ATP induces dephosphorylation of myosin light chain in endothelial cells

T. NOLL, M. SCHÄFER, U. SCHAIVIER-SCHMITZ, AND H. M. PIPER

Physiologisches Institut, Justus-Liebig-Universität, D-35392 Giessen, Germany

Received 17 December 1999; accepted in final form 29 March 2000

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-sensitive 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 acetoxyethyl ester (10 μM). Thus ATP also activates a Ca²⁺-dependent kinase acting on MLC. In summary, ATP simultaneously stimulates a functional antagonism toward both phosphorylation and dephosphorylation of MLC in which the dephosphorylation prevails. In endothelial cells, ATP is the first physiological mediator identified to activate MLC dephosphorylation by a Ca²⁺-independent mechanism.

Address for reprint requests and other correspondence: T. Noll, Physiologisches Institut, Justus-Liebig-Universität, Aulweg 129, D-35392 Giessen Germany (E-mail: thomas.noll@physiologie.med.uni-giessen.de).

Materials and Methods

Cell cultures. Endothelial cells from porcine aorta were isolated and cultured as previously described (25). Confluent cultures of primary endothelial cell were trypsinized in phosphate-buffered saline (composed of (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 either 24-mm round polycarbonate filters (pore size of 0.4 μm), 25-mm round glass coverslips, or 30-mm culture dishes for determination of albumin permeability, [Ca²⁺]i, level, or MLC phosphorylation, respectively. Experiments were performed with confluent endothelial Passage 1 monolayers 4 days after seeding.

Experimental protocols. The basal medium used in incubations was modified Tyrode solution (composition in mM: 150
ATP-INDUCED DEPHOSPHORYLATION OF MYOSIN LIGHT CHAINS

NaCl, 2.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 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 acetoxymethyl 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⁺], [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 (acetoxymethyl 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. The phosphorylation was determined after an initial equilibration.

Basal MLCs from the phosphorylated ones, the latter of which migrate more rapidly under this condition. Electrophoretically separated proteins were transferred 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 ~ P) as follows

MLC phosphorylation = (2 × MLC ~ P) + MLC ~ P × 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 Soden, 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 membranes and were incubated with an anti-MLC antibody (clone MY-21, Sigma Chemical, Deisenhofen, Germany). 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⁺] (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⁺

[Ca²⁺]i was calculated according to the equation

\[ [Ca²⁺]i = K_d × \beta \times (R - R_{min})/(R_{max} - R) \]

with use of \( K_d \), the dissociation constant of fura 2 (8), and \( \beta \), the ratio of the 380-nm excitation signals of ionomycin-[Ca²⁺]. ATP (5–100 μM) reduced 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⁺] (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⁺

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⁺] (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⁺

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⁺] (not shown).
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...
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 Ca$^{2+}$-independent. 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$^{2+}$]i. 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-

---

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).
phorylation, 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 phospha-
different compared with MLC phosphorylation in the presence of ATP. BAPTA alone was added. Data are means ± SD of n = 5 separate experiments of independent cell preparations. At time t = 10 min, MLC phosphorylation in the presence of BAPTA alone was significantly different from control (P < 0.05). At time t = 20 min, MLC phosphorylation in the presence of BAPTA + ATP, BAPTA + calyculin A or BAPTA + ATP + calyculin A was not significantly different compared with MLC phosphorylation in the presence of BAPTA alone (P > 0.05).

tases PP-1 and PP-2A, calyculin A, resulted in a rapid increase in the phosphorylation state of MLC. This result shows that under basal conditions the level of MLC phosphorylation is also controlled by protein phosphatases. Under basal conditions, MLC phosphorylation is thus in a steady state in which the action of MLC kinase is balanced by myosin phosphatases.

Exposure of endothelial cells to ATP caused a fast reduction of MLC phosphorylation. The ATP metabolite adenosine also induced dephosphorylation of MLC. However, the effect of ATP is not mediated by its metabolite, as the action of adenosine but not the one of ATP can be blunted by the adenosine receptor inhibitor 8-PT.

ATP acts on MLC phosphorylation in a dose-dependent manner. This effect persisted for over 20 min. When protein phosphatases were first inhibited by calyculin A and ATP was then applied, it caused an (additional) increment in MLC phosphorylation instead of dephosphorylation. This latter experiment demonstrates that ATP also stimulates a protein kinase acting on the MLC. The finding that ATP, when applied alone, causes a net dephosphorylation must therefore be explained by a strong activation of protein phosphatases overriding the activation of protein kinase. It was tested whether the activation of protein phosphatase, acting on MLC, was Ca^{2+}-dependent. The inhibitor of IP_3-regulated Ca^{2+} release, xestospongin C (5, 15), was used to abolish the ATP-induced rise in [Ca^{2+}]. 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


