Caveolin Interaction with Protein Kinase C

**ISOENZYME-DEPENDENT REGULATION OF KINASE ACTIVITY BY THE CAVEOLIN SCAFFOLDING DOMAIN PEPTIDE**

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**EXPERIMENTAL PROCEDURES**

**Materials—**The vectors for the insect and mammalian expression systems containing PKC-α, -e, or -ζ cDNA were made as described previously (12, 13). MBP 4–14 substrate peptide and peptide e were purchased from Upstate Biotechnology (Lake Placid, NY) and Peninsula Laboratories (Belmont, CA). PKC isoform-specific antisera were purchased from Research and Diagnostic Antibodies (Berkeley, CA). All other reagents not otherwise noted were obtained from Sigma. Caveolin peptides were prepared as described previously (9). The following four peptides were used mostly: the caveolin-1 nonscaffolding domain peptide (NRDPKHLNDVKKIDFEDVIAEPEGTHSF, amino acid residues 53–81); the caveolin-1 scaffolding domain peptide (DKWVCSHALFESIKKYVMYK, amino acid residues 54–73); and the caveolin-3 scaffolding domain peptide (DGVWRVSYTFTTFTVSKYWYR, amino acid residues 55–74). These peptides were made by Research Genetics (Huntsville, AL) and Bio Synthesis (Lewisville, TX).

**Recombinant PKC Isoforms—**PKC-α, -e, and -ζ were overexpressed in H5 (high five) insect cells (Invitrogen, San Diego, CA), as described previously (14). The supernatant prepared from infected insect cells was used as a source of PKC isoforms.

**Sucrose Gradient Centrifugation and Immunoblotting—**PKC isoforms were overexpressed in COS cells using LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD). After completion of the recombinant PKC expression, the cells were sonicated, centrifuged and the supernatant was subjected to sucrose gradient centrifugation. The crude supernatant was layered on a 5–20% sucrose gradient in Buffer A (100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol). The gradients were centrifuged at 40,000 rpm for 16 h at 4 °C and fractions were collected for analysis by SDS-PAGE and immunoblotting. The immunoblotting was performed as described previously (12, 13) with PKC isoform-specific antisera purchased from Upstate Biotechnology (Lake Placid, NY) and Peninsula Laboratories (Belmont, CA).

**Phorbol ester treatment—**The cell inhibitory effect of caveolin on protein kinase C activity was examined by phorbol ester treatment of cells. Phorbol ester was added to the cell culture medium at a final concentration of 10 μM. After 1 h of incubation, the cells were solubilized and subjected to sucrose gradient centrifugation as described above. The fractions containing PKC isoforms were collected for analysis by SDS-PAGE and immunoblotting. The immunoblotting was performed as described previously (12, 13) with PKC isoform-specific antisera purchased from Upstate Biotechnology (Lake Placid, NY) and Peninsula Laboratories (Belmont, CA).

**Cell Preparation—**Cells were solubilized by the addition of a lysis buffer (10 mM Tris, pH 7.4, 1% NP-40, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) and subjected to sucrose gradient centrifugation as described above.

**Immunoelectron Microscopy—**Immunoelectron microscopy was performed as described previously (12, 13). The preparation of the cell for immunoelectron microscopy was performed as described above.

**RESULTS**

**Caveolar localization of protein kinase C and the regulation of caveolar function by protein kinase C are well known. This study was undertaken to examine whether caveolin subtypes interact with various protein kinase C isoforms using the caveolin scaffolding domain peptide. When protein kinase Cα, -e, and -ζ were overexpressed in COS cells followed by subcellular fractionation using the sucrose gradient method, all the isoforms detected in the same fraction as caveolin.**

**The scaffolding domain peptide of caveolin-1 and -3, but not -2, inhibited the kinase activity and autophosphorylation of protein kinase Cα and -ζ, but not of protein kinase C-ε, overexpressed in insect cells. Truncation mutation studies of the caveolin-1 and -3 peptides demonstrated that a minimum of 16 or 14 amino acid residues of the peptide were required for the inhibition or direct binding of protein kinase C. Thus, the caveolin peptide physically interacted with protein kinase C and regulated its function. Further, this regulation occurred in a protein kinase C isoform-dependent manner. Our results may provide a new mechanism regarding the regulation of protein kinase C isoform activity and the molecular interaction of protein kinase C with its putative binding proteins.**

Several studies from independent laboratories have demonstrated that multiple phorbol ester-sensitive, classic protein kinase C (PKC) isoforms are accumulated in caveolae. Enrichment of PKC-α was detected by immunoelectron microscopy (1) as well as by the cell fractionation technique using buoyant density gradient centrifugation (2). PKC-β and -γ were also detected in caveolae as separated by the silica coating method from lung endothelial cells (3). Whether all PKC isoforms, including nonclassic isoforms, are similarly accumulated in caveolae, however, remains uncharacterized. PKC also regulates the function and formation of caveolae. Caveolin, the major structural protein of caveolae, contains a conserved consensus phosphorylation site of PKC as well as of v-Src (4). Phorbol ester treatment of the cell inhibits caveolae-mediated internalization and markedly reduces the number of caveolae (1). Further, activation of PKC-α by phorbol esters dislocates this isoform from caveolae. Thus, PKC is not only present in caveolae, but interacts functionally with caveolae.

It has been suggested that caveolin by itself regulates the function of certain molecules accumulated in caveolae. Caveolin may directly interact with G protein, Src kinase, and Ha-Ras as has been demonstrated using a short stretch of membrane proximal regions of the cytosolic amino terminus caveolin domain (or the caveolin scaffolding domain) (5–8). Further, a small peptide derived from this domain bound G protein directly and regulated its function (9). The specificity of binding caveolin to target molecules has been confirmed, using a random peptide sequence library, by identifying a common amino acid sequence motif (ψXψXXXψ or ψXXXψψψψ; ψ is an aromatic residue), which is contained in many targeting molecules, including PKC, that are found in caveolae (9).

We examined the potential interaction of caveolin and PKC in this study. We first examined the subcellular localization of a representative isoform from each PKC subgroup, i.e. PKC-α (the classic subgroup), -ε (the novel subgroup), and -ζ (the atypical subgroup) (10, 11). We also investigated the functional interaction of caveolin with PKC by the use of the caveolin scaffolding domain peptide. We demonstrate that the PKC isoforms are located similarly in the caveolin fraction. However, these isoforms are regulated by the caveolin scaffolding domain peptide in a caveolin subtype- and a PKC isoform-dependent manner.
Caveolin-rich membrane domains were purified from COS cells by a previously described method using sodium carbonate (15, 16). Briefly, a 5–35% discontinuous sucrose gradient was formed and centrifuged at 39,000 rpm for 16 h at 4 °C. A light-scattering band was confined to the 5–35% sucrose interface. From the top of each gradient, a total of 13 fractions were collected and applied to SDS-polyacrylamide gel electrophoresis (PAGE) (6% gel). Immunoblotting was performed with 1:2000 dilution of an antiserum to the PKC isoenzyme using the ECL detection system (Amersham Corp.).

**Purification of PKC—**Recombinant PKC isoenzymes overexpressed in insect cells were purified with a DEAE-anion exchange column and a phosphorylcholine affinity chromatography column as described previously (17) and modified by us (14).

**PKC Activity Assay—**PKC activity was measured with peptide substrates (MBP 4–14 for PKC-α, peptide ε for PKC-ε and -ζ) as described previously using phosphocellulose disc paper (18).

**Autophosphorylation of PKC—**The reaction mixture (50 μl) contained 20 μM Tris-HCl (pH 7.5), 20 μM ATP, 20 μM MgCl₂, 1 μM CaCl₂, 10 μM phosphol 12-myristate 13-acetate, 0.28 mg/ml phosphatidyl serine, 0.3% Triton X-100, 100 μM [γ-³²P]ATP, and 0.2 μg of the purified PKC. The reaction mixture was incubated at 30 °C for 10 min, and the assay was terminated by the addition of 6 × Laemmli sample buffer. After boiling in a water bath, samples were subjected to 6% SDS-PAGE and autoradiography.

**Peptide Binding Assay—**Caveolin peptides were immobilized on Affi-Gel 10 (Bio-Rad). The resin was incubated in the presence of 2 mM caveolin peptide overnight, followed by inactivating the resin binding sites with ethanolamine. The binding of the caveolin peptide to the resin was monitored by the recovery of the peptide remained in the supernatant, which was similar among the peptides examined (50–65%). After washing of the peptide-bound resin, the resin and the cytosolic solution overexpressing PKC were incubated for 10 h in a buffer containing 20 mM Tris-HCI (pH 7.4), 0.5% Triton X-100, 150 mM NaCl, and protease inhibitors. The resin was washed extensively twice with an excess amount of the buffer, followed by the addition of Laemmli buffer and boiling for 5 min. After centrifugation, the supernatant was applied to SDS-PAGE, followed by immunoblotting using an anti-PKC antiserum.

**RESULTS AND DISCUSSION**

**Subcellular Distribution of PKC Isoenzymes—**PKC isoenzymes (α, ε, and ζ) representing each subgroup (classic, novel, and atypical subgroups) were overexpressed in COS cells, followed by the purification with the sucrose gradient method. A single light-scattering band corresponding to a low density complex was observed, mainly in fractions 5 and 6 of these gradients as previously reported (20). An equal-sized aliquot from each of the 13 sucrose gradient fractions was subjected to SDS-PAGE. The distribution of overexpressed PKC isoenzymes and endogenous caveolin-1 in COS cells was detected by immunoblot analysis. Caveolin-1 was detected only in fractions 5 and 6 (Fig. 1). The PKC isoenzymes were detected in the same fractions as caveolin-1, but also in fractions 10–13. We did not find any major differences in the distribution in the caveolin fraction among the PKC isoenzymes; the relative amount of the PKC isoenzyme accumulated in the caveolin fraction versus that in the non-caveolin fraction was similar among the isoenzymes. Previous studies have demonstrated similar caveolar accumulation of the classic PKC isoenzymes (α, β, and γ) (1, 9).

Taken together, the presence in the caveolin fraction may be a common property among the PKC family members.

**Effect of Caveolin Scaffolding Domain Peptides on PKC Activity—**Because caveolin regulates the function of certain molecules accumulated in caveolae (5–8), we next examined whether caveolin functionally interacts with the above PKC isoenzymes as well. PKC-α, -ε, and -ζ were overexpressed in H5 cells as the dominant positive isoenzyme. The cytosolic prepartation of infected insect cells showed a significant increase in PKC activity from that of noninfected H5 cells (a 200-fold increase for PKC-α, a 14-fold increase for PKC-ε, and a 13-fold increase for PKC-ζ). We evaluated the interaction of caveolin with PKC using the caveolin scaffolding domain peptide (9) because the purified caveolin was easily aggregated at high concentrations (21, 22) and not applicable to our in vitro assays.

The caveolin-1 and -3 scaffolding domain peptides inhibited PKC-α and -ζ dose dependently (Fig. 2). The degree of inhibition was similar between the caveolin-1 and -3 peptides. The caveolin peptide of different lots or the peptide made by different manufactures showed similar inhibitory effect. In contrast, a peptide from the non-scaffolding domain of caveolin-1 and the caveolin-2 scaffolding domain peptide had no inhibitory effect. When PKC-ε was examined, however, we found a different pattern of regulation. The caveolin-1 scaffolding peptide appeared to increase PKC activity slightly, but the other peptides had no effect.

We also examined the specificity of the peptide-mediated inhibition by the use of a series of deletion mutants of the caveolin-1 scaffolding domain peptide: 20-mer (amino acid residues 82–101), 18-mer (amino acid residues 84–101), 16-mer (amino acid residues 86–101), 14-mer (amino acid residues 88–101), and 12-mer (amino acid residues 90–101) peptides. As shown in Fig. 3, 18-mer and 16-mer peptides inhibited PKC-α activity to a similar degree as 20-mers while 14-mers and 12-mers were mostly ineffective, suggesting at least 16 amino acid residues are required to inhibit PKC and/or that the residues 82–88 are critical for inhibition. Similar results were obtained with PKC-ζ (Fig. 3B), suggesting that the regulation of kinase activity by the caveolin peptide is very similar between PKC-α and -ζ.

**Effect on PKC Autophosphorylation—**The above results showed that the caveolin peptide inhibited the kinase activity as assessed by the use of peptide PKC substrates. We examined whether the phosphorylation of PKC protein itself, another index of kinase activation, was regulated by the caveolin peptide. Autophosphorylation of PKC is also a common property among the PKC isoenzymes (19).

The caveolin-1 scaffolding domain peptide inhibited the autophosphorylation of the purified PKC-α and -ζ (Fig. 4A and C, left panel). Inhibition was in a dose-dependent manner as observed in the inhibition of the peptide substrate phosphorylation. Higher caveolin-1 peptide concentrations, however, ap-
peared to be required to inhibit the PKC autophosphorylation than the exogenous peptide substrate phosphorylation (Fig. 2). The caveolin-3 scaffolding domain peptide similarly inhibited the autophosphorylation of PKC-\(\alpha\) and -\(\zeta\). Neither the caveolin-1 non-scaffolding domain peptide nor the caveolin-2 scaffolding domain peptide had inhibitory effect (Fig. 4, right panel). In contrast, PKC-\(\epsilon\) autophosphorylation was unaffected by these peptides. The lack of inhibition on the autophosphorylation of PKC-\(\epsilon\) agreed with the lack of inhibition on its kinase activity (Fig. 2B). Further, inhibition of the autophosphorylation of the purified PKC suggested that the interaction between the peptide and PKC was direct.

**Effect on PDBu Binding**—We also assessed the effect of caveolin peptides on \(^{[3]H}\)PDBu binding, another index of PKC activation. PKC-\(\alpha\) is activated classically by increasing amounts of diacylglycerol in membranes resulting from agonist

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**Fig. 2. Regulation of PKC activity by caveolin peptides.** The cytoplasmic fraction of H5 insect cells overexpressing PKC-\(\alpha\) (A) -\(\epsilon\) (B), and -\(\zeta\) (C) was incubated for assays in the presence of various caveolin peptides (0–10 \(\mu\)M) (open circles, the non-scaffolding domain caveolin-1 peptide, amino acid residues 53–81; closed circles, the caveolin-1 scaffolding domain peptide, residues 82–101; triangles, the caveolin-2 scaffolding domain peptide, residues 54–73; squares, the caveolin-3 scaffolding domain peptide, residues 55–74). Means \(\pm\) S.E. from four independent assays are shown.
induced hydrolysis of inositol phospholipids. As shown in Fig. 5, the caveolin-1 scaffolding domain peptide inhibited the \(^{3}H\)PDBu binding to PKC-\(\alpha\) in a dose-dependent manner. The cytosolic protein prepared from non-infected H5 cells had negligible \(^{3}H\)PDBu binding, which was not affected by the scaffolding domain peptide (data not shown).

These findings suggest that the interference with phorbol ester binding is another mechanism of inhibiting PKC-\(\alpha\) activity although this may not contribute largely because the degree of inhibiting PDBu binding was minor (27% inhibition at the peptide concentration of 10 \(\mu\)M). Interestingly, PKC-\(\alpha\) has a...
caveolin binding motif in the diacylglycerol binding region in the regulatory domain (amino acid residues 58–65).

Direct Interaction with Caveolin Peptide—Finally, we examined whether the caveolin peptide physically interacts with PKC. We tested the binding of PKC-α and -ζ to a series of deletion mutants of the caveolin-3 scaffolding domain peptide. These peptides were immobilized on resin, to which PKC-α and -ζ overexpressed in H5 cells were bound. The peptide bound resin was washed, followed by the elution and detection of PKC-α and -ζ by immunoblotting. Resin without the peptide was used as control.

We demonstrated that PKC activity is regulated by the caveolin peptide through direct interaction. The mechanisms of regulation included the direct inhibition of kinase activity and autophosphorylation of PKC as well as, at least partially, phosphol ester binding to PKC-α. More important, the pattern of regulation depended on the combination of the caveolin subtype and the PKC isoenzyme. We do not know, however, whether all these mechanisms similarly contribute to the regulation of PKC activity by caveolin in intact cells.

We have also demonstrated that the caveolin peptide inhibited a classic (PKC-α) and an atypical (PKC-ζ) isoenzyme, but not a novel (PKC-ε) isoenzyme, suggesting that the caveolin peptide is an isoenzyme-selective inhibitor of PKC. The caveolin interacting motif (ΨΩφΩΩφΩΩ or ΨΩΩΨΩΩΩφΩΩ) is present in the kinase domain of all three PKC isoenzymes. Interestingly, the motif within the kinase domain in PKC-α and -ζ is located in the corresponding region while the motif in PKC-ε is located in a distinct region within the kinase domain.

The caveolin peptide may also be useful to selectively measure the activity of the atypical PKC isoenzymes by inhibiting the classic and novel PKC isoenzymes in tissues or cell homogenates. Alternatively, the peptide may be used to assess the contribution of the classic and novel PKC isoenzymes in kinase reactions that involve various PKC isoenzymes. The effect of the caveolin peptide on the other PKC isoenzymes of each subgroup, such as PKC-β, -γ of the classic subgroup or PKC-α of the atypical subgroup, was not examined in this study and needs to be verified. Biochemical properties are, however, usually maintained within the same subgroup.

The regulation of the enzymatic activity by scaffolding proteins has been best exemplified by A kinase anchoring proteins (AKAP) (23, 24). AKAP79, a member of AKAP in postsynaptic membranes, acts as an anchoring protein for protein kinase A, calcineurin, and PKC. AKAP79 accumulates these enzymes within a microdomain and enables them to process a rapid cascade of phosphorylating/dephosphorylating reactions (23). AKAP79 is also known to inhibit the activity of calcineurin and PKC (24), indicating that the role of AKAP79 is not simply anchoring signaling molecules, but to regulate their activity.

Caveolin also acts as an anchoring protein for signaling molecules, including various kinases, within the plasma membrane. It has been demonstrated that caveolae accumulate an even greater diversity of signaling molecules than AKAP79. Investigators have demonstrated that multiple isoforms of the same molecule family are located in caveolae as well. Examples include the isoforms of nonreceptor tyrosine kinase (Yes, Lck, Src, Fyn, Lyn) (3) and multiple heterotrimeric G proteins (Gq, Gs, Gs, and Gp). These isoforms belong to the same enzyme family, but are known to transmit a distinct signal from each other. How does the segregation of the signaling pathways transmitted by each enzyme isoform occur when multiple isoforms of the same enzyme are similarly located in caveolae? Subpopulations of caveolae may be present within the same cell, which are made of different sets of caveolin subtypes and/or accumulate a distinct set of signaling molecules. Alternatively, caveolin (or caveola) itself may play a role in this segregation. It has been demonstrated that the function of the enzymes found in caveolae is altered when caveolae are disrupted in cells (3) or enzyme activity is assayed in the presence of caveolin fragments or peptides (5–9). These data suggest that caveolin/caveola itself is capable of modifying the enzyme function. Our findings further suggest that this regulation occurs in a caveolin subtype- and an enzyme isoform-dependent manner and that the subtype of caveolin, to which each enzyme isoform is anchored to, may play an important role. Such selective regulation may occur through suppressing the activity of caveolin-inhibitable enzyme isoforms as opposed to non-inhibitable isoforms, which are both located in caveolae. We do not know, however, whether all the enzymes accumulated in caveolae, such as the insulin receptor and other GPI-anchored proteins, are similarly inhibited by caveolin.

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