Online Data Supplement

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Materials and methods

Chemicals

All was purchased from Sigma (St. Louis, MO) except the followings: Endothelial-Growth-Medium-2 from Cambrex (East Rutherford, NJ); Phorbol-12,13-dibutyrate (PDBu), bisindolylmaleimide I (BIM) and Rottlerin (specificity: PKC_{δ} IC_{50}=3-6μM; PKC_{α}, β, γ IC_{50}=30-42μM) from Calbiochem (San Diego, CA); LY333531 (specificity: $PKC_{\beta I}$ IC_{50}= 4.7nM; $PKC_{\beta II}$ IC_{50}= 5.9nM[1]) from A.G. Scientific (San Diego, CA); Goat anti-VE-cadherin and rabbit anti-$PKC_{\beta II}$ and δ from Santa Cruz Biotechnology (Santa Cruz, CA); Donkey anti-goat Cye3 and anti-rabbit Cye2 from Jackson ImmunoResearch Laboratories (West Grove, PA); Hoechst 33258 and pentahydrate-(bis-benzimide) from Invitrogen (Carlsbad, CA). The FRET reagents CKAR, myr-palmCKAR and RFP-$PKC_{\beta II}$ are generous gifts from Drs. Alexandra Newton and Roger Tsien at University of California, San Diego.

OGTT

Oral glucose tolerance tests were performed by administrating a 40% glucose solution (1g/kg) via a feeding tube. The blood glucose level was monitored every 20min for 120min with a glucometer.

Cell Culture

HCMEC were maintained in EGM-2 and their early passages (4-5) were used. Cells were transfected with plasmid DNA with Nucleofector™ II (Amaxa Biosystems, Cologne, Germany) according to the manufacturer's instruction. Briefly, plasmid DNA (3 μg) was mixed with 100 μl of cell suspension (5×10^6 cells/ml) in a cuvette followed by addition of 500 μl EGM-2. After 15-min incubation, cells were washed and allowed to grow for 24-48 hrs. This procedure allowed us to obtain consistently 60-80% transfected cells.

Transendothelial Resistance

The cell-cell adhesive barrier property was determined by measuring TER as described previously[2, 3]. Briefly, HCMEC seeded at $10^5/cm^2$ onto ECIS electrode arrays (Applied Biophysics, Troy, NY) were sub-cultured to confluence with a minimal basal electric resistance of 5,000Ω. A current (1-V, 4,000-Hz) was supplied through a 1-mΩ resistor to approximate a constant-current source. The resistance and capacitive resistance were collected and analyzed with ECMS 1.0 software (CET, Inc., Coralville, IA). For each intervention, the TER value is expressed as the value normalized to the baseline and then averaged from multiple groups and presented as Means±SE.

Immunofluorescence microscopy

Cells were incubated for 2hr at 37°C and vessels overnight at 4°C with an antibody against VE-cadherin (6.6μg/ml) together with an anti-$PKC_{\beta II}$ or anti-PKC_{δ} (2μg/ml), followed by an secondary antibody for 1hr. The specificity of these antibodies for each isoform has been previously reported[4]. Nuclei were labeled with Hoechst. Samples were

viewed under a Zeiss Axiovert 200M microscope equipped with an Apotome system allowing optical sectioning. Samples were mounted on slide in *SlowFade*® Gold (Invitrogen, Carlsbad, CA) and images acquired with a Zeiss 63x oil immersion objective, 1.4 NA, at 300 nm Z-spacing through narrow band pass filters for FITC, Texas Red and DAPI with a Zeiss Axiocam MRm camera. Images were background subtracted and thresholded based on fluorescence intensity values obtained with secondary antibody controls (when one of the primary antibodies was omitted). For co-localization experiments, the 3D data sets were aligned using fiduciary markers (TetraSpeck Fluorescent microsphere standards 0.2 μm, Invitrogen). Images were processed and analyzed with Metamorph 7.1 (Molecular Devices, Downingtown, PA). All images are 3D reconstructions at the indicated depth and displayed in maximum rendering mode.

Immunoblotting

Cell lysates were obtained from confluent HMVEC treated with 200nM PDBu or vehicle for 10min. Subcellular fractionation (surface plasma membrane, intracellular plasma membrane organelles and cytosol fractions) were performed with the plasma membrane protein extraction kit from BioVision (Mountain View, CA) according to the manufacturer's instructions. The samples were electrophorized with SDS-PAGE (10%) and transferred to nitrocellulose membrane that were blocked with 3% bovine serum albumin and incubated overnight (4°C) with primary followed by secondary antibodies. The immunoreactive bands were detected with Supersignal-West-Pico chemiluminescence (Pierce, Rockford, IL) and their intensities quantified with ImageJ (NIH, Bethesda, MA).

FRET

HCMEC were transfected with mpCKAR and RFP-$PKC_{\beta II}$, serum starved for 2hrs and imaged at 37°C, 5% CO_2 in endothelial basal medium (EBM, Cambrex) with a Zeiss 40X oil immersion objective on a Zeiss Axiovert 200M. Joint cells with a minimum of two neighbors expressing both mpCKAR and RFP-$PKC_{\beta II}$ were selected. Both CFP and YFP emissions were collected simultaneously every 15s with a Dual-view module (Photometric, Tucson, AZ), a coolSNAP$_{HQ-2}$ camera and appropriate filters (HQ535/40 and HQ480/30, Chroma Technology Corp., Rockingham, VT) controlled by MetaFluor 7.0 (Molecular Device). Individual images were corrected for photobleaching with MetaFluor Analyst software (Molecular Devices). CFP/FRET images and values were computed and pseudocolor images created with MetaFluor Analyst. Pooled data are expressed as normalized means±SEM.

References used in the supplement

1. Ishii H, Jirousek MR, Koya D, Takagi C, Xia P, Clermont A, Bursell SE, Kern TS, Ballas LM, Heath WF, Stramm LE, Feener EP, King GL. Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor. *Science.* 1996;272:728-731.
2. Breslin JW, Sun H, Xu W, Rodarte C, Moy AB, Wu MH, Yuan SY. Involvement of ROCK-mediated endothelial tension development in neutrophil-stimulated microvascular leakage. *Am J Physiol Heart Circ Physiol.* 2006;290:H741-750.
3. Tinsley JH, Breslin JW, Teasdale NR, Yuan SY. PKC-dependent, burn-induced adherens junction reorganization and barrier dysfunction in pulmonary

microvascular endothelial cells. *Am J Physiol Lung Cell Mol Physiol.* 2005;289:L217-223.

4. Tinsley JH, Teasdale NR, Yuan SY. Involvement of PKCdelta and PKD in pulmonary microvascular endothelial cell hyperpermeability. *Am J Physiol Cell Physiol.* 2004;286:C105-111.

Supplemental Figures

Figure I

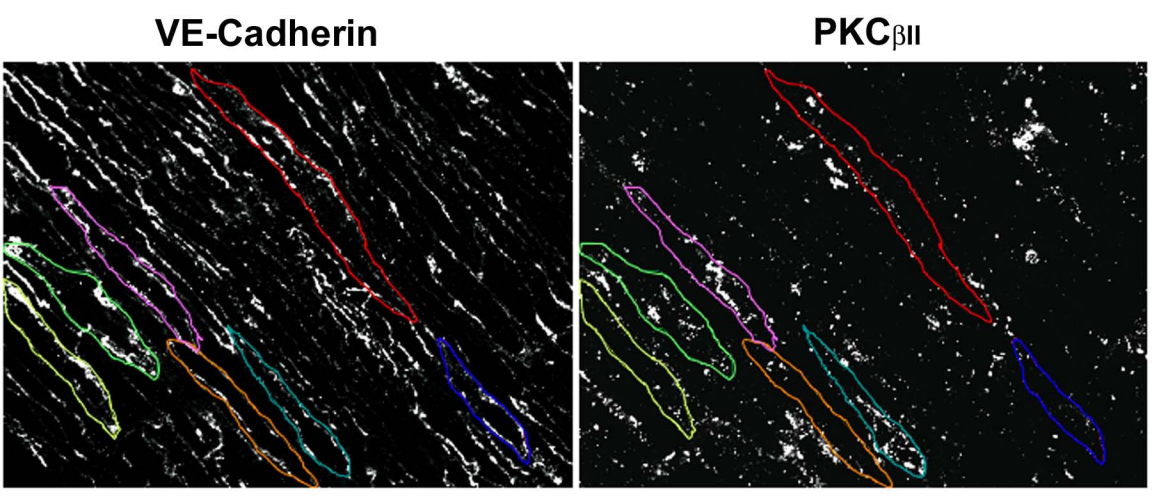


Figure I. *Enface* coronary artery fluorescence quantification**.** Background fluorescence intensity was determined by averaging the fluorescence intensity obtained in images of enface coronary artery (all acquired in the same conditions) in which the primary antibody was omitted. Threshold values were determined by averaging the threshold value necessary to obtain less than 1% fluorescent pixels in background subtracted images of enface coronary artery in which the primary antibody was omitted. For each PKCs, the average fluorescence intensity per cell was compared between lean and diabetic Zucker rats. Regions were drawn using VE-cadherin as a marker of the cell border to delineate individual cells. In this example, 7 different cells are demarcated by colored line. Only cells with continuous VE-cadherin marker were chosen for quantification purposes. These regions were then transferred to the corresponding z-stack of PKC labeled cells and the average fluorescence intensity measured and normalized to the area (for all planes with more than 1% highlighted pixels) and value for each plane averaged for a whole cell.

Figure II

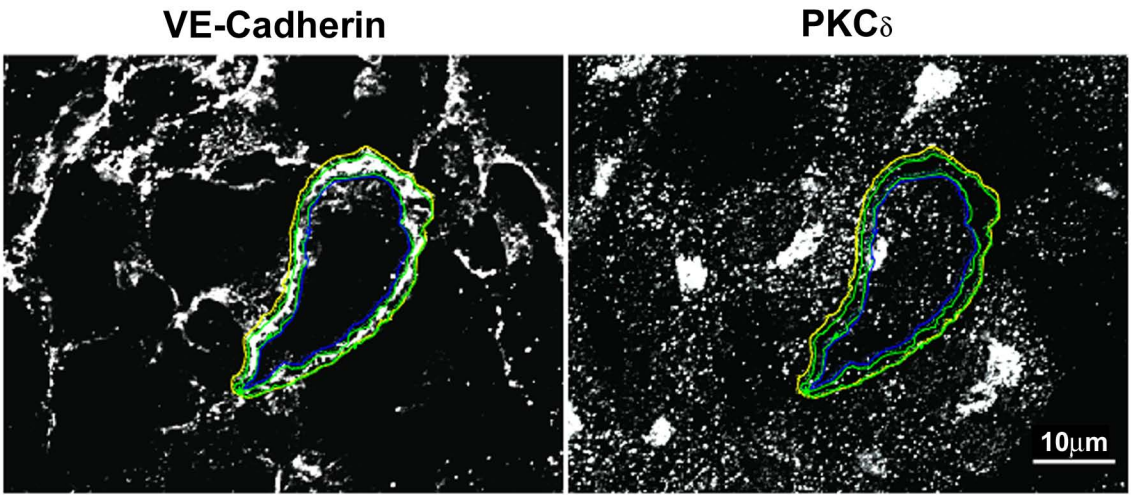
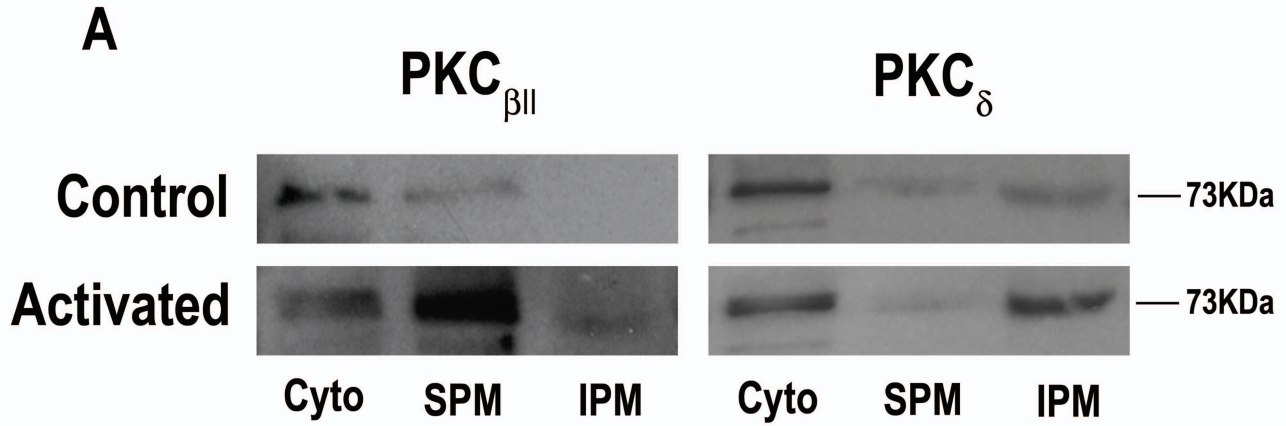


Figure II. Subcellular quantification of fluorescence intensity in HCMEC. Individual cells were selected based on complete junction formation with neighboring cells and continuous VE-cadherin labeling. For $PKC_{\beta II}$ cell selection, in addition to the previous criteria the cells also had to express RFP- $PKC_{\beta II}$. Tree regions were drawn on the optical section with the most VE-cadherin labeling (as shown in this example). The cell junction area was determined using the area marked by VE-cadherin (green dashed line). The cytosolic space was defined by the area internal to the VE-cadherin labeling (blue dashed line). The whole cell area was determined using the outer edge of the VE-cadherin labeling (yellow dashed line). All regions were then transferred to the corresponding z-stack of PKC labeled cells and the average fluorescence intensity measured and normalized to the area (for all planes with more than 1% highlighted pixels) and value for each plane averaged for a whole cell. PKC density is defined by the number of labeled pixels per volume area for each region. The percent colocalization is defined as the percent of PKC labeled pixel colocalizing with VE-cadherin pixel for the cell junction and whole cell volume area.

Figure III


A
PKC βII
PKC δ
Control
Activated
73KDa
73KDa
Cyto
SPM
IPM
Cyto
SPM
IPM

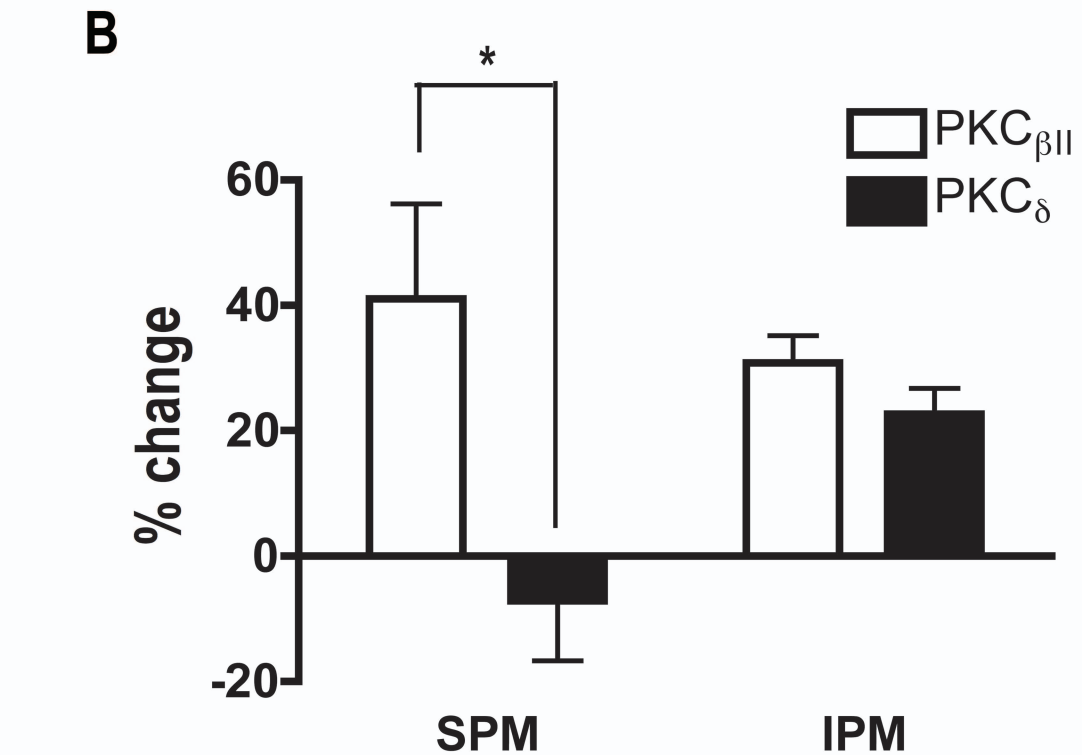

B
*
PKC βII
PKC δ
60
40
20
0
-20
% change
SPM
IPM

Figure III. Subcellular fractionation assays showing $PKC_{\beta II}$ redistribution to surface membrane while PKC_{δ} remaining in cytosolic pool and intracellular membrane. **(A)** Western blots of $PKC_{\beta II}$ and PKC_{δ} associated with the cytosol (Cyto), surface plasma membrane (SPM) and intracellular plasma membrane (IPM) in non-activated and PDBu-activated HCMEC (200nM, 10min). **(B)** Normalized band intensity expressed as % of control. Upon PDBu stimulation, $PKC_{\beta II}$ redistributed to the cell surface plasma membrane while PKC_{δ} was concentrated in the intracellular membranous pool. In the surface membranous fraction, activation of PKC produced a 41% increase and 7% decrease in the band intensity of $PKC_{\beta II}$ and PKC_{δ}, respectively ($*p<0.05$). In the intracellular plasma membranous fraction, $PKC_{\beta II}$ increased to a less extent but PKC_{δ} increased to a larger extent (41% vs. 31% for $PKC_{\beta II}$ and -7% vs 23% for PKC_{δ}), consistent with the imaging data.

Figure IV

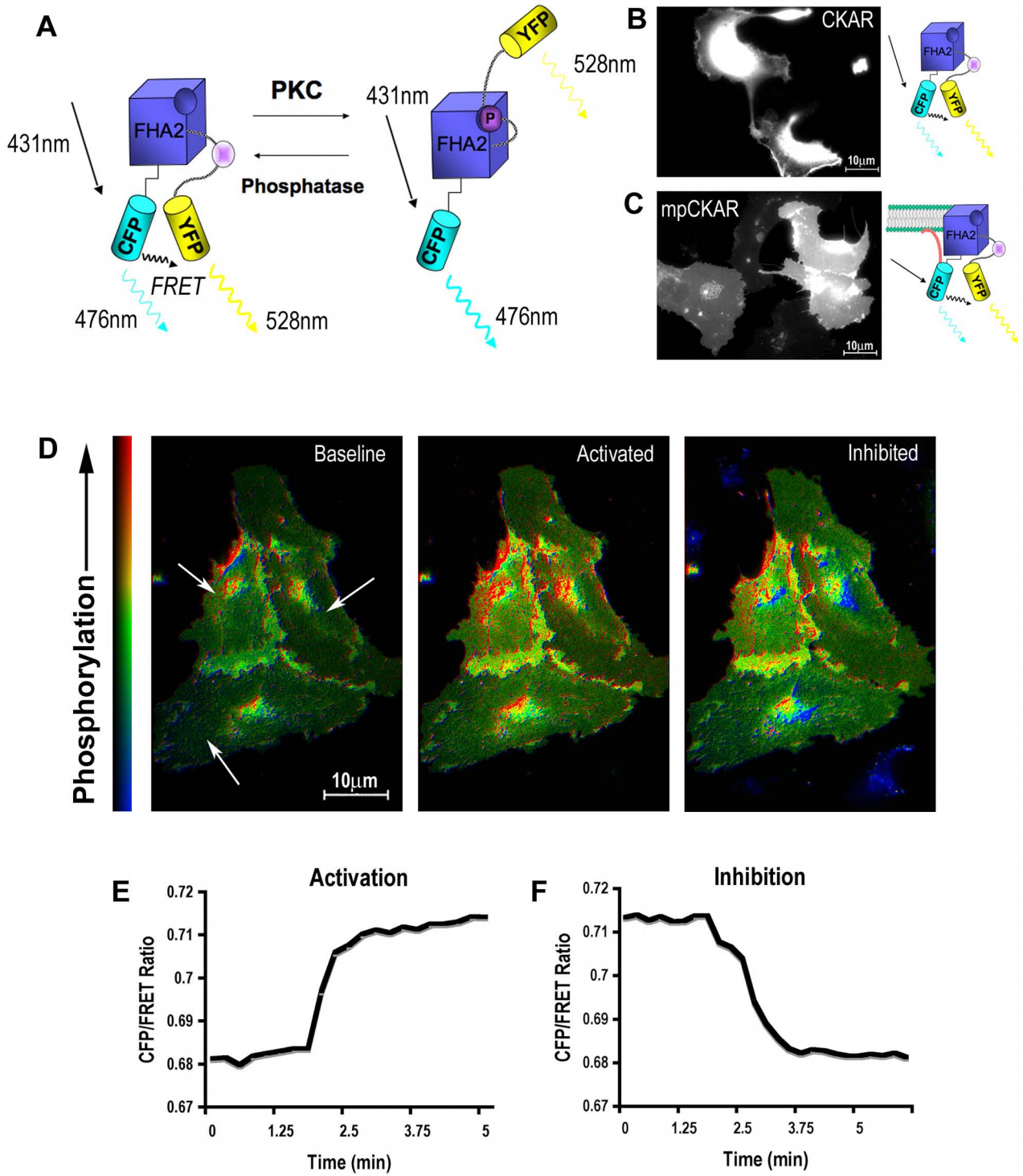

A
PKC
Phosphatase
431nm
FHA2
CFP
YFP
FRET
476nm
528nm
P
B
CKAR
10μm
C
mpCKAR
D
Phosphorylation
Baseline
Activated
Inhibited
E
Activation
CFP/FRET Ratio
Time (min)
F
Inhibition

Figure IV. FRET analysis of PKC-substrate phosphorylation in human coronary endothelial cells. (**A**) Schematic structure of the C kinase activity reporter (CKAR). The reporter is composed of an FHA2 phospho-threonine binding domain containing a PKC substrate sequence. The N and C terminals are conjugated to cyan and yellow fluorescent proteins (CFP & YFP) allowing intra-molecular transfer of fluorescent resonance energy. Substrate phosphorylation induces a conformational change that separates YFP from CFP, reducing FRET. (**B&C)** A membrane-targeting CKAR (mpCKAR) was obtained by adding a sequence encoding for myristoylation and palmitoylation. Both CKAR (Figure **B**) and mpCKAR (**C**) were transfected into HCMEC with high efficiency. Black and white images show CFP channel only (Scale bar=10μm). Since mpCKAR offered a superior spatial resolution over CKAR due to elective expression on cell surface and in distinct cytosolic plasma membrane domains, it was selected for the subsequent experiments. Clusters of mpCKAR within the cytosolic space surrounding the nucleus were identified to be associated with vesicle-like structures and they appeared to constantly move between the cytosol and surface membrane (**Video I**). (**D**) The FRET signals were acquired and computed as CFP/FRET ratio and displayed as pseudocolor images, white arrows point at each individual cell. Typical patterns of CFP/FRET in confluent HCMEC are shown as pseudocolor images at baseline, following PKC activation by PDBu (200nM) and PKC inhibition by BIM (10μM), (Scale bar=10μm). (**E&F)** Changes in CFP/FRET over time following PKC activation and inhibition.

Figure V

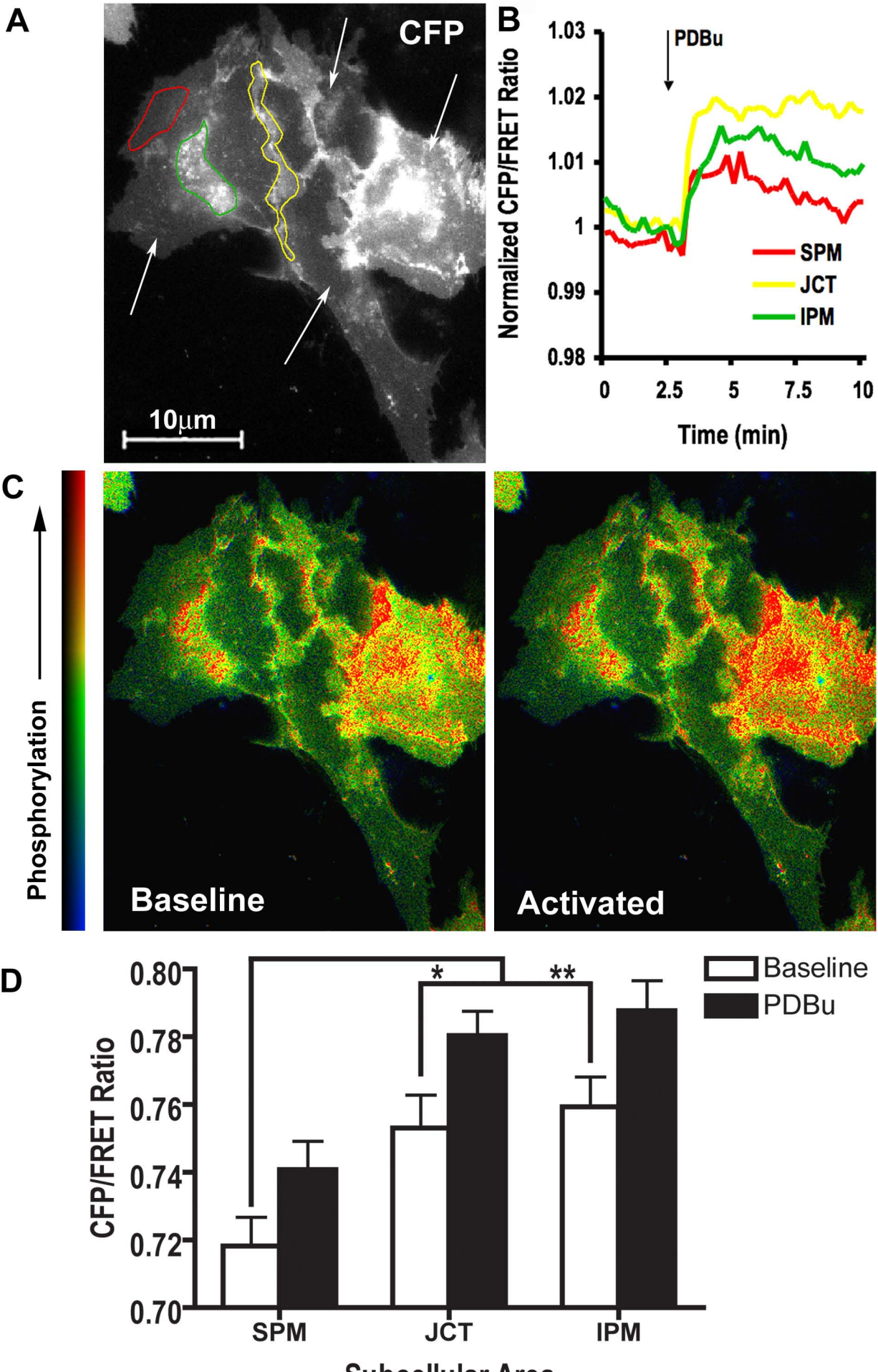

A
CFP
10μm
B
PDBu
Normalized CFP/FRET Ratio
1.03
1.02
1.01
1
0.99
0.98
SPM
JCT
IPM
0
2.5
5
7.5
10
Time (min)
C
Phosphorylation
Baseline
Activated
D
CFP/FRET Ratio
0.80
0.78
0.76
0.74
0.72
0.70
*
**
Baseline
PDBu
SPM
JCT
IPM
Subcellular Area

Figure V. Basal PKC activity in coronary endothelial cells. **(A)** mpCKAR expression in HCMEC (CFP channel), white arrows point to individual cells. Demarcated areas representing the surface plasma membrane (SPM, red), cell-cell junction (JCT, yellow), and intracellular plasma membrane (IPM, green). (**B**) Normalized changes in CFP/FRET over time at 3 sub-cellular locations (colors corresponding to demarcated areas in A). (**C**) Pseudocolor images of FRET before and after PKC activation. (**D**) Pooled analysis of CFP/FRET changes (n=17-26 cells from ≥3 experiments). Basal PKC activity was higher at JCT (*$p<0.05$) and IPM (**$p<0.01$) compared to SPM. PDBu further increased PKC activity ($p<0.05$ vs. basal) to the same extent in all three areas.

Video I. Intracellular plasma membrane shuffling. This video shows a typical HCMEC expressing mpCKAR. Note the shuffling of intracellular vesicles labeled with mpCKAR. Two examples of fast moving vesicles within the cell are indicated by arrows. Images shown were acquired with Zeiss Axiovert 200m, dual view and 40x objective with a 15 second time interval. Only images acquired with the CFP channel are shown and displayed at a rate of 4 frames per second (Scale bar = 10μm).