Interaction of PKC and NOS in signal transduction of microvascular hyperpermeability

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Huang, Qiaobing, and Yuan Yuan. Interaction of PKC and NOS in signal transduction of microvascular hyperpermeability. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2442–H2451, 1997.—Our previous studies have shown that inflammatory mediators increase microvascular permeability through a phospholipase C-nitric oxide synthase (NOS)-guanylate cyclase cascade. The aim of this study is to delineate in more detail the signaling pathway leading to microvascular hyperpermeability. Endothelial cytosolic calcium and the apparent permeability coefficient of albumin (P_a) were measured in isolated and perfused coronary venules. Histamine stimulated a rapid increase in cytosolic calcium followed by a transient elevation in P_a. The NOS inhibitor N′-monomethyl-L-arginine (L-NMMA) and the guanosine 3',5'-cyclic monophosphate-dependent protein kinase G (PKG) inhibitor KT-5823 abolished the hyperpermeability but did not affect the calcium response to histamine. Similarly, the calcium ionophore ionomycin produced a calcium spike preceding venular hyperpermeability. Blockage of the NOS-PKG cascade inhibited the increase in P_a whereas the endothelial calcium was still elevated on administration of ionomycin. Furthermore, the relationship between protein kinase C (PKC) and the calcium-NOS-PKG pathway in modulation of venular permeability was investigated. Stimulation of PKC with phorbol 12-myristate 13-acetate (PMA) dramatically increased basal P_a without significantly changing the cytosolic calcium level. The selective PKC inhibitor bisindolylmaleimide abolished the effect of PMA but did not alter the effect of histamines on P_a. In contrast, both L-NMMA and KT-5823 were able to greatly attenuate the increase in P_a caused by PMA. These results suggest that 1) elevation of endothelial cytosolic calcium is an early signaling event preceding nitric oxide (NO) synthesis in the transduction of endothelial hyperpermeability, and 2) activation of PKC may alter the endothelial barrier function partially through the modulation of NO production.

nitric oxide; protein kinase C; protein kinase G; cytosolic calcium; endothelium

INFLAMMATORY CYTOKINES increase microvascular permeability by binding to their cognate receptors, leading to activation of a series of second messengers in the endothelial cell (12, 14). Different intracellular signaling processes have been proposed, and considerable evidence suggests the role of endothelial cytosolic calcium and protein kinases in the control of endothelial barrier function (14). The calcium theory was supported by studies demonstrating that calcium is required in the maintenance of endothelial integrity (30) and that calcium ionophores facilitate transendothelial flux of water and proteins (7, 27). Histamine, a typical endogenous mediator that produces leaky sites in postcapillary venules during inflammation, has been shown to promote albumin diffusion by stimulating calcium influx (27). The production of inositol phosphates via phospholipase C (PLC) has been considered as an initial event responsible for calcium mobilization (5, 14), yet the specific site of action of calcium has not been identified. In this regard, calcium may modulate the endothelial barrier property by directly acting on structural proteins or indirectly through other second messengers. Within this context, a potential target of elevated intracellular calcium is constitutive nitric oxide synthase (NOS) in the endothelial cell, because calcium is a potent stimulator of the enzyme (28). Indeed, evidence is accumulating that activation of NOS and subsequent production of nitric oxide (NO) and guanosine 3',5'-cyclic monophosphate (cGMP) plays an important role in upregulation of the transport process by which fluid and macromolecules move across the vascular endothelium (1, 16, 21, 23, 26, 32). However, the stimulating effect of calcium on NOS and the regulatory role of NOS in the barrier function have been studied as separate processes, and evidence is limited regarding the sequential linkage between calcium and NO in the signal transduction of microvascular permeability. Moreover, it is not clear whether the calcium-dependent NO-mediated pathway is involved in macromolecular transflux induced by agonists that are different from histamine, such as phorbol 12-myristate 13-acetate (PMA), which is known to exert a hyperpermeability effect through activation of protein kinase C (PKC; 13–15).

Based on the conventional concepts and our recent findings, we hypothesize that the mechanism of agonist-induced microvascular hyperpermeability involves two different pathways characterized by the activation of NOS-protein kinase G (PKG) and upregulation of PKC, respectively (Fig. 1). On one hand, receptor occupancy by histamine and similar agonists stimulates PLC to produce d-myo-inositol 1,4,5-trisphosphate, which causes an internal release and further influx of calcium, leading to upregulation of NOS and NO production. NO stimulates guanylate cyclase (GC) to produce cGMP, a potent activator of cGMP-dependent PKG. The NO-cGMP-PKG system may participate in the barrier regulatory process through modulation of the structure and function of proteins in the endothelial cytoskeleton, intercellular junction, and cell-matrix contact. On the other hand, activated PLC can catalyze, in parallel with the formation of d-myo-inositol 1,4,5-trisphosphate, the production of diacylglycerol (DAG), which in turn causes upregulation of PKC. PKC may alter endothelial permeability by directly acting on the endothelial structural proteins and/or by indirectly modifying the activity of the common signaling protein NOS. The interaction between the calcium-NOS-PKG and
the PKC pathways may play a homogeneous role in modulation of microvascular permeability.

We have previously tested, in part, the above hypothesis by examining the sequence of events from PLC activation to NO production and PKG upregulation during stimulation by various agonists and growth factors (32, 34, 36). This study was undertaken to delineate the signaling process in greater detail with respect to the linkage among calcium, PKC, and the NOS-PKG cascade. Specifically, the aims of this study were 1) to define the contribution and location of the calcium signal in the transduction of histamine-induced microvascular hyperpermeability and 2) to examine the effect of PKC on coronary venular permeability and its relationship with the NO pathway.

MATERIALS AND METHODS

General Preparation

Pigs weighing 9–13 kg were anesthetized with pentobarbital sodium (25 mg/kg iv) and heparinized (250 U/kg iv). After a tracheotomy and intubation the animal was ventilated. A left thoracotomy was performed, and the heart was electrically fibrillated, excised, and placed in 4°C physiological saline. The coronary sinus was cannulated, and 5 ml of India ink-gelatin-physiological salt solution were infused to clearly define venular microvessels. This solution was prepared by adding 0.2 ml of India ink (Koh-I-Noor, Bloomsbury, NJ) and 0.35 g of porcine skin gelatin to 10 ml of warm physiological salt solution and was filtered through P8 filter paper (Fisher Scientific, Pittsburgh, PA). A recent study (10) has demonstrated that the perfusion of the ink-gelatin mixture increases the basal permeability of coronary arterioles from $5.7 \pm 2.3 \times 10^{-7}$ cm/s to $8.7 \pm 3.3 \times 10^{-7}$ cm/s, raising the possibility that the venules exposed to the ink-gelatin solution during isolation were in a state of increased basal permeability. In this regard, the hyperpermeability effects of the agonists reported in this study might be underestimated. However, a large body of evidence supports the argument that the barrier function of the isolated venules is not seriously damaged by the ink-gelatin solution. Information regarding the validation and limitation of the technique has been provided in detail in our previous publications (33).

Solutions and Perfusates

An albumin-physiological salt solution (APSS) was used as a bathing solution while the microvessels were being dissected. It contained the following (in mM): 145.0 NaCl, 4.7 KCl, 2.0 CaCl$_2$, 1.17 MgSO$_4$, 1.2 NaH$_2$PO$_4$, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, and 3.0 3-(N-morpholino)propanesulfonic acid buffer. After 1% bovine serum albumin was added, the solution was buffered to a pH of 7.40 at 4°C and then filtered through a Millex-PF 0.8-µm filter unit (Millipore, Bedford, MA). The APSS used to perfuse the vessels had the same composition as mentioned previously but was buffered to a pH of 7.40 at 37°C. For the cytosolic calcium measurement, the fura 2-acetoxymethyl ester (AM) solution was prepared fresh daily at a concentration of 1 µM in APSS containing 0.1% dimethyl sulfoxide (DMSO). Before use the solution was agitated and filtered through an 0.8-µm pore filter.

Isolated and Perfused Microvessel Preparation

The methods for isolating and cannulating coronary venules have been described in detail in our previous study (33).
Briefly, a suitable venule (length 0.8–1.2 mm, diameter 20–60 µm) was dissected from surrounding myocardium in the dissecting chamber containing APSS at 4°C with the aid of a Zeiss stereo dissecting microscope. The vessel was transferred to a cannulating chamber, which was mounted on a Zeiss axiovert microscope. The isolated vessel was cannulated with a micropipette on each end and secured with 11–0 suture (Alcon, Fort Worth, TX). A third smaller pipette was inserted into the inflow pipette. The vessel was perfused with either APSS through the outer inflow pipette or APSS containing fluorescein isothiocyanate-albumin through the inner inflow pipette. Each cannulating micropipette was connected to a reservoir so that the vessel was perfused at a relatively constant intraluminal pressure and flow rate. The bath solution in the chamber was maintained at 37°C and pH 7.4 throughout the experiments. The image of the vessel was projected onto a Hamamatsu charge-coupled device intensified camera and was displayed on a high-resolution monochrome video monitor and recorded onto a VHS video recorder. Diameter of the vessel was measured on-line with a video caliper (Micrcirculation Research Institute, Texas A & M University, College Station, TX).

Measurement of Venular Permeability

The permeability of the vessel was measured with a fluorescence ratio technique (9). With an optical window of a video photometer positioned over the venules and adjacent space on the monitor, the fluorescent intensity from the window was measured and digitized on-line by a Power Macintosh computer. In each measurement the isolated venule was first perfused with APSS through the outer inflow pipette to establish a baseline intensity. The venular lumen was then rapidly filled with fluorochromes by switching the perfusion to the inner inflow pipette. This produced an initial step increase, followed by a gradual increase, in the intensity of fluorescence. There was a step decrease of intensity when fluorescein isothiocyanate-albumin through the inner inflow pipette. The apparent solute permeability coefficient of the fluorescently labeled molecules were washed out of the extravascular space, and the fluorescence intensities exerted by the calcium indicator at 340 vs. 380 nm was calculated with the computer program and presented as an index of the intracellular calcium level. To obtain the absolute concentration of calcium as a function of the 340/380 nm fluorescence ratio, the standard calibration experiment was conducted as previously described (7, 20).

Chemicals and Drugs

The selective PKC inhibitor bisindolylmaleimide (BIM), the specific PKG inhibitor KT-5823, and the calcium ionophore ionomycin were ordered from Calbiochem (San Diego, CA). PMA, Nα-monomethyl-L-arginine (L-NMMA), Nα-monomethyl-L-arginine (L-NMMA), and 8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP) were also from Calbiochem. The chemicals used to make the perfusate, including fluorescein isothiocyanate-albumin, were purchased from Sigma Chemical (St. Louis, MO). Histamine and sodium nitroprusside (SNP) were also purchased from Sigma. Bovine serum albumin was obtained from United States Biochemical (Cleveland, OH). The calcium indicator fura 2-AM was from Molecular Probes (Eugene, OR). Ionomycin, KT-5823, and BIM were diluted with 0.1% DMSO in physiological salt solution.

Experimental Protocol

In each experiment the cannulated venule was perfused at a constant perfusion pressure gradient of 20 cmH2O. According to our previous investigations (35, 36), this approach produced an approximate intraluminal pressure of 10 cmH2O and a flow velocity of 7 mm/s. The preparation was equilibrated for 45–60 min after cannulation, and the measurements were conducted under a temperature of 36–37°C and a pH of 7.35–7.45. In each vessel, limited (4–6) interventions were applied. The preparations were washed three times and allowed to equilibrate for 10–15 min between interventions. In some vessels the permeability and the calcium response were monitored over 4 h to ensure that the function of the venular endothelium was not significantly altered with time. For each experimental condition, P was measured two to three times at specific time points, and the average value was reported.

Effects of histamine, ionomycin, and PMA on cytosolic calcium. The changes in the endothelial cytosolic calcium were measured with a fluorescence ratio technique with the aid of a microscope photometry system (Photon Technology), which consisted of a Powerfilter high-speed dual-wavelength illuminator, a high-grade quartz fiber optic bundle, a D-104B single channel microscope photometer, and a 710 photon-counting photomultiplier tube (PMT). The cell-permeable form of a fluorescent dye, fura 2-acetoxymethyl ester (fura 2-AM), was used as the calcium indicator. To load the endothelium with the indicator, the porcine venule was cannulated as previously described and perfused for 20 min at a pressure gradient of 20 cmH2O through the inner inflow pipette with APSS containing 1 μM fura 2-AM and 0.1% DMSO. After loading was completed, the dye remaining in the extracellular space was washed off by switching the perfusion to the outer inflow pipette containing APSS for 20 min. The loading and washing procedures were performed in the dark at room temperature. The cannulated vessel was then aligned on the optical axis of a Zeiss axiovert microscope, which was connected to the PMT equipped with the photometry system. Emission fluorescence at 510 nm during excitation at 340 and 380 nm was detected by the PMT photon counting system through the measuring window positioned over the venule and recorded with FelIX, computer software associated with the photometer system. In each experiment, the excitation wavelength alternated between 340 and 380 nm at a rate of 650/s, and the fluorescence intensities at both wavelengths were recorded for 10 min at a 5-s interval. Background correction was automatically controlled by the program through subtraction of the background values from the measured values in real time. Because the wall of isolated venules mainly consisted of endothelial cells, the contribution of other cells to the fluorescence signals was small and therefore ignored. The ratio of fluorescent intensity at 340 vs. 380 nm was calculated with the computer program and presented as an index of the intracellular calcium level. To obtain the absolute concentration of calcium as a function of the 340/380 nm fluorescence ratio, the standard calibration experiment was conducted as previously described (7, 20). However, because the conclusion drawn from the study does not critically depend on the absolute value of intracellular calcium concentrations, the data are primarily represented as the fluorescence ratio.
Venules were treated for 20 min with the PKG inhibitor KT-5823 (10⁻⁶ M), the NOS inhibitor L-NMMA (10⁻⁴ M), or the inactive NOS inhibitor d-NMMA (10⁻⁴ M), and Pₐ was measured during activation of PKC with PMA (10⁻⁶ M). The changes in Pₐ in response to the PKC activator were compared before and after inhibition of NOS or PKG. In each experiment, Pₐ was measured at 5–7 min after administration of the respective agonists.

Data Analysis

For each experimental condition Pₐ was measured two to three times, and the values were averaged and presented. To compare the changes in Pₐ before and after administration of the agonists or inhibitors, the absolute values of Pₐ were also normalized to the control values obtained before the treatment and were reported as percentages of the controls. In the experiments on calcium, the level of cytosolic calcium was presented as the ratio of fluorescence at 340/380 nm. All the data were reported as means ± SE. Analysis of variance was applied to test the significance of the changes in venular permeability in response to the agonists and inhibitors. Fisher’s protected least-significant-difference analysis was used to evaluate the significance of intergroup differences. A value of P < 0.05 was considered significant for the comparisons. For all experiments n is given as the number of vessels studied, with each vessel representing a separate animal.

RESULTS

Data Cogent to Calcium-NOS-PKG Pathway

The changes in endothelial cytosolic calcium were measured in isolated and perfused coronary venules in the presence of various agonists. The ratio of fluorescence intensities acquired at the dual wavelengths represented the intracellular level of free calcium vs. bound calcium. The mean basal fluorescence ratio was 0.67 ± 0.03 (n = 6). Figure 2 shows representative calcium traces from single experiments. A typical pattern of calcium responses to histamine in six vessels was a biphasic increase in the fluorescence ratio with a rapid peak followed by a several-minute plateau (Fig. 2A). The onset and the time course of changes in calcium were closely related to those in Pₐ. Treatment of the vessels with either L-NMMA (Fig. 2B) or KT-5823 (Fig. 2C) did not have a significant effect on the basal level of fluorescence ratios (0.68 ± 0.03 with L-NMMA and 0.66 ± 0.02 with KT-5823). In the majority of vessels the inhibitors did not significantly change the magnitude and the time course of the calcium response to histamine. However, both L-NMMA and KT-5823 abolished the increase in Pₐ caused by histamine. Figure 3 summarizes the effects of L-NMMA, d-NMMA, and KT-5823 on histamine-induced changes in coronary venular permeability. Under control conditions histamine increased Pₐ by 189.21 ± 20.63% from a basal value of 2.70 ± 0.32 × 10⁻⁶ cm/s to 5.09 ± 0.82 × 10⁻⁶ cm/s (n = 5, diameter 55.60 ± 2.66 µm). This hyperpermeability effect was not altered by d-NMMA (n = 4, Pₐ values were 3.70 ± 0.34 × 10⁻⁶ cm/s before and 7.40 ± 0.88 × 10⁻⁶ cm/s after histamine) but was diminished during inhibition of NOS with L-NMMA (Pₐ = 3.12 ± 0.14 × 10⁻⁶ cm/s at control and 2.10 ± 0.52 × 10⁻⁶ cm/s after histamine in the presence of L-NMMA, n = 4), as well as during blockage of PKG.
with KT-5823, where $P_a$ slightly, but not significantly, increased from $2.98 \pm 0.38 \times 10^{-6}$ cm/s to $3.76 \pm 0.36 \times 10^{-6}$ cm/s in response to histamine in the presence of KT-5823 ($n = 4$). Similarly, the calcium ionophore ionomycin induced a calcium spike preceding the increase in $P_a$ ($n = 6$; Fig. 4A). Inhibition of NOS with L-NMMA (Fig. 4C) and PKG with KT-5823 (Fig. 4B) did not affect the elevation in intracellular calcium but prevented the permeability response to ionomycin. As shown in Fig. 5, ionomycin increased $P_a$ by $193.21 \pm 12.08\%$ from a basal value of $2.36 \pm 0.26 \times 10^{-6}$ cm/s to $4.49 \pm 0.26 \times 10^{-6}$ cm/s ($n = 4$, diameter $53.25 \pm 2.93 \mu m$). The effect was inhibited in the presence of L-NMMA ($P_a$ was from $3.06 \pm 0.23 \times 10^{-6}$ cm/s at control to $3.97 \pm 0.27 \times 10^{-6}$ cm/s after ionomycin, $n = 5$, diameter $53.00 \pm 1.92 \mu m$) or KT-5823 ($P_a$ was from $2.50 \pm 0.23 \times 10^{-6}$ cm/s at control to $2.80 \pm 0.13 \times 10^{-6}$ cm/s after ionomycin, $n = 4$, diameter $51.75 \pm 1.75 \mu m$).

It should be noted that neither histamine nor ionomycin significantly altered the venular diameter. Therefore, the $P_a$ values reflected the changes in the barrier function of the endothelium.

### Data Cogent to PKC Pathway

Similar to the results obtained from our previous studies (36), activation of PKC by treating the vessels with the specific activator PMA caused a dramatic increase in coronary venular permeability. Importantly, there were no changes in the level of endothelial cytosolic calcium on administration of PMA ($n = 3$) (Fig. 6). Furthermore, the PKC inhibitor BIM did not significantly alter the basal $P_a$ but abolished the hyperpermeability effect of PMA in a dose-dependent fashion (Fig. 7). Under control conditions PMA caused a dramatic increase in $P_a$ by $290.94 \pm 78.31\%$ ($n = 7$, diameter $63.86 \pm 3.14 \mu m$). This effect was greatly attenuated in the presence of $10^{-7}$ M BIM ($P_a$ increased by $156.08 \pm 18.66\%$, $n = 4$, diameter $59.75 \pm 2.32 \mu m$) and was abolished at higher concentrations of the inhibitor ($139.97 \pm 20.82\%$ at $10^{-6}$ M BIM, $n = 4$, diameter $43.25 \pm 3.08 \mu m$, and $133.15 \pm 4.91\%$ at $10^{-5}$ M BIM, $n = 4$, diameter $58.00 \pm 1.08 \mu m$). In contrast, BIM did not significantly alter the hyperpermeability effects of the agents known to activate the NO pathway (Fig. 8). Under control conditions the increases in $P_a$ on administration of histamine, SNP, and 8-BrcGMP were $189.21 \pm 20.63\%$ ($n = 5$, diameter $55.60 \pm 2.66 \mu m$), $267.59 \pm 63.96\%$ ($n = 4$, diameter $66.50 \pm 11.32 \mu m$), and $176.86 \pm 23.48\%$ ($n = 7$, diameter $54.86 \pm 0.68 \mu m$), respectively. After treatment with BIM, these agonists were still able to increase the $P_a$ value by $184.30 \pm 16.89\%$ ($n = 9$), $223.93 \pm 32.49\%$ ($n = 3$), and $187.44 \pm 5.49\%$ ($n = 5$), respectively. Furthermore,
BIM did not change the time course and the transience of the effect of histamine on venular permeability. Consistent with our previous investigation, neither the agonists nor the inhibitors caused changes in the vessel diameter. The data indicate that inhibition of PKC blocked the effect of PMA but did not affect the hyperpermeability response mediated by the NO pathway.

In contrast, inhibition of the NOS-PKG cascade greatly attenuated the hyperpermeability effect of PMA (Fig. 9). In L-NMMA-treated vessels, PMA caused an increase in $P_a$ from a basal value of $4.28 \pm 0.44 \times 10^{-6}$ cm/s to $7.26 \pm 1.08 \times 10^{-6}$ cm/s ($n = 8$), which was in an extent less than the control response. Similarly, PMA caused a lower but still high $P_a$ in venules treated with KT-5823 $(2.66 \pm 0.24 \times 10^{-6}$ cm/s at control and $4.39 \pm 0.63 \times 10^{-6}$ cm/s after PMA in the presence of KT-5823, $n = 6$). L-NMMA did not exert any inhibitory effect on PMA-induced hyperpermeability ($n = 3$). Taken together, these data suggest that the calcium-NOS-GC-

PKC cascade is an independent but common pathway leading to agonist-induced protein transflux across the coronary venular endothelium, whereas PKC activation mediates the hyperpermeability reaction partially through the NO pathway.

DISCUSSION

This study focused on the interaction of different signaling molecules during modulation of macromolecular permeability in coronary venules. The measurements of cytosolic calcium transience and albumin permeability in the endothelium of isolated venules provided some interesting results. First, histamine and ionomycin caused a rapid elevation in endothelial cytosolic calcium preceding the increase in endothelial permeability to albumin, whereas PMA induced a hyperpermeability response unaccompanied by intracellular calcium changes. These data indicate a heterogeneity of calcium dependence among different hyperpermeability mediators. Second, the NOS and PKG blockers inhibited the increases in venular permeability but
failed to block the calcium spikes during stimulation of histamine and ionomycin. The dissociation of the permeability effect from the calcium response suggests that elevation in endothelial cytosolic calcium is an early signaling event that occurs upstream from NO synthesis. Finally, the relationship between PKC and NOS was examined. Although the PKC inhibitor did not affect the hyperpermeability effects of histamine, SNP, and cGMP, which are known to stimulate the NO pathway, inhibition of NO synthesis or PKG activity was able to attenuate the permeability response to PMA, a PKC activator. Therefore, we suggest that there are at least two independent signaling pathways that contribute to the pathophysiological regulation of coronary venular permeability. One mechanism involves a rapid elevation in endothelial cytosolic calcium followed by NO synthesis and PKG activation. The other is characterized by activation of PKC. However, interactions occur between the two pathways, in that PKC may exert its action partially by modulating the synthesis of NO.

Endothelial Cytosolic Calcium and NO Production in Modulation of Venular Permeability

The control of cytosolic calcium is critical in the physiological and pathophysiological regulation of endothelial function. Calcium is required for the maintenance of endothelial integrity, and alterations in calcium produce dynamic changes in endothelial structure and barrier properties (30). Elevation of endothelial cytosolic calcium has been considered as a primary trigger of transendothelial flux of macromolecules on stimulation by some inflammatory mediators such as histamine (27). Supporting the importance of calcium in acute inflammatory reactions is an in vivo observation that histamine-caused leakage in postcapillary venules of the hamster cheek pouch is dependent of extracellular calcium (17). A possible mechanism for the increase in intracellular calcium is provided by the finding that histamine stimulates inositol phosphate production via a G protein associated with PLC (5). In this instance, PLC hydrolysis of phosphotidylinositol bisphosphate and subsequent calcium mobilization comprise the initial intracellular signaling processes after agonist binding (14). However, the later events that occur downstream from calcium elevation remain to be clarified. The proposed sites of action of calcium include actin-myosin contraction, endothelial cell-cell adhesion, and cell-matrix interaction (6, 14). Although all these processes are very important to the barrier property, they are generally considered as the determinants of endothelial integrity that ultimately control the structure or morphology of the endothelial monolayer (6, 14). In other words, there may be some intermediate molecules that reside within the cell to transduce the signals from calcium to structural proteins in the cytoskeleton and intercellular junction. Within this context, a potential second messenger of

![Fig. 7. Dose-responsive effects of bisindolylmaleimide (BIM), selective PKC inhibitor, on coronary venular permeability. *Significance vs. basal permeability in absence of PMA.](image)

![Fig. 8. Selective PKC inhibitor BIM did not significantly affect hyperpermeability effects of histamine, sodium nitroprusside (SNP), and cGMP. *Significant vs. basal permeability before agonist stimulation. §Significant vs. BIM alone.](image)
calcium is NOS. In view of the potent stimulating effect of calcium on endothelial constitutive NOS (28) and the key role of NOS in mediation of agonist-induced microvascular hyperpermeability (16, 21, 23, 26, 34), it is possible that the conventionally emphasized central effect of calcium relies on the activity of NOS and is transduced by NO. This hypothesis has not been directly tested due to technical difficulties in measuring cytosolic calcium and macromolecular permeability in the intact microvessel.

Our study reports for the first time the measurement of agonist-elicited changes in endothelial calcium and permeability of isolated and perfused intact coronary venules. The method has the advantage of the precise measurement of endothelial cytosolic calcium and albumin permeability in the absence of neutrophils and with little influence from hemodynamic and parenchymal factors (33). Under such conditions, histamine induced a rapid and biphasic rise in intracellular calcium followed by an increase in albumin permeability in the endothelium of coronary venules. The onset and time course of changes in calcium and Pₐ were closely related. Similarly, the calcium spike on administration of ionomycin preceded the elevation in Pₐ in the presence of the ionophore. The pattern of calcium responses to histamine and ionomycin is consistent with that observed in cultured endothelial cells (27) and perfused frog mesenteric capillaries (7). More importantly, we found that histamine- and ionomycin-induced increases in venular permeability were abolished, whereas the calcium responses were not significantly altered by inhibition of NOS or PKG. This indicates that elevation in endothelial cytosolic calcium occurs before NOS activation. Therefore, NO, rather than calcium, is a common mediator toward the downstream of the pathway leading to microvascular hyperpermeability. Although calcium is an important signaling molecule it may not be essential in mediation of permeability responses to certain agonists. Indeed, the present study suggests that PMA-induced increases in coronary venular permeability are not calcium dependent.

A limitation of this study is that the association of calcium and NOS was not directly tested, and it is therefore unconfirmed that the presence of calcium is required in the signaling process triggered by histamine. In this regard, a measurement of the permeability response to the agonist in the absence of intracellular and extracellular calcium would directly address the question. However, depletion of calcium could largely alter the basal permeability of the endothelium by disrupting the cell-cell and cell-matrix attachment, rendering results meaningless. On the other hand, we were unable to measure the activity and products of NOS in isolated and perfused microvessels due to technical difficulties. Therefore, we tested the calcium-NOS-PKG cascade by using pharmacological approaches. Our results support the sequential association between calcium and NOS. A recent study on perfused mesenteric microvessels (7) has also demonstrated that calcium-dependent release of NO is a necessary step to increase microvascular hydraulic conductivity in response to ionomycin and ATP.

One of the major aims of this study was to evaluate the relationship of calcium and NO in the signal transduction of coronary venular hyperpermeability. Specifically, we were interested in testing the hypothesis that elevation in endothelial calcium occurs upstream from NO production, rather than the route of calcium mobilization or the absolute value of intracellular calcium. Therefore, we report the trend of calcium responses in the form of fluorescence ratio at 340/380 nm. Regarding the concentration and source of calcium, previous studies (7, 27, 35) have shown that histamine, ATP, or ionomycin stimulates an increase in endothelial cytosolic calcium from a resting level of 50–100 to 500–1,000 nM, and that the biphasic response of calcium is due to an initial release from intracellular pools followed by an influx of extracellular calcium.

Interactions Between NO and PKC in Modulation of Venular Permeability

The endothelial cell responds to a wide array of messages carried by hormones, neurotransmitters, growth factors, cytokines, and other cells. The efficient operation of the endothelial barrier requires coordination and integration between different signaling pathways that are triggered by various mediators. Our current findings suggest that at least two different signaling events occur in the endothelium of coronary venules that are responsible for agonist-induced changes in the barrier function. We have previously demonstrated that histamine increases venular permeability via a PLC-NOS-GC cascade (34). Recently, we have extended the hypothesis to PKG, a second messenger of cGMP (32, 36). The current study further includes intracellular calcium as an early intermediate located upstream from NO production. We suggest that PLC-calcium-NOS-GC-PKG comprises a signaling cascade in the pathophysiologic regulation of the endothelial
permeability. It is likely that this pathway plays a
dominating role in mediation of histamine-elicited transvenular flux of albumin, because the response
could be abolished or greatly attenuated only by inhibi-
tors of the related enzymes but not affected during
inhibition of cyclooxygenase (34) or PKC.
In addition to the calcium-initiated response, other
mechanisms may be involved in the regulatory process
associated with certain agonists that are different from
histamine. For example, PKC has been known as an
important second messenger in the regulation of micro-
vascular barrier function during stimulation by phor-
bol esters, DAG, thrombin, bradykinin, and platelet-
activating factor (6, 11, 13–15, 22). A significant finding
of the current study is that activation of PKC with the
phorbol ester PMA increased venular permeability via
a mechanism independent of calcium. This is in agree-
ment with other investigations in which PKC activa-
tors caused albumin transflux across the endothelial
monolayer unaccompanied by alterations in the level of
endothelial cytosolic calcium (3). The dissociation of
changes in the endothelial permeability and the intra-
cellular calcium content suggests that the PKC activa-
tors, unlike histamine-type agonists, act as barrier
modulators through a pathway that is not triggered by
calcium mobilization. However, how PKC alters the
barrier function is still a controversial issue. A novel
study in the hamster cheek pouch has demonstrated
that the increase in microvascular permeability during
stimulation of PKC requires the production of NO (25),
indicating that NOS may be a target protein of PKC.
Considering the effect of PKC on the activity of NOS,
albeit some studies showed that pharmacological
inhibition as well as depletion of PKC increased the
expression of NOS mRNA and enhanced the release of
NO in cultured endothelial cells derived from large
vessels (24), opposite data were reported in which PKC
activation promoted NO production in various cells,
including cardiac myocytes and vascular smooth muscle
cells (8, 19, 29). The disparity between the two results
may be explained by the difference in experimental
preparations and conditions. As in the postcapillary
venular endothelium where leaky sites are formed on
inflammatory stimulation (18), there is no direct evi-
dence showing the relationship between PKC activa-
tion and NO production. Unfortunately, our current
technique did not allow us to measure the enzyme
activity and products in isolated and perfused venules.
Nevertheless, studies with pharmacological approac-
hes have revealed that the hyperpermeability effect of
PKC-activating agents could be blocked by NOS inhibi-
tors (13, 16, 22, 25, 26), supporting the concept that
PKC displays its signaling effect by modulating the
activity of NOS in the endothelium. Our observation
that inhibition of NOS attenuated PMA-augmented
permeability is favorable to the regulatory relationship
between PKC and NO. In fact, we emphasize that
PKC-mediated changes in venular permeability in-
volve, in part, the production of NO. Because inhibition
of NOS did not totally abolish the effect of PMA,
mechanisms that are independent of NO should be
considered.
The molecular mechanism responsible for the upregu-
lation of PKC has not been specified. A potential
reason for the lack of understanding is that the enzyme
consists of at least eight isoforms that may respond to
different signals and exert different actions on the
downstream proteins (14). Generally, it is accepted that
PLC hydrolysis of phosphatidylinositol bisphosphate
produces DAG, leading to PKC activation (2). In this
study, we used a phorbol ester that is known to directly
stimulate PKC bypassing PLC, rendering difficulties in
verification of the link between PLC and PKC in the
signaling pathway leading to microvascular permeabil-
ity. On the other hand, we found that PLC was an early
signal before NO production in the mediation of histo-
mine's effect on venular permeability (34), which was
not affected by the PKC inhibitors. This is interesting
because if PLC activation causes PKC upregulation,
then blockage of PKC should at least attenuate the
histamine-elicited, PLC-mediated response. An expla-
nation for the insignificant effect of the PKC inhibitor is
that PKC is not necessarily activated after PLC activa-
tion by histamine. Alternatively, different isoforms of
PKC are involved in the signaling process, of which the
one turned on by PLC may not have a significant effect
on the endothelial permeability.
In summary, we provide evidence for a sequential
linkage between the elevation in endothelial cytosolic
calcium and the activation of NOS during mediation of
agonist-elicited venular hyperpermeability. The study
further supports the regulatory importance of the
PLC-calcium-NOS-NO cascade and the PKC path-
way in the process of microvascular exchange. During
inflammation, activated PKC may modulate the endo-
thelial barrier function directly and/or indirectly
through stimulating the NOS activity. Through this
interaction, the two pathways may act in concert to
regulate microvascular permeability.

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REFERENCES
medial hydraulic conductance of aorta, possibly by release of
EDRF. Am. J. Physiol. 264 (Heart Circ. Physiol. 33): H26–H32,
1993.
2. Bell, R. M. Protein kinase C activation by second messengers.
3. Buchan, K. W., and W. Martin. Modulation of barrier function of
bovine aortic and pulmonary artery endothelial cells: disso-
ciation from cytosolic calcium content. Br. J. Pharmacol. 107:
solic and membrane-derived protein kinase C activity by stauro-
sporine and other kinase inhibitors. FEBS Lett. 362: 139–142,
1995.


