Extracellular ATP induces assembly and activation of the myosin light chain phosphatase complex in endothelial cells


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Abstract

Objectives: Extracellular ATP stabilizes the endothelial barrier and inactivates the contractile machinery of endothelial cells. This inactivation relies on dephosphorylation of the regulatory myosin light chain (MLC) due to an activation of the MLC phosphatase (MLCP). To date, activation and function of MLCP in endothelial cells are only partially understood.

Methods: Here, the mechanism of extracellular ATP-mediated activation of MLCP was analyzed in human endothelial cells from umbilical veins. Cells were transfected with the endogenous protein phosphatase 1 (PP1)-specific inhibitor-2 (I-2).

Results: Overexpression of I-2 led to inhibition of PP1 activity and abrogation of the ATP-induced dephosphorylation of MLC. This indicates that the PP1 catalytic subunit is the principal phosphatase catalyzing the MLC dephosphorylation induced by extracellular ATP. As demonstrated by immunoprecipitation analysis, extracellular ATP recruits the PP1δ catalytic subunit and the myosin phosphatase targeting subunit (MYPT1) to form a complex. ATP stimulated dephosphorylation of MYPT1 at the inhibitory phosphorylation sites threonine 850 and 696. However, extracellular ATP failed to stimulate MYPT1 dephosphorylation in I-2-overexpressing cells.

Conclusions: The present study shows for the first time that, in endothelial cells, extracellular ATP causes activation of MLCP through recruitment of PP1δ and MYPT1 into a MLCP holoenzyme complex and PP1-mediated reduction of the inhibitory phosphorylation of MYPT1.

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Keywords: ATP; Myosin light chain phosphatase; Myosin protein targeting subunit; Protein phosphatase inhibitor-2

1. Introduction

Vascular endothelial cells form a dynamic barrier and actively control the traffic of water, solutes and plasma proteins between the vascular lumen and the interstitial space. Failure of endothelial barrier function can occur when endothelial cells are exposed to inflammatory mediators that are generated during sepsis or conditions of metabolic disturbance such as ischemia–reperfusion. Loss of barrier function results from the opening of gaps between adjacent cells as a consequence of both a loss of cell adhesion and activation of the endothelial contractile machinery [1]. Generation of contractile forces by endothelial cells can cause adjacent cells to retract from each other [2,3]. The importance of this actin-myosin based contractile apparatus for dynamic adaptation of endothelial barrier function under physiological conditions as well as for the development of barrier failure has been well established [3–5]. The endothelial contractile machinery has therefore become a target for new strategies to control disease states with pathologically increased endothelial permeability. During the past

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decade many efforts have been undertaken to analyze the mechanisms that activate the contractile machinery, such as those provoked by inflammatory mediators like thrombin [6–8] and activated neutrophils [9,10], or to identify signaling mechanisms that may counteract an activation of the contractile machinery [5,11–14]. For future therapeutic aims the analysis of the mechanisms that de-activate the endothelial machinery is of equal or greater importance.

In previous studies, we demonstrated that extracellular ATP stabilizes barrier function of endothelial monolayers and effectively antagonizes the thrombin-induced loss of barrier function due to a robust reduction of myosin light chain (MLC) phosphorylation and isometric force production [5,15–17]. Results with pharmacological inhibitors have indicated that the ATP-induced MLC dephosphorylation is based on a potent activation of myosin light chain phosphatase (MLCP). This conclusion was supported by a recent study from Kolosova et al. [18]. The aim of the present study was to analyze the mechanism by which extracellular ATP controls activation of the MLCP complex in endothelial cells.

In smooth muscle cells, structure and function of MLCP have been studied extensively [19–24]. It was shown that the MLCP holoenzyme consists of a catalytic subunit PP1, a myosin phosphatase targeting subunit MYPT1, and a small 20-kDa subunit with yet unknown function. MYPT1 targets PP1 to its substrate myosin and is involved in the formation of an active MLCP complex. Phosphorylation of MYPT1 at threonine 850 (T850) or threonine 696 (T696) may regulate the functional role of this docking protein. Phosphorylation of these sites reduces the activity of the MLCP holoenzyme [25]. It is well established that endothelial cells express the δ isoform of the catalytic subunit of PP1 [26,27], which is known to be the integral catalytic phosphatase in smooth muscle MLCP. Expression and complex formation of MYPT1 and PP1δ were also found in different endothelial cell types [28].

In the present study we investigated three main aspects of the activation of endothelial MLCP by extracellular ATP: (i) whether PP1 is the principal catalytic phosphatase involved, (ii) whether MLCP is activated through recruitment of PP1δ to MYPT1, (iii) whether such recruitment is accompanied by a dephosphorylation of MYPT1 at the above-mentioned strategic sites. To analyze the role of the PP1 catalytic subunit specifically, PP1 was blocked by a targeted molecular approach. This was accomplished by overexpression of the PP1-specific inhibitor-2 (I-2) in endothelial cells by use of an adenoviral vector.

2. Materials and methods

2.1. Materials

HRP-conjugated anti-mouse, anti-rabbit IgG antibodies and γ-[32P]-ATP were from Amersham Biosciences (Freiburg, Germany); HEK 293 cells were from the American Type Culture Collection (Rockville, USA); Falcon plastic tissue culture dishes were from BD Biosciences, Adeno-X Rapid Titer Kit was from BD Clontech Laboratories, anti-Inhibitor-2 antibody was from BD Transduction Laboratories (Heidelberg, Germany); ATP was from Boehringer (Mannheim, Germany); Y-27632 was from Biotrend (Cologne, Germany); calyculin A and okadaic acid were from Calbiochem (Bad Soden, Germany); Dynabeads protein G were from Dynal Biotech GmbH (Hamburg, Germany); fetal calf serum, penicillin–streptomycin, and trypsin–EDTA were from GIBCO Life Technologies (Egggenstein, Germany); polyvinylidene difluoride membrane (PVDF) was from Millipore (Eschborn, Germany); Taq DNA polymerase, and DNA purification system were from Promega (Mannheim, Germany); endothelial cell basal medium plus supplement pack from PromoCell (Heidelberg, Germany); Complete™ was from Roche (Mannheim, Germany); anti-MLC antibody, recombinant inhibitor-2 (I-2), phosphorylase b, phosphorylase kinase, MLC, adenosine, diithiothreitol, phenylmethylsulfonyl fluoride (PMSF), 8-phenyltheophylline (8-PT), and thioglycolate were from Sigma (Deisenhofen, Germany); AdEasy Adenoviral Vector System was from Stratagene (LaJolla, USA); HRP-conjugated rabbit antischwein antibodies, anti-PP1, anti-MYPT1, anti-phospho-MYPT1 (T696 and T850), recombinant PP1α and MYPT1 were from Upstate Biotechnology (Hamburg, Germany).

2.2. Cell cultures

The investigation conforms with the principles outlined in the Declaration of Helsinki (Cardiovascular Research 1997;35:2–3). Human endothelial cells were isolated from umbilical cords and cultured according to Peters et al. [29]. The harvested cells were cultured in PromoCell™ endothelial cell basal medium supplemented with 10% (vol./vol.) FCS, 0.4% (vol./vol.) endothelial growth supplement with heparin, 0.1 ng/ml human EGF, 1.0 μg/ml hydrocortisone, 1 ng/ml human bFGF and 2% (vol./vol.) penicillin/streptomycin in a fully humidified atmosphere at 37 °C and 5% CO2. Confluent cultures of primary endothelial cells were trypsinized in phosphate-buffered saline PBS [composition in mM: 137 NaCl, 2.7 KCl, 1.5 KH2PO4, and 8.0 Na2HPO4, at pH 7.4, supplemented with 0.05% (wt./vol.) trypsin, and 0.02% (wt./vol.) EDTA] and seeded at a density of 7 × 104 cells/cm² on 30-mm or 100-mm culture dishes. Experiments were performed with confluent endothelial monolayers of passage 1, 3 days after seeding.

2.3. Experimental protocols

The basal medium used in incubations was modified Tyrode’s solution (composition in mM: 150 NaCl, 2.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 1.0 CaCl2, and 30.0 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; pH 7.4, 37 °C). Basal MLC phosphorylation was determined after an initial equilibration period of 10 min. Agents were added as
indicated. Stock solution of ATP, adenosine, and thrombin were prepared immediately before use with basal medium. Stock solutions of calyculin A, Y-27632 and 8-phenylthio-phelline (8-PT), were prepared with dimethyl sulfoxide (DMSO). Appropriate volumes of these solutions were added to the cells yielding final solvent concentrations <0.1% (vol./vol.). The same final concentrations of DMSO were included in all respective control experiments. Stock solutions of all other substances were prepared in basal medium (composition as described above). Appropriate volumes of these solutions were added to the cells. Identical additions of basal medium were included in all respective control experiments.

2.4. Determination of MLC phosphorylation

The MLC phosphorylation was determined by glycerol-polyacrylamide gel electrophoresis and Western blot analysis using an anti-MLC antibody as previously described [15]. This procedure allows separation of non-phosphorylated from phosphorylated MLC protein, the latter of which migrates more rapidly. Briefly, electrotheroretically separated proteins were transferred on PVDF membranes and incubated with an anti-MLC antibody followed by a peroxidase-conjugated anti-IgM antibody. Blots were scanned densitometrically, and the percentage of MLC phosphorylation (expressed as % of total MLC) was calculated from the densitometric values of non-(MLC), mono-(MLC−P), and di-phosphorylated MLC (MLC−PP) as follows:

$$\text{MLC phosph.} = \frac{2 \times (\text{MLC} - \text{PP}) + (\text{MLC} - P)}{\text{Total MLC}} \times 100$$

As all MLC can become diphosphorylated, MLC phosphorylation varies between 0 and 200%.

2.5. Immunoprecipitation and Western blotting

Confluent endothelial monolayers grown in 10-cm dishes were stimulated as indicated in the text. Cells were lysed for 10 min on ice (composition of lysis medium: 1% (vol./vol.) Triton X-100, 0.5% (vol./vol.) Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, protease inhibitor Complete™, 50 mM Tris/HCl pH 7.4). The precipitate was solubilized in 2 ml dialysis buffer (10 mM Tris/HCl; pH 7.4) for 2.5 h at 30 °C. Precipitation was centrifuged (1000 × g, 5 min, 4 °C). The supernatant was transferred into a fresh tube and incubated with MYPT1-specific antibodies pre-immobilized on magnetic protein G-Sepharose beads for 1.5 h at 4 °C. Pre-immobilization of MYPT1 antibodies were performed according to manufacturer’s instructions. After incubation, beads were washed three times with PBS (pH 7.4) containing 0.1% (vol./vol.) Tween 20. The beads were collected and bound proteins were either eluted in Laemmli sample buffer [30] for Western blot analysis using antibodies against MYPT1, PP16 and MLC, or used for determination of protein phosphatase activity.

2.6. Generation of recombinant adenovirus coding for human I-2

Total RNA was isolated from biopsies of human left ventricle as described by Chomczynski and Sacchi [31]. The human I-2 cDNA sequence was amplified by PCR with Taq DNA polymerase using following primers, forward: 5′-CTGATCTTCCAAATTGCCGCTGACG-3′ (nt −3 to nt +15); reverse: 5′-CTTCTGGAGTGAAATGACTAGGCACAAG-3′ (nt +189 to nt +209 downstream of the I-2 translational stop codon), introducing BglII and XhoI restriction sites (underlined), respectively. The amplified PCR product was purified using the DNA purification system. The recombinant adenovirus (Ad I-2) was generated by cloning the human I-2 sequence into a pAdTrack-CMV shuttle vector of the AdEasy Adenoviral Vector System as described [32]. The DNA sequence was established by sequencing. A similar adenovirus lacking the human I-2 sequence, only expressing green fluorescent protein (GFP), served as control. Adenoviral plasmids were purified, linearized, and transfected into HEK 293 cells for production of high-titer adenoviral stocks according to the manufacturer’s instructions. Adenoviral titer was determined using the Adeno-X Rapid Titer Kit.

2.7. Adenovirus infection of HUVEC

Cells were grown on 35-mm tissue culture dishes as described above. Preconfluent cultures were infected with Ad I-2 or control virus at a multiplicity of infection (MOI) of 3 to 12. After 24 h, cells were stimulated and harvested as described above.

2.8. Preparation of [32P]-labeled substrate

[32P]-labeled phosphorylase a was prepared as described [6,33] with some modifications. Briefly, phosphorylase b (5 mg/ml) and phosphorylase kinase (200 U/ml) were incubated in a 2 ml incubation mixture (composition: 20 mM MgCl2, 31 mM β-mercaptoethanol, 0.5 mg/ml BSA, 1 mM CaCl2, 1 mM ATP, 1 mM γ-[32P]-ATP, and 50 mM Tris/HCl; pH 7.4) for 2.5 h at 30 °C. The radioactive-labeled phosphorylase a was precipitated by addition of 2 volumes of ice-cold saturated ammonium sulfate solution, 20 min incubation on ice and centrifugation for 30 min at 12,000 × g at 4 °C. The precipitate was solubilized in 2 ml dialysis buffer (10 mM Tris/HCl; pH 7.4, 1 mM EDTA) dialyzed at room temperature against 2 × 2 liter dialysis buffer and finally stored at 4 °C. Radioactive labeling was determined by measuring the product in a liquid scintillation counter.

2.9. Protein phosphatase assay

The phosphatase activity assay was performed as described by Neumann et al. [34]. Diluted protein fractions were pre-incubated in a total volume of 30 μl for 10 min at
30 °C in the presence of 5 nM okadaic acid, an inhibitor of phosphatase type 2 at that concentration [27], or 0.5 μM human recombinant I-2. The reaction was started by addition of 20 μl [32P]-labeled phosphorylase a [6,33] in an incubation mixture containing 50 mM Tris/HCl; pH 7.4, 12.5 mM caffeine, 0.25 mM EDTA, 1.25 mM MnCl₂, 0.25% (vol./vol.) β-mercaptoethanol. After incubation of 20 min at 30 °C the reaction was terminated on ice by addition of 20 μl 50% (wt./vol.) ice-cold TCA and 30 μl 2% (wt./vol.) BSA. After 15 min on ice, the suspension was centrifuged for 5 min and 70 μl of the supernatant was measured in a liquid scintillation counter. Reactions were carried out in triplicate. To ensure linear rates of dephosphorylation, the extent of dephosphorylation was restricted to <25%.

2.10. Inhibitor-2 assay activity assay

Activity of I-2 in endothelial cells was determined according to Sakashita et al. [35] with some modifications. Briefly, endothelial cells were incubated for 5 min on ice with lysis buffer (composition: 100 mM NaCl, 5 mM MgCl₂, 0.5 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 1 mM DTT, protease inhibitor Complete™, and 50 mM Tris/HCl; pH 7.4). The lysed cells were boiled for 10 min at 100 °C and centrifuged at 14,000 ×g for 10 min. Aliquots of the supernatant were incubated with 2.5 μU recombinant catalytic subunit of PP1α for 10 min at 30 °C and the phosphorylase phosphatase activity was determined as described above.

2.11. Detection of protein phosphatases in a cytoskeletal fraction

The content of PP1 in a myosin-enriched cell fraction was determined as previously described [5]. Briefly, confluent endothelial monolayers grown on 10-cm dishes were stimulated as described above. Afterwards monolayers were rinsed once with PBS to remove the incubation medium, 200 μl of homogenization buffer (0.1 mM EDTA, 28 mM mercaptoethanol, 0.5 μg/ml protease inhibitor Complete™, Tris/HCl; pH 7.4) was added, and dishes were cooled immediately to −80 °C. Afterwards the cells were scraped and homogenized. Homogenates were incubated with a high salt buffer (0.6 M NaCl, 0.1% (vol./vol.) Tween 20, 0.5 μg/ml protease inhibitor Complete™) for 1 h at 4 °C and were centrifuged at 4500 ×g for 30 min at 4 °C. Supernatants were diluted 10-fold with assay buffer (0.1 mM EDTA, 28 mM mercaptoethanol, Tris/HCl; pH 7.0) and centrifuged again at 8200 ×g for 40 min at 4 °C. The pellet, containing the myosin-enriched fraction, was used for further determinations.

2.12. Cell fractionation and ROCK activity assay

Dishes were rinsed twice with PBS, and Triton-insoluble particulate fractions were prepared as previously described by Patil et al. [36]. The fractions were pre-incubated for 15 min at 30 °C in presence of 2 μM Y-27632 or DMSO as a control in a final volume of 30 μl in assay buffer containing (4.5 mM β-glycerophosphate, 5 mM MgCl₂, 1 mM KCl, 1 mM Na₂VO₄, 1 mM DTT, 0.2 mM EDTA, 0.1 mM PMSF, 5 μM ATP, 20 mM MOPS; pH 7.4.). The reaction was started by addition of 20 μg dephosphorylated myelin basic protein (MBP) and 0.1 μCi of γ[32P]-ATP. After incubation for 20 min at 30 °C the reaction was stopped by transferring aliquots onto P-81 Whatman filter, and the filters were immediately rinsed and subsequently washed 5 times with 0.75% phosphoric acid. Incorporation of 32P into the substrate was assessed by liquid scintillation counting. Specific Rho-kinase activity was estimated as the Y-27632 inhibitable part of the total kinase activity.

2.13. Statistical analysis

Data are given as means±SD of n experiments using independent cell preparations. The comparison of means between groups was performed by one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test. Changes of parameters within the same group were assessed by multiple ANOVA analysis. Probability (P) values of less than 0.05 were considered significant.

3. Results

3.1. Overexpression of inhibitor-2 in endothelial cells

To analyze whether PP1 is involved in ATP-induced dephosphorylation of MLC, HUVEC were infected either with an adenovirus construct encoding the sequence of human inhibitor-2 plus GFP (I-2-transfected cells) or the GFP sequence only (control-transfected cells). Infection with either construct stimulated expression of I-2 and/or GFP in a time- and titer-dependent manner. Transfection efficiency (number of GFP expressing cells/total cell number determined by fluorescence microscopy) was on average 95% after 24 h (data not shown). In non-control-transfected cells the endogenous expression of I-2 was low as indicated by the faint band in Fig. 1A. A significant increase of I-2 expression was observed when cells were incubated in the presence of the I-2 virus between 3 and 12 MOI for 24 h (Fig. 1A and B). To verify that the I-2 protein expressed in transfected cells represents an active inhibitor, I-2 was harvested as a temperature-resistant protein in the supernatants of heat-treated homogenates of I-2- or control-transfected cells. Aliquots of the supernatants were tested in a phosphatase assay using recombinant PP1α catalytic subunit. As shown in Fig. 1C, the inhibitory effect of I-2 on the activity of the recombinant PP1 increased with increasing I-2 protein expression. At 12 MOI, recombinant PP1 was significantly inhibited. Unless stated otherwise, this latter infection protocol was applied in all experiments.
3.2. MLC phosphorylation and PP1 activity in I-2-over-expressing cells

Previously, we demonstrated [15] that extracellular ATP reduces MLC phosphorylation in a time- and concentration-dependent manner. Here we tested the effect of ATP under the same conditions in I-2-transfected cells. In control-transfected cells, MLC phosphorylation was 51% of control under basal conditions (Fig. 2A). Exposure of these cells to 10 μM ATP for 10 min reduced MLC phosphorylation to 27%. In I-2-transfected cells, MLC phosphorylation was significantly increased to 76% under basal conditions (n=3; P<0.05). In these transfected cells ATP failed to reduce MLC phosphorylation. These data show that dephosphorylation of MLC by ATP depends on PP1. PP1 activity was...
determined in the cytoskeletal fraction of control- or I-2-transfected cells (Fig. 2B). Stimulation of control-transfected cells with ATP increased PP1 activity to 162% compared to control. In I-2-transfected cells, basal PP1 activity was reduced to 57%. In these cells ATP failed to increase PP1 activity.

3.3. MYPT1 phosphorylation in I-2-overexpressing cells

To test whether PP1 is involved in the control of the phosphorylation state of MYPT1, the effect of ATP on MYPT1 phosphorylation at T850 was determined in cells transfected with I-2 or the control virus. In control-transfected cells, ATP induced a 0.5-fold reduction of MYPT1 phosphorylation within 10 min (Fig. 3). The extent of dephosphorylation in non-transfected cells was similar (Fig. 5). In I-2-transfected cells, the basal phosphorylation level of MYPT1 was elevated with the increase in I-2 expression, and ATP was unable to reduce MYPT1 phosphorylation.

3.4. Effect of extracellular ATP on the assembly of the PP1 holoenzyme complex

In order to gain more insight into the ATP-induced activation of the PP1 holoenzyme, we immunoprecipitated the phosphatase complex by using a MYPT1-specific antibody and determined the phosphatase activity of the complex. As shown in Fig. 4A, stimulation of HUVEC with ATP resulted in an increase in phosphatase activity. Phosphatase activity of the complex formed in non-stimulated as well as in ATP-stimulated cells was completely blocked by addition of a recombinant I-2 to the in vitro assay, indicating that the phosphatase activity of the complex is due to the PP1 catalytic subunit. Western blot analysis
showed that in unstimulated cells PP1δ and MLC were co-immunoprecipitated with MYPT1 (Fig. 4B), indicating that complexes of PP1δ and MYPT1 with the substrate MLC are formed under basal conditions. Stimulation of the cells with ATP for 10 min led to an increase in PP1δ and MLC in the MYPT1 immunoprecipitation, indicating that ATP causes an activation of PP1 when recruited to its docking protein MYPT1 and an increase in affinity of the phosphatase holoenzyme for the substrate MLC.

3.5. Phosphorylation of MYPT1 in the presence of ATP or thrombin

Assembly and activity of the MLCP complex may be controlled by phosphorylation of MYPT1 at T850 and T696. Exposure of cells to ATP for 10 min caused a reduction of MYPT1 phosphorylation at T850 and T696 by 47 and 30%, respectively (Figs. 5, 6). Stimulation of the cells with 0.2 U/ml thrombin for 10 min provoked an increase of MYPT1 phosphorylation at T850 and T696 of 3.0 and 3.5-fold, respectively. When, however, the cells were exposed to both ATP plus thrombin, the thrombin-induced effect on MYPT1 phosphorylation was abrogated. Thrombin-induced MYPT1 phosphorylation was also completely blocked by Y-27632, a direct inhibitor of Rho-kinase, confirming that both phosphorylation sites are targeted by Rho-kinase signaling. As a control, when endothelial cells were exposed for 30 min to 10 nM calyculin A, an inhibitor of PP1 and PP2A, MYPT1 phosphorylation at either site was increased to almost the same level as in the presence of thrombin.

In a set of experiments it was tested whether dephosphorylation of MYPT1 is due to extracellular ATP or to its degradation product adenosine. To analyze this question, two different procedures were followed. First, endothelial cells were exposed to ATP in the presence of 10 μM 8-phenyltheophylline, a pan-specific adenosine-receptor antagonist. As shown in Fig. 5, 8-PT did not affect ATP-induced reduction of MYPT1 phosphorylation at T850; likewise, addition of 8-PT alone had no effect on MYPT1 phosphorylation at T850. Similar results were obtained for MYPT1 phosphorylation at T696 (data not shown). Second, ATP degradation to adenosine was blocked by ARL 67156, an ectonucleotidase inhibitor. Also under this condition, the effect of ATP on MYPT1 dephosphorylation on either phosphorylation site was not altered (data not shown).

![Fig. 5](image_url)
MYPT1 phosphorylation at threonine 696 (T696). HUVEC were exposed to various treatments: ATP, thrombin, or ATP plus thrombin for 10 min. In a second set of experiments, cells were exposed to 10 μM Y-27632 (Y), a Rock inhibitor, or Y plus Thr for 10 min. In a third set of experiments, cells were exposed to 10 μM calyculin A (Caly), an inhibitor of protein phosphatase 1 (PP1). PP1 is known to be the catalytic subunit of MLCP [19]. Extracellular ATP enhanced the recruitment of PP1 to the docking protein MYPT1, indicating that more of the MLCP holoenzyme is formed.

The binding of MLC to the MYPT1-containing complex is also increased. Western blot analysis showed a 6.5-fold increase in the MLC/MYPT1 ratio as compared to a 1.9-fold increase in PP1δ/MYPT1. Irrespective of possible differences in the non-linearities of immunostaining for MLC and PP1δ, the increase in MLC association with MLCP correlates with dephosphorylation of MYPT1, suggesting an increase in affinity of the PP1 holoenzyme for MLC.

Analysis of the I-2 sensitive phosphatase activity of this immunoprecipitated MLCP complex revealed that the relative increase in activity (1.7-fold) closely corresponds to the increase in recruitment of PP1δ protein into this complex (1.9-fold). These data indicate that extracellular ATP stimulates the activity of the MLCP complex mainly by recruitment of the PP1δ catalytic subunit into the complex.

As a specific substrate of MLCP, MLC co-immunoprecipitates with MYPT. It is unclear to what extent this is due to a molecular interaction of MLC with the catalytic subunit PP1δ or to an interaction with MYPT1. Experimentally, the co-immunoprecipitation of MLC gives rise to the question whether the immunoprecipitates could contain even other phosphatases attached to the actin part of actomyosin. This cannot be excluded from our analysis. The close correspondence between the increase in PP1δ recruitment and in I-2 sensitive phosphatase activity, however, renders these possible contaminants of minor importance.

The function of the phosphatase docking protein MYPT1 is regulated by phosphorylation. It has been shown in other cells that the phosphorylation of MYPT1 can be mediated by various kinases, including Rock, PKC, ILK, and ZIP kinase [21,37–39]. It was shown recently that stimulation of endothelial cells with thrombin induces a Rock-mediated increase in phosphorylation of MYPT1 at T850 and T696 [40]. Studies by Toth et al. [39] and Velasco et al. [41] showed that...
phosphorylation of MYPT1 reduces its binding to PP1 or MLC. For these reasons it was of interest to analyze MYPT1 phosphorylation in the present study.

Extracellular ATP induced dephosphorylation of MYPT1 at the inhibitory phosphorylation sites threonine 850 and 696. Thrombin induced MYPT1 phosphorylation at both sites in a ROCK-dependent way. ATP effectively antagonized this effect of thrombin. These data explain, on a molecular level, the previously reported antagonism between extracellular ATP and thrombin [5]. In that earlier study we demonstrated in endothelial monolayers that ATP reduces MLC phosphorylation, isometric tension and macromolecule permeability, and antagonizes very effectively the thrombin-induced increases in these parameters.

I-2 overexpression was used to identify the type of phosphatase that causes dephosphorylation of MYPT1 during stimulation with extracellular ATP. Basal phosphorylation of MYPT1 increased with increasing expression of I-2. Under the same conditions, the effect of extracellular ATP on MYPT1 phosphorylation was abolished. These data show that PP1 is also the principal phosphatase responsible for the dephosphorylation of MYPT1. The studies do not identify the exact location of PP1 in this signaling pathway. The most plausible assumption is that PP1 exerts a catalytic effect on MYPT1 when recruited to it. It is also possible, however, that plausible assumption is that PP1 exerts a catalytic effect on the exact location of PP1 in this signaling cascade.

In summary, this study has identified the mechanism by which external ATP causes a strong activation of myosin light chain phosphatase in endothelial cells. Understanding this mechanism is of (path)physiological significance because MLCP is a potent regulator of endothelial barrier function.

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