

Activated Protein Kinase C Isoforms Target to Cardiomyocyte Caveolae Stimulation of Local Protein Phosphorylation

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Abstract—Protein kinase C (PKC) isoforms constitute an important component of the signal transduction pathway used by cardiomyocytes to respond to a variety of extracellular stimuli. Translocation to distinct intracellular sites represents an essential step in the activation of PKC isoforms, presumably as a prerequisite for stable access to substrate. Caveolae are specialized subdomains of the plasma membrane that are reported to concentrate key signaling proteins and may represent a locus for PKC action, given that PKC activators have been reported to dramatically alter caveolae morphology. Accordingly, this study examines whether PKC isoforms initiate signaling in cardiomyocyte caveolae. Phorbol ester-sensitive PKC isoforms were detected at very low levels in caveolae fractions prepared from unstimulated cardiomyocytes; phorbol 12-myristate 13-acetate (PMA) (but not 4 α -PMA, which does not activate PKC) recruited calcium-sensitive PKC α and novel PKC δ and PKC ϵ to this compartment. The subcellular localization of the phorbol ester-insensitive PKC λ isoform was not influenced by PMA. Endothelin also induced the selective translocation of PKC α and PKC ϵ (but not PKC δ or PKC λ) to caveolae. Multiple components of the extracellular signal-regulated protein kinase (ERK) cascade, including A-Raf, c-Raf-1, mitogen-activated protein kinase kinase, and ERK, were detected in caveolae under resting conditions. Although levels of these proteins were not altered by PMA, translocation of phorbol ester-sensitive PKC isoforms to caveolae was associated with the activation of a local ERK cascade as well as the phosphorylation of a \approx 36-kDa substrate protein in this fraction. Finally, a minor fraction of a protein that has been designated as a receptor for activated protein kinase C resides in caveolae and (along with caveolin-3) could represent a mechanism to target PKC isoforms to cardiomyocyte caveolae. These studies identify cardiomyocyte caveolae as a meeting place for activated PKC isoforms and their downstream target substrates. (*Circ Res.* 1999;84:980-988.)

Key Words: protein kinase C ■ caveolae ■ cardiomyocyte

Caveolae were first identified as flask-shaped invaginations on the surface of epithelial and endothelial cells >40 years ago. Investigations of the molecular structure and function of caveolae have only recently advanced with the advent of methods to obtain large quantities of this specialized plasma membrane microdomain and the identification of caveolin, the distinct coat protein that decorates the internal membrane surface of caveolae. Caveolin comprises a multi-gene family of at least 3 immunologically distinct 21- to 24-kDa isoforms that serve as marker proteins for this organelle. Caveolin-1 is most abundantly expressed in adipocytes, endothelial cells, and fibroblasts; caveolin-2 shows a similar pattern of protein expression; caveolin-3 is the muscle-specific isoform.¹⁻³ Caveolae are most conspicuous in highly differentiated cell types in which their role in the transport of macromolecules such as albumin and low-density lipoproteins from the blood to the tissue space (transcytosis), in the delivery of small molecules such as folate from the

extracellular space to the cytoplasm (potocytosis), and in signal transduction by receptors for growth factors and hormones has been the focus of intense recent investigation in several laboratories.

There is recent growing evidence that caveolae may act as structurally and biochemically distinct plasma membrane compartments that localize and regulate the function of a subset of transmembrane signaling events (reviewed in Reference 4). This hypothesis is based on the identification of multiple components of signaling cascades in caveolae at steady state or after ligand-induced activation. Further studies identify caveolin as a plasma membrane docking protein that sequesters certain cytoplasmically oriented signaling molecules in their inactive form, thereby serving to promote the efficient and rapid coupling of agonist-occupied receptors to effector mechanisms.⁵⁻⁷ Caveolin has been shown to directly interact with G protein α subunits,⁵ Ras,⁶ Src,⁷ endothelial nitric oxide synthase,⁸ and the epidermal growth factor

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receptor.⁹ In each case, the interaction has been mapped to the same cytosolic membrane-proximal region of caveolin (termed the "caveolin-scaffolding domain").⁷ Using the scaffolding domain of caveolin as a receptor, 2 related aromatic amino acid-rich protein sequences that function as caveolin binding motifs have been identified; these motifs exist in most G protein α subunits, in numerous G protein-coupled receptors, in several peptide growth factor receptors, and in the kinase domains of many tyrosine and serine/threonine protein kinases,^{4,10} including protein kinase C (PKC) isoforms.¹⁰ Indeed, several studies identify conventional, calcium-sensitive PKC isoforms as constituent components of caveolar membranes at steady state,¹¹⁻¹⁵ whereas 1 recent study identified regulated (phorbol 12-myristate 13-acetate [PMA]-induced) affiliation of PKC α with the caveolin-enriched fraction of MDCK cells¹⁶ (although phorbol ester-induced loss of PKC α from caveolae also has been reported¹²). Although most cells coexpress multiple PKC isoforms, curiously, analyses of endogenously expressed PKC isoforms thus far have been restricted largely to the conventional, calcium-sensitive PKC α and PKC β isoforms. Nevertheless, there is evidence that PKC activators cause caveolae to flatten out and prevent the caveolae uptake of small molecules such as folate via potocytosis,^{12,17} suggesting that localization of PKC isoform(s) to caveolae is associated with functionally important consequences in certain cell types. Accordingly, the goal of the present study was to determine whether caveolae form a signaling module for PKC isoforms in cardiomyocytes.

Materials and Methods

Materials

Antibodies and their sources were as follows: mouse monoclonal anti-caveolin-1 and anti-caveolin-3, mouse monoclonal anti-c-Raf-1, rabbit polyclonal affinity-purified anti-mitogen-activated protein kinase kinase (MEK), mouse monoclonal anti-annexin II, and mouse monoclonal anti-receptor for activated protein kinase C (RACK) (Transduction Laboratories); rabbit polyclonal affinity-purified anti-A-Raf and rabbit polyclonal affinity-purified anti-extracellular signal-regulated protein kinase (ERK) (Santa Cruz Biotechnology); mouse monoclonal anti-plasma membrane calcium ATPase (PMCA; Affinity Bioreagents, Inc); and mouse monoclonal anti-Golgi 58-kDa protein IgG1 (Sigma). The mouse monoclonal anti-PKC α antibodies were from Upstate Biotechnology, the mouse monoclonal anti-PKC β was from Transduction Laboratories, the rabbit polyclonal anti-PKC δ antibodies were from GIBCO-BRL, and the polyclonal anti-PKC ϵ was a generous gift from Dr Doriano Fabbro (CIBA-Geigy). ¹²⁵I-labeled goat anti-rabbit IgG F(ab')₂ fragment and ¹²⁵I-labeled sheep anti-mouse IgG F(ab')₂ fragment were purchased from DuPont NEN. The enhanced chemiluminescence (ECL) kit was from Amersham Pharmacia Biotech. All other chemicals were reagent grade.

Cell Culture

Cardiac myocytes were isolated from hearts of 2-day-old Wistar rats by a trypsin dispersion procedure according to a protocol that incorporates a differential attachment procedure to enrich for cardiac myocytes as described previously.¹⁸ The yield of myocytes typically is 2.5 to 3 × 10⁶ cells per neonatal heart. Cells were plated at a density of 0.5 × 10⁶ cells per milliliter in fibronectin-coated 100-mm culture dishes. Although the preplating step effectively decreases fibroblast contamination of the cultures, a small number of cells with proliferative capability such as cardiac fibroblasts persists in myocardial cell cultures. Proliferation of these cells was further curtailed with an

irradiation protocol.¹⁸ Experiments were performed after 5 to 6 days of culture in MEM (GIBCO-BRL) with 10% FCS, 5 × 10⁻⁶ mol/L hypoxanthine, and 12 mmol/L NaHCO₃.

Purification of Caveolin-Rich Membrane Fractions

Two procedures were used to prepare caveolin-enriched fractions. The first method followed the detergent-free purification scheme described by Song et al.⁶ All steps were carried out at 4°C. Briefly, cells (from 5 dishes, 100 mm-diameter each) were washed twice with ice-cold PBS and then scraped into 0.5 mol/L sodium carbonate, pH 11.0 (0.5 mL per dish). Cells from the 5 dishes were combined (total volume, ≈2.5 mL) for each preparation. To disrupt cellular membranes, homogenization was carried out sequentially with a loose-fitting Dounce homogenizer (10 strokes), a Polytron tissue grinder (three 10-second bursts), and a sonicator (three 20-second bursts). The homogenate was then adjusted to 40% sucrose by adding an equal volume of 80% sucrose prepared in Mes-buffered saline (MBS; 25 mmol/L Mes, pH 6.5, and 0.15 mol/L NaCl) and placed on the bottom of an ultracentrifuge tube. A 5% to 35% discontinuous sucrose gradient was formed above the sample (3 mL of 5% sucrose and 4 mL of 35% sucrose, both in MBS containing 250 mmol/L sodium carbonate) and centrifuged at 38 000 rpm for 16 to 18 hours in a rotor (model SW40, Beckman Instruments). For profiles, 1-mL fractions starting at the top of each gradient were collected to yield a total of 12 fractions. Alternatively, the light scattering band confined to the 5% to 35% interface (fractions 3 to 4, which contains caveolin but excludes most other cellular proteins) was diluted 3-fold with MBS and centrifuged at 40 000g for 1 hour to pellet the caveolae, which were then solubilized with SDS-PAGE sample buffer.

In some experiments, caveolae also were prepared according to the method that relies on their resistance to solubilization by the nonionic detergent Triton X-100 at low temperatures (essentially as described by Sargiacomo et al¹⁹). In this case, cells were scraped into MBS containing (in mmol/L) sodium vanadate 0.1, NaF 25, and phenylmethylsulfonyl fluoride 1; 10 μg/mL leupeptin; 10 μg/mL aprotinin; and 1% Triton X-100. The extract was homogenized with 10 strokes of a loose-fitting Dounce homogenizer, adjusted to 40% sucrose, and placed on the bottom of an ultracentrifuge tube. A 5% to 35% discontinuous sucrose gradient was formed above the lysate (in MBS containing protease inhibitors but lacking Triton X-100), and the Triton X-100-insoluble caveolin-enriched complexes that form a flocculent band at the 5% to 35% interface were collected, diluted 3-fold in MBS containing protease inhibitors (but lacking Triton X-100), sedimented by centrifugation (40 000g for 1 hour), and solubilized in SDS-PAGE sample buffer.

Transmission Electron Microscopy

Pellets were fixed with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.2) for an hour on ice. Samples were then rinsed in 0.1 mol/L sodium cacodylate buffer, postfixed with 1% OsO₄ in cacodylate buffer, and en bloc stained with 1% aqueous uranyl acetate. Samples were dehydrated in graded ethanol and embedded in Ladd Lx112 embedding medium (Ladd Research Industries, Inc). Thin sections were cut on an ultramicrotome RMC MT-7000, stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (Jeol 1200).

Immunoblot Analysis

Samples (generally 7 to 10 μg of protein from the caveolae fraction/lane) were separated by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose, which was cut longitudinally for incubations with various primary antibodies. Some experiments used cardiomyocyte soluble and particulate fractions that were prepared according to standard methods as described previously.²⁰ The immunodetection of PKC isoforms was according to methods described in previous publications.^{20,21} All other antibodies were diluted in 50 mmol/L Tris, pH 7.5, 0.2 mol/L NaCl containing 5% nonfat dry milk, 0.05% Tween 20, and 0.02% NaN₃. Primary antibodies were used at final dilutions of 1:5000 (caveolin-1 and caveolin-3), 1:2500

(RACK-1), 1:2000 (annexin II), 1:1000 (MEK and 58-kDa protein), 1:500 (ERK), 1:300 (PMCA), 1:250 (c-Raf-1), and 1:100 (A-Raf), and bound primary antibodies were visualized with the appropriate ¹²⁵I-labeled secondary antibodies (see Figures 1, 2, 3, 5, and 6) or ECL according to the manufacturer's instructions (Figure 4). Signals were quantified with a phosphor imager (model 445SI PhosphorImager, Molecular Dynamics, for ¹²⁵I-labeled secondary antibodies) or densitometric analysis (for experiments that used ECL to detect antigen-antibody complexes). Each of the antibodies was initially screened with total cell lysates to ensure that they reacted with a band (or bands) of the appropriate molecular mass in cardiomyocytes. For the polyclonal antibodies (PKC isoforms, A-Raf, MEK, and ERK), preliminary experiments established that the immunoreactive bands were specific (ie, immunoreactivity was completely blocked by an excess of the respective competing antigen peptide).

Assay of ERK Activity in SDS-Polyacrylamide Gels Containing Myelin Basic Protein (MBP)

Caveolae from control cells and cells exposed to 300 nmol/L PMA for the indicated intervals were assayed for ERK activity with MBP as substrate by "in-the-gel"-kinase assays as described previously.²⁰

Metabolic Labeling of Proteins in Cardiomyocyte Caveolae

For *in vivo* phosphorylation assays, the monolayer was rinsed with P_i-free MEM (4 mL per 100 mm-diameter dish) and then incubated for 4 to 5 hours at 37°C in P_i-free MEM supplemented with [³²P]orthophosphate (0.15 to 0.26 mCi/mL; 4 mL per dish). To study the effects of PKC activation, PMA (300 nmol/L) was added during the final 30 minutes of the labeling period as indicated. At the end of the treatment interval, the radioactive medium was aspirated, dishes were rinsed with ice-cold PBS (4 mL per dish), and cells were scraped into MBS containing (in mmol/L) sodium vanadate 0.1, NaF 25, and phenylmethylsulfonyl fluoride 1; 10 μg/mL leupeptin; 10 μg/mL aprotinin; and 1% Triton X-100. This was followed by the isolation of caveolae (according to the method described above), electrophoresis (8% polyacrylamide gels), and autoradiography to detect PMA-dependent incorporation of ³²P into the resolved caveolar proteins.

Results

We initially used the sodium carbonate-based detergent-free method of Song et al⁶ to purify caveolae from quiescent cultured neonatal rat ventricular myocytes. The fractionation scheme is based on the resistance of integral membrane proteins to solubilization in sodium carbonate as well as the low buoyant density of caveolae due to their high cholesterol and glycosphingolipid content. After solubilization in sodium carbonate, cell lysates were adjusted to 40% in sucrose and then overlaid with a 5% to 35% discontinuous sucrose gradient. A small amount of protein moves up the gradient during centrifugation for 16 hours at 285 000*g* and can be visualized as a sharp light-scattering band at the 5% to 35% interface of the discontinuous sucrose gradient (fractions 3 to 4). Figure 1 illustrates that these fractions exclude the bulk of the total cell protein that remains in the 40% sucrose layer (fractions 8 to 12) or sediments to the bottom of the tube as an insoluble pellet (P). Western blot analysis demonstrates that the muscle-specific caveolin-3 isoform is recovered in fractions 3 to 4; small amounts of caveolin-3 also are recovered in the insoluble pellet, perhaps as a result of incomplete cell lysis (Figure 1D). Consistent with recent results reported by others,²² this fraction contains abundant immunoreactivity for the PMCA but excludes the 58-kDa protein, a marker for the Golgi²³ (Figure 1E). By transmission

electron microscopy, the caveolin-rich membrane domains appear as ≈50- to 100-nm vesicular structures that resemble the plasmalemmal vesicles (caveolae) that are seen in transmission micrographs of whole cells (Figure 1F). There also are some curved membrane fragments (similar to the morphology observed for caveolin-enriched domains isolated from other cell types,^{13,19}) but no morphologically recognizable contaminants. In agreement with a previous study,¹ caveolin-1 (the immunologically distinct form of this protein, which is abundant in adipocytes, endothelial cells, and fibroblasts) is not detected in this preparation (even when 5 times as much protein is loaded on the gel; data not shown).

Cardiomyocytes are known to contain at least 2 proteins that at least theoretically could serve to anchor or regulate PKC isoforms in caveolin-enriched domains. The first is the muscle-specific caveolin-3 isoform, the scaffolding domain sequence of which was recently shown to directly interact in *in vitro* binding assays with the phorbol ester-sensitive PKCα isoform.¹⁵ A second class of proteins that potentially could anchor PKC isoforms in caveolae is the RACK proteins. According to this formulation, individual PKC isoforms translocate to distinct intracellular loci as a result of specific high-affinity interactions between unique sequences in individual PKC isoforms and their respective anchoring RACK proteins.²⁴ Because RACK proteins were first identified in the Triton X-100 insoluble fraction of neonatal rat hearts, and RACK immunoreactivity was recently reported to be detectable in the caveolae fraction of kidney epithelial cells,¹² we next examined whether RACK proteins could constitute a class of proteins that bind PKC in caveolae. Figure 1C shows that the bulk of the RACK protein is recovered as a 36-kDa protein in the heavy fractions. Nevertheless, a minor component of total cellular RACK immunoreactivity (≈0.05%) also is detectable in cardiomyocyte caveolae.

Having identified at least 2 potential PKC anchoring proteins in cardiomyocyte caveolae, caveolin-enriched complexes were isolated from basal and PMA-stimulated (300 nmol/L for 30 minutes) cultured neonatal rat ventricular myocytes and probed for PKC isoform immunoreactivity. Previous studies established that neonatal rat ventricular myocytes coexpress multiple PKC isoforms, including the phorbol ester-/calcium-sensitive PKCα, the phorbol ester-sensitive novel PKCδ and PKCε, and the atypical PKCλ isoforms.^{21,25,26} These isozymes differ in their cofactor requirements for enzymatic activation, their substrate specificity, and their subcellular localization in cardiomyocytes and other cell types.²⁷ Figure 2 shows that no PKC immunoreactivity could be detected in the caveolae fraction from control cells when ¹²⁵I-labeled secondary antibodies were used as the method to detect antigen-antibody complexes (although it was possible to visualize small amounts of immunoreactivity for each PKC isoform when blots were developed with the more sensitive ECL method; see Figure 4). Immunoreactivity for all 4 PKC isoforms was recovered in great abundance in the heavy gradient fractions (data not shown). Immunoreactivity for each of the phorbol ester-sensitive PKC isoforms increased dramatically in caveolae in response to treatment with PMA (but not 4α-PMA, which does not activate PKC). Incubation with PMA did not lead to the recruitment of the

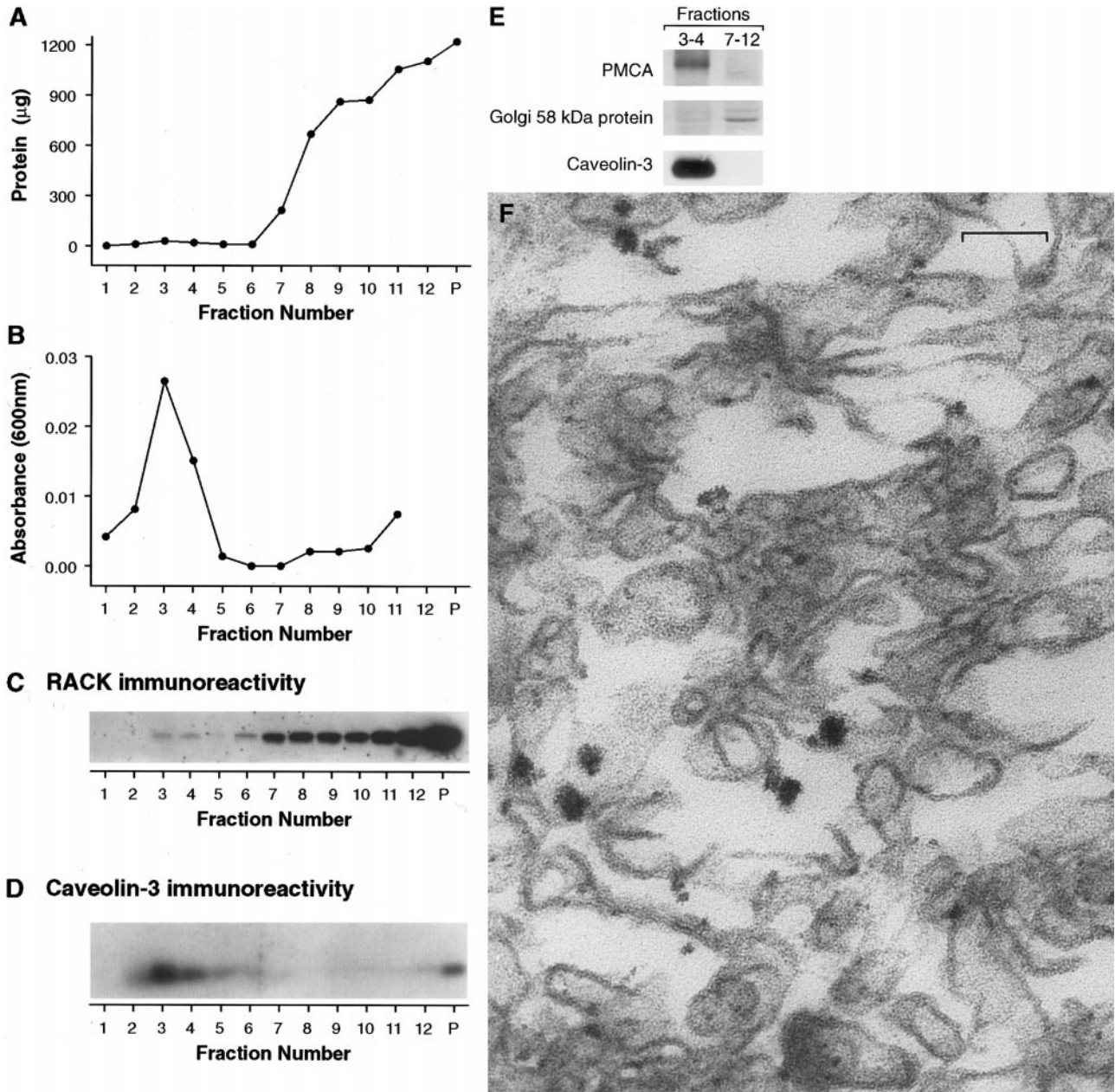


Figure 1. Isolation of caveolin-rich domains from cultured neonatal rat ventricular myocytes. Myocytes lysed in sodium carbonate were subjected to sucrose density centrifugation as described in Materials and Methods. One-milliliter fractions were collected from the top of the gradient and were analyzed for protein content (A) and light scattering (B). Aliquots containing equal amounts of protein (or a volume equal to that of the fraction with the least amount of detectable protein for “protein-free” fractions 1 and 2) were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose, and probed for RACK (C) and caveolin-3 immunoreactivity (D). Note that the light-scattering band (fractions 3 to 4) contains substantial amounts of caveolin-3 but excludes >99% of the cellular protein. Fractions 3 to 4 represent the 5% to 35% sucrose interface, fractions at the bottom of the gradient represent the 40% sucrose cushion, and P indicates the insoluble pellet. The profile illustrated is from a resting culture but is representative of results obtained in control cultures and cultures treated with PMA for 30 minutes (ie, the recovery of RACK/caveolin and protein in the light-scattering fractions was not altered by treatment with PMA). E, Aliquots of protein pooled from fractions 3 to 4 (lane 1) or 7 to 12 (lane 2) were probed for PMCA (140 K), the Golgi 58-kDa protein, or caveolin-3 as indicated. Only the regions of the gel to which the indicated proteins migrate are shown. F, Electron micrograph of the caveolin-3-enriched fraction isolated from cardiomyocytes. Caveolae appear as 50- to 100-nm vesicular structures and some curved membrane sheets. The morphology is similar to that observed for caveolin-enriched domains in other cell types. Bar=0.1 µm.

atypical PKCλ to the caveolin-enriched domain (data not shown). These results indicate that the effects of PMA are due to the specific activation of phorbol ester-sensitive PKC isoforms (rather than to a nonspecific effect on membrane structure).

The time course for the PMA-induced translocation of PKC isoforms to the caveolin-enriched fraction is shown in Figures 2B and 3. For each PKC isoform, immunoreactivity was detected at 5 minutes, became further elevated by 15 minutes, and remained relatively constant for at least the

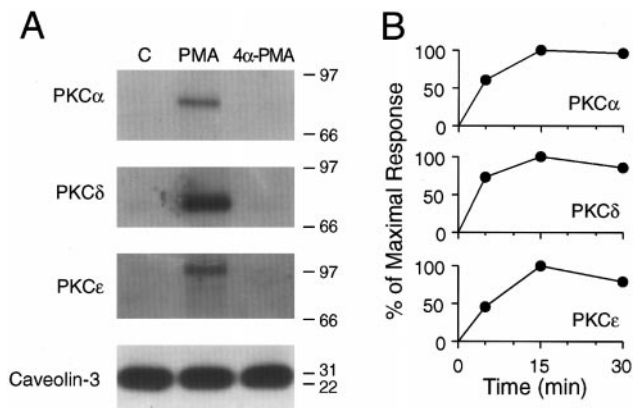


Figure 2. PKC isoforms translocate to caveolae in response to stimulation with PMA. Myocytes were incubated at 37°C in the absence or presence of PMA or 4 α -PMA (both at 300 nmol/L) for various time periods and then were processed according to the detergent-free method to prepare caveolae. Equal amounts of caveolae proteins (7 μ g/lane) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-PKC or anti-caveolin-3 antibodies as described in Materials and Methods. A, Representative autoradiograms of caveolin-enriched fractions. Positions of molecular mass standards are indicated on the right (in kilodaltons). C indicates control. B, Time course of PMA-dependent translocation of PKC isoforms to caveolae. Results are representative of data from 4 separate experiments.

subsequent 2 hours of stimulation with PMA. The PMA-induced recruitment of PKC isoforms to the caveolae fraction occurred in the absence of any changes in the yield of caveolin-enriched vesicles or caveolin-3 recovery (Figure 2B, bottom). It should be noted that the caveolin-enriched fraction of cells treated with PMA for 30 minutes contained between 10% and 20% of total cellular PKC immunoreactivity for each phorbol ester-sensitive isoform. Although this represents an enormous enrichment in phorbol ester-sensitive PKC isoforms in the caveolae compartment (which excludes >99% of the cellular proteins), the heavy fractions retain the vast majority of each PKC isoform. Figure 3 shows that prolonged treatment with PMA ultimately leads to the disappearance of PKC isoforms from the caveolin-enriched domain. For each isoform, the time course of this process

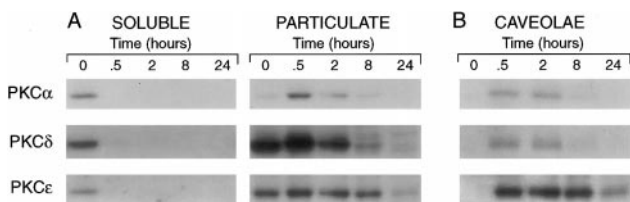


Figure 3. Kinetics of PMA-induced PKC isoform downregulation in cardiomyocyte caveolae. Myocytes were incubated at 37°C in the absence or presence of PMA (300 nmol/L) for the indicated time periods and then either were partitioned into soluble and particulate fractions (A) or were processed according to the detergent-free method to prepare caveolae (B). Proteins (30 μ g/lane soluble, 80 μ g/lane particulate, or 9 μ g/lane caveolae) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with the indicated anti-PKC antibodies as described in Materials and Methods. Results are representative of data from 3 separate experiments. Any minor differences in immunoreactivity were not consistent across the series of experiments and therefore are not considered to be significant.

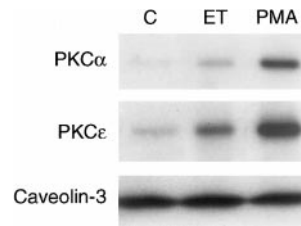


Figure 4. PKC isoforms translocate to caveolae in response to stimulation with endothelin. Myocytes were incubated in the absence or presence of endothelin (ET; 100 nmol/L for 2 minutes) or PMA (300 nmol/L for 15 minutes) and then were processed according to the detergent-free method to prepare caveolae. Equal amounts of caveolae proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-PKC or anti-caveolin-3 antibodies as described in Materials and Methods. Results are representative of data from 3 separate experiments. An effect of endothelin to selectively translocate to the caveolae fraction PKC α and PKC ϵ (but not PKC δ) also was observed when cells were stimulated with endothelin for shorter (1-minute) and somewhat longer (5-minute) intervals. C indicates control.

parallels the kinetics of downregulation in the particulate fraction of the cell. Thus, there is a substantial downregulation of PKC α and PKC δ by 8 hours, and this process is complete by 24 hours. Consistent with previous observations in cardiomyocyte preparations,^{20,26} the downregulation of PKC ϵ is a slower process, with the abundance of PKC ϵ immunoreactivity reduced to \approx 20% of the initial level only at the 24-hour time point. Collectively, these results indicate that stimulation with PMA leads to the temporary stable association of phorbol ester-sensitive PKC isoforms with the caveolae fraction of neonatal rat ventricular myocytes.

Further experiments using endothelin provide evidence that the translocation of PKC isoforms to caveolae is a component of the physiological activation process. Endothelin is known to stimulate phosphoinositide hydrolysis and induce the diacylglycerol-dependent translocation of novel PKC isoforms (PKC δ and PKC ϵ) from the soluble to the particulate fraction of cardiomyocytes.^{20,26} Figure 4 demonstrates that endothelin promotes the rapid association of PKC α and PKC ϵ with the caveolae fraction (70% and 100% over basal, respectively). Although these responses to endothelin are relatively modest compared with the 10-fold increase in PKC isoform immunoreactivity in the caveolae fraction elicited by PMA, this result is consistent with previous studies demonstrating that direct pharmacological activation of PKC isoforms with PMA is more robust than physiological activation through G protein-coupled receptors.²⁰ Endothelin does not alter the level of immunoreactivity for the phorbol ester-sensitive PKC δ isoform in caveolae (data not shown). This result was somewhat surprising, but there is a precedent for a translocation/activation process in cardiomyocytes that is selective for PKC α and PKC ϵ (and does not involve PKC δ).²⁸ Consistent with previous reports that the subcellular distribution of PKC λ is not altered by endothelin,^{20,26} endothelin does not promote the translocation of the phorbol ester-insensitive PKC λ isoform to caveolae.

The recruitment of activated PKC isoforms to caveolae could lead to the activation of downstream targets at this site. A key signaling cascade that might be activated by PKC

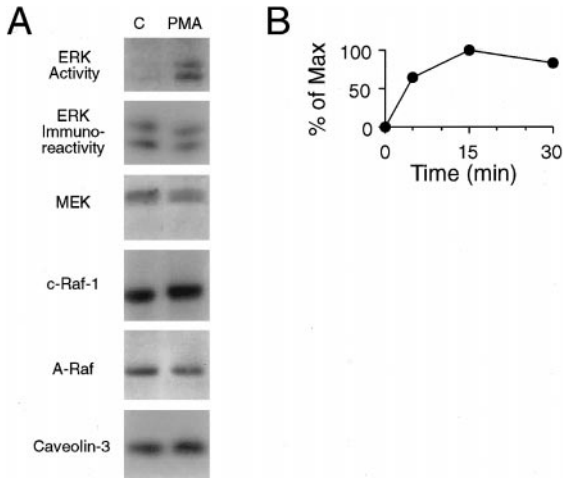


Figure 5. Caveolae contain components of the ERK cascade; the 42- and 44-kDa isoforms of ERK become activated in response to PMA. Myocytes were incubated at 37°C in the absence or presence of PMA (300 nmol/L) for various time periods and then were processed according to the detergent-free method to prepare caveolae. Equal amounts of caveolae proteins were either subjected to the in-the-gel phosphorylation reaction or probed with the indicated antibodies as described in Materials and Methods. A, Representative experiment demonstrating equivalent immunoreactivity for A-Raf, c-Raf-1, MEK, ERK, and caveolin-3 in caveolin-enriched fractions from control myocytes and myocytes treated with 300 nmol/L PMA for 15 minutes; ERK activity is markedly enhanced in the PMA-treated myocytes. B, Time course for PMA-dependent activation of ERK in cardiomyocyte caveolae. Results are representative of data from 3 separate experiments. C indicates control.

isoforms in caveolae is the ERK subfamily of mitogen-activated protein kinases; this pathway involves the sequential phosphorylation and activation of Raf, MEK, and the 42- and 44-kDa isoforms of ERK. Only a few sporadic reports have investigated the presence of components of the ERK cascade in caveolae, and the results have been inconsistent. Thus, there is evidence that epidermal growth factor induces the transient appearance and activation of c-Raf-1 in a detergent-free caveolae fraction from Rat-1 fibroblasts.²⁹ However, MEK and the 44-kDa isoform of ERK are reported to be excluded from caveolin-enriched fractions (when detergent is used in the preparation of the complex¹³). Although the 42-kDa isoform of ERK has been detected in caveolae prepared from resting human fibroblasts and mouse lung tissue^{13,30} (and there is very recent evidence that it becomes activated in response to stimulation with PDGF in human fibroblasts),³¹ a recent study reports the release of ERK from the caveolin-enriched fraction of MDCK cells on activation with PMA.¹⁶ Accordingly, studies illustrated in Figure 5 examined the extent to which components of the ERK signaling cascade partition to the caveolin-enriched domain of neonatal ventricular myocytes. Since certain signaling molecules may be recruited to caveolae (or conversely may disappear from caveolae) after agonist stimulation, samples were prepared under resting conditions and after stimulation with PMA.

Previous studies established that neonatal myocytes express 2 isoforms of Raf, A-Raf and c-Raf-1, and that both are activated (albeit with distinct kinetics) by PKC activators.³²

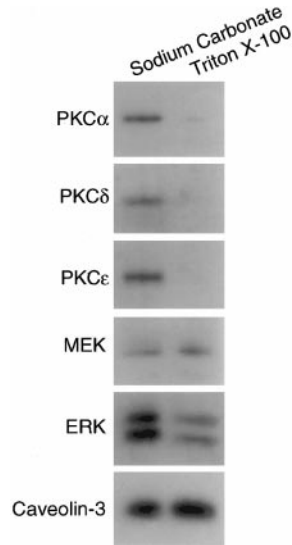


Figure 6. Immunodetection of PKC isoforms, MEK, and ERK in caveolin-enriched fractions prepared using sodium carbonate-based (detergent-free) and Triton X-100-based purification schemes. Myocytes were incubated with PMA (300 nmol/L) for 30 minutes at 37°C before the preparation of caveolin-enriched fractions according to detergent-free or Triton X-100-based methods. Equivalent amounts of caveolin-enriched fractions were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the indicated antibodies.

Figure 5 shows that A-Raf and c-Raf-1, as well as MEK and the 42- and 44-kDa isoforms of ERK, cosegregate to caveolae and that these species are present in equal abundance in the caveolin-enriched fractions of resting and PMA-treated cardiomyocytes. To determine whether the colocalization of these signaling molecules represents a mechanism to facilitate signaling through an ERK phosphorylation cascade localized to the caveolae membrane domain, we measured in-the-gel MBP kinase activity in caveolae from control and PMA-stimulated cardiomyocytes. Figure 5 shows that ERK is activated in response to PMA (2.2 ± 0.08 -fold over basal, $n=3$, $P<0.05$) and that the kinetics of ERK activation parallels the time course for PKC translocation to caveolae (ie, the increase in ERK activity is detectable by 5 minutes and reaches a maximal level at 15 minutes, which persists for an additional 15 minutes of stimulation with PMA).

The detection of MEK and both isoforms of ERK in caveolae contrasts with the previous inability to detect these species in this compartment.¹³ To determine whether technical issues related to differences in the method used to prepare caveolae could explain these discrepant results, we performed immunoblot analysis on caveolae prepared according to both sodium carbonate- and Triton X-100-based purification schemes. Figure 6 shows that the presence of detergent does not alter the abundance of MEK in caveolae. In contrast, detergent modestly reduces the abundance of ERK, dramatically reduces the abundance of PKC α , and totally eliminates PKC δ and PKC ϵ from this compartment. These results indicate that the association of PKC isoforms and (to a lesser extent) ERK with caveolae is weak (or lipid dependent) such that these species are removed by the Triton X-100-based extraction protocol.

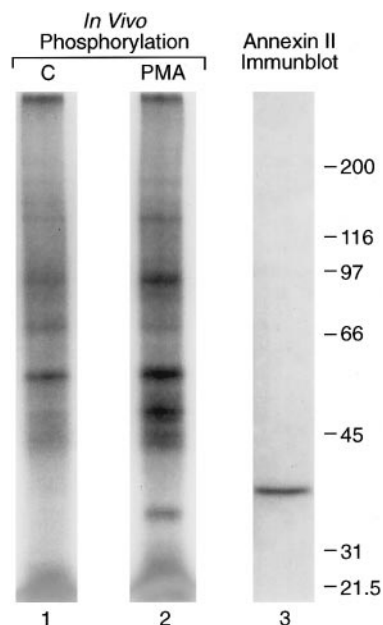


Figure 7. PMA leads to the phosphorylation of proteins in caveolin-enriched complexes. Shown is a representative autoradiogram of caveolae fractions (5 μ g/lane) from 32 P-labeled myocytes incubated without (lane 1) or with PMA (300 nmol/L for 30 minutes at 37°C, lane 2). To compare the relative mobilities of the \approx 36-kDa protein phosphorylated in the caveolin-enriched fraction and the heavy chain of annexin II (with a reported apparent molecular mass of 36 to 39 kDa), caveolae proteins were transferred to nitrocellulose and probed for annexin II immunoreactivity in parallel (lane 3). Numbers on the right indicate the positions of the molecular mass standards (in kilodaltons). Results are representative of data from 2 separate experiments. C indicates control.

Finally, to determine whether activation of PKC leads to the phosphorylation of resident caveolae proteins, neonatal cardiomyocytes were metabolically labeled with [32 P]orthophosphate and then left untreated or stimulated with PMA for 30 minutes before the isolation of caveolin-enriched complexes. Figure 7 shows that stimulation with PMA enhances the phosphorylation state of proteins in the 80- to 90-kDa range (which could at least in part represent phosphorylation of the MARCKS protein, an 80-kDa protein that is a known PKC substrate and is abundant in the immature rat heart^{26,33}) and several \approx 42- to 50-kDa species, and leads to the appearance of a smaller \approx 36-kDa phosphoprotein in the caveolae fraction. With respect to the \approx 36-kDa phosphoprotein, the heavy chain of annexin II is a known PKC substrate with a similar molecular mass (36 to 39 kDa) that has been reported to compartmentalize to caveolae^{13,19}; we performed further studies to determine whether this lower molecular mass phosphoprotein species in caveolae fractions from PMA-treated cardiomyocytes is the heavy chain of annexin II. Although Figure 7 (far right lane) shows that annexin II immunoreactivity is detectable in cardiomyocyte caveolae, its mobility is considerably slower than the lower molecular mass phosphoprotein species in caveolae fractions from PMA-treated cardiomyocytes. These results argue that the \approx 36-kDa protein substrate for PKC in cardiomyocyte caveolae is not the heavy chain of annexin II.

Discussion

Although several laboratories have initiated a systematic analysis of the signaling molecules resident in caveolae, the literature on PKC isoform localization to caveolin-enriched domains is largely limited to an analysis of PKC α , the data are inconsistent (with phorbol esters variably recruiting PKC α to caveolae or inducing a loss of PKC α from this compartment^{12,16}), and there is very limited information on the molecular composition of caveolae in cardiomyocytes. Studies reported herein establish that under basal conditions the level of immunoreactivity for various PKC isoforms in cardiomyocyte caveolae is at the limits of detection using the conventional visualization methods and that these molecules are selectively recruited to caveolae after pharmacological and physiological stimuli (PMA and endothelin). Several factors may reconcile our findings and the discrepant data already published in the literature. First, the observations that the association of PKC isoforms with caveolae does not withstand treatment with Triton X-100 and that PKC isoforms are detected in unstimulated cardiomyocytes only when a sensitive ECL method is applied to the analysis identify 2 technical factors (the nature of the extraction scheme used before the isolation of caveolae and the method for detection) that may explain at least some of the prior inconsistent results. As demonstrated in this study and recently suggested by others, the precise nature of the agonist stimulation protocol, which if sufficiently intense would lead to a loss of PKC isoform immunoreactivity due to the downregulation of the enzyme, also may influence the recovery of PKC isoforms in the caveolae fraction.¹⁶ Finally, it is possible that caveolae from different cell types act in a specialized fashion to concentrate and functionally regulate cytoplasmically oriented signaling molecules.

There are at least 3 mechanisms that potentially could mediate the recruitment of PKC isoforms to caveolin-enriched domains. First, on the basis of recent studies demonstrating that PKC α interacts with the scaffolding domain of caveolin-3 (and caveolin-1, but not the structurally distinct homologous region of caveolin-2¹⁵), it is reasonable to speculate that activation of PKC isoforms might lead to a conformational change that exposes an aromatic amino acid-rich consensus caveolin-binding domain that facilitates targeting to caveolae. Second, despite the partitioning of only a minor component of total cellular RACK immunoreactivity to caveolae, RACK proteins still could constitute a second class of proteins that bind PKC only in the presence of PKC activators in this compartment. Third, proteins such as the annexins have been reported to bind negatively charged phospholipids such as phosphatidylserine and act as PKC binding proteins.^{34,35} Available evidence suggests that this process involves protein-protein interactions that are facilitated or stabilized by lipid cofactors (such as phosphatidylserine). This final mechanism would be particularly susceptible to treatment with 1% Triton X-100 (which removes resident lipid-modified signaling proteins such as G protein $\beta\gamma$ complexes and Ras from caveolae⁶) and therefore might be particularly pertinent to the results reported herein. Finally, it should be noted that these mechanisms may not be mutually exclusive. Pertinent to this point is the observation

that PMA recruits all phorbol ester-sensitive PKC isoforms (α , δ , and ϵ) to the caveolae fraction, whereas endothelin induces a rapid increase in the association of PKC α and PKC ϵ (but not PKC δ) with the caveolae fraction. This observation is consistent with the notion that activated PKC isoforms are recruited to caveolae via mechanisms that are distinct for isozymes.

These studies demonstrate that PMA induces the phosphorylation of several proteins in the caveolin-enriched domain. Although this further establishes caveolae as a target for PKC isoform actions, there are several issues that require further study. First, we have yet to determine whether PKC activators lead to the phosphorylation of proteins already associated with caveolae in their unphosphorylated form in the absence of PKC activators or whether PKC activation leads to the de novo association of proteins that are susceptible to PMA-dependent phosphorylation within the caveolae compartment. Second, the identity of the phosphorylating kinase was not determined. In this regard, the *in vivo* phosphorylation experiments cannot discriminate a direct effect of PKC isoforms to phosphorylate resident substrate proteins from an indirect effect secondary to PKC isoform-dependent activation of downstream kinases. In particular, a casein kinase II-like enzyme, which is activated by PKC, has been reported to be the dominant serine kinase in the caveolae fraction³⁶ (although this conclusion was based on an analysis of kinase activity in caveolae prepared from resting cells and does not entirely rule out a dominant function for PKC isoforms after their recruitment to caveolae by PMA). The evidence that stimulation with PMA leads to the local activation of ERK establishes ERK as yet an additional candidate kinase in this compartment. Third, the substrates phosphorylated in cardiomyocyte caveolae have yet to be identified. This study considered the possibility that 1 of the phosphoproteins in caveolae from PMA-stimulated cardiomyocytes is the heavy chain of annexin II. Indeed, annexin II immunoreactivity was detected in cardiomyocyte caveolae. However, the electrophoretic mobilities of annexin II and the phosphoprotein species in the caveolae fraction differ substantially, suggesting that annexin II is not a major substrate for the PKC-induced phosphorylation in caveolae. The identification of the proteins phosphorylated in cardiomyocyte caveolae should provide insights into the functional consequences of PKC-activated signaling events in this compartment.

These studies are the first to identify cardiomyocyte caveolae as a meeting place for activated PKC isoforms and their targets, including the phosphorylation cascade that results in the activation of ERK. As such, this report lends further credence to the notion that caveolae represent "signaling processing centers," which orchestrate signaling events at the cell surface that influence cell function. Nevertheless, the full functional significance of this event remains to be determined. Because PKC activators have been reported to dramatically alter the morphology and functional activity of caveolae,^{12,17} and caveolae act as a processing center for multiple receptor-dependent signaling events (which likely include adrenergic receptors, cholinergic receptors, and their downstream targets),^{37,38} it is tempting to speculate that activation of PKC can influence subsequent signal output

from other receptors. Results reported herein also are consistent with recent evidence that components of the ERK cascade are concentrated in caveolae as a mechanism to facilitate agonist-dependent activation of this pathway.^{30,31} Indeed, the identification of all of the biochemical machinery for the ERK phosphorylation cascade in caveolae raises the possibility that the ERK signaling pathway performs specific functions in this location that cannot be accomplished elsewhere in the cell.

There is evidence that caveolin-1 acts as a general "kinase inhibitor" and a negative regulator of mitogenic growth at least in part through an effect to inhibit cellular activation through the Ras/ERK pathway.³⁹ Thus, it will be interesting to determine whether caveolae (and caveolin-3, which is structurally homologous to caveolin-1) also influences hypertrophic growth responses in cardiomyocytes. Apart from a potential role in the control of cardiomyocyte growth, PKC isoforms also phosphorylate an array of substrate proteins that fulfill functions that are unique to cardiomyocytes, including modulation of the rate of spontaneous contractile activity, regulation of intracellular calcium ion concentration, ischemic preconditioning of the heart, and the genesis of arrhythmias. The recent identification of a "loss-of-function" mutation in the caveolin-3 gene as the molecular basis for some forms of autosomal dominant limb-girdle muscular dystrophy⁴⁰ emphasizes the importance of caveolae in muscle cell biology. Studies of patients in this kindred (which are likely to be accompanied by studies in genetically altered mice with specific mutations or targeted disruption of the caveolin-3 gene) will delineate the importance of PKC (and other second-messenger molecules) targeting to caveolae in the contractile or growth response of the heart.

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