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Phosphodiesterase Inhibitor Modulation of Brain Microvascular Endothelial Cell Barrier Properties

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Abstract

Background and Purpose—Brain microvascular disorders, including cerebral microscopic hemorrhage, have high prevalence but few treatment options. To develop new strategies for these disorders, we analyzed effects of several phosphodiesterase (PDE) inhibitors on human brain microvascular endothelial cells (HBEC).

Methods—We modified barrier properties and response to histamine of HBEC using cilostazol (PDE-3 inhibitor), rolipram (PDE-4 inhibitor), and dipyridamole (non-specific PDE inhibitor).

Results—Cilostazol and dipyridamole altered distribution of endothelial F-actin. Cilostazol increased expression of tight junction protein claudin-5 by 118 % compared to control ($p < .001$). Permeability to albumin was decreased by cilostazol (21% vs control, $p < .05$), and permeability to dextran (70Kd) was decreased by both cilostazol (37% vs control, $p < .001$) and dipyridamole (44% vs control, $p < .0001$). Cilostazol increased trans-endothelial electrical resistance (TEER) after 12 hours by 111% compared to control ($p < .0001$). Protein kinase A (PKA) inhibitors H89 and KT5720 attenuated the TEER increase by cilostazol. Transient increased permeability in response to histamine was significantly mitigated by cilostazol, but not other PDE inhibitors.

Conclusions—These findings demonstrate distinctive effects of cilostazol and other PDE inhibitors on HBEC, including enhanced barrier characteristics and mitigation of response to histamine. PKA-mediated effects of cilostazol were prominent in this model. These *in vitro* findings are consistent with therapeutic potential of PDE inhibitors in human brain microvascular disorders.

Keywords

cell culture; endothelial; microcirculation; phosphodiesterase; histamine

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Introduction

Microvascular disorders of the brain are increasingly recognized as a major public health issue. These disorders are part of the spectrum of cerebrovascular disease ranging from clinical ischemic and hemorrhagic stroke to the largely subclinical cerebral white matter disease (CWMD) [1–4]. Both CWMD and cerebral microscopic hemorrhage are widely prevalent in the aging population and include a substantial capillary component [5–7]. Currently there is no specific treatment for these disorders. A recently proposed model of cerebral microscopic hemorrhage suggested that transient loss of endothelial barrier function might be an underlying process [5].

Effective stroke prevention should consider both thrombosis (pathological generation of clot) and hemostasis (maintenance of blood within the vasculature). Current ischemic stroke prevention therapy is typically anti-thrombotic, with little concern for hemostasis. This attitude is becoming increasingly untenable given the high prevalence of hemorrhagic phenomena with ischemic stroke, the coexistence of which has been termed “mixed cerebrovascular disease” [4, 8].

Cilostazol has been evaluated in ischemic stroke prevention clinical trials [9–11] and relies on phosphodiesterase (PDE) inhibition as its principal mechanism [12]. Cyclic nucleotide PDEs are enzymes that regulate the cellular levels of second messengers cAMP and cGMP by controlling their degradation [13–15]. Cilostazol is a well-known PDE-3 inhibitor [12] while dipyridamole, another ischemic stroke prevention agent [16, 17], is a nonspecific PDE inhibitor [13]. While the platelet effects of these agents are well-known, their effectiveness in protecting and enhancing endothelial barrier function has received limited attention. Other PDE inhibitors (eg, PDE-5 inhibitor tadalafil) have been shown to improve functional recovery in experimental stroke [18].

The current study is designed to assist development of new therapeutic strategies for brain microvascular disorders, with particular reference to the population of patients with coexisting ischemic and hemorrhagic processes. We studied the effectiveness of these PDE inhibitors (cilostazol and dipyridamole) along with rolipram (a PDE-4 inhibitor)[13] in modulating endothelial barrier properties in vitro.

Materials and Methods

Cell culture preparations

HBEC (Applied Cell Biology Research Institute, Kirkland, WA) were grown on tissue culture plates pre-coated with attachment factor (Invitrogen Corporation, Carlsbad, CA). Endothelial cells demonstrated typical cobblestone morphology and immunoreactivity for von Willebrand factor (Dako Corporation, Carpinteria, CA) and uptake of acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (Biomedical Technologies, Stoughton, MA). Cell passaging was performed using passage reagent group (Cell Systems, Kirkland, WA), and cells from passages 6–10 were used for experiments.

HBEC were maintained in Medium 131 (Invitrogen Corporation, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 5% microvascular growth supplement (MVGS) (Invitrogen Corporation, Carlsbad, CA), and 1% penicillin-streptomycin solution (Invitrogen Corporation, Carlsbad, CA); at the beginning of experiments, 50µM forskolin [19] (Sigma-Aldrich, St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) was added to all cell culture preparations. Rolipram (AG Scientific, CA), cilostazol (Otsuka Pharmaceutical, Japan), and dipyridamole

(Boehringer Ingelheim, Germany), dissolved in DMSO, were added to cell culture preparations at concentrations of 10, 20 and/or 30 μM . PDE7 inhibitor BRL50481 (Sigma-Aldrich, St. Louis, MO) was used at 1, 10 and 30 μM . PDE3 inhibitor cilostamide (Sigma-Aldrich, St. Louis, MO) was used at 30 μM . DMSO concentrations were the same throughout all cell culture groups (0.1%). We changed medium at intervals of 48 hours, and cells were treated up to 3 days to enhance barrier function [20].

PKA inhibitors, 10 μM H89 (Calbiochem, San Diego, CA) [21–23] and 1 μM KT5720 [19] (Sigma-Aldrich, St. Louis, MO), and PKG inhibitor, 1 μM KT5823 (Calbiochem, San Diego, CA) [24, 25], were initially dissolved in distilled water (H89) or in DMSO (the latter two). Cells were pretreated with H89, KT5720, or KT5823 for 0.5–1 hour before addition of PDE inhibitors. BRL50481 was added in the same way as cilostazol. 8-(4-Chlorophenylthio)-2'-O-methyl-cAMP (BIOLOG Life Science Institute, Bremen, Germany) was dissolved in DMSO and used at 10 μM [26, 27]. Histamine (Sigma-Aldrich, St. Louis, MO) was used at 200 $\mu\text{g/ml}$ working concentration. Mock treatment used vehicle (PBS diluted in M131 medium) without histamine. Histamine was added after 3 days of treatment with PDE inhibitors.

Measurement of Trans-Endothelial Electrical Resistance (TEER)

TEER across the HBEC monolayer was measured using Electric Cell-substrate Impedance Sensing (ECIS) system Model 1600R (Applied BioPhysics, Troy, NY). Cells were grown on ECIS arrays (8W10E), with each well containing ten gold microelectrodes. Experiments were performed after the cells reached confluence (confirmed by stabilized TEER at baseline) with basal TEER values over 1000 Ω . The long-term resistance increase was monitored with the multi-frequency option (62.5, 125, 250, 400, 500, 1000, 2000, 4000, 8000, 16000, 32000, and 64000 Hz). According to the manufacturer's instructions, data were displayed at 400 Hz (corresponding to establishment of cell-cell junctions). Histamine-induced decline was also measured at 400Hz. Resistance values of empty wells were measured and subtracted from TEER data. TEER at representative time points from three independent experiments were pooled and plotted against time. ECIS data were adjusted (normalized) to control.

Western blot and cAMP studies

Cells were collected in RIPA Lysis and Extraction Buffer with Halt Protease Inhibitor Cocktail and phosphatase inhibitor (Thermo Scientific, Waltham, MA). Protein concentration was measured by Bradford assay (Thermo Scientific, Waltham, MA). Protein was mixed with Novex Tris-Glycine SDS Sample Buffer and Reducing Agent (both from Invitrogen Corporation, Carlsbad, CA) before loading to 10% polyacrylamide gel (Invitrogen, Carlsbad, CA) and subject to electrophoresis (75 Volts). Protein was transferred to PVDF (Polyvinylidene Difluoride) membrane (Millipore, Bedford, MA) at 30 volts at 4°C overnight. Membrane was blocked in 5% milk for 1 hour at room temperature, and later incubated with mouse anti-claudin-5 monoclonal antibody (Invitrogen Corporation, Carlsbad, CA). After incubation with primary antibody, membrane was washed with TBST (1% Tween 20) and then incubated with secondary antibody: goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA). Membrane was washed 3 times with TBST, 10 minutes each. Membrane was incubated in SuperSignal West Pico Chemilumin (Thermo Scientific, Waltham, MA) for 5 minutes before imaging. Membrane was stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Waltham, MA) and re-probed for actin with anti-actin goat polyclonal IgG and donkey anti-goat IgG-HRP (both Santa Cruz Biotechnology, Santa Cruz, CA). Images were quantified using ImageJ (NIH). Intracellular levels of cAMP were measured by enzyme immunoassay (R&D systems, Minneapolis, MN), according to instructions from the manufacturer.

F-actin staining

F-actin was stained with rhodamine phalloidin (Cytoskeleton, Inc., Denver, CO). Briefly, cells were quickly and gently washed with PBS for 30 seconds and immediately fixed with 10% (v/v) formaldehyde and 3% methanol for 10 min at room temperature. Cells were then (1) permeabilized with 0.5 % Triton X-100 in PBS for 5 min, (2) incubated with rhodamine phalloidin for 30 min in the dark, (3) incubated with DAPI (Roche Diagnostics, Indianapolis, IN) for 10 min in the dark. Images were taken with a fluorescent microscope and Olympus Camera.

Permeability Assay

Fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA; Invitrogen Corporation, Carlsbad, CA) stock solution concentration was 5 mg/ml. FITC-labeled dextran (molecular size: 70kDa, Invitrogen, Carlsbad, CA) stock solution was 50 mg/ml. HBECs were cultured on 12 mm diameter transwell insert (pore size 0.4 μ m) (Corning, Lowell, MA) and treated for 3 days total. Prior to permeability assay, stock solution was diluted 100 times with M131 medium and added to the upper chambers. Upper chamber was sampled at the beginning; bottom chamber was sampled periodically (15 minutes e.g.) for at least 3 consecutive time points. Fluorescence intensity was measured using Chameleon Mikrowin-2000 microplate fluorescent reader (Bioscan, Washington, DC). Permeability coefficient (P, mm/s) was calculated using the following equation: $P = [V/(A \times C_0)] \times [dC/dt]$, where V is the receiver volume (the volume of bottom chamber), A is the surface area of the endothelial monolayer, C_0 is the concentration of the donor solution (the initial concentration of the upper chamber), and dC/dt is the rate of diffusion across the monolayer [28]. Permeability coefficient of endothelial cell monolayer (P_e) was calculated using the following equation: $1/P = 1/P_e + 1/P_f$, where P_f is the permeability coefficient of the transwell membrane without cells [29].

Statistical Analysis

Statistical analysis was performed using analysis of variance with Tukey's tests for individual comparisons of groups. A p-value of <.05 was considered statistically significant.

Results

F-actin staining showed cilostazol and dipyridamole (30 μ M) modified actin cytoskeleton distribution. Both cilostazol and dipyridamole induced more concentrated actin in the central perinuclear region (Figure 1). Moreover, cilostazol induced F-actin mesh that was more extensive and uniformly distributed throughout cell. Dipyridamole induced F-actin distribution that concentrated in the cell periphery (cortical actin).

Protein immunoblotting studies demonstrated that after three days cilostazol (30 μ M) increased tight junction protein claudin-5 expression by 118% ($p < .01$ vs control), while rolipram and dipyridamole (30 μ M) had no effect (Figure 2). Permeability studies demonstrated permeability to albumin and dextran of control was $1.1 \pm 0.2 \times 10^{-6}$ cm/s and $3.8 \pm 0.5 \times 10^{-6}$ cm/s, respectively. Cilostazol (30 μ M) decreased endothelial permeability to both albumin (21% less than control, $p < .05$) (Figure 3A) and dextran (37% less than control, $p < .001$). Dipyridamole (30 μ M) also reduced dextran permeability (44% less than control, $p < .001$) (Figure 3B).

After 2 hour treatment, cilostazol (30 μ M) significantly increased TEER compared to control. After 12 hours, cilostazol increased TEER by 111% compared to control ($p < .0001$). Rolipram and dipyridamole did not significantly increase TEER (Figure 4A). Protein kinase A (PKA) inhibitors H89 (Figure 4B) and KT5720 (Figure 4C) substantially attenuated

elevated TEER induced by cilostazol. However, PKG inhibitor KT5823 did not modify increased resistance induced by cilostazol, and Epac activator 8-(4-Chlorophenylthio)-2'-O-methyl-cAMP (10 μ M) did not significantly increase TEER (data not shown). Use of PDE7 inhibitor BRL50481 (1, 10, and 30 μ M) did not significantly modify TEER (data not shown). Cilostamide (30 μ M), another PDE3 inhibitor, also increased TEER (25 $\pm$ 9%, 29 $\pm$ 8%, 30 $\pm$ 9%, 29 $\pm$ 9%, 30 $\pm$ 11%, 30 $\pm$ 12%, and 29 $\pm$ 13% increase at 6, 7, 8, 9, 10, 11, and 12 hours after treatment, respectively; $p < .05$ vs control in all cases, pooled data of three independent experiments performed in triplicate). Levels of intracellular cAMP were similar for cilostazol-, rolipram-, and dipyridamole-treated cells (data not shown).

Histamine (200 μ g/ml) induced a transient decline in TEER, with maximum decline between 1 and 2 minutes in all treatment and control groups (Figure 5A). Compared to control, HBEC treated with cilostazol maintained an overall higher TEER both before and after histamine treatment (Figure 5A). Mock treatment (vehicle without histamine) produced no decline in TEER (data not shown). After treatment for 3 days, cilostazol (30 μ M), but not dipyridamole or rolipram, increased TEER (32% higher than control, $p < .0001$) (Figure 5B). When comparing the lowest point (nadir) of TEER during histamine treatment, cilostazol produced a dose-dependent increase, with 30 μ M increasing nadir by 40% compared to the nadir of control ($p < .0001$) (Figure 5B). Percent changes in TEER following treatment with 30 μ M cilostazol reflect decline from 3224 $\pm$ 380 ohms ($p < .001$ vs control) to 2759 $\pm$ 312 ohms ($p < .001$ vs control, pooled data of three independent experiments performed in triplicate).

Discussion

We analyzed effects of cilostazol, dipyridamole, and rolipram, on barrier properties of HBEC *in vitro*. Cilostazol and dipyridamole decreased dextran (para-cellular) permeability and modified actin cytoskeleton distribution. Cilostazol decreased albumin (trans-cellular) permeability and increased tight junction protein claudin-5 expression. Cilostazol most potently and persistently increased TEER (mediated by PKA), producing an overall higher TEER throughout the course of response to histamine. These findings demonstrate distinctive effects of cilostazol and other PDE inhibitors on modulating endothelial barrier properties.

It is well-established that cAMP elevating agents increase TEER [30, 31]. However, the duration of increase has been largely limited. In one study, cAMP-dependent increase in TEER induced by adenosine lasted for a maximum of only 2 hours, after which the resistance declined dramatically [32]. Another study showed that TEER induced by forskolin and rolipram increased within one hour range [31]. Our study demonstrated treatment with rolipram increased TEER (compared to baseline), but TEER began to decline at approximately 2 hours. In contrast, treatment with cilostazol produced a sustained increase in TEER lasting for at least 12 hours. This suggests a mechanism different from that of rolipram may be involved in mediating the enhancement of barrier functions induced by cilostazol.

The effects of cilostazol in this study are likely mediated via PDE3. Cilostazol is a potent PDE3 inhibitor [12] and PDE3 is known to be expressed by HBEC [33]. Moreover, another PDE3 inhibitor (cilostamide) increased TEER in the current study. However, cilostazol may also inhibit PDE5, PDE7 (IC₅₀ of 4.4 and 21.4 μ M, respectively) [34], and adenosine uptake (IC₅₀ of 5–10 μ M) [35]. In our study, cilostazol was most effective at 30 μ M, higher than its IC₅₀ for PDE3 (0.20 μ M for PDE3A and 0.38 μ M for PDE3B in human recombinant phosphodiesterases [36]; 1 to 10 μ M in intact cells or hearts [35]). At this concentration, cilostazol may inhibit other targets. It is unlikely that cGMP dependent

PDE5[13] was involved, because cGMP inhibitor KT5823 did not modify effects of cilostazol; moreover, cilostazol has been shown to increase cAMP, but not cGMP levels in platelets [37]. It is also unlikely that inhibition of adenosine uptake was the mechanism, since dipyridamole (which also inhibits adenosine uptake [38]) did not increase TEER. It is also unlikely PDE7 mediated these effects, because PDE7 inhibitor BRL50481 did not increase TEER, despite known presence of PDE7 in HBEC and in rat brain[13].

Elevation of intracellular cAMP is known to induce barrier formation in cultured brain endothelial cells [29, 39, 40], and treatment of brain endothelial cells with PDE inhibitors strengthens monolayer integrity [41, 42]. More specifically, Ishiguro et al showed that cilostazol protected brain endothelial cells against in vitro ischemia (OGD) and enhanced VE-cadherin via cAMP/PKA [43]. Ishiguro et al later showed blood-brain barrier protective effects of cilostazol in an in vivo model of experimental stroke, and also showed evidence of endothelial cell protection by cilostazol in vitro [44]. Easton and Dvorini-Zis showed that rolipram (at 100uM) blocked histamine-induced p-selectin expression by brain endothelial cells [45]. Folcik et al showed that rolipram improved blood-brain barrier permeability in vivo during experimental autoimmune encephalomyelitis [46]. Guo et al showed that dipyridamole protected brain endothelial cells against OGD-induced MMP-9 release [47]. Mackic et al examined the response of brain endothelial cells to Cereport, a bradykinin B2 agonist, showing that increased permeability was inhibited by rolipram and increased by zaniprist; the latter was attributed to cGMP-mediated effects [48]. The study of Mackic et al is particularly relevant to the current investigation [48]. We have previously shown that rolipram induces only minor changes in permeability of brain endothelial cell monolayer treated with forskolin [49]. Moreover, dipyridamole is known to have cGMP-mediated effects [38]. Therefore, our findings for rolipram (no effects on response to histamine) and dipyridamole (increased response to histamine) appear to be consistent with the work of Mackic et al [48].

Our results showed PKA inhibitors (H89 and KT5720) strongly attenuated the increased TEER induced by cilostazol. This suggests cilostazol increases TEER through PKA-dependent pathways. H89 has been found also to inhibit cilostazol-induced vasodilator-stimulated phosphoprotein (VASP) phosphorylation in platelets and platelet aggregation [37]. In a recent study cilostazol activated PKA and Epac1 pathways, leading to increased integrin expression and endothelial adhesion [50]. We used 8-(4-Chlorophenylthio)-2'-O-methyl-cAMP, a potent, specific and membrane-permeant activator of Epac, to see if Epac activation would lead to similar results observed with cilostazol. We have previously shown that this Epac activator reduced tPA expression in our cell culture system [51]. However, this Epac activator was unable to increase TEER, suggesting Epac activation was not responsible for cilostazol mediated effects. These findings suggest PKA activation is regularly involved in those cilostazol-mediated effects. Since PDE3 activates and is a substrate for PKA [52] and function of PDE3 is dependent on PKA and actin cytoskeleton [53], the effect of cilostazol in our study is likely mediated by PDE3. We also measured cAMP levels and found comparable levels for all three PDE inhibitors, suggesting that the difference in efficacy may not be related to overall cAMP-elevating potential. This is consistent with the understanding that cAMP signaling is compartmentalized, regulated both spatially and temporally, and that global measurements of cAMP may not represent the complexity of the cAMP signals [53, 54]. Different PDE inhibitors inherently differ from each other in their specificity to PDE targets, generating unique cAMP patterns and alterations of signaling pathways.

PKA plays an important role in regulating endothelial barrier properties via cytoskeletal rearrangement [55], and endothelial cytoskeleton is essential for endothelial permeability [23, 56]. We observed that dipyridamole treatment produced more pronounced cortical

actin, which is necessary for maintenance of endothelial barrier integrity [56]. Consistently, dipyridamole improved barrier function in terms of decreased permeability to dextran (para-cellular permeability). Cilostazol improved barrier function, with decreased permeability to both albumin and dextran (trans-cellular and para-cellular permeability, respectively [29, 57]) and increased TEER. This may be due to enhanced tight junctions, as evidenced by upregulated claudin-5 expression. Claudin-5 is a key tight junction protein linked to barrier properties *in vitro* and *in vivo* and is the most prominent tight junction protein induced by cAMP in brain endothelial cells [58, 59]. Cilostazol has been shown to reduce brain edema and hemorrhagic transformation *in vivo* by inhibiting decreased expression of claudin-5 [44]. Moreover, cilostazol has been shown to protect the blood-brain barrier *in vitro* by increasing VE-cadherin expression in brain endothelial cells via cAMP/PKA-dependent pathways [43].

We observed mitigation of histamine-mediated effects on endothelial resistance by cilostazol. Histamine, a mediator of inflammation, is released by mast cells and circulating basophils, resulting in increased endothelial permeability and vascular leakage [60, 61]. Histamine induces a rapid and transient increase in barrier permeability, as shown by a transient decrease in TEER [62, 63]. The precise pathway by which histamine increases permeability is incompletely understood. Potential mechanisms include calcium mobilization and activation PKC, myosin light chain phosphorylation by myosin light chain kinase (MLCK) and actin-myosin contraction, and alterations in actin cytoskeleton [60, 64–66]. Cilostazol may interfere with the effects on histamine on multiple levels. Cilostazol inhibits the redistribution of the actin cytoskeleton and junctional proteins under hypoxia/reoxygenation conditions [23]. It also inhibits calcium mobilization, which attenuates the histamine-induced contraction in smooth muscle of the peripheral middle cerebral artery in rabbits [67]. In the current study, increased TEER induced by cilostazol was maintained after histamine so that absolute TEER level after decline remained higher than the baseline control level.

The implications of our findings are limited by the *in vitro* nature of the study and the characteristics of our *in vitro* model. Therefore, extrapolations of our findings to the *in vivo* setting are of necessity limited and must be done with caution. Specifically, we utilized passaged HBEC, with forskolin treatment to improve basal barrier properties [22, 68]. In addition, the concentration of cilostazol in our system (30 μ M) may be higher than that found in clinical use. After oral administration, concentration of cilostazol has been shown to be 2–10 μ M in plasma but may be higher in certain tissues because of lipophilicity [69, 70]. Nonetheless, 30 μ M cilostazol has been used in prior studies [37, 69, 71]. Our study is consistent with the protective effects against ischemic-reperfusion injury in mouse cerebrum [72] as well as therapeutic efficacy of cilostazol in stroke clinical trials [9–11]. It is noteworthy that use of cilostazol was associated with fewer ischemic strokes and hemorrhagic events than aspirin in the stroke clinical trials [11], suggesting a beneficial impact on both thrombosis and hemostasis. Rolipram was used at 10 μ M *in vivo* and *in vitro* to increase intracellular cAMP levels [73]. Dipyridamole used at 5 μ M significantly attenuated ICAM-1 and MMP-9 levels after inflammatory challenge [47] and has been used at concentration of 100 μ M *in vitro* [37].

In conclusion, cilostazol and other PDE inhibitors modified multiple aspects of brain endothelial barrier properties *in vitro*, including TEER, permeability, tight junction protein expression, and actin cytoskeleton. In addition, cilostazol modified brain endothelial barrier response to histamine injury, suggesting a protective effect on vascular integrity. These *in vitro* findings are consistent with a potential therapeutic role for PDE inhibitors in the treatment of cerebral microvascular diseases.

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Figure 1. Phalloidin rhodamine staining (200X magnification) of F-actin of human brain microvascular endothelial cells (control) (**A**), and microvascular endothelial cells treated with cilostazol (**B**), rolipram (**C**), and dipyridamole (**D**). Cilostazol and dipyridamole treatment produced more pronounced actin in nuclear region (shown with vertical arrow); dipyridamole also increased cortical actin (shown with horizontal arrow). All PDE inhibitors used at 30 μ M. Scale bar is 50 μ M. A representative image is shown for each treatment.

Figure 2. Effect of PDE inhibitors (30 μ M) on change in tight junction protein claudin-5 expression. Cilostazol significantly increased claudin-5 expression. Signal intensity ratio between of claudin-5 and actin was normalized to control. Pooled results from three independent experiments. Values represent mean; error bars represent standard error (* $p < .05$ vs control).

3A

3B

Figure 3.

Effect of PDE inhibitors (30 μ M) on endothelial permeability to albumin (trans-cellular permeability marker) and on dextran (para-cellular permeability marker). Permeability to albumin was significantly decreased by cilostazol (A) while permeability to dextran was significantly decreased by both cilostazol and dipyridamole (B). Data were pooled from three independent experiments and normalized to control. Values represent mean; error bars represent standard error (* p <.05 vs control).

4A

4B

4C

Figure 4.

Effect of PDE inhibitors on trans-endothelial electrical resistance (TEER). (A) Cilostazol most potently and persistently increased TEER measured after 1 hour. Protein kinase A inhibitors H89 (B) and KT5720 (C) attenuated TEER increase induced by cilostazol at 1 hour after treatment. For A, data were pooled from three independent experiments and normalized to control at time 0; for B and C, data were pooled from three independent experiments and normalized to control. Values represent mean; error bars represent standard error (* $p < .05$ vs control at each corresponding time point).

(A)

(B)

Figure 5.

Pretreatment for 3 days with PDE inhibitors modified the histamine-induced decline in TEER. Histamine (200 μg/ml) induced a transient decline in TEER (A). Both before and after histamine (including at TEER nadir), only 30 μM cilostazol produced higher TEER than control (B). Data were pooled from three independent experiments and normalized to control at time 0. Values represent mean; error bars represent standard error (comparing TEER before histamine: *p < .05 vs control; comparing nadirs: † p < .05 vs control).

Figure 6.
Schematic representation of findings.