Published in final edited form as:

J Cell Physiol. 2019 May ; 234(5): 5863–5879. doi:10.1002/jcp.26419.

Differential mechanisms of adenosine- and ATP γ S-induced microvascular endothelial barrier strengthening

Róbert Bátori¹, Sanjiv Kumar¹, Zsuzsanna Bordán¹, Mary Cherian-Shaw¹, Anita Kovács-Kása¹, Justin A. MacDonald², David J. R. Fulton^{1,3}, Ferenc Erdősi^{4,5}, Alexander D. Verin^{1,6}

¹Vascular Biology Center, Augusta University, Augusta, Georgia

²Department of Biochemistry & Molecular Biology, Smooth Muscle Research Group, University of Calgary, Calgary, Alberta, Canada

³Department of Pharmacology, Augusta University, Augusta, Georgia

⁴Department of Medical Chemistry, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

⁵MTA-DE Cell Biology and Signalling Research Group, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

⁶Department of Medicine, Augusta University, Augusta, Georgia

Abstract

Maintenance of the endothelial cell (EC) barrier is critical to vascular homeostasis and a loss of barrier integrity results in increased vascular permeability. While the mechanisms that govern increased EC permeability have been under intense investigation over the past several decades, the processes regulating the preservation/restoration of the EC barrier remain poorly understood. Herein we show that the extracellular purines, adenosine (Ado) and adenosine 5'-[γ -thio]-triphosphate (ATP γ S) can strengthen the barrier function of human lung microvascular EC (HLMVEC). This ability involves protein kinase A (PKA) activation and decreases in myosin light chain 20 (MLC20) phosphorylation secondary to the involvement of MLC phosphatase (MLCP). In contrast to Ado, ATP γ S-induced PKA activation is accompanied by a modest, but significant decrease in cyclic adenosine monophosphate (cAMP) levels supporting the existence of an unconventional cAMP-independent pathway of PKA activation. Furthermore, ATP γ S-induced EC barrier strengthening does not involve the Rap guanine nucleotide exchange factor 3 (EPAC1) which is directly activated by cAMP but is instead dependent upon PKA-anchor protein 2 (AKAP2) expression. We also found that AKAP2 can directly interact with the myosin phosphatase-targeting protein MYPT1 and that depletion of AKAP2 abolished ATP γ S-induced increases in transendothelial electrical resistance. Ado-induced strengthening of the HLMVEC barrier required the coordinated activation of PKA and EPAC1 in a cAMP-dependent manner. In

Correspondence: Alexander D. Verin. Vascular Biology Center, Augusta University, Augusta, GA., averin@augusta.edu.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

summary, ATP γ S-induced enhancement of the EC barrier is EPAC1-independent and is instead mediated by activation of PKA which is then guided by AKAP2, in a cAMP-independent mechanism, to activate MLCP which dephosphorylates MLC20 resulting in reduced EC contraction and preservation.

Keywords

adenosine; ATP γ S; endothelial barrier protection; myosin light chain; PKA

1 | INTRODUCTION

Endothelial cells (ECs) form a semiselective barrier between the interior space of blood vessels and underlying tissues contributing to important physiological processes, like regulation of vascular tone, leukocyte infiltration (Song et al., 2017; Wittchen et al., 2005) and maintenance of normal tissue-fluid homeostasis (Mehta & Malik, 2006). Disruption of the endothelial barrier can lead to severe disease such as acute lung injury (ALI). ALI is characterized by a significant pulmonary inflammatory response resulting in a protein-rich pulmonary edema due to the loss of microvascular endothelial barrier integrity, secondary to endothelial dysfunction (Matthay & Zimmerman, 2005). Despite years of research, the mechanisms that govern EC barrier preservation are not completely understood.

Extracellular purines, adenosine (Ado) and adenosine triphosphate (ATP), function as intercellular signaling molecules (Hasko & Cronstein, 2013; Hasko & Cronstein, 2004; Patil, Kaplan, & Minnear, 1997) initiating their effects by ligating cell surface P1 and P2 purinergic receptors, respectively (Ralevic & Burnstock, 1998). P1 receptors can be further divided into four subclasses, namely A1, A2A, A2B, and A3 Ado receptors (Sheth, Brito, Mukherjea, Rybak, & Ramkumar, 2014). The A1 and A3 receptors are coupled to G_i proteins that inhibit adenylyl cyclase, while A2A and A2B are G_s protein-coupled receptors that can therefore increase intracellular cyclic adenosine monophosphate (cAMP) level (Sheth et al., 2014). The physiologic effects of extracellular ATP and its stable, very slowly hydrolysable analog, adenosine 5'-[γ -thio]-triphosphate (ATP γ S), are mediated by P2 receptors (Burnstock & Ralevic, 2014; Ralevic & Burnstock, 1998). P2 receptors are divided into two primary subclasses: P2X receptors are extracellular ATP-gated, calcium-permeable, nonselective cation channels, while P2Y receptors are G-protein-coupled receptors. Among the eight known purinergic P2Y receptors, P2Y1, 2, 4, 11, and 12 can be activated by ATP γ S in mammals (Burnstock, 2004). P2Y1, 2, 4, 6 and 11 are coupled to G_{q/11} and activate PLC β (Burnstock, 2004). P2Y12, 13, and 14 are coupled to G_i proteins (Burnstock, 2004; Burnstock, 2008a; 2008b; Erlinge & Burnstock, 2008; Ralevic & Burnstock, 1998). P2Y11 is also coupled to G_s protein which activates adenylyl cyclase thus increasing cAMP level (Torres, Zambon, & Insel, 2002). While P2Y4 receptors are preferentially coupled to G_q, coupling to G_i proteins has also been reported in specific cell types (Filippov, Simon, Barnard, & Brown, 2003). Despite evident similarity between ATP and ATP γ S, there are potentially important differences in their engagement with P2 receptor subtypes (Burnstock, 2004). For example, in contrast to ATP γ S, ATP cannot activate P2Y12 receptors (Burnstock, 2004). Further, P2Y2 receptors can be activated with relatively low concentrations of ATP

(<1 μ M) and higher concentration of ATP γ S (>10 μ M), while P2Y₁₁ receptors are more selective to ATP γ S (Burnstock, 2004).

The second messenger cAMP is best known for its role as an important mediator of smooth muscle (SM) relaxation but it also promotes EC barrier integrity, at least in part, via activation of the protein kinase A (PKA) pathway (Garcia, Davis, & Patterson, 1995; Patterson & Garcia, 1994; Patterson, Lum, Schaphorst, Verin, & Garcia, 2000). Increasing cAMP levels represents a classical means of PKA activation, however, several studies have demonstrated that PKA activation may also occur independent of changes in cAMP levels via coupling of specific trimeric G proteins with scaffolding proteins known as PKA-anchoring proteins (AKAPs), resulting in release of the PKA catalytic subunit from its regulatory subunit, thus to kinase activation (Niu et al., 2001). Recent studies also demonstrate that AKAP9 and 12 may contribute to cAMP-dependent EC barrier regulation (Sehrawat et al., 2011; Weissmuller et al., 2014). Barrier preservation mediated by cAMP can also involve Rap guanine nucleotide exchange factor 3, also known as an exchange factor directly activated by cAMP 1 (EPAC1; Birukova et al., 2010). It has been reported that both, PKA and EPAC1 can activate Ras-related C3 botulinum toxin substrate 1 (Rac1), a small GTPase (Birukova et al., 2010; Lu et al., 2010), which is commonly implicated in EC barrier strengthening due to actin cytoskeletal rearrangement (Nerlich et al., 2009), and regulation of myosin light chain phosphatase (MLCP; Shibata et al., 2015). In SM cells EPAC1 can also modulate MLCP activity via a Rap1–RhoA–ROCK1-mediated pathway (Lakshmikanthan et al., 2014).

Our group has previously reported that extracellular ATP significantly enhanced barrier integrity via G_{i2}-coupled P2Y receptors in human pulmonary artery ECs (HPAEC) and this effect is dependent, at least in part, on activation of both PKA and MLCP (Kolossova et al., 2005). MLCP consists of a protein phosphatase-1 (PP1) catalytic subunit, myosin phosphatase-targeting subunit 1 (MYPT1) and a 20 kDa subunit, M20, with an unknown function (Grassie, Moffat, Walsh, & MacDonald, 2011; Hartshorne, Ito, & Erdodi, 2004). The activity of MLCP can be regulated by phosphorylation of the regulatory subunit MYPT1 at Ser and Thr residues. Phosphorylation of MYPT1 by Rhokinase (ROCK) at Thr696 and Thr853 inhibits enzyme activity (according to the numbering of human isoform; Ichikawa, Ito, & Hartshorne, 1996; Khromov, Choudhury, Stevenson, Somlyo, & Eto, 2009; Kimura et al., 1996), whereas phosphorylation of MYPT1 at Ser695/Ser852 or dual phosphorylation at Ser695/Thr696 and/or Ser852/Thr853 in response to cAMP-dependent PKA activation prevents the inhibitory phosphorylation of MYPT1 by ROCK (Wooldridge et al., 2004), thus activating (de-inhibiting) MLCP.

The expression of purinergic receptors among different EC types is variable. Human umbilical vein EC (HUVEC) mainly express P2Y₁, P2Y₂, and P2Y₁₁ receptors, while the expression level of P2Y₄ and P2Y₆ is approximately seven-fold lower. We have previously observed differences in the expression of some P2Y receptors in HPAEC and human lung microvascular EC (HLMVEC; Zemskov, Lucas, Verin, & Umapathy, 2011), however, the complete profile of P2Y receptors in HLMVEC has not been reported. HPAEC also express A_{2B} and, to a lesser extent, A_{2A}, but not A₁ and A₃ Ado receptors (Umapathy et al., 2010). Ado receptor expression profile in HLMVEC has not been assessed.

While the EC barrier-protective effects of extracellular ATP γ S and Ado in macrovascular EC (HPAEC) and in murine models of ALI have been described (Kolosova et al., 2005; Kolosova et al., 2008; Umapathy et al., 2010), the ATP γ S- and Ado-induced barrier-enhancing mechanisms in the human microvasculature remain largely unknown. The goal of the present study was to define and compare the molecular mechanisms linking Ado- and ATP γ S-induced purinergic receptor activation and barrier strengthening in HLMVECs.

2 | MATERIALS AND METHODS

2.1 | Materials

Chemicals and vendors were as follows: fetal bovine serum, antibiotic–antimycotic solution, sodium pyruvate, L-glutamine were purchased from Gibco (Gaithersburg, MD); Dulbecco's modified Eagle's medium (DMEM), ProLong Gold Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI), and siPORT™ Amine Transfection Agent were from Thermo Fisher Scientific (Waltham, MA); Duolink® proximity ligation assay kit, penicillin–streptomycin, and trypsin-ethylenediaminetetraacetic acid (EDTA) solution, anti-P2Y13, horseradish-peroxidase (HRP)-conjugated anti-mouse IgG, and HRP-conjugated anti-rabbit IgG, phosphatase inhibitor cocktail, and Ado were obtained from Sigma-Aldrich (St. Louis, MO). Endothelial basal medium-2 (EBM-2) and EGM™-MV BulletKit™ were obtained from Clonetics (San Diego, CA). Dynabeads® Protein G were from Life Technologies (Grand Island, NY). Protease inhibitor cocktail, Set III, and anti- β -tubulin were obtained from EMD Millipore (Darmstadt, Germany). X-tremeGENE HP DNA transfection reagent was from Roche (Indianapolis, IN). Four to twenty percent of Mini-PROTEAN® TGX™ Gel, 0.2- μ m pore size polyvinylidene fluoride (PVDF) membrane, Laemmli sample buffer and Trans-Blot® Turbo™ Transfer System, iScript™ cDNA synthesis kit, and SYBR Green were purchased from Bio-Rad (Hercules, CA). Enhanced chemiluminescence (ECL) reagent (Pierce, Rockford, IL). TRIzol reagent was from Life Technologies. Polyclonal antibodies for dual MYPT1 phosphorylation (pMYPT1^{Ser696/Thr697}) were generated and affinity-purified as previously described (Sutherland, MacDonald, & Walsh, 2016). Anti-ppMLC^{T18/S19}, anti-PKA α , and anti-EPAC1 were from Cell Signaling Technology (Beverly, MA). AKAP2 antibody was from Bethyl Laboratories Inc. (Montgomery, TX). Anti-c-myc, anti-HA antibody, rabbit anti-P2Y4-antibody, mouse anti-G_{i2}-antibody, normal rabbit serum, nonspecific (scrambled) small interfering RNA (siRNA), EPAC1, P2Y4, and P2Y13 siRNA were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). AKAP2, P2Y14, and PKA α specific siRNA duplexes were from Dharmacon Research (Lafayette, CO). P2Y1, P2Y11, and P2Y12 receptors specific siRNAs were obtained from Ambion (Austin, TX). Anti-P2Y2, anti-P2Y12, and anti-P2Y14 antibody were from Abcam (Cambridge, MA). Anti-P2Y1 and anti-P2Y11 antibody were from Alomone Labs (Jerusalem, Israel). HyBlot® ES autoradiography film was from Denville Scientific Inc. (Holliston, MA). ATP γ S was purchased from Tocris (Mineapolis, MN). cAMP assay EIA kit was from Cayman Chemicals (Ann Arbor, MI). PKA activity assay kit was from Enzo Life Science (Farmingdale, NY).

2.2 | Cell cultures

Primary HLMVECs were purchased from Lonza and were utilized at passages 4–9 (Walkersville, MD). Cells were cultured in EBM-2 supplemented with 5% (vol/vol) FBS, 0.2 ml of hydrocortisone, 2 ml of human FGF-B, 0.5 ml of vascular endothelial growth factor, 0.5 ml of long-arm insulin-like growth factor-1 (R3-IGF-1), 0.5 ml of ascorbic acid, 0.5 ml of human epidermal growth factor (EGF), 0.5 ml of GA-1000, and 0.5 ml of heparin solutions.

Human Embryonic Kidney cell line (HEK293) was obtained from American Type Tissue Culture Collection (Rockville, MD). The cells were cultured in Dulbecco's modified minimum essential medium (DMEM) supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine and 1% (vol/vol) antibiotic–antimycotic solution. Cells were maintained at 37°C, 5% CO₂ in a humidified atmosphere.

2.3 | Immunoprecipitation

HEK293 cells were lysed in 0.1% (vol/vol) Triton X-100, 150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 20 mM EDTA and 0.5% (vol/vol) and 0.5% (vol/vol) protease inhibitor mix containing buffer. Immunoprecipitations of ectopically expressed c-myc-MYPT1 or HA-AKAP2 from HEK293 were carried out using anti-c-myc and anti-HA antibody, respectively, coupled with protein-G Sepharose magnetic beads. After 3 hr of incubation at 4°C the beads were washed three times with 0.1% (vol/vol) Triton X-100, 0.5 mM NaCl, 50 mM Tris–HCl (pH 7.4), 20 mM EDTA, and 0.5% (vol/vol) protease inhibitor mix containing washing buffer, then boiled in 100 µl 2× Laemmli buffer for 5 min at 100°C. Immunoprecipitates were subsequently subjected to immunoblotting with specific antibodies as described in figure legends.

2.4 | Immunoblotting

HLMVEC cells were washed once with ice-cold PBS on ice, then lysed in 2× Laemmli sample buffer, scraped and sonicated. All of the samples were boiled for 6 min at 100°C and applied for western blotting. Protein samples were separated on 4–20% TGX™ gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.20-µm pore size PVDF membrane with Trans-Blot® Turbo™ Transfer System 25 V, 1 mA for 30 min. The membranes were blocked with 5% (wt/vol) nonfat dry-milk powder solution in TBS-Tween 20 (TBST) and incubated with specific antibodies overnight at 4°C. After incubation with the primary antibodies, the membranes were washed three times with TBST and incubated with HRP-conjugated secondary antibody. Immunoreactive proteins were visualized by ECL-based method on autoradiography films. ImageJ software (Research Services Branch, National Institute of Health, Bethesda, MD) was used for densitometric analyses.

2.5 | Expression plasmid and transfection protocol

HEK293 cells were transfected in 10 cm cell culture dishes with AKAP2 coding pReceiver-M45-AKAP2-C-3xHA+IRES-eGFP and MYPT1 coding pcDNA3.1-FLAG-MYPT1 (GeneCopoeia) expression vectors using X-tremeGENE™ HP DNA transfection reagent according to the manufacturer's instructions. In 600 µl serum-free medium, 6 µg DNA was

added and the mixture was gently suspended, then 18 μ l X-tremeGENE™ HP DNA transfection reagent was added to the mixture and incubated for 24 min at room temperature. Then the transfection mixture was added to the cells in complete media containing 10% FBS and incubated for 48 hr at 37°C. Overexpression of AKAP2 and MYPT1 was assessed in whole cell lysate by immunoblot analysis.

2.6 | Gene silencing

HLMVEC cells seeded on six-well plates were transfected with nonspecific (scrambled), EPAC1, AKAP2, PKA α , or P2Y receptor specific siRNAs in 50 nM final concentration using siPORT™ Amine transfection reagent according to the manufacturer's instructions. Nonspecific siRNA was used as negative control. Briefly, siPORT™ Amine transfection reagent and different siRNAs were diluted in Opti-MEM in separate tubes. After 5 min of incubation at room temperature diluted transfection reagent was added to the diluted siRNA, followed by incubation at room temperature for 20 min. When the transfection complex was formed, the transfection mixture was added to HLMVECs in serum-free media. After 6 hr of incubation the serum-free media was changed to complete EBM-2 supplemented with the components of EGM™-MV BulletKit™ and the cells were incubated for 72 hr at 37°C. Then, the transfected cells were used for further transendothelial electrical resistance (TER) measurements and western blot analysis.

2.7 | Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis was conducted using iScript cDNA Synthesis Kit and 1 μ g RNA template. Diluted cDNA (7.5 $\times$) was used for qPCR reactions. qPCR was performed using a Qiagen Rotor-Gene Q system (Qiagen, Hilden, Germany) and iQ™ SYBR Green Supermix (forward and reverse primers are listed in Supplementary Information Table 1). Data were analyzed using tools developed internally.

2.8 | PKA activity measurement

HLMVECs were incubated in the absence or presence of 50 μ M Ado or ATP γ S for 30 min, then HLMVECs were washed three times with 1 ml ice-cold PBS on ice and lysed in 20 mM MOPS, 50 mM β -glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 2 mM EDTA, 1% NP-40, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) and 1% (vol/vol) protease inhibitor cocktail containing lysis buffer. PKA activities were measured using a commercial kit (Enzo Life Sciences) according to the manufacturer's instructions. Briefly, 96-well assay plates precoated with PKA-specific substrate were incubated with the extracted protein (1 μ g/well) in the presence of ATP and PKA activity was revealed with a phospho-specific substrate antibody.

2.9 | cAMP measurement

HLMVECs cultured in six-well plates were treated at 90% confluency with 100 μ M Ado or ATP γ S for 30 min. After washing with 1 ml PBS HLMVECs were incubated in 250 μ l, 0.1 M HCl at room temperature for 20 min, then scraped, and the mixture was further processed

by pipetting up and down until the suspension became homogeneous. The cell lysate was centrifuged at 1,000g for 10 min, then cAMP was measured from the supernatant by using a cyclic AMP EIA kit (Cayman Chemicals) according to the manufacturer's instructions.

2.10 | Transendothelial electrical resistance measurement

TER of HLMVEC monolayers was measured as previously described (Batori et al., 2017). Briefly, HLMVECs ($2.5\text{--}3 \times 10^4$ cells/well) were seeded on 8W10E electric cell-substrate impedance sensing arrays and experiments were performed on the wells that achieved at least 1,000 Ω of baseline steady-state resistance. Six hours before treatment media was changed to complete EBM-2 and changes in the initial resistance were measured using Model 1600R station controller (Applied BioPhysics, Troy, NY). After treatment with different agonists the collected data were normalized to the initial resistance values and plotted as normalized resistance.

2.11 | Proximity ligation assay (PLA)

HLMVECs cultured in 12-well plates on glass coverslips were fixed with 4% paraformaldehyde solution for 10 min, permeabilized with 0.1% Triton X-100 for 10 min and blocked with 5% BSA in PBS. The PLA was carried out according to the manufacturer's instructions. Briefly, to visualize the interaction between P2Y₄ and G_{i2} the samples were stained with mouse anti-G_{i2} (1:500) and rabbit anti-P2Y₄ (1:500) antibodies overnight, at 4°C prior to incubation with PLA probes for 1 hr at 37°C. Then, ligase was added to hybridize the probes and after the rolling-circle amplification (RCA) the labeled probes were hybridized to the RCA product. After washing three times with PBS the samples were mounted with ProLong Gold Antifade Mountant with DAPI and prepared for fluorescent microscopy.

2.12 | Statistical analysis

Data are presented as mean $\pm$ SEM of at least three independent experiments. Statistical analyses of differences were performed using by the two-tailed Student *t* test for two groups or by analysis of variance followed by the Newman–Keuls post hoc test. Results with $p < 0.05$ were considered statistically significant GraphPad Prism 5.01 software (GraphPad Software Inc., La Jolla, CA).

3 | RESULTS

3.1 | Ado and ATP γ S enhances HLMVEC barrier function

To examine the effects of Ado and ATP γ S on HLMVEC barrier function, we challenged HLMVECs with increasing concentrations (25–200 μ M) of Ado (Figure 1a) or ATP γ S (Figure 1b) and measured TER. Both Ado and ATP γ S-induced TER increase of HLMVECs in a dose-dependent manner, reflecting HLMVEC barrier strengthening with maximal effect at 100 μ M. As such, we used 50 and 100 μ M Ado or ATP γ S in subsequent experiments.

3.2 | Expression of P1 and P2Y purinergic receptors in HLMVECs

We and others have demonstrated various expression patterns of purinergic receptors in EC of different origin (Feoktistov et al., 2002; Zemskov et al., 2011), thus we investigated the expression of G-protein-coupled P1 and P2Y receptors in HLMVEC. Our quantitative reverse-transcription PCR (RT-PCR) analysis showed that HLMVEC mainly expressed A2B receptors with negligible expression of other receptors (Figure 2a). This expression pattern differs significantly compared with HUVEC (Feoktistov et al., 2002) and also varies that of observed in HPAEC (Umapathy et al., 2010) suggesting differences in P1 receptor signaling in ECs from different vascular beds. Analysis of P2Y receptors profile revealed that HLMVECs express messenger RNAs (mRNAs) for all eight receptor types (Figure 2b) with preferential expression of P2Y1, 4, 6, 13, and 14 mRNAs, while the expression level of other receptor subtypes was lower.

3.3 | Identification of G-protein-coupled receptors involved in ATP γ S- and Ado-induced HLMVEC barrier enhancement

Since five P2Y receptors may be activated by ATP γ S, in the next set of experiments we identified the ones which are involved in the ATP γ S-induced increase in TER. HLMVECs were treated with siRNA specific to P2Y1, 2, 4, 11, and 12 for 72 hr, then TER was measured in the presence or absence of ATP γ S. We have demonstrated that depletion of P2Y4 and P2Y12, but not other receptors resulted in significant decrease in TER, indicating that these receptors are involved in ATP γ S- induced HLMVEC barrier enhancement (Figure 3). P2Y12 is solely G_i-coupled receptor (Erb & Weisman, 2012). P2Y4 is the best known to be coupled to G_q (Erb & Weisman, 2012), however, some recent literature data revealed G_i coupling for P2Y4 as well (Filippov et al., 2003). Our PLA experiments (Supporting Information Figure S1) demonstrated that P2Y4 receptor is in close proximity (<40 nm) to G_{i2} in HLMVEC confirming G_i-coupling of this receptor. While none of the P2Y receptors, which are not coupled to G_i, are involved in ATP γ S-induced barrier enhancement (Figure 3), it is reasonable to assume that ATP γ S-induced HLMVEC barrier enhancement is mediated through G_i signaling. To further validate our hypothesis, we depleted G_{i2} from HLMVECs using siRNA approach, then treat ECs with ATP γ S and measure the level of MLC20 phosphorylation at Thr18/Ser19, a known EC contractility index (Goeckeler & Wysolmerski, 1995). We found that depletion of G_{i2} significantly attenuated ATP γ S-induced decrease of MLC20 phosphorylation (Supporting Information Figure S2), confirming that G_{i2}-mediated signaling is involved in ATP γ S-induced EC cytoskeletal rearrangement leading to decrease of EC contractility and barrier strengthening.

Interestingly, in contrast to ATP γ S, Ado-induced HLMVEC barrier strengthening likely involved G_s- and G_q-coupled A2B receptors (Figure 2a), suggesting that mechanisms of Ado- and ATP γ S-induced EC barrier strengthening are drastically different in the microvasculature.

3.4 | Ado and ATP γ S increase PKA activity by different signaling mechanisms

We have previously shown that PKA activation is necessary for Ado- and ATP-induced barrier strengthening in HPAEC (Kolosova et al., 2005; Umapathy et al., 2010). The primary activation pathway for PKA involves the binding of four cAMP molecules to the regulatory

subunit leading to dissociation of the holoenzyme (Nguyen et al., 2013; Tasken et al., 1997). While the G_s -mediated cAMP-dependent activation of PKA by Ado is well described, cAMP-independent mechanisms of PKA activation are by comparison poorly understood. Our data indicate (Figure 4) that microvascular EC barrier strengthening likely involved G_i -mediated, cAMP-independent mechanisms. Ado treatment induced immediate elevation of cAMP levels in HLMVEC (Figure 4a), culminating at 30–45 min which paralleled with Ado-induced TER increase (Figure 1a). In contrast, ATP γ S-induced HLMVEC barrier enhancement accompanied by modest, but significant cAMP decrease started as early as 2 min (Figure 4b), supporting the involvement of G_i -mediated signaling in ATP γ S-induced HLMVEC barrier enhancement. In a parallel experiment, we examined the effect of Ado and ATP γ S on PKA activity in HLMVEC homogenates (Figure 4c). We found that both compounds induced significant activation of PKA after 30 min of treatment. Activation of PKA in EC homogenates is accompanied by increased MYPT1 phosphorylation at Ser695/Thr696, which are established PKA phosphorylation sites (Grassie et al., 2012; Sutherland et al., 2016; Figure 4d, inset). While these sites can be phosphorylated by PKA as well as by PKG in vitro (Wooldridge et al., 2004), our results (Supporting Information Figure S3) indicated that PKG is not involved in Ado or ATP γ S effects on HLMVEC permeability, therefore, it is unlikely that Ser695/Thr696 are phosphorylated in response to these agonists in human microvascular EC. These data strongly suggest that ATP γ S activates PKA via an unconventional G_i -mediated pathway. Since PKA-mediated phosphorylation of MYPT1 is involved in the regulation of MLCP activity, at least in vitro (Wooldridge et al., 2004), these data also suggest that the effects of PKA on ATP γ S-induced cytoskeletal rearrangement and HLMVEC barrier enhancement are MLCP dependent.

3.5 | Differential involvement of EPAC1 and PKA in Ado- and ATP γ S-induced HLMVEC barrier enhancement

To directly evaluate the role of PKA activity in HLMVEC cytoskeletal rearrangement and barrier strengthening induced by either Ado or ATP γ S, we next examined the effects of PKAc depletion on the phosphorylation state of MLC20 in the presence or absence of these purinergic agonists (Figure 5). In control, nonspecific siRNA transfected cells both Ado and ATP γ S decreased the phosphorylation level of MLC20 at Thr18/Ser19 phosphorylation sites, as anticipated (Figure 5a–c). However, depletion of PKAc abolished ATP γ S-, but not Ado-induced MLC20 dephosphorylation (Figure 5a), suggesting the involvement of another (perhaps more complex) signaling pathway that is stimulated by Ado. Since the other cAMP effector, EPAC1 has been reported to be involved in SM relaxation and regulation of MLC20 dephosphorylation (Lakshmikanthan et al., 2014; Zieba et al., 2011), we investigated the involvement of EPAC1 in Ado-induced MLC20 dephosphorylation in HLMVEC. Predictably, EPAC1 depletion had no effect on cAMP-independent ATP γ S-induced MLC20 dephosphorylation (Figure 5b), but surprisingly, had no effect on Ado-induced cAMP-dependent MLC20 dephosphorylation either. However, simultaneous depletion of PKAc and EPAC1 in double silencing experiment (Figure 5c) abolished Ado-induced MLC20 dephosphorylation indicating that PKA and EPAC1 activities are both required to achieve MLC20 dephosphorylation in cAMP-dependent Ado model.

To examine the role of PKA and EPAC1 in HLMVEC barrier strengthening induced by Ado and ATP γ S we first depleted PKAc, then challenged HLMVECs with Ado or ATP γ S and measured TER changes. We found that PKAc silencing significantly attenuated ATP γ S- but not Ado-induced effects on TER (Figure 6a). Depletion of EPAC1 had no effect on TER changes induced by either Ado or ATP γ S (Figure 6b). Similar to the effects on MLC20 phosphorylation, simultaneous depletion of PKAc and EPAC1 attenuated Ado-induced TER increase indicating that PKA effects on EC barrier properties are EPAC1-dependent (Figure 6c). Simultaneous depletion of PKAc and EPAC1 decreased ATP γ S-induced increase in TER (Figure 6c) in the similar extent as depletion of PKAc alone (Figure 6a) as anticipated indicating that EPAC1 is not involved in G $_i$ -mediated ATP γ S-induced EC barrier enhancement. These data suggest that while PKA alone is sufficient to exert barrier-enhancing effect in ATP γ S model, PKA-mediated Ado-induced EC barrier strengthening required simultaneous EPAC1 activation. Conversely, EPAC1-mediated effects of Ado on EC barrier are PKA dependent.

3.6 | AKAP2-MLCP axis is involved in ATP γ S-, but not Ado-induced barrier-enhancing effect in HLMVEC

Scaffolding PKA-binding AKAP proteins exerted their activities mainly via directing PKA to specific subcellular location (targets) and serving as a platform for biochemical interactions (Colledge & Scott, 1999). It has been shown that cAMP-independent PKA activation may involve AKAPs (Niu et al., 2001). Specific AKAPs (e. g., AKAP110) interact with G proteins (Niu et al., 2001), whereas other AKAPs like AKAPs 9 and 12 have been shown to be involved in EC barrier regulation in cAMP-dependent manner (Sehrawat et al., 2011; Weissmuller et al., 2014). In the next set of experiments, we examined the effects of AKAPs depletion on Ado- and ATP γ S-induced TER increase and MLC20 phosphorylation. Depletion of AKAP2 in HLMVECs attenuated the effect of ATP γ S, but not Ado, on TER (Figure 7a). The results are indicative of the specific involvement of AKAP2, but not AKAP9 (Figure 7b) or AKAP12 (Figure 7c) in HLMVEC barrier regulation and further suggest a dominant role for AKAP2 in cAMP-independent PKA activation. AKAP2 depletion experiments also revealed a direct involvement of AKAP2 in ATP γ S-but not in Ado-induced MLC20 dephosphorylation (Figure 8).

Our data (Figures 5 and 6) suggest that PKA affects EC barrier properties through MLC20 dephosphorylation, supporting the involvement of both AKAP2 and MLCP in PKA-mediated EC barrier regulation. Direct interaction of AKAPs and PP1-containing holoenzymes has been reported previously (Schillace, Voltz, Sim, Shenolikar, & Scott, 2001); however, the interaction of AKAP(s) with MYPT1 has not been described in the literature. Therefore, we investigated whether AKAP2 or other AKAPs could interact with MYPT1. HEK293 cells were transfected with c-myc-MYPT1 (Kim et al., 2012) and coimmunoprecipitation experiment was performed. Our results (Figure 9a) demonstrated that MYPT1 could be co-immunoprecipitated with AKAP2 but not with AKAP9 or AKAP12, suggesting the existence of a specific functional complex between AKAP2 and MLCP. Reciprocal experiments where HA-AKAP2 was overexpressed (Figure 9b) and provided coimmunoprecipitation of MYPT1 further supported an interaction (direct or indirect). Moreover, our results provided evidence of PKAc and G $_{i2}$ coimmunoprecipitation

with AKAP2 in HLMVECs (Figure 9b) suggesting the scaffolding role AKAP2 in G_i-mediated PKA activation. Collectively, our data strongly suggest the existence of a novel mechanism of G_i-mediated cAMP-independent activation of PKA which involves AKAP2 as part of a larger PKA/AKAP2/MLCP signaling axis that mediates ATP γ S-induced EC barrier strengthening.

4 | DISCUSSION

EC form the inner layer of the blood vessel, and as such the barrier integrity of EC acts as the gatekeeper for controlling the passage of fluids, macromolecules, and immune cells between the inner space of blood vessels and the surrounding interstitium (Mehta & Malik, 2006). The lung EC barrier can become compromised due to unbalancing of the contractile and tethering forces in EC, which leads to pulmonary edema and is the primary pathology of ALI (Matthay & Zimmerman, 2005). We and others have previously shown that extracellular purines, ATP and its degradation product, Ado, are able to preserve EC barrier in pulmonary artery EC (PAEC; Kolosova et al., 2005; Lu et al., 2010; Umapathy et al., 2010). We have also demonstrated previously that Ado and a very slowly hydrolysable ATP analog, ATP γ S, attenuated LPS-induced lung inflammation and vascular leak in murine models of ALI (Gonzales et al., 2014; Kolosova et al., 2008). Furthermore, we shown that barrier protection/enhancement induced by Ado or ATP in HPAEC involves the activation of PKA and MLCP leading to decreased contractile responses in ECs (MLC20 dephosphorylation, stress fibers dissolution), an increase in cortical actin and the strengthening of intercellular contacts (Kim et al., 2012; Kolosova et al., 2005; Umapathy et al., 2010). However, the upstream signaling pathways leading to PKA and MLCP activation induced by purinergic agonists in HPAEC are largely unknown. These pathways are diverse and include activation of the A2A receptors, G_s engagement and cAMP increases (for Ado) and P2Y-mediated G_{i2} and G_q activation leading to PKA/MLCP activation in cAMP-independent manner (for ATP; Kolosova et al., 2005; Umapathy et al., 2010).

In the present study, we characterized and compared the signaling pathways in HLMVECs mediating Ado- and ATP γ S-induced EC barrier-strengthening. Our data demonstrate that both Ado and ATP γ S have similar functional effects on the EC barrier in vitro. Since ATP γ S is very slowly hydrolysable, it cannot activate Ado receptors. Further, the formation of pro-inflammatory “inflammasomes” apparently requires ATP hydrolysis (Baron et al., 2015), and therefore, ATP γ S is unlikely to increase EC inflammatory responses. Indeed, we have previously shown that ATP γ S decreases LPS-induced inflammation in murine model of ALI (Kolosova et al., 2008).

There are no prior studies in the literature reporting on the mRNA expression profile of Ado (P1) and P2 receptors in HLMVEC. To provide a comprehensive analysis we measured the mRNA expression of each receptor subtype using qRT-PCR. A2B was found to be the most abundant P1 receptor in HLMVECs, while the expression of other receptors is very low or none (see Figure 2). In contrast, our published data demonstrated that large vessel EC (HPAEC) predominantly express A2A and A2B receptors with A2A expression approximately two times lower compared with the A2B isotype (Umapathy et al., 2010). Differential expression of P1 receptors has also been observed in human macrovascular

(HUVEC) and microvascular EC (HMEC-1) where they are proposed to contribute to the functional heterogeneity of human macrovascular and microvascular EC (Feoktistov et al., 2002). We have shown that A2A, but not A2B is functionally important in Ado-induced barrier enhancement in HPAEC (Umapathy et al., 2010). A2B is considered a low-affinity receptor and is ~50 times less sensitive than other receptors requiring supraphysiological conditions for its activation (Fredholm, Irenius, Kull, & Schulte, 2001). However, Qing et al. provided evidence that both A2A and A2B, but not A1 or A3 receptors are involved in the barrier-enhancing effects of Ado in bovine PAEC (Lu et al., 2010). Genetic ablation of the A2B receptor exacerbated the loss of barrier function and increased pulmonary inflammation in bleomycin ALI model, but decreased inflammatory responses in a chronic model of lung injury (Zaynagetdinov et al., 2010; Zhou et al., 2011). Collectively, these findings strongly suggest that the expression level and functional significance of P1 receptor subtypes is dissimilar in different disease states and highly dependent on the vascular bed where the EC were obtained and further emphasizes a critical need to examine the signaling mechanisms of P1-mediated barrier-enhancement in HLMVEC.

A2, but not A1 or A3 Ado receptors are coupled to G_s trimeric G-protein, which activate adenylyl cyclase (Klinger, Freissmuth, & Nanoff, 2002). Our results demonstrate that Ado-induced increases in TER in HLMVEC (see Figure 1) are accompanied by increased cAMP production (see Figure 4) supporting the involvement of the G_s-coupled A2B receptor in HLMVEC barrier strengthening.

Based on our qPCR analysis all eight P2Y receptors are expressed in HLMVECs in different ratios (see Figure 2b), five P2Y receptors (P2Y 1, 2, 4, 11, and 12) can be activated by different concentrations of ATP γ S in mammals (Burnstock, 2004). However, there are some inconsistencies in the literature with regard to the selectivity of P2Y receptors toward their agonists. Molecular cloning and characterization of rat P2Y4 (rP2Y4) and human P2Y4 (hP2Y4) receptors revealed, that these proteins share ~83% sequence homology, furthermore, ATP and ATP γ S are potent agonists of rP2Y4 receptors (Bogdanov, Wildman, Clements, King, & Burnstock, 1998). Kennedy, Qi, Herold, Harden, and Nicholas (2000) demonstrated that ATP did not activate hP2Y4, and behaved more as a competitive antagonist of hP2Y4 and a potent agonist of rP2Y4, although they did not investigate the effect of ATP γ S against the hP2Y4. In contrast, Bilbao, Santillan, and Boland (2010) provided evidence that stimulation of P2Y2/P2Y4 receptors by ATP increased the proliferation rate of human breast cancer MCF-7 cells by a PI3K/Akt-dependent signaling mechanism indicating the involvement of these receptors and demonstrating that they can be activated by ATP in human cells. The latter results are consistent with our recent findings on P2Y receptor-depleted cells. We show that depletion of P2Y4 and P2Y12 receptors resulted in a significant decrease in TER following ATP γ S stimulation. This data demonstrates a primary role of both receptors in ATP γ S-induced HLMVEC barrier enhancement. Since both receptors are coupled to G_i-proteins (Burnstock, 2004) it was anticipated that they would decrease or not impact cAMP levels following agonist stimulation. However, endothelial barrier preservation is often associated with elevated cAMP level (Aslam et al., 2014; Umapathy et al., 2010) followed by PKA activation (Patterson et al., 2000). These findings led us to measure the cAMP levels and PKA activity of Ado- and ATP γ S-treated HLMVECs. Upon Ado stimulation, we detected a significant elevation in cAMP levels and

increased PKA activity which is consistent with the expression of G_s-coupled A2B receptors. At the same time, ATP γ S treatment resulted in a slight, but significant decrease in cAMP levels further supporting the involvement of G_i-coupled P2Y₄ and P2Y₁₂ receptors in ATP γ S response. We need to emphasize that modest decrease in cAMP may be explained by the fact that ATP γ S is able to activate G_s-coupled P2Y₁₁ receptor, which is not involved in the ATP γ S-induced EC barrier enhancement but contributes to the net effect on cAMP. Importantly, that despite the observed decrease in cAMP levels, we found that the activity of PKA was increased upon ATP γ S treatment. This was somewhat surprising; however, some published studies support the existence of a cAMP-independent model of PKA activation (Ferraris, Persaud, Williams, Chen, & Burg, 2002; Kolosova et al., 2005; Murthy, Zhou, Grider, & Makhoul, 2003; Sriwai, Zhou, & Murthy, 2008).

The signaling mechanisms involved in PKA-mediated EC barrier protection/enhancement are not completely understood but likely involved PKA-mediated MYPT1 phosphorylation leading to MLCP activation (Kolosova et al., 2005). Using a dual phospho-MYPT1 specific antibody MYPT1^{pS695/T696}, we found that PKA activation upon treatment with both, Ado and ATP γ S led to increased phosphorylation of MYPT1, thus promoting MLCP-mediated dephosphorylation of its substrate, MLC20. Further, Ado and ATP γ S equally induced MLC20 dephosphorylation. Surprisingly, depletion of PKA abolished ATP γ S-, but not Ado-induced MLC20 dephosphorylation suggesting the involvement of additional mechanisms. The cAMP-mediated signaling mechanisms involve two major downstream targets, PKA (Patterson et al., 2000) and EPAC1 (Birukova et al., 2010; Bogatcheva, Zemskova, Kovalenkov, Poirier, & Verin, 2009). Recent publications indicate that EPAC1 activation leads to ROCK1 inactivation through a Rap1-RhoA-ROCK1 pathway (Lakshmikanthan et al., 2014) that results in decreased inhibitory phosphorylation of MYPT1 (Muranyi et al., 2005). Therefore, it seemed to be plausible to test the effect of EPAC1 depletion on Ado and ATP γ S responses. Our data (see Figure 5) demonstrated that EPAC1 depletion alone does not prevent the decrease in MLC20 phosphorylation induced by either Ado or ATP γ S in HLMVEC, while combined depletion of both PKAc and EPAC1 had a significant effect. Collectively, these data indicate that Ado-induced MLC20 dephosphorylation requires both PKA- and EPAC1-mediated signaling (see Figure 10). We hypothesize that both of these signaling pathways promote increases in MYPT1 phosphorylation at PKA-specific sites accompanied by decreases in MYPT1 phosphorylation at ROCK1 sites coupled with MLCP deinhibition and decreased ROCK1-mediated MLC20 phosphorylation. In contrast, PKA, but not EPAC1, is involved in the regulation of ATP γ S-induced dephosphorylation of MLC20 which is consistent with the ability of ATP γ S to induce HLMVEC cytoskeletal remodeling in a cAMP-independent manner (see Figure 10).

Consistent with the data on MLC20 phosphorylation, TER measurements indicate that depletion of PKAc attenuated ATP γ S-, but not Ado-induced EC barrier enhancement, depletion of EPAC1 alone had no effect on either Ado- or ATP γ S-induced TER increase, and simultaneous depletion of PKA and EPAC1 attenuated the TER increase after treatment with both agonists. We found that there was not much difference between PKAc depletion and PKAc/EPAC1 depletion in the response to ATP γ S, suggesting that increases in TER are EPAC1-independent (see Figure 6), and consistent with the ability of ATP γ S to enhance the HLMVEC barrier in a cAMP-independent manner.

Our group has previously demonstrated that ATP-induced barrier enhancement in HPAEC involves the cAMP-independent activation of PKA (Kolosova et al., 2005). In the current study, we found that ATP γ S also induces the cAMP-independent activation of PKA in HLMVEC. Models for cAMP-independent activation of PKA have been proposed by several groups. One possible pathway for cAMP-independent PKA activation is the PAR-1 mediated association of nuclear factor- κ B with PKA (Zieger, Tausch, Henklein, Nowak, & Kaufmann, 2001). Another pathway reported by Niu et al. (2001) involves the G₁₃/AKAP110 axis. We demonstrated that AKAP2 depletion abolished ATP γ S-induced TER response in HLMVECs, but had no effect on Ado-induced TER increase. AKAPs are known to be involved in the temporal and spatial regulation of PKA activity via their interactions with different targets (Colledge & Scott, 1999). It also has been reported that specific AKAPs interact with calcineurin (PP2B; Lester, Faux, Nauert, & Scott, 2001) and interactions with AKAP220 contribute to the regulation of PP1C, the catalytic subunit of MLCP (Schillace et al., 2001). Thus, we next tested whether AKAP2 can interact with MYPT1, the regulatory subunit of MLCP, in a HEK293 model system. We found that ectopically expressed c-myc-MYPT1 coimmunoprecipitates with AKAP2, but not AKAP9 or 12 suggesting the existence of a specific AKAP2/MYPT1 complex (see Figure 9a). These results were confirmed by reciprocal immunoprecipitation experiments using anti-AKAP2 antibodies (see Figure 9b). Our data indicate that AKAP2 is involved in ATP γ S-induced HLMVEC barrier enhancement and suggest that an AKAP2-dependent mechanism regulates the cAMP-independent activation of PKA and the close interaction of MYPT1. We speculate that AKAP2 serves as an adapter protein facilitating PKA-induced phosphorylation of MYPT1 leading to MLCP activation and that this mechanism mediates the cAMP-independent strengthening of the EC barrier in response to ATP γ S (see Figure 10), however, this hypothesis requires further investigation.

This study has clarified the signaling pathways involved in actions of Ado and ATP γ S to promote barrier strengthening in microvascular EC. Our data strongly suggest that both Ado and ATP γ S utilize PKA-dependent mechanisms and MLCP-mediated MLC20 dephosphorylation. While Ado-induced barrier-enhancing signaling is cAMP-dependent, ATP γ S-induced HLMVEC barrier enhancement is cAMP-independent and likely involves AKAP2-mediated activation of PKA. Characterization of these two distinct mechanisms of EC barrier strengthening induced by extracellular purines might contribute to the development of new therapeutic tools in the treatment of ALI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This study was supported by the National Institute of Health Program Project (Grant HL101902), and Canadian Institutes of Health Research (MOP-133494, to J. A. M.)

Funding information

National Heart, Lung, and Blood Institute, Grant/Award Number: HL101902; Canadian Institutes of Health Research, Grant/Award Number: MOP-133494

REFERENCES

- Aslam M, Tanislav C, Troidl C, Schulz R, Hamm C, & Gunduz D (2014). cAMP controls the restoration of endothelial barrier function after thrombin-induced hyperpermeability via Rac1 activation. *Physiological Reports*, 2(10), e12175 10.14814/phy2.12175 [PubMed: 25344477]
- Baron L, Gombault A, Fanny M, Villeret B, Savigny F, Guillou N, ... Couillin I (2015). The NLRP3 inflammasome is activated by nanoparticles through ATP, ADP and adenosine. *Cell Death & Disease*, 6, e1629 10.1038/cddis.2014.576 [PubMed: 25654762]
- Batori R, Becsi B, Nagy D, Konya Z, Hegedus C, Bordan Z, ... Erdodi F (2017). Interplay of myosin phosphatase and protein phosphatase-2A in the regulation of endothelial nitric-oxide synthase phosphorylation and nitric oxide production. *Science Reports*, 7, 44698 10.1038/srep44698
- Bilbao PS, Santillan G, & Boland R (2010). ATP stimulates the proliferation of MCF-7 cells through the PI3K/Akt signaling pathway. *Archives of Biochemistry and Biophysics*, 499(1–2), 40–48. 10.1016/j.abb.2010.05.001 [PubMed: 20450878]
- Birukova AA, Burdette D, Moldobaeva N, Xing J, Fu P, & Birukov KG (2010). Rac GTPase is a hub for protein kinase A and Epac signaling in endothelial barrier protection by cAMP. *Microvascular Research*, 79(2), 128–138. 10.1016/j.mvr.2009.11.007 [PubMed: 19962392]
- Bogatcheva NV, Zemskova MA, Kovalenkov Y, Poirier C, & Verin AD (2009). Molecular mechanisms mediating protective effect of cAMP on lipopolysaccharide (LPS)-induced human lung microvascular endothelial cells (HLMVEC) hyperpermeability. *Journal of Cellular Physiology*, 221(3), 750–759. 10.1002/jcp.21913 [PubMed: 19725051]
- Bogdanov YD, Wildman SS, Clements MP, King BF, & Burnstock G (1998). Molecular cloning and characterization of rat P2Y4 nucleotide receptor. *British Journal of Pharmacology*, 124(3), 428–430. 10.1038/sj.bjp.0701880 [PubMed: 9647463]
- Burnstock G (2004). Introduction: P2 receptors. *Current Topics in Medicinal Chemistry*, 4(8), 793–803. 10.2174/1568026043451014 [PubMed: 15078211]
- Burnstock G (2008a). Purinergic receptors as future targets for treatment of functional GI disorders. *Gut*, 57(9), 1193–1194. 10.1136/gut.2008.151134 [PubMed: 18719131]
- Burnstock G (2008b). Purinergic signalling and disorders of the central nervous system. *Nature Reviews. Drug Discovery*, 7(7), 575–590. 10.1038/nrd2605 [PubMed: 18591979]
- Burnstock G, & Ralevic V (2014). Purinergic signaling and blood vessels in health and disease. *Pharmacological Reviews*, 66(1), 102–192. 10.1124/pr.113.008029 [PubMed: 24335194]
- Colledge M, & Scott JD (1999). AKAPs: From structure to function. *Trends in Cell Biology*, 9(6), 216–221. 10.1016/S0962-8924(99)01558-5 [PubMed: 10354567]
- Erb L, & Weisman GA (2012). Coupling of P2Y receptors to G proteins and other signaling pathways. *Wiley Interdisciplinary Reviews: Membrane Transport Signaling*, 1(6), 789–803. 10.1002/wmts.v1.6 [PubMed: 25774333]
- Erlinge D, & Burnstock G (2008). P2 receptors in cardiovascular regulation and disease. *Purinergic Signalling*, 4(1), 1–20. 10.1007/s11302-007-9078-7
- Feoktistov I, Goldstein AE, Ryzhov S, Zeng D, Belardinelli L, Voyno-Yasenetskaya T, & Biaggioni I (2002). Differential expression of adenosine receptors in human endothelial cells: Role of A2B receptors in angiogenic factor regulation. *Circulation Research*, 90(5), 531–538. 10.1161/01.RES.0000012203.21416.14 [PubMed: 11909816]
- Ferraris JD, Persaud P, Williams CK, Chen Y, & Burg MB (2002). cAMP-independent role of PKA in tonicity-induced transactivation of tonicity-responsive enhancer/ osmotic response element-binding protein. *Proceedings of the National Academy of Sciences USA*, 99(26), 16800–16805. 10.1073/pnas.222659799
- Filippov AK, Simon J, Barnard EA, & Brown DA (2003). Coupling of the nucleotide P2Y4 receptor to neuronal ion channels. *British Journal of Pharmacology*, 138(2), 400–406. 10.1038/sj.bjp.0705043 [PubMed: 12540532]
- Fredholm BB, Irenius E, Kull B, & Schulte G (2001). Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. *Biochemical Pharmacology*, 61(4), 443–448. 10.1016/S0006-2952(00)00570-0 [PubMed: 11226378]

- Garcia JG, Davis HW, & Patterson CE (1995). Regulation of endothelial cell gap formation and barrier dysfunction: Role of myosin light chain phosphorylation. *Journal of Cellular Physiology*, 163(3), 510–522. 10.1002/(ISSN)1097-4652 [PubMed: 7775594]
- Goeckeler ZM, & Wysolmerski RB (1995). Myosin light chain kinase-regulated endothelial cell contraction: The relationship between isometric tension, actin polymerization, and myosin phosphorylation. *The Journal of Cell Biology*, 130(3), 613–627. 10.1083/jcb.130.3.613 [PubMed: 7622562]
- Gonzales JN, Gorshkov B, Varn MN, Zemskova MA, Zemskov EA, Sridhar S, ... Verin AD (2014). Protective effect of adenosine receptors against lipopolysaccharide-induced acute lung injury. *American Journal of Physiology, Lung Cellular and Molecular Physiology*, 306(6), L497–L507. 10.1152/ajplung.00086.2013 [PubMed: 24414256]
- Grassie ME, Moffat LD, Walsh MP, & MacDonald JA (2011). The myosin phosphatase targeting protein (MYPT) family: A regulated mechanism for achieving substrate specificity of the catalytic subunit of protein phosphatase type 1delta. *Archives of Biochemistry and Biophysics*, 510(2), 147–159. 10.1016/j.abb.2011.01.018 [PubMed: 21291858]
- Grassie ME, Sutherland C, Ulke-Lemee A, Chappellaz M, Kiss E, Walsh MP, & MacDonald JA (2012). Cross-talk between Rho-associated kinase and cyclic nucleotide-dependent kinase signaling pathways in the regulation of smooth muscle myosin light chain phosphatase. *The Journal of Biological Chemistry*, 287(43), 36356–36369. 10.1074/jbc.M112.398479 [PubMed: 22948155]
- Hartshorne DJ, Ito M, & Erdodi F (2004). Role of protein phosphatase type 1 in contractile functions: Myosin phosphatase. *The Journal of Biological Chemistry*, 279(36), 37211–37214. 10.1074/jbc.R400018200 [PubMed: 15136561]
- Hasko G, & Cronstein B (2013). Regulation of inflammation by adenosine. *Frontiers in Immunology*, 4, 85. 10.3389/fimmu.2013.00085 [PubMed: 23580000]
- Hasko G, & Cronstein BN (2004). Adenosine: An endogenous regulator of innate immunity. *Trends in Immunology*, 25(1), 33–39. 10.1016/j.it.2003.11.003 [PubMed: 14698282]
- Ichikawa K, Ito M, & Hartshorne DJ (1996). Phosphorylation of the large subunit of myosin phosphatase and inhibition of phosphatase activity. *The Journal of Biological Chemistry*, 271(9), 4733–4740. 10.1074/jbc.271.9.4733 [PubMed: 8617739]
- Kennedy C, Qi AD, Herold CL, Harden TK, & Nicholas RA (2000). ATP, an agonist at the rat P2Y(4) receptor, is an antagonist at the human P2Y(4) receptor. *Molecular Pharmacology*, 57(5), 926–931. [PubMed: 10779375]
- Khromov A, Choudhury N, Stevenson AS, Somlyo AV, & Eto M (2009). Phosphorylation-dependent autoinhibition of myosin light chain phosphatase accounts for Ca²⁺ sensitization force of smooth muscle contraction. *The Journal of Biological Chemistry*, 284(32), 21569–21579. 10.1074/jbc.M109.019729 [PubMed: 19531490]
- Kim KM, Csontos C, Czokora I, Fulton D, Umapathy NS, Olah G, & Verin AD (2012). Molecular characterization of myosin phosphatase in endothelium. *Journal of Cellular Physiology*, 227(4), 1701–1708. 10.1002/jcp.22894 [PubMed: 21678426]
- Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, ... Kaibuchi K (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science*, 273(5272), 245–248. 10.1126/science.273.5272.245 [PubMed: 8662509]
- Klinger M, Freissmuth M, & Nanoff C (2002). Adenosine receptors: G protein-mediated signalling and the role of accessory proteins. *Cellular Signalling*, 14(2), 99–108. 10.1016/S0898-6568(01)00235-2 [PubMed: 11781133]
- Kolosova IA, Mirzapoiazova T, Adyshev D, Usatyuk P, Romer LH, Jacobson JR, ... Verin AD (2005). Signaling pathways involved in adenosine triphosphate-induced endothelial cell barrier enhancement. *Circulation Research*, 97(2), 115–124. 10.1161/01.RES.0000175561.55761.69 [PubMed: 15994434]
- Kolosova IA, Mirzapoiazova T, Moreno-Vinasco L, Sammani S, Garcia JG, & Verin AD (2008). Protective effect of purinergic agonist ATPgammaS against acute lung injury. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, 294(2), L319–L324. 10.1152/ajplung.00283.2007 [PubMed: 17993588]

- Lakshmikanthan S, Zieba BJ, Ge ZD, Momotani K, Zheng X, Lund H, ... Chrzanowska-Wodnicka M (2014). Rap1b in smooth muscle and endothelium is required for maintenance of vascular tone and normal blood pressure. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 34(7), 1486–1494. 10.1161/ATVBAHA.114.303678
- Lester LB, Faux MC, Nauert JB, & Scott JD (2001). Targeted protein kinase A and PP-2B regulate insulin secretion through reversible phosphorylation. *Endocrinology*, 142(3), 1218–1227. 10.1210/endo.142.3.8023 [PubMed: 11181538]
- Lu Q, Harrington EO, Newton J, Casserly B, Radin G, Warburton R, ... Rounds S (2010). Adenosine protected against pulmonary edema through transporter- and receptor A2-mediated endothelial barrier enhancement. *American Journal of Physiology, Lung Cellular and Molecular Physiology*, 298(6), L755–L767. 10.1152/ajplung.00330.2009 [PubMed: 20228181]
- Matthay MA, & Zimmerman GA (2005). Acute lung injury and the acute respiratory distress syndrome: Four decades of inquiry into pathogenesis and rational management. *American Journal of Respiratory Cell and Molecular Biology*, 33(4), 319–327. 10.1165/rcmb.F305 [PubMed: 16172252]
- Mehta D, & Malik AB (2006). Signaling mechanisms regulating endothelial permeability. *Physiological Reviews*, 86(1), 279–367. 10.1152/physrev.00012.2005 [PubMed: 16371600]
- Muranyi A, Derkach D, Erdodi F, Kiss A, Ito M, & Hartshorne DJ (2005). Phosphorylation of Thr695 and Thr850 on the myosin phosphatase target subunit: Inhibitory effects and occurrence in A7r5 cells. *FEBS Letters*, 579(29), 6611–6615. 10.1016/j.febslet.2005.10.055 [PubMed: 16297917]
- Murthy KS, Zhou H, Grider JR, & Makhlof GM (2003). Inhibition of sustained smooth muscle contraction by PKA and PKG preferentially mediated by phosphorylation of RhoA. *American Journal of Physiology, Gastrointestinal and Liver Physiology*, 284(6), G1006–G1016. 10.1152/ajpgi.00465.2002 [PubMed: 12736149]
- Nerlich A, Rohde M, Talay SR, Genth H, Just I, & Chhatwal GS (2009). Invasion of endothelial cells by tissue-invasive M3 type group A streptococci requires Src kinase and activation of Rac1 by a phosphatidylinositol 3-kinase-independent mechanism. *The Journal of Biological Chemistry*, 284(30), 20319–20328. 10.1074/jbc.M109.016501 [PubMed: 19473989]
- Nguyen E, Gausdal G, Varennes J, Pendino F, Lanotte M, Doskeland SO, & Segal-Bendirdjian E (2013). Activation of both protein kinase A (PKA) type I and PKA type II isozymes is required for retinoid-induced maturation of acute promyelocytic leukemia cells. *Molecular Pharmacology*, 83(5), 1057–1065. 10.1124/mol.112.081034 [PubMed: 23455313]
- Niu J, Vaiskunaite R, Suzuki N, Kozasa T, Carr DW, Dulin N, & Voyno-Yasenetskaya TA (2001). Interaction of heterotrimeric G13 protein with an A-kinase-anchoring protein 110 (AKAP110) mediates cAMP-independent PKA activation. *Current Biology*, 11(21), 1686–1690. 10.1016/S0960-9822(01)00530-9 [PubMed: 11696326]
- Patil S, Kaplan JE, & Minnear FL (1997). Protein, not adenosine or adenine nucleotides, mediates platelet decrease in endothelial permeability. *The American Journal of Physiology*, 273(5 Pt 2), H2304–H2311. 10.1152/ajpheart.1997.273.5.H2304 [PubMed: 9374767]
- Patterson CE, & Garcia JG (1994). Regulation of thrombin-induced endothelial cell activation by bacterial toxins. *Blood Coagulation & Fibrinolysis*, 5(1), 63–72. 10.1097/00001721-199402000-00010 [PubMed: 8180340]
- Patterson CE, Lum H, Schaphorst KL, Verin AD, & Garcia JG (2000). Regulation of endothelial barrier function by the cAMP-dependent protein kinase. *Endothelium*, 7(4), 287–308. 10.3109/10623320009072215 [PubMed: 11201526]
- Ralevic V, & Burnstock G (1998). Receptors for purines and pyrimidines. *Pharmacological Reviews*, 50(3), 413–492. [PubMed: 9755289]
- Schillace RV, Voltz JW, Sim AT, Shenolikar S, & Scott JD (2001). Multiple interactions within the AKAP220 signaling complex contribute to protein phosphatase 1 regulation. *The Journal of Biological Chemistry*, 276(15), 12128–12134. 10.1074/jbc.M010398200 [PubMed: 11152471]
- Sehrawat S, Hernandez T, Cullere X, Takahashi M, Ono Y, Komarova Y, & Mayadas TN (2011). AKAP9 regulation of microtubule dynamics promotes Epac1-induced endothelial barrier properties. *Blood*, 117(2), 708–718. 10.1182/blood-2010-02-268870 [PubMed: 20952690]

- Sheth S, Brito R, Mukherjea D, Rybak LP, & Ramkumar V (2014). Adenosine receptors: Expression, function and regulation. *International Journal of Molecular Sciences*, 15(2), 2024–2052. 10.3390/ijms15022024 [PubMed: 24477263]
- Shibata K, Sakai H, Huang Q, Kamata H, Chiba Y, Misawa M, ... Ikebe M (2015). Rac1 regulates myosin II phosphorylation through regulation of myosin light chain phosphatase. *Journal of Cellular Physiology*, 230(6), 1352–1364. 10.1002/jcp.24878 [PubMed: 25502873]
- Song J, Zhang X, Buscher K, Wang Y, Wang H, Di Russo J, ... Sorokin L (2017). Endothelial basement membrane laminin 511 contributes to endothelial junctional tightness and thereby inhibits leukocyte transmigration. *Cell Reports*, 18(5), 1256–1269. 10.1016/j.celrep.2016.12.092 [PubMed: 28147279]
- Sriwai W, Zhou H, & Murthy KS (2008). G(q)-dependent signalling by the lysophosphatidic acid receptor LPA(3) in gastric smooth muscle: Reciprocal regulation of MYPT1 phosphorylation by Rho kinase and cAMP-independent PKA. *The Biochemical Journal*, 411(3), 543–551. 10.1042/BJ20071299 [PubMed: 18237278]
- Sutherland C, MacDonald JA, & Walsh MP (2016). Analysis of phosphorylation of the myosin-targeting subunit of myosin light chain phosphatase by Phos-tag SDS-PAGE. *American Journal of Physiology Cell Physiology*, 310(8), C681–C691. 10.1152/ajpcell.00327.2015 [PubMed: 26864694]
- Tasken K, Skälhegg BS, Tasken KA, Solberg R, Knutsen HK, Levy FO, ... Jahnsen T (1997). Structure, function, and regulation of human cAMP-dependent protein kinases. *Advances in Second Messenger and Phosphoprotein Research*, 31, 191–204. 10.1016/S1040-7952(97)80019-5 [PubMed: 9344252]
- Torres B, Zambon AC, & Insel PA (2002). P2Y₁₁ receptors activate adenylyl cyclase and contribute to nucleotide-promoted cAMP formation in MDCK-D(1) cells. A mechanism for nucleotide-mediated autocrine-paracrine regulation. *The Journal of Biological Chemistry*, 277(10), 7761–7765. 10.1074/jbc.M110352200 [PubMed: 11788591]
- Umapathy NS, Fan Z, Zemskov EA, Alieva IB, Black SM, & Verin AD (2010). Molecular mechanisms involved in adenosine-induced endothelial cell barrier enhancement. *Vascular Pharmacology*, 52(5-6), 199–206. 10.1016/j.vph.2009.12.008 [PubMed: 20045081]
- Weissmuller T, Glover LE, Fennimore B, Curtis VF, Macmanus CF, Ehrentraut SF, ... Colgan SP (2014). HIF-dependent regulation of AKAP12 (gravin) in the control of human vascular endothelial function. *FASEB Journal*, 28(1), 256–264. 10.1096/fj.13-238741 [PubMed: 24029533]
- Wittchen ES, Worthylake RA, Kelly P, Casey PJ, Quilliam LA, & Burridge K (2005). Rap1 GTPase inhibits leukocyte transmigration by promoting endothelial barrier function. *The Journal of Biological Chemistry*, 280(12), 11675–11682. 10.1074/jbc.M412595200 [PubMed: 15661741]
- Wooldridge AA, MacDonald JA, Erdodi F, Ma C, Borman MA, Hartshorne DJ, & Haystead TA (2004). Smooth muscle phosphatase is regulated in vivo by exclusion of phosphorylation of threonine 696 of MYPT1 by phosphorylation of Serine 695 in response to cyclic nucleotides. *The Journal of Biological Chemistry*, 279(33), 34496–34504. 10.1074/jbc.M405957200 [PubMed: 15194681]
- Zaynagetdinov R, Ryzhov S, Goldstein AE, Yin H, Novitskiy SV, Goleniewska K, ... Feoktistov I (2010). Attenuation of chronic pulmonary inflammation in A2B adenosine receptor knockout mice. *American Journal of Respiratory Cell and Molecular Biology*, 42(5), 564–571. 10.1165/rcmb.2008-0391OC [PubMed: 19556606]
- Zemskov E, Lucas R, Verin AD, & Umapathy NS (2011). P2Y receptors as regulators of lung endothelial barrier integrity. *Journal of Cardiovascular Disease Research*, 2(1), 14–22. 10.4103/0975-3583.78582 [PubMed: 21716747]
- Zhou Y, Schneider DJ, Morschl E, Song L, Pedroza M, Karmouty-Quintana H, ... Blackburn MR (2011). Distinct roles for the A2B adenosine receptor in acute and chronic stages of bleomycin-induced lung injury. *Journal of Immunology*, 186(2), 1097–1106. 10.4049/jimmunol.1002907
- Zieba BJ, Artamonov MV, Jin L, Momotani K, Ho R, Franke AS, ... Somlyo AV (2011). The cAMP-responsive Rap1 guanine nucleotide exchange factor, Epac, induces smooth muscle relaxation by down-regulation of RhoA activity. *The Journal of Biological Chemistry*, 286(19), 16681–16692. 10.1074/jbc.M110.205062 [PubMed: 21454546]

Zieger M, Tausch S, Henklein P, Nowak G, & Kaufmann R (2001). A novel PAR-1-type thrombin receptor signaling pathway: Cyclic AMP-independent activation of PKA in SNB-19 glioblastoma cells. *Biochemical and Biophysical Research Communications*, 282(4), 952–957. 10.1006/bbrc.2001.4683 [PubMed: 11352644]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

FIGURE 1.

Adenosine and ATP γ S enhance the EC barrier in HLMVEC. HLMVECs were treated with increasing concentrations (10–200 μM) of adenosine (a) or ATP γ S (b) and TER was recorded. Results are presented as mean $\pm$ SEM of three independent experiments. Arrows indicate the time points of Ado and ATP γ S administration. Ado: adenosine; ATP γ S: adenosine 5'-[γ -thio]-triphosphate; EC: endothelial cell; HLMVEC: human lung microvascular endothelial cell; TER: transendothelial electrical resistance

FIGURE 2.

Relative expression of adenosine (P1) and P2Y receptor mRNAs in HLMVEC. Receptor mRNA expression was determined by quantitative RT-PCR. Bar graph represents the normalized level of adenosine (P1) receptors (A1, A2A, A2B, and A3) (a) or P2Y receptors (b) by 18S rRNA and are presented as arbitrary unit (AU). mRNA: messenger RNA; rRNA: ribosomal RNA; RT-PCR: reverse-transcription polymerase chain reaction

FIGURE 3.

ATP γ S improves endothelial barrier function via P2Y4 and P2Y12 receptors in HLMVEC. Endothelial cells were treated either with nonspecific siRNA (nsRNA) or silencing RNA specific to different P2Y receptors for 72 hr and TER was measured upon challenge with 50 μ M ATP γ S. Depletion of receptors was determined by western blotting with specific antibody using β -tubulin as loading control (insets). Arrows indicate the time points of ATP γ S administration. ATP γ S: adenosine 5'-[γ -thio]-triphosphate; HLMVEC: human lung

microvascular endothelial cell; TER: transendothelial electrical resistance; siRNA: small interfering RNA

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

FIGURE 4.

Effect of ATP γ S and adenosine on cAMP levels and PKA activity in HLMVEC. HLMVECs were treated with 100 μ M Ado (a) or ATP γ S (b) for 30 min and the levels of cAMP were measured by competitive enzyme linked immunosorbent assay (Cyclic AMP EIA Kit).

Results are shown as mean $\pm$ SEM of three individual experiments, with three parallels each time. * p < 0.05, ** p < 0.01, *** p < 0.001 versus control (0 time point, one-way ANOVA, the Newman–Keuls post hoc test). (c) HLMVECs were treated with 100 μ M Ado or ATP γ S for 30 min and PKA activity was measured by colorimetric protein kinase activity assay.

Results are shown as mean $\pm$ SEM of three individual experiments. * p < 0.05, Veh versus Ado or Veh versus ATP γ S, one-way ANOVA, the Newman–Keuls post hoc test. (d) Changes in the level of MYPT1^{S695/T696} was assessed by western blotting upon vehicle, 50 μ M Ado or ATP γ S treatment (upper panel). Bar graphs represent the changes in the level of MYPT1^{S695/Thr696} determined by densitometric analysis of immunoblots from four independent experiments (mean $\pm$ SEM, * p < 0.05, Veh versus Ado or Veh versus ATP γ S, one-way ANOVA, the Newman–Keuls post hoc test). Ado: adenosine; ANOVA: analysis of variance; ATP γ S: adenosine 5'-[γ -thio]-triphosphate; cAMP: cyclic adenosine

monophosphate; HLMVEC: human lung microvascular endothelial cell; PKA: protein kinase A

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

FIGURE 5.

Effect of PKA and EPAC1 depletion on adenosine and ATP γ S-induced MLC20 dephosphorylation. HLMVECs were transfected with nonspecific siRNA (nsRNA), PKA α (a), EPAC1 (b), specific silencing RNA or with both together (c). Three days after transfection the cells were treated with 50 μ M ATP γ S or Ado for 30 min and the level of ppMLC20^{T18/S19}, depletion of PKAc and EPAC1 was determined by western blot analysis. The bar graphs represent the changes in the level of ppMLC20^{T18/S19}. Densitometric analysis of blots from three to seven independent experiments (mean $\pm$ SEM). ** p < 0.01, *** p < 0.001 versus nonspecific siRNA (nsRNA)-treated vehicle control, one-way ANOVA, the Newman–Keuls post-hoc test). Ado: adenosine; ANOVA: analysis of variance; ATP γ S: adenosine 5'-[γ -thio]-triphosphate; EPAC1: exchange factor 3; HLMVEC: human lung

microvascular endothelial cell; ns: not significant; MLC20: myosin light chain 20; PKA: protein kinase A; siRNA: small interfering RNA

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

FIGURE 6.

Effect of PKAc and EPAC1 depletion on adenosine- and ATP γ S-induced EC barrier enhancement. HLMVECs were transfected with nonspecific siRNA (nsRNA), PKAc α specific (a) or EPAC1 specific (b) silencing RNAs. Three days after transfection HLMVECs were treated with 50 μ M Ado (left panel) or ATP γ S (right panel) and changes in TER was recorded. Arrows indicate the time points of ATP γ S and Ado administration. Data are presented as mean $\pm$ SEM. Depletion of PKA catalytic subunit or EPAC1 (insets) was confirmed by western blot analysis. Ado: adenosine; ANOVA: analysis of variance; ATP γ S:

adenosine 5'-[γ -thio]-triphosphate; EPAC1: exchange factor 3; HLMVEC: human lung microvascular endothelial cell; PKA: protein kinase A; TER: transendothelial electrical resistance

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

FIGURE 7.

AKAP2 contributes to ATP γ S-induced endothelial barrier function. a–c, Effect of AKAP2, AKAP9, and AKAP12 silencing on HLMVEC barrier function. HLMVECs were transfected with nonspecific siRNA (nsRNA) or AKAP2, AKAP9, and AKAP12 specific silencing RNA. Three days after transfection HLMVECs were treated with 50 μ M ATP γ S (right panels) or 50 μ M Ado (left panels) and changes in TER was recorded. Arrows indicate the time points of ATP γ S and Ado administration. Depletion of AKAPs (insets) were confirmed by western blot analysis. Data are presented as mean $\pm$ SEM of four independent

experiments. Ado: adenosine; ATP γ S: adenosine 5'-[γ -thio]-triphosphate; HLMVEC: human lung microvascular endothelial cell; siRNA: small interfering RNA; TER: transendothelial electrical resistance

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

FIGURE 8.

Effect of AKAP2 depletion on adenosine and ATP γ S-induced MLC20 dephosphorylation. HLMVECs were transfected with nonspecific siRNA (nsRNA) and AKAP2-specific silencing RNA. Three days after transfection the cells were treated with vehicle, 50 μ M Ado or ATP γ S for 30 min and the level of ppMLC20^{T18/S19}, further depletion of AKAP2 was determined by western blot analysis. The bar graphs represent the changes in the level of ppMLC20^{T18/S19}. Densitometric analysis of blots from four independent experiments (mean $\pm$ SEM). * p < 0.05, ** p < 0.01, *** p < 0.001 versus nonspecific siRNA-treated vehicle control, one-way ANOVA, the Newman–Keuls post hoc test). ANOVA: analysis of variance; ATP γ S: adenosine 5'-[γ -thio]-triphosphate; HLMVEC: human lung microvascular endothelial cell; MLC20: myosin light chain 20; ns: not significant; siRNA: small interfering RNA

FIGURE 9.

Identification of an AKAP2-MYPT1 complex. (a) c-myc-MYPT1-containing plasmid was transfected into HEK293 cells. After 48 hr harvested cell lysate was immunoprecipitated (IP) with control IgG or anti-myc antibody. IPs were subjected to western blot analysis with specific antibodies to myc-tag (MYPT1) and AKAP 2, 9, and 12. (b) AKAP2 with HA-tag was overexpressed in HEK293 cells and after 48 hr the IP was carried out as described in Section 2. The samples were subjected to immunoblotting with antibodies against HA-tag (AKAP2), MYPT1, PKAc, and G $_{i2}$

FIGURE 10.

Schematic representation of PKA-, EPAC1-, and AKAP2-PKA-mediated signaling pathways induced by adenosine and ATP γ S in HLMVECs. We hypothesize that adenosine stimulates increased cAMP levels in HLMVECs through A2B receptors which leads to the activation of two downstream targets, PKA and EPAC1. In contrast, ATP γ S dependent signaling is mediated by P2Y4 and P2Y12 receptors leading to the cAMP-independent activation of PKA that requires AKAP2 expression. The effect of both, adenosine and ATP γ S converge at the level of MLCP via increased phosphorylation of the regulatory subunit MYPT1 resulting in MLCP deactivation, which leads to MLC20 dephosphorylation and finally to EC barrier strengthening. ATP γ S: adenosine 5'-[γ -thio]-triphosphate; cAMP: cyclic adenosine monophosphate; EC: endothelial cell; EPAC1: exchange factor 3; HLMVEC: human lung microvascular endothelial cell; MLC20: myosin light chain 2; MLCP: myosin light chain phosphatase; PKA: protein kinase A