ATP induces dephosphorylation of myosin light chain in endothelial cells

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Noll, T., M. Schäfer, U. Schavier-Schmitz, and H. M. Piper. ATP induces dephosphorylation of myosin light chain in endothelial cells. Am J Physiol Cell Physiol 279: C717–C723, 2000.—In cultured porcine aortic endothelial monolayers, the effect of ATP on myosin light chain (MLC) phosphorylation, which controls the endothelial contractile machinery, was studied. ATP (10 μM) reduced MLC phosphorylation but increased cytosolic Ca2+ concentration ([Ca2+]i). Inhibition of the ATP-evoked [Ca2+]i rise by xestospongin C (10 μM), an inhibitor of the inositol trisphosphate-dependent Ca2+ release from endoplasmic reticulum, did not affect the ATP-induced dephosphorylation of MLC. MLC dephosphorylation was prevented in the presence of calyculin A (10 nM), an inhibitor of protein phosphatases PP-1 and PP-2A. Thus ATP activates MLC dephosphorylation in a Ca2+-independent manner. In the presence of calyculin A, MLC phosphorylation was incremented after addition of ATP, an effect that could be abolished when cells were loaded with the Ca2+ chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetyoxymethyl ester (10 μM). Thus ATP also activates a Ca2+-dependent kinase acting on MLC. In summary, ATP simultaneously activates MLC dephosphorylation by a Ca2+-independent mechanism.

MATERIALS AND METHODS

Endothelial cells possess a contractile apparatus, resembling the one found in smooth muscle cells (18, 27). They contain the contractile elements actin and myosin and accompanying regulatory proteins (18). Phosphorylation of the regulatory myosin light chain (MLC) leads to activation of the endothelial contractile elements (26). An important second messenger regulating MLC phosphorylation is cytosolic Ca2+ concentration ([Ca2+]i); this controls the activity of endothelial Ca2+-/calmodulin-dependent MLC kinase (23, 26, 27). It has been shown that mediators like thrombin (6, 19) and histamine (12, 13) can cause hyperpermeability of the endothelial barrier by eliciting an increase in MLC phosphorylation and endothelial cell contraction via a receptor-mediated transient rise in cytosolic Ca2+ (6, 20). Endothelial cells also express protein phosphatases PP-1 and PP-2A (3, 10, 24), which can also influence the phosphorylation state of MLC. We and others showed that inhibition of these protein phosphatases leads to hyperpermeability of the endothelial barrier (3, 10, 24). At present, little is known about the functional antagonism between MLC kinase and myosin phosphatases when these are stimulated simultaneously in endothelial cells.

We found recently (16) that the purine receptor agonist ATP causes a transient rise in [Ca2+], and yet a reduction of barrier permeability in endothelial monolayers, derived from different mammalian species and vascular provinces such as porcine aorta, porcine pulmonary artery, bovine aorta, and human umbilical vein. The effects were specific for the nucleotide and could not be imitated by adenosine. The observations have suggested that, in endothelial cells, ATP simultaneously activates the antagonistic mechanisms controlling the phosphorylation state of MLC, i.e., a Ca2+-dependent protein kinase and a Ca2+-independent protein phosphatase acting on MLC. The present study was undertaken to analyze these mechanisms in intact endothelial cells. The results demonstrate that ATP predominantly activates dephosphorylation of MLCs. ATP is the first physiological mediator for which such a Ca2+-independent activation has been shown in endothelial cells.
NaCl, 2.7 KCl, 1.2 KH₂PO₄, 1.0 CaCl₂, and 30.0 HEPES; pH 7.4, 37°C) supplemented with 5% (vol/vol) heat-inactivated newborn calf serum (10 min, 60°C). Basal MLC phosphorylation was determined after an initial equilibration period of 10 min. Agents were added as indicated. Stock solution of ATP was prepared with basal medium immediately before use. Stock solutions of calyculin A, ML-7, xestospongin C, and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxyethyl ester (BAPTA-AM) 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 also 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.

Free [Ca²⁺]i, [Ca²⁺], was determined with the use of the fluorescent Ca²⁺ indicator fura 2. Confluent endothelial monolayers cultured on round glass coverslips were incubated in medium 199 supplemented with 5% (vol/vol) heat-inactivated newborn calf serum and addition of 5 μM fura 2-AM (acetoxyethyl ester of fura 2) at 20°C in the dark. After a 50-min incubation, extracellular fura 2-AM was removed by medium change. This was followed by a 20-min incubation period in the same medium before measurements were started. The coverslips were then mounted in a fluorescence microscope (IX 70; Olympus, Hamburg, Germany). [Ca²⁺], was analyzed using a TILL Photonics (Martinsried, Germany) imaging system. During incubations, the excitation wavelength was alternated between 340 and 380 nm (bandwidth of 8 nm). Emitted light was detected at 510 nm. Fura 2 fluorescence was calibrated according to the method described by Grynkiewicz et al. (8). For this purpose, the cells were exposed to 5 μM ionomycin in modified Tyrode solution containing either 3 mM Ca²⁺ or 5 mM EGTA to obtain the maximum (Rₘₐₓ) and the minimum (Rₘᵢₙ) of the ratio of fluorescence, respectively. [Ca²⁺], was calculated according to the equation

\[
[Ca^{2+}]_i = K_d \times \beta \times (R - R_{\text{min}})/(R_{\text{max}} - R)
\]

with use of Kₐ, the dissociation constant of fura 2 (8), and β, the ratio of the 380-nm excitation signals of ionomycin-treated cells at 5 mM EGTA and at 3 mM Ca²⁺.

Determination of MLC phosphorylation. The phosphorylation of MLC was determined by glycerol-polyacrylamide gel electrophoresis and immunoblot analysis using an anti-MLC antibody as described previously (17). Cells cultured on 30-mm dishes were incubated as indicated in the text. After pretreatment, the incubation medium was rapidly removed and the reaction was terminated by addition of ice-cold tri-chloroacetic acid (1.2 mM). Precipitated proteins were transferred into Eppendorf reaction tubes and centrifuged for 10 min at 14,000 g at 4°C. The sediments were washed twice with ice-cold diethyl ether. After evaporation of the ether at room temperature, the sediments were suspended in 30 μl of lysis buffer (8.8 M urea, 10 mM dithiothreitol, 5 mM thioglycolate, 0.6 mM phenylmethylsulfonyl fluoride, 10 μM cantharidin, 60 mM imidazol, 20 mM Tris, and 23 mM glycine, pH 8.8). The lysate was centrifuged at 14,000 g for 5 min. Afterward, 30 μl of a saturated sucrose-bromphenol blue solution were added and 10 μl of lysate (20–40 μg protein) per lane were run at 400 V and 18°C on a 10% polyacrylamide-40% glycerol gel. Before the lysates were loaded, preelectrophoresis was performed at 400 V for 1.5 h. This procedure allows separation of nonphosphorylated MLCs from the phosphorylated ones, the latter of which migrate more rapidly under this condition. Electrophoretically separated proteins were transblotted on polyvinylidene difluoride membranes and were incubated with an anti-MLC antibody (clone MY-21, Sigma Chemical, Deisenhofen, Germany) followed by an alkaline phosphatase-coupled second antibody as previously described (14). The blots were scanned, and the percentage of MLC phosphorylation (expressed as percentage of total MLC) was calculated from the blot areas of non- (MIC), mono- (MLC-P), and diphosphorylated MLC (MLC ~ PP) as follows

\[
\text{MLC phosphorylation} = \frac{(2 \times \text{MLC} \sim \text{PP}) + \text{MLC} \sim \text{P}}{\text{total MLC}} \times 100
\]

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

Materials. Falcon plastic tissue culture dishes were from Becton Dickinson (Heidelberg, Germany); ATP was from Boehringer (Mannheim, Germany); BAPTA-AM, calyculin A, ML-7, and xestospongin C were from Calbiochem (Bad Dösen, Germany); Transwell polycarbonate filter inserts (24 mm diameter, 0.4 μm pore size) were from Costar (Bodenheim, Germany); newborn calf serum, medium 199, penicillin-streptomycin, and trypsin-EDTA were from Gibco Life Technologies (Eggenstein, Germany); fura 2-AM was from Molecular Probes (Leiden, The Netherlands); polyvinylidene difluoride was from Millipore (Eschborn, Germany); dithiothreitol, phenylmethylsulfonyl fluoride, and thiglycolate were from Sigma Chemical. All other chemicals were of the best available quality, usually analytical grade.

Statistical analysis. Data are given as means ± SD of n = 6 experiments using independent cell preparations. Statistical analysis of data was performed according to Student's unpaired t-test. Probability (P) values of <0.05 were considered significant.

RESULTS

Effect of ATP on MLC phosphorylation and [Ca²⁺]. Addition of 10 μM ATP reduced MLC phosphorylation of aortic endothelial monolayers from a basal level of 36 ± 4% of total MLC to 6 ± 3% within 20 min (Fig. 1). The onset of MLC dephosphorylation coincided with the ATP-induced rise in [Ca²⁺]. ATP (5–100 μM) reduced the level of net MLC phosphorylation in a concentration-dependent manner (Fig. 2).

To test whether the effect of ATP on MLC dephosphorylation is, in part, transmitted via adenosine receptors, 8-phenyltheophylline (8-PT), an adenosine receptor antagonist, was applied. Presence of 8-PT (10 μM) left the reduction of MLC phosphorylation by ATP (10 μM) unchanged: 4 ± 3% after 20 min (not significant vs. ATP alone). 8-PT alone had no effect on MLC phosphorylation (not shown). Addition of adenosine (10 μM) also reduced MLC phosphorylation within 20 min to 5 ± 3%. This effect was inhibited in the presence of 8-PT (10 μM): 32 ± 5% MLC phosphorylation after 20 min in the presence of 8-PT plus ATP. Adenosine did not change [Ca²⁺]i (not shown).

Effects of Ca²⁺ chelation, ML-7, and calyculin A on MLC phosphorylation in the absence of ATP. First, it was tested whether intracellular Ca²⁺ chelation influences MLC phosphorylation under basal conditions. For that purpose, the cells were loaded with the Ca²⁺-best available quality, usually analytical grade.

TILL Photonics (Martinsried, Germany) imaging system. During incubations, the excitation wavelength was alternated between 340 and 380 nm (bandwidth of 8 nm). Emitted light was detected at 510 nm. Fura 2 fluorescence was calibrated according to the method described by Grynkiewicz et al. (8). For this purpose, the cells were exposed to 5 μM ionomycin in modified Tyrode solution containing either 3 mM Ca²⁺ or 5 mM EGTA to obtain the maximum (Rₘₐₓ) and the minimum (Rₘᵢₙ) of the ratio of fluorescence, respectively. [Ca²⁺], was calculated according to the equation

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\]
chelator BAPTA-AM (10 μM) (Fig. 3). When endothelial cells were incubated in the presence of the chelator, MLC became progressively dephosphorylated. This finding indicates that under basal conditions MLC phosphorylation is regulated in a Ca\(^{2+}\)-dependent manner.

Second, it was tested whether ML-7, an inhibitor of MLC kinase, affects the basal state of MLC phosphorylation. Addition of ML-7 (10–100 μM) caused a dose-dependent reduction of MLC phosphorylation (Fig. 4). At 100 μM ML-7, MLC became completely dephosphorylated within 10 min. This finding indicates that MLC phosphorylation under basal conditions is controlled by MLC kinase.

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Fig. 1. Effect of ATP on cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and myosin light chain (MLC) phosphorylation of aortic endothelial monolayers. A: increase in [Ca\(^{2+}\)]\(_i\) in response to ATP (10 μM). A representative single recording is shown. B: MLC dephosphorylation in response to ATP (10 μM). Means ± SD of n = 5 separate experiments of independent cell preparations are given. At time ≥1 min, MLC phosphorylation was significantly different from control (P < 0.05).

C: Western blot analysis of MLC of endothelial cells exposed to ATP (10 μM) at time 0 and at 1, 10, 20, and 30 min. To enhance net dephosphorylation of MLC, endothelial cells were exposed to ML-7 (100 μM for 30 min), an inhibitor of MLC kinase. To induce net phosphorylation of MLC, cells were exposed to calyculin A (Caly; 10 nM for 60 min), an inhibitor of phosphatases. A blot of a representative experiment is given. The bands represent, from top to bottom, the nonphosphorylated (MLC), monophosphorylated (MLC-P), and diphosphorylated (MLC-PP) MLC, respectively.

Fig. 2. Dose-dependent effect of ATP on endothelial MLC phosphorylation. Data are means ± SD of n = 5 separate experiments of independent cell preparations. At time ≥1 min, MLC phosphorylation was significantly different from control (P < 0.05).

Fig. 3. Effect of intracellular Ca\(^{2+}\) chelation on endothelial MLC phosphorylation under basal conditions. Endothelial cells were incubated in an extracellular medium containing 1.2 mM Ca\(^{2+}\) (control) or in the presence of 1.2 mM extracellular Ca\(^{2+}\) plus 10 μM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA). Data are means ± SD of n = 5 separate experiments of independent cell preparations. At time ≥5 min, MLC phosphorylation was significantly different from control (P < 0.05).
kinase contributes actively to the basal state of MLC phosphorylation.

Third, it was tested whether an inhibitor of protein phosphatases influences MLC phosphorylation under basal conditions. The effects of calyculin A (1–10 nM), an inhibitor of PP-1 and PP-2A isoenzymes (9), was analyzed. As shown in Fig. 5, the inhibitor caused an increase in MLC phosphorylation in a dose-dependent manner. This finding indicates that the basal state of MLC phosphorylation is also influenced by protein phosphatase activity.

Effect of ATP on MLC phosphorylation in the presence of xestospongin C. It was studied whether the ATP-induced net dephosphorylation of MLC is triggered by the concomitant increase in the ATP-induced rise in [Ca^{2+}]. To test this, endothelial cells were incubated for 20 min in the presence of xestospongin C (10 μM), an inhibitor of the inositol trisphosphate (IP3)-stimulated Ca^{2+} release from endoplasmic reticulum, before ATP (10 μM) was added to the cells. If the ATP-induced MLC dephosphorylation is Ca^{2+} independent, ATP will induce dephosphorylation of MLC also under those conditions, which prevent the ATP-induced Ca^{2+}-release. As shown in Fig. 6, pretreatment with xestospongin C abolished the ATP-induced rise in [Ca^{2+}], but did not affect the ATP-induced reduction of MLC phosphorylation (Fig. 7). These results indicate that the ATP-induced MLC dephosphorylation is Ca^{2+} independent.

Effect of ATP on MLC phosphorylation in calyculin A-pretreated cells. As shown in the preceding section, ATP given alone caused a net dephosphorylation of MLC. Now, it was analyzed whether ATP influences MLC phosphorylation also in the presence of a protein phosphatase inhibitor, i.e., 10 nM calyculin A. ATP (10 μM) was added 15 min after the addition of calyculin A, when MLC phosphorylation had started to rise, indicating the inhibitory action of calyculin A (Fig. 8). In contrast to its effect when given alone, ATP enhanced MLC phosphorylation in cells pretreated with a phosphatase inhibitor. The results indicate that ATP also activates a MLC kinase activity that only becomes apparent, however, when the predominant phosphatase activation is prevented.

Effect of ATP plus calyculin A on MLC phosphorylation in Ca^{2+}-depleted cells. It was tested whether the ATP-induced stimulation of MLC phosphorylation in the presence of calyculin A depends on [Ca^{2+}]. For this purpose, endothelial cells were incubated in the presence of both BAPTA and calyculin A (Fig. 9). Under these conditions, ATP no longer increased MLC phos-

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**Fig. 4.** Dose-dependent effect of MLC kinase inhibitor ML-7 on endothelial MLC phosphorylation under basal conditions. A: data are means ± SD of n = 5 separate experiments of independent cell preparations. At time ≥1 min, MLC phosphorylation was significantly different from control (P < 0.05). B: Western blot analysis of MLC of endothelial cells exposed to ML-7 (100 μM) at time 0 and at 1, 10, 20, and 30 min. A blot of a representative experiment is given. The bands represent, from top to bottom, the nonphosphorylated (MLC), monophosphorylated (MLC-P), and the diphosphorylated (MLC-PP) MLC, respectively.

**Fig. 5.** Dose-dependent effect of protein phosphatase inhibitor calyculin A on endothelial MLC phosphorylation under basal conditions. Data are means ± SD of n = 5 separate experiments of independent cell preparations. *P < 0.05 vs. control. Because MLC can become diphosphorylated, MLC phosphorylation varies between 0 and 200% (see Determination of MLC phosphorylation in MATERIALS AND METHODS).
Phosphorylation, indicating that the MLC kinase activity stimulated by ATP is Ca\(^{2+}\) dependent.

**DISCUSSION**

This is the first study to analyze the effect of the physiological mediator ATP on the phosphorylation state of MLC, the regulatory protein of the contractile machinery in endothelial cells. The major finding is that ATP simultaneously stimulates a functional antagonism toward phosphorylation and dephosphorylation of MLC, in which the dephosphorylation of MLC prevails. Protein kinase activation is caused by the rise in cytosolic Ca\(^{2+}\) elicited by ATP; activation of MLC dephosphorylation is a Ca\(^{2+}\)-independent effect of ATP.

Under basal culture conditions, MLC of porcine aortic endothelial cells was found to be partially phosphorylated. Exposure of these cells to an inhibitor of MLC kinase (ML-7) quickly reduced the extent of MLC phosphorylation. This indicates that under basal conditions, MLC kinase is one of the factors determining the state of MLC phosphorylation. The observation that intracellular Ca\(^{2+}\) chelation can mimic the effect of ML-7 indicates that the Ca\(^{2+}\)/calmodulin-dependent MLC kinase is involved in phosphorylation of MLC under basal conditions. Exposure of the endothelial cells to the specific inhibitor of the protein phosphatase inhibitor calyculin A. Control cells were exposed to neither ATP nor calyculin A. Endothelial cells were exposed to calyculin A (10 nM; ●). Where indicated, ATP (10 μM; ▲) was added to the calyculin A-treated cells. Data are means ± SD of n = 5 separate experiments of independent cell preparations. At time ≥10 min, MLC phosphorylation in the presence of calyculin A alone was significantly different from control (P < 0.05). *P < 0.05 vs. calyculin A alone. Because MLC can become diphosphorylated, MLC phosphorylation varies between 0 and 200% (see Determination of MLC phosphorylation in MATERIALS AND METHODS).

**Fig. 6.** Effect of xestospongin C (Xe C) on ATP-induced increase of [Ca\(^{2+}\)]\(_i\) in aortic endothelial cells. Control cells (○) were exposed to neither ATP nor xestospongin C. Other cells were exposed to xestospongin C (10 μM; ▲) for 20 min before additional administration of ATP (10 μM; ▲). Results were compared with effects of ATP alone (10 μM; ●). Means ± SD of n = 5 separate experiments of independent cell preparations are given. *P < 0.05 vs. control.

**Fig. 7.** Effect of xestospongin C on ATP-induced endothelial MLC phosphorylation. Control cells (○) were exposed to neither ATP nor xestospongin C. Other cells were exposed to xestospongin C (10 μM; ▲) for 20 min before additional administration of ATP (10 μM; ▲). Results were compared with effects of ATP alone (10 μM; ●). Means ± SD of n = 5 separate experiments of independent cell preparations are given. *P < 0.05 vs. control.

**Fig. 8.** Influence of ATP on endothelial MLC phosphorylation in the presence of protein phosphatase inhibitor calyculin A. Control cells (○) were exposed to neither ATP nor calyculin A. Endothelial cells were exposed to calyculin A (10 nM; ●). Where indicated, ATP (10 μM; ▲) was added to the calyculin A-treated cells. Data are means ± SD of n = 5 separate experiments of independent cell preparations. At time ≥10 min, MLC phosphorylation in the presence of calyculin A alone was significantly different from control (P < 0.05). *P < 0.05 vs. calyculin A alone. Because MLC can become diphosphorylated, MLC phosphorylation varies between 0 and 200% (see Determination of MLC phosphorylation in MATERIALS AND METHODS).
 ATP alone ([Ca^{2+}])_i. Under these conditions, a dephosphorylation of MLC in response to ATP was still observed. This shows that the calyculin A-sensitive protein phosphatases activated by ATP are Ca^{2+} independent. This is in accordance with previous findings that these endothelial cells express constitutively the Ca^{2+}-independent phosphatase isoforms PP-1 and PP-2A (3, 10, 24), which are specifically inhibited by calyculin A at 10 nM (9). Recently, Verin et al. (22) found that endothelial cells can also express the (inducible) Ca^{2+}/calmodulin-dependent protein phosphatase type 2B, which can contribute to the control of MLC dephosphorylation in the presence of thrombin.

It was also tested whether the protein kinase activated by ATP depends on the ATP-induced Ca^{2+} rise. For this purpose, endothelial cells were first Ca^{2+} depleted and then ATP and calyculin A were added simultaneously. The fact that, under this condition, ATP no longer caused an increase in MLC phosphorylation shows that the ATP-induced activation of protein kinase is indeed dependent on the Ca^{2+} rise. ATP stimulates both a phosphorylation of MLC by a Ca^{2+}-dependent activation of MLC kinase and a dephosphorylation of MLC by Ca^{2+}-independent protein phosphatases. The dephosphorylation prevails.

In endothelial cells, as in cells from other tissues, little is known about signal transduction leading to activation of myosin phosphatases. Activation of PP-1 and PP-2A could be due to reduction of basal Rho kinase activation, since the latter is part of an inhibitory mechanism (4). In smooth muscle cells, PP-1 and PP-2A are also activated via protein kinase A or G (1, 21), by mechanisms that have not been fully analyzed. Further work is required to clarify whether ATP activates myosin phosphatase through one of these or yet another mechanism.

The results of this study are of particular interest for two main reasons. First, they describe a novel effect of ATP on endothelial cells. ATP is an important vascular mediator. Its extracellular concentration is normally kept low by ectonucleotidases (7, 28) but may increase substantially at the sites of thrombus formation (11) in hypoxic myocardium (2) or close to ATP-releasing nerve endings. The present findings also explain why ATP was found to reduce endothelial barrier permeability despite a cytosolic Ca^{2+} rise (16). Second, ATP is one of the few known physiological mediators and the first described for endothelial cells that strongly induces MLC dephosphorylation. The results of this study indicate that this effect is due to an activation of myosin phosphatases. The vast majority of other physiological mediators investigated in smooth muscle cells acts through an inhibition mechanism if affecting myosin phosphatases. The identification of a physiological mechanism of myosin phosphatase activation is of particular interest for endothelial pathophysiology as its knowledge may lead to new therapeutic principles for stabilizing the endothelial barrier by dephosphorylation of endothelial MLC.
REFERENCES


