cells = 83.1 ± 4.0%, ovoid-shaped cells = 81.5 ± 2.7%), indicating that most CAV-1 is localized near PFK. However, the overlap of PFK with CAV-1 diminished from the spindle-shaped cells (53.7 ± 4.3%) to the ovoid-shaped cells (35.9 ± 2.1%) (Fig. 5). These results indicate a wider distribution of PFK than CAV-1 within the A7r5 cell, similar to the observed PFK distribution in freshly isolated cells. However, the overlap of PFK with CAV-1 appears to be different among the different cell phe-
notypes with the highest degree of overlap in the in vivo phenotype (freshly isolated cells) and the lowest overlap in the ovoid-shaped A7r5 cell phenotype. Because image acquisition parameters and image processing were the same for images of all cell types and morphologies, differences in the degree of colocalization are unlikely to result from technical artifacts such as pixel misregistration. These results suggest that CAV-1 normally associates with one pool of PFK, whereas PFK is capable of localizing to a variety of loci within the cell.

Cellular colocalization of PFK and CAV-1 was determined by analysis of the immunofluorescence intensity emitted by labeled protein at every pixel in within the AOI. Analysis performed on the entire cellular area (whole cell; Fig. 6) resulted in correlation coefficients ($R^2$; means ± SE) of 0.71 ± 0.02, 0.77 ± 0.02, and 0.68 ± 0.02 for the correlation of CAV-1 and PFK in VSM freshly isolated cells, spindle-shaped A7r5 cells, and ovoid-shaped A7r5 cells, respectively. These results further indicate a substantial cellular colocalization of PFK with CAV-1 in VSM cells, regardless of the cell type. However, colocalization among subcellular regions of the cell was different among the different cell types (results described below).

To determine whether colocalization of PFK and CAV-1 is similar among regions of the VSM cell, we used the same approach to determine and compared the correlation of PFK and CAV-1 fluorescence intensity in AOIs of different subcellular locations. The AOIs were created by random selection of rectangular areas on specific membrane regions, specific cytoplasmic regions, and specific perinuclear regions of the cell. The perinuclear region analysis was not performed on freshly isolated VSM cells because their nuclei were not visually identifiable as in the case of the A7r5 cells. Colocalization of PFK and CAV-1 in specific membrane regions (Fig. 6) of the freshly isolated VSM cell resulted in an $R^2 = 0.74 ± 0.04$, indicating that CAV-1 has a primarily peripheral localization and that most CAV-1 located at the plasma membrane is localized near PFK. Colocalization in cytoplasmic regions of freshly isolated cells (Fig. 6) also occurred, but to a lesser extent ($R^2 = 0.39 ± 0.06$), consistent with the wider distribution of PFK than CAV-1 obtained from the image overlay.
analysis (Fig. 4). Colocalization at specific membrane regions of the spindle-shaped A7r5 cells ($R^2 = 0.49 \pm 0.04$) and the ovoid-shaped A7r5 cells ($R^2 = 0.20 \pm 0.04$) occurred to a substantially lesser extent than in freshly isolated VSM cells ($R^2 = 0.74 \pm 0.04$) in a decreasing manner from the in vivo phenotype (freshly isolated cells) to the cultured phenotype (ovoid-shaped A7r5 cells) (Fig. 6). These results are consistent with previously reported lower concentrations of caveolae formation in VSM culture cells than in vivo cells (59, 60). Moreover, colocalization of PFK and CAV-1 at specific cytoplasmic regions ($R^2 = 0.38 \pm 0.04$) and specific perinuclear regions ($R^2 = 0.55 \pm 0.03$) of the ovoid-shaped A7r5 cells was higher than colocalization at their respective membrane regions ($R^2 = 0.20 \pm 0.04$) (Fig. 6). However, colocalization at specific membrane regions and cytoplasmic regions remained higher than colocalization at perinuclear regions for the freshly isolated cells and to a lesser extent for the spindle-shaped A7r5 cells. These results indicate a progressive shift in the pattern and the colocalization of PFK and CAV-1 from a mainly peripheral (membrane) localization to a mainly cytoplasmic localization in the in vivo phenotype (freshly isolated cells) compared with the spindle-shaped and ovoid-shaped A7r5 cells of the cultured phenotype, which is consistent with a pheno-

Fig. 3. Confocal microscopy images of freshly isolated VSM cells labeled with anti-PFK and anti-CAV-1. Immunofluorescence labeling techniques were used to label the glycolytic-specific enzyme PFK (green) and the specific membrane microdomains known as caveolae (CAV-1; red). CAV-1 and PFK exhibit a similar, but not entirely identical, overlap (yellow).

Fig. 4. Overlap of PFK and CAV-1 in freshly isolated VSM cells. Specific quantification of overlap between PFK and CAV-1 was determined by overlay of the PFK and CAV-1 images from each respective cell using MetaMorph software. Results are expressed as means ± SE of 11 fields.
type-dependent variation in the distribution and expression of CAV-1. Finally, the finding that colocalization of PFK and CAV-1 occurred at the plasma membrane as well as at different cytoplasmic regions suggests that PFK and CAV-1 might interact physically regardless of the localization.

Variations in the relative proportion of PFK to CAV-1 in VSM cells of different phenotypes. Cellular colocalization of PFK and CAV-1 was originally measured using epifluorescence microscopy and determined by analysis of the immuno-fluorescence intensity emitted by each protein at every pixel in within the AOI. Bitmaps of red (CAV-1) and green (PFK) were separately created, and the fluorescence intensity of each red pixel array was plotted against each green pixel array, resulting in a pixel-by-pixel plot of red and green fluorescence intensity. Representative plots of a single cell from each VSM cell group are shown in Fig. 7. The fluorescence intensity emitted by CAV-1 (x-axis) among all cell groups was plotted on the same range (Fig. 7). However, there is an increase in the fluorescence intensity emitted by PFK (y-axis) from the freshly isolated VSM cell to the spindle-shaped A7r5 cells to the ovoid-shaped A7r5 cells (Fig. 7). The slope (m) of the linear regression plot from each cell represents the relative proportion of PFK to CAV-1 (PFK/CAV-1 fluorescence ratio). The average ratio of PFK to CAV-1 fluorescence from each cell group was determined (Fig. 8). Results indicate that the ratio of PFK fluorescence to CAV-1 fluorescence of the spindle-shaped A7r5 cells ($m = 3.02 \pm 0.27; P = 0.0013$) and the ovoid-shaped A7r5 cells ($m = 4.26 \pm 0.15; P = 2.5 \times 10^{-6}$) were substantially higher than in freshly isolated VSM cells ($m = 1.04 \pm 0.09$) in an increasing manner from the in vivo state (freshly isolated cells) to the cultured state (ovoid-shaped A7r5 cells) (Fig. 8).

Coimmunoprecipitation of PFK and CAV-1 further validates the colocalization of these proteins. Western blot analysis was used to confirm the specificity of labeling of the antibodies.
used for immunofluorescence microscopy. Tissue lysate from hog carotid arteries was analyzed by SDS-PAGE. Nitrocellulose membranes were probed with the same antibodies and the same concentrations used for immunofluorescence labeling during confocal microscopy (Fig. 9). The antibodies exhibited strong specificity for their expected target proteins.

Coimmunoprecipitation experiments were used to validate at the in vitro level the observed results using intact cells. Control experiments demonstrated that there is some degree of nonspecific binding of PFK to the protein G-Sepharose matrix (Fig. 10). However, the presence of polyclonal anti-caveolin immunoprecipitating antibody resulted in an increased binding of PFK to the immunoprecipitating complex, indicating that the caveolin protein has the ability to immunoprecipitate PFK (Fig. 10). These studies validate the immunofluorescence colocalization results by demonstrating coimmunoprecipitation of PFK and CAV-1 from the VSM cells. The physical nature of the interaction remains to be elucidated, given that PFK could bind caveolin directly or through an accessory protein.

**DISCUSSION**

**Metabolic compartmentation in VSM cells and the role of the plasma membrane.** Extensive evidence indicates a coupling of metabolic cascades to specific processes in different types of cells, and this concept is now emerging as a more general feature of cellular organization. Compartmentation has been described in a wide variety of cells (2, 9, 30, 33–35, 67) (see Introduction for details). However, little work has been done to identify the physical basis for compartmentation.

Previous work by our laboratory has shown that a compartmentation of carbohydrate metabolism exists in VSM cells. Using 13C NMR to examine the products of metabolism from glycolysis and gluconeogenesis (which share 8 common enzymes), we have determined that glycolysis and gluconeogenesis occur in separate compartments within the VSM cell and that their respective intermediates do not mix freely within the cytoplasm (19, 20, 22, 33–35). Here we used 13C NMR to demonstrate that compartmentation of glycolysis from gluconeogenesis also exists in cultured A7r5 VSM cells (Fig. 1). In VSM cells, 13C-labeled glucose is metabolized to lactate via glycolysis, whereas 13C-labeled FBP, a glycolytic intermediate, is metabolized mostly to glucose (gluconeogenesis) rather than to lactate (glycolysis) (33, 34). Therefore, spatial separation of glycolytic and gluconeogenic enzymes and close proximity of the metabolic intermediates to the localized enzymes must be the basis of compartmentation. Caveolae have been proposed to provide scaffolding for compartmented signal transduction, and thus we proposed that caveolae might provide scaffolding for localization of glycolytic enzymes. We hypothesized that glycolytic enzymes are specifically localized to caveolae, whereas gluconeogenic enzymes are localized to noncaveolae domains in VSM. Therefore, the goal of the present study was to use confocal microscopy to determine whether the specific glycolytic enzyme PFK and the caveolae-specific protein CAV-1 were colocalized.

Confocal microscopy reveals a similar pattern of localization of one pool of PFK with CAV-1 by possible physical interaction between the proteins. Our results indicated a wider distribution of PFK than CAV-1 within the VSM cell, regard-
less of phenotype. The wider distribution of PFK fluorescence in this study is consistent with considerable evidence that suggests that glycolytic enzymes are found not only in association with the plasma membrane (11, 18, 21, 35, 44, 45) but also may be in association with the microtubules (16, 26, 28, 29, 53, 62, 63) and F-actin (3, 25, 27), resulting in different glycolytic cascades associated to different cellular domains. Presumably, a similar distribution of one pool of PFK with CAV-1 may provide the structural basis for one compartment of glycolysis from other metabolic pathways.

The pattern of localization of CAV-1 fluorescence significantly overlapped with PFK fluorescence, indicating that most CAV-1 is localized near PFK in freshly isolated VSM cells (Fig. 4). The pattern and localization of PFK and CAV-1 was also studied in a culture cell model (A7r5) that displayed two apparent phenotypes of smooth muscle cells distinguishable by shape. Results indicated a phenotype-dependent variation in the distribution and expression of PFK and CAV-1. Previous

![Western blot analysis demonstrates the labeling specificity of the antibodies used for immunofluorescence. Tissue lysate from hog carotid arteries was analyzed by SDS-PAGE.](image)

Fig. 9. Western blot analysis demonstrates the labeling specificity of the antibodies used for immunofluorescence. Tissue lysate from hog carotid arteries was analyzed by SDS-PAGE. Nitrocellulose membranes were probed with the corresponding antibodies at the same concentrations used for immunofluorescence labeling. Total protein in the amount of 0.05 and 0.2 mg was loaded into lanes 1 and 2, respectively. Western blot development resulted in identification of the proteins of interest at the expected sizes, demonstrating the labeling specificity of the antibodies. The lysate concentration used for lane 2 was higher than that used for lane 1 because of the smaller percentage of CAV-1 protein present on cells compared with PFK. Furthermore, the mouse monoclonal anti-CAV-1 IgG is not as efficient for Western blot analysis as it is for immunofluorescence labeling. Molecular weights are given. The gel shown is a representative example of 2 experiments.

![Caveolae and glycolytic enzyme localization.](image)

Fig. 10. CAV-1 significantly coimmunoprecipitates PFK from tissue lysates of hog carotid arteries. Two different dilutions of tissue lysate (5 mg/ml, lanes 2 and 3; 10 mg/ml, lanes 4 and 5) were immunoprecipitated in either the absence (lanes 2 and 4) or presence (lanes 3 and 5) of anti-CAV-1 IgG. Immune complex proteins were separated in a 10% polyacrylamide gel, transferred to nitrocellulose, and probed with anti-PFK IgG or anti-CAV-1 IgG. In addition, an equivalent amount of protein G-Sepharose and anti-CAV-1 IgG was analyzed in lane 1. Results indicated some degree of nonspecific binding of PFK to the protein G-Sepharose (Prot G-Seph) matrix with increasing concentration of lysate (lane 4). However, the presence of anti-CAV IgG resulted in an increased binding of PFK to the immunoprecipitating complex, indicating that the caveolin protein has the ability to immunoprecipitate PFK. Molecular weights are given. The gel shown is a representative example of 3 experiments.
studies have shown that VSM cells shift between two differentiated states with distinct morphological and functional properties, a contractile and a synthetic (proliferative) phenotype (60). Smooth muscle cells adopt a more immature state (a synthetic phenotype) after vascular injury and in early atherosclerosis (60). This dedifferentiation includes a loss of myofilaments and the formation of a prominent endoplasmic reticulum (ER) and Golgi complex. The cells lose their contractility and become more proliferative and more prominent to migration from the media to the intima of the vascular vessel. A similar modification has been shown to take place when VSM cells are put in culture (59, 60).

Overall, cellular colocalization of PFK with CAV-1 was significant in all cell groups (Fig. 6). Analysis of the colocalization in specific subcellular regions suggested a shift in the pattern and the colocalization of PFK and CAV-1 from a mainly peripheral (membrane) localization to a mainly cytoplasmic localization increasingly between the in vivo phenotype (freshly isolated cells) and the ovoid-shaped cells of the cultured phenotype among the VSM cell groups (Fig. 6). This particular shift in the pattern of localization is consistent with reports of a more prominent ER and Golgi complex in the proliferative phenotype of cultured cells (60). Moreover, CAV-1 was recently reported to be abundant in cis-Golgi and to have a cis-Golgi targeting domain (36). Thyberg (60) reported a reduced number of caveolae in smooth muscle when the cells shifted from a contractile to a proliferative (synthetic) phenotype. Those results revealed that the density of caveolae was more than sixfold higher in contractile cells than in synthetic proliferative cells. However, levels of CAV-1 remained unchanged, with a redistribution of the protein from the plasma membrane to the perinuclear cytoplasm (60). Caveolin-1 is found in the Golgi apparatus and at the cell surface of most normal tissue culture cells. As much as 90% of the cellular CAV-1 is at the cell surface, and immunogold labeling indicates that the majority of this pool is localized to caveolae (37). It has been suggested that surface CAV-1 also recycles through the Golgi apparatus by using a novel pathway that involves the direct movement of the molecule from caveolae to the lumen of the ER and on to the Golgi apparatus. A cytoplasmic pool of CAV-1 in complex with multiple chaperones may be an intermediate step in this pathway (61). Immunofluorescence cannot distinguish between the recycling and the newly synthesized pools of CAV-1 in the Golgi apparatus. The CAV-1 in the Golgi apparatus is either in route to the cell surface from its site of synthesis in the ER or has arrived from a recycling pathway (61). Therefore one can argue that the shift in the distribution of caveolin is due to newly synthesized CAV-1 in the ER and not necessarily to a massive mobilization of CAV-1 from the membrane to the cytoplasmic compartment. However, the fluorescence intensity emitted by CAV-1 (x-axis) among all cell groups was within a consistent range (Fig. 7), suggesting that the levels of CAV-1 remained unchanged and that the differences in the fluorescence intensity and correlation among the subcellular areas of the cells are indeed due to a redistribution of CAV-1 and PFK proteins. These results suggest that the proposed movement of CAV-1 through the recycling pathway may be a way to relocate the scaffolding of metabolic proteins according to shifts in the metabolic demand of the cell.

Our results also demonstrated that the relative proportion (m) of PFK to CAV-1 was higher in cultured cells than in freshly isolated VSM cells increasingly from the in vivo phenotype (freshly isolated cells) to the cultured state (ovoid-shaped A7r5 cells) (Fig. 8). We found an increase in the fluorescence intensity emitted by PFK (y-axis) between the freshly isolated VSM cell and both the spindle-shaped A7r5 cells and the ovoid-shaped A7r5 cells (Fig. 8). Although no characterization of phenotype, other than simple morphometric analysis, was done on any of the cells used, it is reasonable to speculate that the A7r5 cells are of a more proliferative phenotype than freshly isolated cells, which may be presumed to be of the contractile phenotype. Furthermore, the ovoid-shaped A7r5 cells may be presumed to be of a more proliferative phenotype than the spindle-shaped A7r5 cells. Our results suggest a higher glycolytic rate of VSM cells in their proliferative state (ovid-shaped A7r5 cells) than in their contractile state (spindle-shaped A7r5 cells and freshly isolated cells). This phenotype-dependent variation in the proportion of PFK to CAV-1 is likely to be functionally important as well as dependent on the mobilization of CAV-1 from the cell membrane to cytoplasm-associated membranes. These results suggest once more that the mobilization of CAV-1 may be a way to fulfill a shift in the metabolic demand of the cell. A recent study by Martens et al. (38) demonstrated that the voltage-gated potassium channel Kv1.5 colocalizes with caveolin on the cell surface and redistributes with caveolin after microtubule disruption. In addition, immunolocalization of caveolae with the use of caveolin antibodies demonstrated that the Kv1.5 channel protein copurifies with intact caveolae (38). Our results demonstrate that colocalization of PFK and CAV-1 occurred at the plasma membrane as well as different cytoplasmic regions and that PFK redistributes with CAV-1 in a phenotype-dependent way, suggesting a possible physical interaction in between PFK and CAV-1 regardless of the localization.

Coimmunoprecipitation of PFK and CAV-1. Our results demonstrated coimmunoprecipitation of PFK and CAV-1, which provides additional confirmation of our confocal microscopy results demonstrating significant colocalization of CAV-1 and PFK in VSM cells regardless of cellular localization. Although these results provide additional clear evidence of some interaction between the proteins, the nature of the interaction between these proteins remains to be elucidated, because PFK could be bound to caveolin directly or through an accessory protein. Moreover, our studies are consistent with studies in differentiated myotubes that have demonstrated, under certain metabolic conditions, that PFK-M forms a stable complex with CAV-3 (50). Therefore, our immunoprecipitation results may represent direct interaction, as has been shown in myotubes, or indirect associations via an intermediary protein.

The results of our current experiments support the hypothesis that PFK and CAV-1 share a common localization in the VSM cell, which would be a necessary arrangement if caveolae were responsible for part of the structural organization and compartmentation of glycolysis.

ACKNOWLEDGMENTS

We thank Dr. Sparks from Premium Standard Foods, Inc. in Milan, MO, for donation of pig tissue used for research. We thank Dr. Mayandi Sivaguru for advice and assistance with the confocal microscopy. We also thank Dr.
REFERENCES

40. Oka N, Yamamoto M, Schwencke C, Kawabe J, Ebina T, Ohno S, Coutet J, Lisanti MP, and Ishikawa Y. Caveolin interaction with protein...


