MEK/MAPK as a signaling element in ATP control of endothelial myosin light chain

D. Klingenberg, D. Günþüz, F. Härtel, K. Bindewald, M. Schäfer, H. M. Piper, and T. Noll. MEK/MAPK as a signaling element in ATP control of endothelial myosin light chain. Am J Physiol Cell Physiol 286: C807–C812, 2004; 10.1152/ajpcell.00002.2003.—Phosphorylation of endothelial myosin light chains (MLC) is a key mechanism in control of endothelial contractile machinery. Extracellular ATP influences endothelial MLC phosphorylation by either activation of Ca\(^{2+}\)-dependent MLC kinase or Ca\(^{2+}\)-independent MLC phosphatase. Here, the role of the MEK/MAPK pathway in this signaling was investigated in porcine aortic endothelial cells. Phosphorylation of ERK2 and phosphorylation of MLC were analyzed in cultured aortic endothelial cells. ATP (10\(\mu\)M) increased ERK2 phosphorylation from basal 17 ± 3 to 53 ± 4%, an effect suppressed in the presence of the MEK inhibitors PD-98059 (20\(\mu\)M) or U0126 (10\(\mu\)M). Phosphorylation of ERK2 was not dependent on the ATP-induced cytosolic Ca\(^{2+}\) rise, because it was unaltered when this was suppressed by the Ca\(^{2+}\) chelator BAPTA (10\(\mu\)M) or xestospongin C (3\(\mu\)M), an inhibitor of the inositol 1,4,5-trisphosphate-sensitive Ca\(^{2+}\) release mechanism of the endoplasmic reticulum. Phosphorylation of ERK2 was neither induced by the adenosine analog 5'-N-ethylcarboxamido)adenosine (1\(\mu\)M) nor inhibited in the presence of the adenosine receptor antagonist 8-phenyltheophylline (10\(\mu\)M). ATP increased MLC kinase activity, and this was blocked in presence of PD-98059. ATP also increased MLC phosphatase activity, which was not inhibited by PD-98059. The MEK/MAPK pathway is a Ca\(^{2+}\)-independent part of ATP signaling toward MLC kinase but not of ATP signaling toward MLC phosphatase.

THE ENDOTHELIAL LINING of blood vessels forms a barrier to plasma solutes and macromolecules. One of the key mechanisms regulating endothelial barrier function is the endothelial contractile machinery. Generation of mechanical forces within the endothelial cell layer can lead to opening of intercellular gaps and thereby changes in endothelial barrier function (10). The contractile machinery of endothelial cells is similar in protein composition and biochemical control to that of smooth muscle cells (18, 21, 22). As in smooth muscle cells, phosphorylation of myosin light chains (MLC) controls activation of the contractile elements (2, 9, 19). The phosphorylation state of MLC is regulated by both MLC kinase and MLC phosphatase activity. Several stimuli activating MLC kinase or inhibiting MLC phosphatase have already been characterized. These stimuli favor an increase in barrier permeability. For the first time, we recently identified a receptor-mediated stimulus, exogenous ATP, which activates MLC phosphatase and stabilizes endothelial barrier function (12). It acts via purinergic receptors on the endothelial surface (11). This finding has defined a new principle for endothelial barrier control that may be exploited therapeutically. It also assigns a new functional role to ATP accumulating in the vicinity of endothelial cells. At this location, extracellular ATP may be increased, e.g., by a release from endothelial cells themselves in response to shear stress, from platelets activated in the course of developing vascular injury, or from autonomous nerve fibers (1, 3, 23).

The analysis of intracellular signaling in endothelial cells has revealed that ATP induces a dephosphorylation of MLC most likely by a Ca\(^{2+}\)-independent activation of MLC phosphatase (13). However, ATP can also activate MLC kinase by a Ca\(^{2+}\)-dependent mechanism. In the present study we investigated the role of the signaling pathway of mitogen-activated protein kinases (MAPK), also named extracellular signal-regulated protein kinases (ERK), which are organized in modules containing protein kinases activated by sequential phosphorylation. ERK is activated by MAPK kinases (MEK), which in turn are activated by phosphorylation of MEK kinases. As shown previously (4, 15), the MEK/ERK pathway can be activated in response to extracellular ATP in endothelial cells. This pathway has been reported to participate not only in control of endothelial proliferation but also cell migration and endothelial barrier function (7, 20). This previous knowledge led to the hypothesis that the MEK/ERK pathway is also part of the signaling of ATP toward the control of MLC phosphorylation in endothelial cells. In a number of other cells, it was shown that the MEK/ERK pathway is located upstream of MLC kinase (8, 14).

From this finding the question arose as to whether in endothelial cells the MLC kinase-mediated part of ATP signaling can be blocked by inhibitors of the MEK/ERK pathway. This would be of interest as it might allow to further enhance the impact of MLC phosphatase on MLC regulation by exogenous ATP. In the present study cultured porcine aortic endothelial cells were used as experimental model. The analysis was focused on ERK2 because ERK1 and -2 are usually coregulated in endothelial cells.

MATERIALS AND METHODS

**Cell cultures.** Endothelial cells from porcine aorta were isolated and cultured as previously described (17). Confluent cultures of primary endothelial cells were trypsinized in trypsin-EDTA seeded at a density of 7 x 10^4 cells/cm^2 on 30-mm culture dishes. Experiments were performed with confluent endothelial monolayers of passage 1, cultured for 4 days in M199 medium supplemented with 20% newborn calf serum. In a set of experiments endothelial cells were serum-starved for 24 h before experimental incubations.

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**Experimental protocols.** The basal medium used in experiments 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 HEPES; pH 7.4, 37°C) supplemented with 5% (vol/vol) heat-inactivated newborn calf serum (10 min, 60°C). Agents were added as indicated. Stock solutions of 2'-amino-3'-methoxyflavone (PD-98059), 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadine (U1216), 5'-(N-ethylcarbazolamido)adenosine (NECA), 1,2-bis(2-aminophenoxy)-ethane-N,N',N',N'-tetraacetic acid BAPTA-AM, xestospongin C, calyculin A, and 5-iodonaphthalene-1-sulfonil homopiperazine (ML-7) were prepared with dimethyl sulfoxide (DMSO). Final solvent concentrations were <0.1% (vol/vol). The same final concentrations of DMSO were included in all respective controls.

**Determination of phosphorylated and nonphosphorylated ERK.** The phosphorylation of ERK2 was determined by SDS-PAGE and Western blot analysis. Experimental incubations of cultures were terminated by a rapid removal of the medium and addition of lysis buffer [50 mM Tris-HCl, pH 6.7, 2% (wt/vol) SDS, 2% (vol/vol) mercaptoethanol, 1% (wt/vol) sodium orthovanadate] supplemented with benzamidase (5 U/ml). Equal amounts of lysed cellular proteins (60 μg protein/slot) were applied to SDS-PAGE [12% (wt/vol) total acrylamide concentration; 1:100 bisacrylamide-acrylamide ratio] and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 3% (wt/vol) bovine serum albumin and probed with an anti-ERK2 antibody (0.2 μg/ml). The signal was visualized with a peroxidase-conjugated anti-rabbit antibody (0.1 μg/ml). With this method ERK2 is separated into the phosphorylated and the nonphosphorylated proteins, the latter of which migrates more rapidly. Blots were scanned densitometrically, and the ERK2 phosphorylation is given as percentage of total ERK2.

To determine the phosphorylated forms of ERK1/2 and ERK5 as well as the nonphosphorylated form of ERK5, we lysed cells in lysis buffer (composition as described above), and equal amounts of lysed cellular proteins (60 μg protein/slot) were separated in a SDS-PAGE [10% (wt/vol) total acrylamide concentration; 3:3:100 bisacrylamide-acrylamide ratio] followed by protein transfer onto PVDF-membranes. The membranes were probed with an anti-phospho-ERK1/2 (P-Thr180/Pyr182) antibody (0.2 μg/ml), which recognizes the dual-phosphorylated form of ERK1 and -2, an anti-ERK1/2 antibody (0.2 μg/ml), an anti-phospho-ERK5 (1:1,000), and an anti-ERK5 antibody (1:1,000), which recognizes the nonphosphorylated form of ERK5. The signals were visualized with a peroxidase-conjugated anti-rabbit antibody (0.1 μg/ml), and bands were densitometrically evaluated.

**Determination of MLC phosphorylation.** The phosphorylation of MLC was determined by glycerol-urea polyacrylamide gel electrophoresis and Western blot analysis as described (16). Experimental incubations of cultures were terminated by a rapid removal of the medium and addition of lysis buffer (8.8 M urea, 10 mM dithiothreitol, 5 mM thioglycolate, 10 μM dithioretil, thioglycolate, and phorbol 12-myristate 13-acetate (PMA) were from Sigma-Aldrich (Taufkirchen, Germany); PVDF membrane was from Millipore (Schemborn, Germany); anti-phospho MAPK antibody (pTEpY); rabbit polyclonal IgG antibody was from Promega (Mannheim, Germany); and anti-p42 MAPK antibody (rabbit polyclonal IgG antibody) was from Santa Cruz Biotechnology (Heidelberg, Germany). All other chemicals were of the best available quality, usually analytic grade.

**Statistical analysis.** Data are given as means ± SD of 5 experiments with independent cell preparations. The comparison of means between groups was performed by one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post hoc test. Changes in parameters within the same group were assessed by multiple ANOVA analysis. *P* values of <0.05 were considered significant.

**RESULTS**

**Phosphorylation of ERK1/2 and ERK5.** To measure the activation state of ERK, we determined the degree of phosphorylation of these proteins by two different SDS-PAGE protocols followed by Western blot analysis. **Protocol 1** is based on standard resolution of cell proteins by 10% SDS-PAGE. **Protocol 2** allows the gel electrophoretic separation of the nonphosphorylated from the phosphorylated (activated) form of ERK because of its retarded gel mobility. This protocol has the advantage that ERK phosphorylation can be determined independently of the total amount of protein loaded on each lane. Under basal experimental conditions, in cells not serum-starved, 17 ± 3% of ERK2 was constantly phosphorylated (Fig. 1). Addition of ATP (10 μM) caused a rapid but transient rise of ERK2 phosphorylation with a maximum of 53 ± 4% after 5 min. As shown by Western blots, with the use of an antibody that detects both phosphorylated isoforms, phosphorylation of ERK1 and -2 is coregulated by ATP. Therefore, in the ongoing study we focused our analysis on phosphorylation of ERK2.

To test whether the response to extracellular ATP is similar in serum-starved endothelial cells, we also exposed endothelial cells that were serum-starved for 24 h to 10 μM ATP for the same period of time. In these cells ERK2 phosphorylation was not detectable. Addition of ATP (10 μM) induced an increase in ERK2 phosphorylation to 58 ± 7% after 5 min (*P* < 0.05; *n* = 5). These data show that extracellular ATP also stimulates ERK2 phosphorylation in serum-starved cells.

The effect of ATP was dose dependent (Fig. 2) and comparable in strength to that of the PKC activator phorbol 12-
myristate 13-acetate (PMA; 200 nM). Two chemically distinct inhibitors of MEK1/2 were used to block the MEK/ERK pathway: PD-98059 (20 μM) or U0126 (10 μM). These inhibitors reduced the basal level of ERK2 phosphorylation but also abolished the ATP effect (Fig. 3). These data show that ATP can stimulate ERK2 activation via MEK in endothelial cells.

Recently, it was shown that micromolar concentrations of PD-98059 and U0126 inhibit not only MEK1/2 but also MEK5 (5) and thereby inhibit ERK5. ERK5 is a member of the MAPK family that was shown to be selectively activated by MEK5 (6). Therefore, we tested whether extracellular ATP could activate ERK5. As shown by Western blot analysis (Fig. 4), MEK5 was slightly phosphorylated under basal conditions in porcine aortic endothelial cells. Exposure of endothelial cells to extracellular ATP (10 μM) did not cause a change in ERK5 phosphorylation during 30 min of incubation, a time interval in which ERK2 is activated by ATP.

The increase in phosphorylation of ERK2 by ATP could not be antagonized by a blocking dose of the adenosine receptor antagonist 8-PT (10 μM) (Fig. 5). The stable adenosine analog NECA (1 μM) had no effect on ERK2 phosphorylation. These results show that the activation of ERK2 in the presence of exogenous ATP is not due to generation of adenosine from ATP.

Whether changes of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) affect ERK2 phosphorylation has been investigated. We previously showed (12) that addition of ATP causes a rapid and transient rise of [Ca\(^{2+}\)]\(_i\) in endothelial cells that can be abolished 1) by a prior loading of the cells with the Ca\(^{2+}\) chelator BAPTA-AM or 2) by inhibiting the inositol 1,4,5-trisphosphate (IP\(_3\))-sensitive Ca\(^{2+}\) release mechanism of the endoplasmic reticulum with the specific inhibitor xestospongin C (13). As demonstrated in Fig. 6, treatment of the cells with either BAPTA (10 μM) or xestospongin C (3 μM) did not alter the effect of ATP on ERK2 phosphorylation. The results show,

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Fig. 1. Effect of ATP on ERK1/2 phosphorylation of porcine aortic endothelial cells. Representative Western blot shows separation of phosphorylated ERK2 (P-ERK 2) and nonphosphorylated ERK2 (A) and coregulation of ERK1 and -2 phosphorylation in the presence of 10 μM ATP (B) for 0, 2.5, 5, 10, 20, and 30 min. C: time course of ATP-stimulated ERK2 phosphorylation (densitometric evaluation of A). Data are means ± SD of 5 separate experiments with independent cell preparations. *P < 0.05 vs. control.

Fig. 2. Concentration-dependent effect of ATP and effect of phorbol 12-myristate 13-acetate (PMA; 200 nM) on ERK2 phosphorylation. C, control. Data are means ± SD, measured after 5 min in the presence of ATP or PMA, of 5 separate experiments with independent cell preparations. *P < 0.05 vs. control.

Fig. 3. Effect of ATP (10 μM) on ERK2 phosphorylation after 30 min in the presence of MEK inhibitors PD-98059 (PD; 20 μM) or U0126 (UO; 10 μM). Data are means ± SD, measured after 5 min in the presence of ATP, of 5 separate experiments with independent cell preparations. *P < 0.05 vs. control; n.s., values not significantly different from each other.
therefore, that ERK2 activation is independent of the rise in $[Ca^{2+}]$, elicited by ATP.

**Activation of MLC kinase and MLC phosphatase.** Under basal conditions, the degree of MLC phosphorylation was stable at $65 \pm 7\%$ (Fig. 7). Preincubation of endothelial cells with 20 $\mu$M PD-98059, a dose that blunted basal ERK2 phosphorylation, only slightly reduced basal MLC phosphorylation to $57 \pm 8\% \; (P > 0.05; \text{not significantly different from untreated control})$, indicating that under basal conditions MLC phosphorylation is not significantly affected by the ERK2 pathway. To test the influence of the MEK/ERK pathway on MLC kinase, we applied a protocol to block MLC phosphatase activity in intact cells. For this purpose, the phosphatase inhibitor calyculin A was added and the subsequent rise in MLC phosphorylation was monitored over time. The dose of 10 nM calyculin A represents a blocking concentration of MLC phosphatase. In dose-finding experiments, we confirmed that this concentration was the smallest to provide a maximum rise of spontaneous MLC phosphorylation. We showed previously (13) that the continuous slow rise in MLC phosphorylation in the presence of calyculin A alone is due to basally active MLC kinase. In the presence of the MEK inhibitor PD-98059 (20 $\mu$M), this spontaneous rise of MLC phosphorylation was significantly attenuated. This finding indicates that the MEK/ERK pathway controls the basal activity of MLC kinase. When ATP (10 $\mu$M) was added in presence of calyculin A, ATP caused a rapid additional rise of MLC phosphorylation, indicating activation of MLC kinase. This rapid additional rise was completely abolished in the presence of PD-98059. The finding shows that the activation of MLC kinase by ATP is mediated by the MEK/ERK pathway.

In our previous investigations (13), we also showed that in the absence of phosphatase inhibitors, ATP causes a dephosphorylation of MLC and that this is due to activation of a predominant MLC phosphatase. Dephosphorylation of MLC under ATP stimulation was again observed in the present study (Fig. 8). The additional presence of the MEK inhibitor PD-98059 caused a further decrease of MLC phosphorylation. These data indicate that inhibition of the MEK/ERK pathway can enhance the ATP-stimulated dephosphorylation of MLC. To test whether this effect is due to an increase in phosphatase activity, we inhibited MLC kinase by exposure to ML-7 (50 $\mu$M) in an additional set of experiments. As shown in Fig. 8, ML-7 caused a dephosphorylation of MLC, due to the basal activity of MLC phosphatase. With the additional presence of ATP, MLC were further dephosphorylated. When PD-98059 was added under the latter conditions, the level of MLC dephosphorylation was not altered. These results indicate that the MLC phosphatase activation by ATP is not mediated via MEK/ERK.

We also tested whether ATP reduces MLC phosphorylation in serum-starved endothelial cells to extracellular ATP and analyzed MLC phosphorylation. In cells serum-starved for 24 h, MLC phosphorylation was reduced from $65 \pm 7$ to $43 \pm 5\% \; (P < 0.05; n = 5)$. Addition of 10 $\mu$M ATP further reduced MLC phosphorylation to $19 \pm 3\%$ after 10 min ($P < 0.05; n = 5$). These data show that extracellular ATP also provokes MLC dephosphorylation in serum-starved endothelial cells.

**DISCUSSION**

The present study was performed to identify the role of the MEK/ERK pathway in the biochemical control of endothelial...
contractile elements via MLC. In a previous study, we showed that ATP simultaneously activates MLC kinase in a Ca^{2+}-dependent manner and MLC phosphatase in a Ca^{2+}-independent manner. The main finding of the present study is that Ca^{2+}-independent activation of the MEK/ERK pathway mediates ATP-induced activation of MLC kinase but not that of MLC phosphatase.

Under basal experimental conditions, ERK2 was partially phosphorylated in the endothelial cells, indicating that it is partially active. Consistent with this finding is the result that inhibition of MEK1/2, the kinases upstream of ERK2, reduced the basal phosphorylation state of ERK2. Stimulation of the cells with exogenously applied ATP led to a transient and dose-dependent increase in ERK2 phosphorylation, which was blocked by MEK inhibitors. Phosphorylation of ERK2 coincided with phosphorylation of ERK1, indicating that activation of both ERK isoforms are cocontrolled by extracellular ATP. The maximum effect of ATP was comparable in magnitude to the ERK2 activation observed in presence of PMA, an activator of the upstream kinase PKC. This comparison shows that ATP has a strong effect on ERK2. ATP also stimulated an increase in ERK phosphorylation in endothelial cells that had been serum-starved for 24 h. This finding shows that the stimulatory effect of extracellular ATP on ERK2 phosphorylation is independent of serum starvation.

We also investigated whether the ATP effect could be explained by a stimulation through its metabolite adenosine. This was not the case, because the adenosine receptor antagonist 8-PT failed to inhibit the ATP effect on ERK2 phosphorylation and addition of the stable nonselective adenosine analog NECA had no effect. Endothelial cells express purinergic receptors for ATP (11). In the present study, the purine receptor mediating the ATP effect was not further characterized. As we showed in a previous study by pharmacological means, the purine receptor mediating the ATP effect on endothelial permeability belongs to the P2Y family (11). Activation of the MEK/ERK pathway by ATP was not dependent on the transient rise in [Ca^{2+}], that is triggered by the addition of ATP. This rise is due to a release of Ca^{2+} from the endoplasmic reticulum via the IP_3-sensitive release channel. Because this channel can be specifically inhibited by xestospongin C, the effect of ATP stimulation could be analyzed without the Ca^{2+} rise occurring (13). The activation of ERK2 remained unaffected by these changes in experimental conditions. Similarly, a suppression of Ca^{2+} rise by loading the cells with the Ca^{2+} chelator BAPTA did not affect ERK2 activation. These results, therefore, show that the activation of the MEK/ERK pathway by ATP is independent of its effect on [Ca^{2+}], control.

To characterize the activation of MLC kinase in intact cells, we used an indirect protocol in which MLC phosphorylation was monitored in presence of a blocking concentration of the phosphatase inhibitor calyculin A. As reported previously (13), ATP was found to activate MLC kinase. This activation of MLC kinase could be inhibited by the MEK inhibitors PD-98059 or U0126, showing that it depends on the MEK/ERK pathway.

Fig. 7. Effect of ATP on myosin light chain (MLC) phosphorylation under MLC phosphatase inhibition by calyculin A. Endothelial cells were either nontreated or pretreated with the MEK inhibitor PD-98059 (20 μM) for 30 min and then exposed to calyculin A (Caly; 10 nM), ATP (10 μM), or Caly + ATP. A: representative Western blots show MLC phosphorylation. The bands represent, from top to bottom, the nonphosphorylated (MLC), monophosphorylated (MLC~P), and diphosphorylated protein (MLC~PP), respectively. B: MLC phosphorylation in the absence of PD-98059 (+PD), ATP stimulates MLC phosphorylation. Bottom: MLC phosphorylation in the presence of PD-98059 (+PD). ATP does not stimulate MLC phosphorylation any more. *P < 0.05, Caly vs. Caly + ATP.

Fig. 8. Effects of ATP on MLC phosphorylation in the presence of MEK inhibitor PD-98059 (20 μM), MLC kinase inhibitor ML-7 (50 μM), and combinations of the agents. Cells were preincubated for 30 min in the presence of the inhibitors, and then ATP (10 μM) was added. Data are means ± SD, measured after 10 min in the presence of ATP, of 5 separate experiments with independent cell preparations. *P < 0.05; n.s., values not significantly different from each other.
In our previous study (13), we demonstrated that ATP activates MLC kinase in a Ca$^{2+}$-dependent way. On the basis of the results of the present study, it can now be established that the Ca$^{2+}$-dependent step is downstream of MEK/ERK. Most likely, it is the MLC kinase itself, which is known in other cells to be a Ca$^{2+}$/calmodulin-dependent enzyme. Activation of MLC phosphatase is the predominant effect of ATP on MLC. We now find that this signaling effect, which we showed previously to be Ca$^{2+}$ independent, is not mediated via MEK/ERK. The MEK/ERK pathway is therefore, exclusively involved in the signaling part of ATP toward MLC kinase.

In the search for agents that can modulate endothelial permeability, agents causing a dephosphorylation of MLC, and thereby a reduction of tension within the endothelial cell layer, are of therapeutic interest because they may be used to stabilize endothelial barrier function and protect organs from edema. ATP is the only receptor-mediated agonist known to date that strongly activates MLC phosphatase. As an adjunct to this therapeutic principle, agents preventing endothelial MLC kinase activation are also of interest because their presence could foster the ability of an activation of MLC phosphatase to cause a marked dephosphorylation of MLC. Apart from inhibitors of MLC kinase, such as ML-7, we now find that inhibitors of MEK/ERK may also be used for this purpose.

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