

# Differential Phosphodiesterase Activity Contributes to Restrictive Endothelial Barrier Function during Angiogenesis

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Angiogenic endothelial hyperpermeability is abruptly diminished between days 4.5 and 5.0 of the 18-day lifespan of the chick chorioallantoic membrane. Here, we evaluated phosphodiesterase (PDE) activity during the differentiation of barrier function. At day 4.5, rolipram-mediated inhibition of cAMP-specific PDE IV reduced FITC–dextran extravasation. Moreover, inhibition of PDE III by HL 725, but not PDE I by 8-IBMX, decreased the temporal angiogenic endothelial hyperpermeability. Reduced FITC–dextran was also observed at day 4.5 after application of KT 5823, a selective inhibitor of cGMP-specific protein kinase G (PKG), LY 83583, an inhibitor of soluble guanylate cyclase, or LNMMA, an inhibitor of nitric oxide synthase. At day 5.0, Rp-cAMPS-mediated inhibition of cAMP-specific protein kinase A (PKA) diminished barrier function and interstitial accumulation of FITC–dextran was increased. In all cases, the mean widths of interendothelial separation remained uniform. Together, the results support the concept that differentiation of restrictive angiogenic endothelial barrier function *in vivo* includes inactivation of PDE III and PDE IV with consequent up-regulation of cAMP/PKA signaling and down-regulation of the cGMP/PKG pathway.

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**Key Words:** cAMP; cGMP; phosphodiesterase; angiogenic endothelium; permeability.

## INTRODUCTION

During chorioallantoic membrane (CAM) angiogenesis, restrictive endothelial barrier properties of the expanding microvascular network conform to precise temporal modulation. Although neovascularization is continuous until day 12 of the 18-day CAM lifespan (Ausprunk *et al.*, 1974), hyperpermeability characterizes the angiogenic endothelium only through day 4.5. Specifically, differentiation of restrictive barrier function occurs abruptly between days 4.5 and 5.0 (Rizzo *et al.*, 1995). Previously, we reported that day 4.5 hyperpermeability was abolished by exogenous activation of cAMP signaling (DeFouw and DeFouw, 1999). Day 5.0 restrictive barrier properties, on the other hand, were diminished in response to exogenous stimulation of the cGMP pathway (DeFouw and DeFouw, 2000a). Here, we tested the concept that temporal modulation of endogenous phosphodiesterase (PDE) activity contributes to the differentiation of CAM endothelial barrier properties.

Evidence from studies of nonangiogenic endothelium also supports the notion that cAMP signaling bolsters restrictive barrier function while cGMP signaling leads to endothelial barrier dysfunction. For example, overexpression of an endogenous protein kinase A (PKA) inhibitor *in vitro*, by adenoviral gene transfer, abrogated cAMP-mediated protection against inflammatory barrier

dysfunction (Lum *et al.*, 1999). Furthermore, preservation of pulmonary microvascular barrier properties *in vivo* (Bernard *et al.*, 1994; Chetham *et al.*, 1997) and basal microvessel permeability *in situ* (He *et al.*, 2000) have been linked with endothelial cell cAMP activity. Although mechanisms responsible for this barrier protection remain uncertain, cAMP/PKA-mediated phosphorylation of either filamin, an actin cross-linking protein, or myosin light chain kinase (MLCK), with consequent kinase inhibition, has been proposed as a means for preventing interendothelial gap formation (Hastie *et al.*, 1997, 1998; Gilbert-McClain *et al.*, 1998).

Proinflammatory mediators, such as histamine (Yuan *et al.*, 1993) and bradykinin (Mayhan, 1992), increase microvascular permeability to fluid and macromolecules by elevating intracellular NO or cGMP levels. Further, cGMP/protein kinase G (PKG)-mediated hyperpermeability has been linked with myosin light chain phosphorylation (Yuan *et al.*, 1997). Alternatively, PDE II and PKG competitively bind cGMP in both adult and angiogenic endothelium (Hempel *et al.*, 1996; He *et al.*, 2000; DeFouw and DeFouw, 2000a). Hence, cGMP-activated PDE II might degrade cAMP and thereby disrupt endothelial barrier function. In either case, widening of the junctional clefts would be expected since interendothelial gaps developed in adult microvessels within minutes of histamine application (Fox *et al.*, 1980; Wu and Baldwin, 1992).

In the current study, selective pharmacologic inhibition of specific PDE isoforms, nitric oxide synthase (NOS), soluble guanylate cyclase (GC), or PKG before differentiation of restrictive barrier function, and PKA after barrier differentiation, was performed. The results serve to establish PDE III and PDE IV function along with NOS/NO/GC/cGMP/PKG signaling during hyperpermeability. Further, PDE inactivation apparently enables prevalence of the cAMP/PKA pathway after the differentiation of restrictive barrier function.

## METHODS

Three-day-old white Leghorn chick embryos (Truslow Farms, Chestertown, MD) were removed from their shells, placed into plastic weigh boats, and

maintained in a humidified environment at 37°. At day 4.5 or 5.0 of gestation, the embryos were transferred to a humidified, temperature-controlled chamber for microscopic observation of the CAM.

### *FITC-Dextran Microinjection and Image Analysis*

Direct intravital observations of the CAM capillary networks were performed by techniques established in our laboratory (Rizzo *et al.*, 1995; DeFouw and DeFouw, 1999). A graded series of FITC-dextran 20, 40, and 70 (Sigma Chemical Co., St. Louis, MO), prepared as 5% solutions in chick Ringers, served to monitor CAM endothelial permeability. The tracers were microinjected via the vitelline vein at a dose of 100  $\mu\text{g/g}$ . Since the tracer volume was less than 2% of the total embryonic blood volume, minimal hemodynamic alteration would be expected (Wagman *et al.*, 1990).

Real-time imaging of the FITC-dextran perfusion patterns employed the 10 $\times$  objective of an Olympus BH2 microscope, equipped with an epi-illumination system optimized for fluorescein. Video signals were delivered by a low-light Dage MTI series 66 SIT camera to image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD). For each CAM preparation, three fluorescent images of the microvascular networks were selected randomly. During image acquisition, the camera's automatic control circuits were disengaged and maintained at predetermined manual levels.

The integrated optical intensity (IOI) method (Kim *et al.*, 1993) was used to measure extravasation of the FITC-dextran. In this case, average interstitial fluorescence intensity values were measured within test areas ( $5 \times 5$  pixels,  $100 \mu\text{m}^2$ ), centered in the intercapillary spaces. Eight test areas were measured on each CAM image, which yielded 24 interstitial measurements for each embryo.

### *Modulation of the cAMP or cGMP Pathways*

The established protocol of injecting pharmacologic agents into the allantoic cavity was used to modulate cyclic nucleotide signaling (DeFouw and DeFouw, 1999, 2000a). Stock solutions of the injectates (Sigma or

Calbiochem–Novabiochem, La Jolla, CA) were prepared as follows: rolipram ( $10^{-3}$  M), 8-IBMX ( $10^{-3}$  M), and KT 5823 ( $10^{-3}$  M) in DMSO; Rp-cAMPS ( $2 \times 10^{-2}$  M), HL 725 ( $10^{-2}$  M), and LNMMA ( $10^{-1}$  M) in deionized water; and LY 83583 ( $10^{-3}$  M) in ethanol. The stock solutions were diluted in chick Ringers (pH 7.4) to their respective injectate concentrations. These concentrations and their selective inhibitory functions were based both on our previous trials and on observations reported by others (Mayhan, 1992; Yuan *et al.*, 1993; Bernard *et al.*, 1994; Hempel *et al.*, 1996; Wu *et al.*, 1996; Fujii *et al.*, 1997; Houslay, 1998).

The allantoic cavity injections (10  $\mu$ l each) were delivered singularly by fine-tip micropipettes at day 4.5 as follows: rolipram ( $10^{-4}$  M), 8-IBMX ( $10^{-5}$  and  $10^{-4}$  M), HL 725 ( $10^{-9}$  and  $10^{-8}$  M), KT 5823 ( $10^{-6}$  and  $10^{-5}$  M), LNMMA ( $10^{-6}$  and  $10^{-5}$  M), and LY 83583 ( $10^{-6}$  and  $10^{-5}$  M). At day 5.0, injections of Rp-cAMPS ( $10^{-6}$  M) were delivered. Injections of chick Ringers at days 4.5 and 5.0 served as controls and the IOI values for the control groups have been recorded previously (DeFouw and DeFouw, 1999).

### Statistical Analyses

A minimum of four embryos per pharmacologic injectate was evaluated. Ten minutes postinjection, a single FITC–dextran tracer was infused via the vitelline vein. Mean interstitial IOI values were recorded from each CAM preparation, and group means were compared with one-way analysis of variance and the Bonferroni post hoc test.

### Endothelial Junction Morphometry

CAM preparations that received injections of rolipram at day 4.5, Rp-cAMPS at day 5.0, or chick Ringers at days 4.5 and 5.0 were also prepared for electron microscopy by immersion in 2% glutaraldehyde prepared in 10 mM phosphate-buffered saline (PBS). Following postfixation in osmium tetroxide (1% in PBS), the CAM samples were routinely embedded in Epon. Ultrathin sections (60–90 nm) were stained with uranyl acetate and lead citrate for viewing with a Philips EM 300 microscope operating at 60 kV.

Assessment of the capillary interendothelial junc-

tions was based on our previously established morphometric protocol (DeFouw and DeFouw, 2000b). Junctional clefts (25–30 clefts per group) were photographed at approximately 35,000 $\times$ . Only those clefts that could be visualized from the luminal to the abluminal cell surface, and in which the respective cell membranes were clearly visible for >90% of the cleft length, were recorded. Widths of interendothelial separation were measured at sites separated by a fixed distance, which equated to approximately every 60 nm along the length of each observed cleft. Mean widths for each cleft were recorded and group means were compared as described above.

## RESULTS

Since hyperpermeability characterizes the angiogenic CAM endothelium at day 4.5 of gestation (Rizzo *et al.*, 1995), temporal cGMP signaling would be expected (DeFouw and DeFouw, 2000a). Whether PKG or PDE III represents a downstream target during the temporal hyperpermeability was tested here.

First, the cGMP/PKG pathway was evaluated by application of KT 5823, a selective PKG inhibitor. Normal extravasation of FITC–dextran 40 was significantly reduced after PKG inhibition (Fig. 1). Temporal cGMP/PKG activity was also suggested by the inhibition of either NOS or GC. Hence, respective applications of LNMMA and LY 83583 decreased FITC–dextran 40 efflux (Fig. 2). Together, these results are consistent with endogenous NOS/NO/GC/cGMP/PKG signaling in the hyperpermeable CAM endothelium at day 4.5.

Based on the competitive affinities of PKG and PDEs for cGMP (Hempel *et al.*, 1996; He *et al.*, 2000; DeFouw and DeFouw, 2000a), the availability of cGMP for specific inhibition of PDE III might be limited at day 4.5. Accordingly, HL 725, a selective PDE III inhibitor, decreased FITC–dextran 40 efflux (Fig. 3). Therefore, cGMP-mediated PKG activation, rather than PDE III inhibition, likely prevails at day 4.5. Temporal PDE III activity, in turn, might contribute to cAMP degradation. On the other hand, selective PDE I inhibition by 8-IBMX failed to reduce CAM endothelial hyperper-

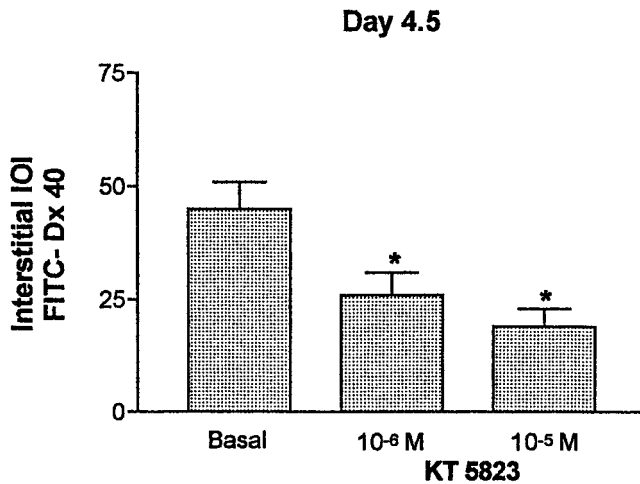


FIG. 1. Levels of FITC-dextran 40 accumulation in the pericapillary interstitium are presented as interstitial integrated optical intensities (IOI). Selective PKG inhibition by KT 5823 injection into the allantoic cavity served to decrease tracer extravasation at day 4.5 of the 18-day CAM lifespan ( $*P < 0.001$ ).

meability at day 4.5 (Fig. 3). Thus, endogenous PDE I activity in the angiogenic endothelium seems unlikely.

The highly specific cAMP phosphodiesterase, PDE IV, is essential for normal regulation of cAMP activity (Houslay, 1998). Assuming that cAMP/PKA down-regulation contributes to CAM endothelial hyperper-

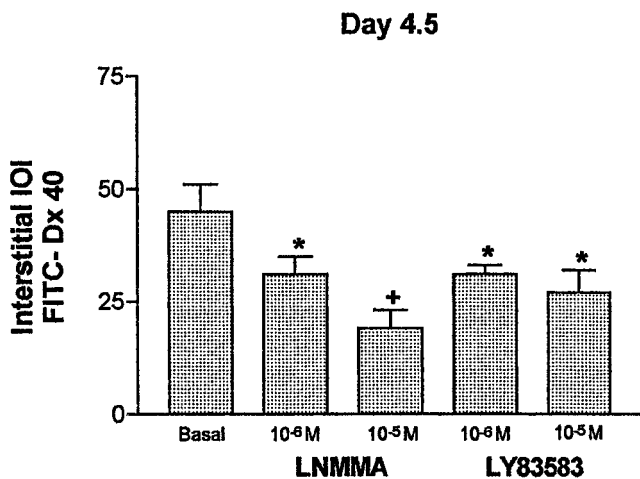


FIG. 2. During relative hyperpermeability at day 4.5, inhibition of NOS by LNMMA or soluble GC by LY83583 significantly reduced FITC-dextran 40 extravasation as reflected by the respective IOI values ( $*P < 0.001$ ; differences between LNMMA concentrations with  $P < 0.01$ ).

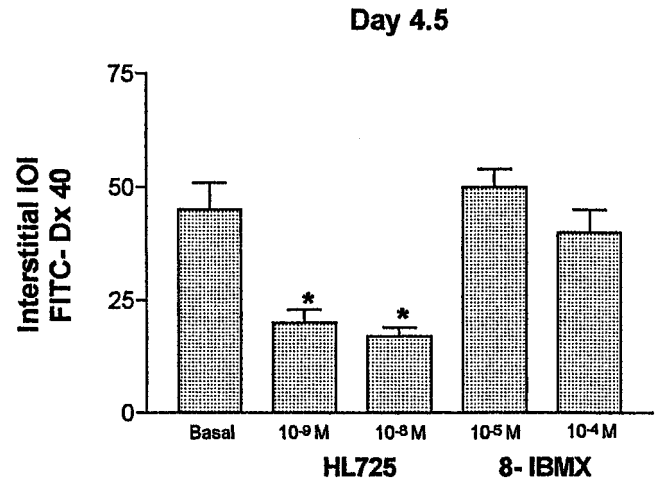


FIG. 3. Selective PDE inhibition at day 4.5 was characterized by specific changes in temporal angiogenic hyperpermeability. As shown by the respective IOI values, PDE III inactivation by HL725 reduced FITC-dextran 40 efflux ( $*P < 0.001$ ), while inhibition of PDE I by 8-IBMX failed to alter angiogenic endothelial hyperpermeability.

meability (DeFouw and DeFouw, 1999), endogenous PDE IV activity would be expected at day 4.5. Accordingly, rolipram-mediated inhibition of PDE IV uniformly decreased the efflux of FITC-dextran 20, 40, and 70 to levels that approached those normally observed at day 5.0 (Fig. 4). Likewise, application of Rp-cAMPS, a selective PKA inhibitor, at day 5.0 served to dedifferentiate barrier function to the less restrictive state normally observed at day 4.5, and increased interstitial accumulation of FITC-dextran 20, 40, and 70 was observed (Fig. 5). Together, these results support the concept that down-regulation of endogenous PDE IV activity likely contributes to enhanced cAMP/PKA signaling that is essential for differentiation of restrictive barrier properties at day 5.0.

An attempt was also made to establish morphologic correlates to the observed changes of angiogenic endothelial permeability. Rolipram-mediated inhibition of PDE IV at day 4.5 served to decrease hyperpermeability without reducing mean widths of interendothelial separation (Table 1). Furthermore, mean widths of the junctional clefts were not increased after cAMP/PKA inhibition and consequent increased macromolecular efflux at day 5.0 (Table 1). Hence, permeability functions of the angiogenic endothelium associated

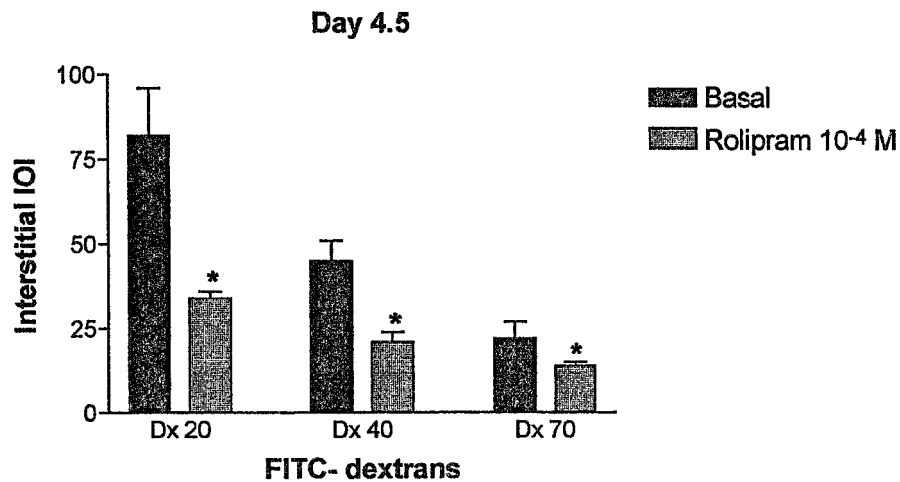


FIG. 4. Although relative hyperpermeability prevails at day 4.5, the angiogenic endothelium displays permselective regulation of macromolecular extravasation. After inactivation of cAMP-specific PDE IV by rolipram injection into the allantoic cavity, permselectivity was retained while restriction to FITC-dextran 20, 40, and 70 efflux was uniformly increased (\* $P < 0.001$ ).

with differential cAMP/PKA signaling did not include modulation of junctional cleft widths.

## DISCUSSION

PDE III activity at day 4.5 was detected here and consequential cAMP degradation likely contributed to the temporal hyperpermeability. Since the hyperper-

meability was also cGMP/PKG-dependent, differential affinities of cGMP for PKG and PDE III likely favored PKG activation, rather than cGMP-mediated PDE III inhibition at day 4.5. Competitive inhibition of PKG by KT 5823, which served to reduce the day 4.5 hyperpermeability, would also increase the availability of cGMP for PDE III binding. Consequent PDE III inhibition likely contributed to the KT 5823-induced reduction of FITC-dextran extravasation. That HL 725-mediated PDE III inhibition reduced macromolecular

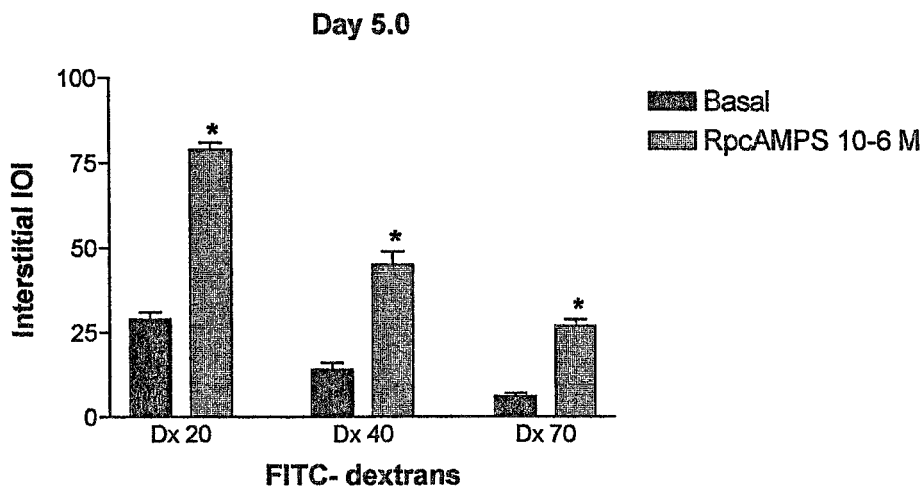


FIG. 5. Differentiation of restrictive barrier function is complete in the angiogenic CAM endothelium by day 5.0. Dedifferentiation of barrier properties is indicated here after inactivation of cAMP/PKA signaling by Rp-cAMPS application. Extravasation of FITC-dextran 20, 40, and 70 was increased in a permselective manner as shown by respective IOI values (\* $P < 0.001$ ).

TABLE 1  
Widths of Capillary Interendothelial Clefts<sup>a</sup>

	Day 4.5		Day 5.0	
	Control	Rolipram	Control	Rp-cAMPS
Widths (nm)	19.78 ± 3.67	20.08 ± 5.41	19.19 ± 3.51	22.52 ± 7.17

<sup>a</sup> Values represent means ± SD.

efflux is consistent with this interpretation. Interestingly, previous application of KT 5823 at day 5.0 (DeFouw and DeFouw, 2000a) afforded enhanced PDE II activation with consequent cAMP degradation and dedifferentiation of barrier function. Together, these results provide support to the concept that the affinity of cGMP for PKG was greater than that for either PDE II or PDE III in the CAM endothelium.

In addition to PDE III, PDE IV activity was detected by rolipram application at day 4.5. Hence, temporal degradation of cAMP likely plays a significant role in CAM endothelial hyperpermeability. Interestingly, previous application of rolipram had minimal effect on the restrictive CAM endothelial barrier at day 5.0 (DeFouw and DeFouw, 2000a). Hence, endogenous down-regulation of PDE IV apparently contributes to sustained cAMP signaling after differentiation of CAM endothelial barrier function.

CAM angiogenesis has been linked with endothelial-specific vascular endothelial growth factor (VEGF) activity (Wilting *et al.*, 1993, 1996). In addition to its mitogenic effect, VEGF is well documented as a permeability-enhancing agent *in vivo* (Mukhopadhyay *et al.*, 1998; Murohara *et al.*, 1998; Van Bruggen *et al.*, 1999). Furthermore, VEGF-mediated hyperpermeability has been associated with the endothelial NOS/NO/GC/cGMP signaling cascade (Wu *et al.*, 1996; Fujii *et al.*, 1997; Wu *et al.*, 1999). Recently, we reported VEGF-mediated tyrosine phosphorylation of CAM endothelial receptor tyrosine kinases (VEGFR-1 and VEGFR-2) at both days 4.5 and 5.0 (DeFouw and DeFouw, 2000c). Thus, VEGF-induced activation of cGMP signaling in the CAM endothelium seems likely. Although such signaling is consistent with hyperpermeability at day 4.5, it would not be expected after differentiation of restrictive barrier function at day 5.0. That VEGFR-1 and -2 expression was lessened at day 5.0 (DeFouw and DeFouw, 2000c) might reflect

a means for lowering endogenous cGMP signaling. Moreover, inhibition of PDE V, a cGMP-specific phosphodiesterase, increased macromolecular extravasation at day 5.0 (DeFouw and DeFouw, 2000a). Therefore, temporal PDE V-mediated suppression of endogenous cGMP signaling might also contribute to restrictive CAM endothelial barrier function.

Although endothelial cGMP/PKG signaling prevailed at day 4.5, PKG target proteins involved in the temporal hyperpermeability remain less certain. Endothelial cell contraction and subsequent interendothelial gap formation have been attributed to PKG-mediated MLC phosphorylation (Yuan *et al.*, 1997). However, interendothelial gaps are not endogenous to the CAM endothelium at day 4.5. Since the hyperpermeable barrier retains an observable degree of permselectivity (Fig. 4), the absence of essentially nonrestrictive interendothelial gaps seems reasonable. On the other hand, cGMP/PKG-mediated microvascular hyperpermeability has also been linked with disassociation of junctional adhesion molecules from the actin cytoskeleton (Park *et al.*, 1999). If this occurred in the CAM endothelium, cGMP/PKG signaling might serve to diminish junctional cleft sieving properties, without inducing interendothelial gap formation. Accordingly, differentiation of restrictive CAM endothelial barrier function equated with enhanced permselectivity characterized by proportionate increases in restriction of the graded FITC-dextran series (Figs. 4 and 5). That this enhanced restriction is dependent on both increased temporal expression and homotypic adhesion of VE-cadherin (Cruz and DeFouw, 1999; DeFouw and DeFouw, 2000b), a molecular component of the junctional sieve (Lampugnani *et al.*, 1992), is also consistent with this interpretation.

Cyclic GMP-mediated alterations of endothelial permeability might also include modulation of microvascular tone. It was reported previously, however, that

the chick extraembryonic microcirculation, which includes the CAM, is not innervated (Bowers, 1989), and vasomotor activity was recognized only after day 6 (Girad, 1973). Moreover, the present FITC-dextran injection volumes would have minimal hemodynamic effects (Wagman *et al.*, 1990). Thus, modulation of CAM microvascular tone, in conjunction with fluctuations of cGMP activity, would not be expected.

Unlike cGMP, which has numerous intracellular target proteins, cAMP acts almost exclusively via PKA activation. Although cAMP/PKA activity has been implicated in protective signaling against endothelial barrier dysfunction (Bernard *et al.*, 1994; Chetham *et al.*, 1997; Lum *et al.*, 1999; He *et al.*, 2000), downstream elements in the cascade remain less certain. It was reported that cAMP/PKA-mediated phosphorylation of endothelial filamin, an actin cross-linking protein, prevented H<sub>2</sub>O<sub>2</sub>-mediated interendothelial gap formation (Hastie *et al.*, 1997, 1998). In addition, cAMP might inhibit MLCK and thereby diminish endothelial cell contraction (Gilbert-McClain *et al.*, 1998). Without contraction, interendothelial gap formation and increased macromolecular flux would be prevented (Schnittler *et al.*, 1990; He and Curry, 1993). Alternatively, increased sites of tight adhesion along the junctional clefts of nonangiogenic endothelium have been reported after cAMP activation (Adamson *et al.*, 1998). Although enumeration of such sites was not performed here, elevated cAMP activity was associated with increased VE-cadherin expression and homotypic adhesion (Cruz and DeFouw, 1999; DeFouw and DeFouw, 2000b) without decreases in the mean widths of interendothelial separation. Hence, enhanced homotypic adhesion might serve to tighten sieving properties of the junctional clefts and thereby contribute to the proportionate increase of permselective restriction offered by the endothelial barrier at day 5.0.

In conclusion, differentiation of angiogenic endothelial barrier function *in vivo* correlates with the downregulation of PDE III and PDE IV activities. Prior hyperpermeability, on the other hand, is dependent, in part, on preferential affinities of cGMP for PKG rather than PDE III.

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