Urocortin-Induced Decrease in Ca\textsuperscript{2+} Sensitivity of Contraction in Mouse Tail Arteries Is Attributable to cAMP-Dependent Dephosphorylation of MYPT1 and Activation of Myosin Light Chain Phosphatase

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Abstract—Urocortin, a vasodilatory peptide related to corticotropin-releasing factor, may be an endogenous regulator of blood pressure. In vitro, rat tail arteries are relaxed by urocortin by a cAMP-mediated decrease in myofilament Ca\textsuperscript{2+} sensitivity through a still unclear mechanism. Here we show that contraction of intact mouse tail arteries induced with 42 mmol/L KCl or 0.5 \mu mol/L noradrenaline was associated with a \( \approx \) 2-fold increase in the phosphorylation of the regulatory subunit of myosin phosphatase (SMPP-1M), MYPT1, at Thr696, which was reversed in arteries relaxed with urocortin. Submaximally (pCa 6.1) contracted mouse tail arteries permeabilized with \( \alpha \)-toxin were relaxed with urocortin by \( 39 \pm 3\% \) at constant [Ca\textsuperscript{2+}], which was associated with a decrease in myosin light chain (MLC\textsubscript{20}\textsuperscript{Ser19}), MYPT1\textsuperscript{Thr696}, and MYPT1\textsuperscript{Thr850} phosphorylation by 60\%, 28\%, and 52\%, respectively. The Rho-associated kinase (ROK) inhibitor Y-27632 decreased MYPT1 phosphorylation by a similar extent. Inhibition of PP-2A with 3 \mu mol/L okadaic acid had no effect on MYPT1 phosphorylation, whereas inhibition of PP-1 with 3 \mu mol/L okadaic acid prevented dephosphorylation. Urocortin increased the rate of dephosphorylation of MLC\textsubscript{20}\textsuperscript{Ser19} \( \approx \) 2.2-fold but had no effect on the rate of contraction under conditions of, respectively, inhibited kinase and phosphatase activities. The effect of urocortin on MLC\textsubscript{20}\textsuperscript{Ser19} and MYPT1 phosphorylation was blocked by Rp-8-CPT-cAMPS and mimicked by Sp-5,6-DCl-cBIMPS. In summary, these results provide evidence that Ca\textsuperscript{2+}-independent relaxation by urocortin can be attributed to a cAMP-mediated increased activity of SMPP-1M which at least in part is attributable to a decrease in the inhibitory phosphorylation of MYPT1. (Circ Res. 2006;98:1159-1167.)

Key Words: arteries ■ calcium sensitivity ■ urocortin ■ PKA ■ myosin phosphatase

Urocortin, (now known as urocortin 1 [UCN1]) is a 40 amino acid polypeptide that belongs to the corticotropin-releasing factor (CRF) family.\textsuperscript{1} Urocortin-like immunoreactivity\textsuperscript{2} and expression of the peripheral subtype of CRF receptors, CRF-2R,\textsuperscript{3} has been detected in the circulatory system. Urocortin, which has a higher affinity for CRF-2R than CRF itself, relaxes blood vessels both in vitro\textsuperscript{4} and in vivo,\textsuperscript{5} causing a CRF-2R-mediated decrease in the mean arterial blood pressure.\textsuperscript{6} Interestingly, in CRF-2R-deficient mice, the resting blood pressure was elevated.\textsuperscript{6} These findings suggest that urocortin is an endogenous regulator of blood pressure and blood flow. In addition, urocortin, the plasma levels of which are increased in human heart failure,\textsuperscript{7} has beneficial effects in experimental heart failure.\textsuperscript{8}

The mechanism by which urocortin relaxes blood vessels appears to be complex, eg, vasodilation has been reported to be both endothelium dependent and independent.\textsuperscript{3,4,9,10} The endothelium-independent vasodilation was suggested to be mediated by activation of the cAMP/protein kinase A (PKA) signaling cascade,\textsuperscript{2,11} which is in line with the observation that activation of CRF-2R increases cAMP levels in Ltk cells.\textsuperscript{12} In some vessels, activation of PKA was associated with activation of potassium channels and membrane potential hyperpolarization,\textsuperscript{3,9} which will then lead to a decrease in intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]). However, in rat tail arteries, relaxation occurred without a decrease in global [Ca\textsuperscript{2+}],\textsuperscript{11} ie, by a phenomenon known as Ca\textsuperscript{2+} desensitization of myofilaments.\textsuperscript{13}

The contractile state of smooth muscle is mainly governed by phosphorylation of the regulatory light chains of myosin (MLC\textsubscript{20}), which is determined by the opposing actions of the Ca\textsuperscript{2+}–calmodulin–activated myosin light chain kinase (MLCK) and myosin phosphatase (SMPP-1M). Within this
scheme, a decrease in Ca\(^{2+}\) sensitivity will ensue when either MLCK is inhibited or SMPP-1M is activated in a Ca\(^{2+}\)-independent manner.\(^{13}\) PKA can decrease the activity of MLCK in vitro, but the physiological relevance is not clear (reviewed by Somlyo and Somlyo\(^{13}\)). SMPP-1M was initially thought to be a permanently active unregulated enzyme.\(^{14}\) It is now well established that SMPP-1M is the target of several intracellular signaling cascades that modulate its activity. Thus, G protein–mediated inhibition of SMPP-1M is responsible for Ca\(^{2+}\) sensitization observed in the presence of contractile agonists.\(^{13,15}\) Furthermore, it was shown that cyclic nucleotides, whereby most studies focused on cGMP,\(^{13}\) can increase the activity of SMPP-1M in vascular smooth muscle\(^{16-19}\) through a not fully understood mechanism.

Among several mechanisms that can decrease the activity of SMPP-1M,\(^{13}\) the Rho/Rho-associated kinase pathway emerged as a central mechanism,\(^{14,13}\) which leads to phosphorylation of the regulatory subunit MYPT1 at Thr696 and Thr850 in vitro.\(^{20}\) To what extent vasoconstrictors increase phosphorylation of these sites is still a matter of debate (reviewed by Ito et al\(^{14}\)). Clearly, if urocortin decreased phosphorylation of these sites, the activity of SMPP-1M should increase and relaxation should ensue. The aim of this study was to test this hypothesis and to further provide evidence that the increase in SMPP-1M activity is mediated by cAMP signaling.

### Materials and Methods

#### Tension Measurements

Dissection, permeabilization with *Staphylococcus aureus* α-toxin, and tension measurements of intact and permeabilized mouse tail arteries were performed as described.\(^{11,21}\)

#### Determination of Protein Phosphorylation

At the desired time points, arteries mounted on wires and subjected to the same experimental protocol as for force measurements were shock frozen and processed as described before.\(^{22}\) Western blots were probed with antibodies against MYPT1 and MLC\(_{20}\) or phosphospecific antibodies against p-MYPT1\(^{\text{Thr696}}\) (eg, see Feng et al\(^{20}\)), p-MYPT1\(^{\text{Thr850}}\), and p-MLC\(_{20}\)\(^{\text{Ser19}}\). Immuno-reactive bands were detected with enhanced chemiluminescence and evaluated by densitometry, as described by Wirth et al.\(^{23}\) The ratio of the optical densities between total MYPT1 or MLC\(_{20}\) and the respective phospho forms was taken as a measure of phosphorylation.

#### Statistics

Results are expressed as means±SEM. Statistical significance was evaluated using paired, time-matched control experiments with the Student *t* test, with *P*<0.05 being considered significant.

Further details regarding the experimental protocols, materials used, and data analysis methods can be found in the online data supplement available at http://circres.ahajournals.org.

### Results

**Urocortin-Induced Relaxation of Intact Mouse Tail Arteries Is Associated With Dephosphorylation of MYPT1\(^{\text{Thr696}}\)**

KCI (42 mmol/L) induced a submaximal contraction amounting to 66±2% of force elicited by 10 μmol/L noradrenaline. KCl-induced contraction was similar in arteries with and without endothelium (2.16±0.23 N/m versus 2.43±0.93 N/m, *n*=5 each, *P*=0.76). Adding urocortin cumulatively at the plateau of the KCl-induced contraction relaxed the arteries with *p*\(_D_2\) (−log *ED*\(_{50}\)) values of 9.02±0.02 and 8.86±0.01 (*n*=5 each, *P*=0.98) in preparations with and without endothelium, respectively (Figure 1a). As shown in Figure 1, stimulation with KCl (42 mmol/L) or noradrenaline (0.5 μmol/L) resulted in a ∼200% increase in MYPT1\(^{\text{Thr696}}\) phosphorylation above resting levels (*P*<0.01 each), which was decreased by addition of urocortin (100 μmol/L) to 127±18% in KCl-preconstricted (*n*=16, *P*<0.05) and 114±26% in noradrenaline-preconstricted (*n*=9, *P*<0.05) arteries. These values were not different from resting phosphorylation (KCl, *P*=0.16; noradrenaline, *P*=0.62).
These experiments suggest that urocortin relaxes arteries by decreasing SMPP-1M activity. To test this hypothesis, arteries were permeabilized with the pore-forming protein α-toxin, in which Ca\(^{2+}\)-independent effects of agonists on force and SMPP-1M activity can be more directly assessed.\(^{19,21,24}\) This is because the quasintracellular [Ca\(^{2+}\)] can be rigorously controlled with high concentrations of Ca buffers, although the pharmacomechanical coupling remains functionally intact.\(^{21}\)

**Urocortin Decreased Force and MLC\(_{20}^{\text{Ser19}}\) Phosphorylation at Constant Submaximal [Ca\(^{2+}\)] in α-Toxin–Permeabilized Arteries in a Receptor- and PKA-Dependent Manner**

Submaximal contractions amounting to 29.0±3.6% (n=6) of maximal force at pCa 4.3 were elicited by pCa 6.1. Addition of urocortin (100 nmol/L) after 15 minutes, ie, at the plateau of the contraction relaxed the preparations by 39.0±2.8% (n=6) of of submaximal force before addition of urocortin was not different compared with the tension decline of 3.3±2.6% in control arteries (Figure 2a and 2b). When added cumulatively, the P$_D$ value of 8.54±0.2 (n=5) was comparable to that in the intact arteries (Figure 1 in the online data supplement). Maximal force was not affected by urocortin, indicating that urocortin decreases myofilament Ca\(^{2+}\) sensitivity. Submaximal force before addition of urocortin was not different between groups (P=0.42). Urocortin-induced relaxation was associated with a decrease in MLC\(_{20}^{\text{Ser19}}\) phosphorylation by $\sim$60% (Figure 2c and 2d). We note that no immunoreactivity with the p-MLC\(_{20}^{\text{Ser19}}\) antibody could be detected at pCa >8 (Figure 2c), which is in line with the notion that Ser19 is specifically phosphorylated in a Ca\(^{2+}\)-dependent manner.\(^{13}\)

We then tested (1) whether the effect on force and MLC\(_{20}^{\text{Ser19}}\) phosphorylation was receptor mediated and (2) whether it could be attributed to activation of PKA. Preincubation for 15 minutes with (and in the continued presence of) the CRF receptor antagonist 9-41-CRF had no effect on Ca\(^{2+}\)-activated force (n=5, P=0.75) but inhibited relaxation induced by urocortin (Figure 3a). Antisuavagine-30, a CRF-2R receptor antagonist,\(^{25}\) similarly blocked urocortin-induced relaxation and MLC\(_{20}^{\text{Ser19}}\) dephosphorylation (Figure 3b through 3d).

Preincubation with the PKA inhibitor Rp-8-CPT-cAMPS completely inhibited relaxation (Figure 4a and 4b) and dephosphorylation of MLC\(_{20}^{\text{Ser19}}\) (Figure 4c and 4d), whereas addition of the PKA activator Sp-5,6-DCI-cBIMPS at the plateau of the submaximal contraction (pCa 6.1) mimicked the effect of urocortin (Figure 4b). Relaxation was also inhibited by the peptide inhibitor of PKA (supplemental Figure III).

**Urocortin and Sp-5,6-DCI-cBIMPS Dephosphorylate MYPPT1 in Permeabilized Arteries**

Consistent with a previous report,\(^{15}\) MYPPT1 is phosphorylated on Thr696 and Thr850 in submaximally (pCa 6.1) activated arteries (Figure 5a and 5b). Addition of urocortin decreased phosphorylation of Thr696 and Thr850 respectively, 52% and 68% (Figure 5a and 5b). The decrease in MYPPT1 phosphorylation was completely prevented by pretreatment with Rp-8-CPT-cAMPS (Figure 5a and 5b). Sp-5,6-DCI-cBIMPS decreased Thr696 and Thr850 phosphorylation by, respectively, 52% and 68% (Figure 5a and 5b). Thus, the larger relaxing effect of Sp-5,6-DCI-cBIMPS (Figure 4b) is associated with a larger decrease in MYPPT1 phosphorylation, particularly of Thr696.

In line with the in vitro observation that both sites are phosphorylated by Rho-associated kinase (ROK),\(^{20}\) we found that the relatively specific ROK inhibitor Y-27632 (10 μmol/L) decreased MYPPT1\(^{\text{Thr696/850}}\) phosphorylation to a similar extent as urocortin (Figure 5c and 5d) and nearly maximally relaxed the preparations. Subsequent addition of urocortin did not enhance relaxation (data not shown).

To exclude that dephosphorylation is the result of cAMP-dependent activation of a type 2A phosphatase (PP-2A),\(^{26}\) arteries were preincubated with 3 mmol/L okadaic acid, which specifically inhibits PP-2A. This prevented neither the
Urocortin-induced dephosphorylation of MYPT1 nor its relaxation (Figure 5e and 5f), whereas inhibition of PP-1 with 3 μmol/L okadaic acid completely blocked MYPT1 dephosphorylation (Figure 5e).

**Urocortin Increases the Rates of Relaxation and Dephosphorylation Under Conditions of Inhibited MLCK**

To test whether the urocortin-induced decrease in MYPT1 phosphorylation results in an increased activity of SMPP-1M, we measured the rates of relaxation and dephosphorylation of MLC$_{20}^{\text{Ser19}}$ in α-toxin–permeabilized arteries, in which relaxation of maximally activated arteries (pCa 4.3) was induced by incubation in relaxing solution containing 10 mmol/L EGTA and ML-9 (200 μmol/L in force and 20 μmol/L in phosphorylation determinations). Under this condition, relaxation is expected to be mainly determined by the activity of SMPP-1M and cross-bridge detachment$^{24,27}$ (for experimental details, see the online data supplement). In arteries pretreated with urocortin, which affected neither force nor MLC$_{20}^{\text{Ser19}}$ phosphorylation at pCa 4.3, the half-time of relaxation ($t_{1/2}$) was decreased from 128±6 seconds to 90±5 seconds at 11°C.
and from 29 ± 5 seconds to 23 ± 2 seconds at 22°C (n = 3), which gives a Q₁₀ value of ∼4.4. Pretreatment with 8-Br-cAMP (300 μmol/L) decreased t₁/₂ in a similar manner (n = 4, P < 0.05). As in rabbit femoral arteries, the tension decline in the mouse tail arteries was biphasic, with an initial quasilinear phase and a subsequent exponential decay (Figure 6a). The acceleration of relaxation seen with urocortin was caused by a decrease in the duration of the linear phase by 26% and an increase in the rate constants of the linear and exponential phase by 54% and 39% (determined at 11°C, n = 5, all parameters P < 0.05; Figure 6).

The increase in the rate of relaxation was associated with an increase in the rate of MLC₂₀ Ser₁₉ dephosphorylation. The time course of apparent dephosphorylation (Figure 7c) could be well fitted with a monoexponential function with rate constants 0.047 sec⁻¹ in control, and 0.10 sec⁻¹ in urocortin-treated arteries (n = 5 each, P < 0.001). Pretreatment with 30 μmol/L Rp-8-CPT-cAMPS completely inhibited the effect of urocortin on MLC₂₀ Ser₁₉ dephosphorylation determined 15 seconds after Ca²⁺ removal (Figure 7d). In contrast, Sp-5,6-DCI-cBIMPS mimicked its effect (Figure 7d). Knowing that cAMP decreased MLCK activity in vitro, we examined whether urocortin decreased the rate of contraction in the presence of 10 μmol/L microcystin-LR (MC-LR), which completely inhibits SMPP-1M activity. As shown in Figure 8, urocortin did not affect the rate of contraction at pCa 6.95, which, under this condition, induced a maximal contraction; t₁/₂ was 132 ± 18 seconds and 117 ± 9 seconds for control and urocortin pretreated arteries, respectively (Figure 8; for experimental details, see the online data supplement).

These results indicate that urocortin via cAMP increases the activity of SMPP-1M. If this were attributable to a decrease in the inhibitory phosphorylation of MYPT₁, as suggested by the steady-state experiments (cf, Figure 5), dephosphorylation of MYPT₁ should precede dephosphorylation of MLC₂₀. MYPT₁ phosphorylation determined at pCa 4.3 was significantly lower in arteries pretreated with Sp-5,6-DCI-cBIMPS compared with control arteries (Figure 7d). Fifteen seconds after removal of Ca²⁺ with the ML-9 and EGTA-containing relaxing solution MYPT₁ phosphorylation was not different from the values at pCa 4.3 in both groups. We note that the lower degree of MYPT₁ phosphorylation had no effect on steady-state force or MLC₂₀ Ser₁₉ phosphorylation, indicating that at maximal pCa 4.3 MLCK activity is much larger than SMPP-1M activity irrespective of the phosphorylation state of MYPT₁. This is also supported by the previous observation that GTPγS, which increases Ca²⁺ sensitivity by inhibiting SMPP-1M, has no effect on maximal force in tonic smooth muscles.

Discussion
Consistent with previous in vivo⁴,⁶ and in vitro⁴,⁹,¹⁰ findings, urocortin potently relaxes mouse tail arteries in an endothelial-independent manner. We proposed previously¹¹ that
urocortin-induced relaxation was caused by a PKA-mediated decrease in the apparent Ca\(^{2+}\) sensitivity of contraction. The mechanism by which this occurs is, however, not fully understood. Here, we present evidence that urocortin decreases Ca\(^{2+}\) sensitivity by a cAMP-mediated increase in the activity of SMPP-1M, which, at least in part, is attributable to a reduction of the inhibitory phosphorylations of the regulatory subunit of SMPP-1M, MYPT1

As previously reported (reviewed by Ito et al\(^{14}\)), MYPT1\(^{Thr96}\) is phosphorylated under resting conditions. Phosphorylation was further increased during the maintained phase of both noradrenaline- and KCl-induced contractions, which is in line with the observation that the maintained phase of phenylephrine- and KCl-induced contraction of arteries requires the activation of Rho.\(^{28}\) Because stimulation with KCl was performed in the presence of blockers of \(\alpha\)- and \(\beta\)-adrenoceptors, it is unlikely that the increase in MYPT1\(^{Thr96}\) phosphorylation was attributable to a depolarization-induced release of noradrenaline from sympathetic varicosities in the vascular wall. We note that contradictory results have been reported with regard to the agonist-induced phosphorylation of Thr96, ie, both an increase and no change in the level of Thr96 phosphorylation in response to agonists have been reported in different types of smooth muscle (for review, see article by Ito et al\(^{14}\) and references therein). The reason for this discrepancy is not clear at present. Urocortin reversed the activation-induced increase in phosphorylation to the basal levels but not below them. Thus, not only the NO/cGMP\(^{39}\) but also the agonist/cAMP pathway may block the agonist-induced increase in Thr96 phosphorylation.

It was previously reported that Rho may be activated by Ca\(^{2+}\) in rabbit aorta.\(^{30}\) and, hence, if urocortin decreased [Ca\(^{2+}\)], inactivation of the Rho/ROK cascade could ensue. However, phosphorylation of MYPT1\(^{Thr96}\) and MYPT1\(^{Thr850}\) was also decreased in \(\alpha\)-toxin–permeabilized preparations in which the quasi-[Ca\(^{2+}\)] \(^{0}\) can be kept constant by high concentrations of EGTA. Dephosphorylation occurred irrespective of whether urocortin was preincubated (cf, Figure 7d) or added to preconstricted arteries (cf, Figure 5), whereby Thr850 appears to be dephosphorylated to a greater extent than Thr96. These results indicate that MYPT1 dephosphorylation cannot be ascribed to a urocortin- or cAMP-induced decrease in [Ca\(^{2+}\)]. Dephosphorylation of MYPT1 by urocortin-induced relaxation was caused by a PKA-mediated decrease in the apparent Ca\(^{2+}\) sensitivity of contraction. The mechanism by which this occurs is, however, not fully understood. Here, we present evidence that urocortin decreases Ca\(^{2+}\) sensitivity by a cAMP-mediated increase in the activity of SMPP-1M, which, at least in part, is attributable to a reduction of the inhibitory phosphorylations of the regulatory subunit of SMPP-1M, MYPT1.

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tin was associated with a decrease in submaximal but not maximal \( \text{Ca}^{2+} \)-induced contraction and \( \text{MLC}_{20} \) phosphorylation in the \( \alpha \)-toxin–permeabilized arteries. The effects were mediated by CRF receptor activation.

The degree of \( \text{MYPT1}^{\text{Thr96/Thr95}} \) phosphorylation is determined by the activity of ROK and other \( \text{MYPT1} \) kinases,\(^3\) and a not-yet-identified \( \text{MYPT1} \) phosphatase(s), as well as by interaction\(^2\) of the PKG/\( \text{PKA} \) phosphorylation site \( \text{MYPT1}^{\text{Ser695}} \) with \( \text{MYPT1}^{\text{Thr96}} \). It was shown that cAMP inactivates Rho in cultured smooth muscle cells\(^3\) and in nonmuscle cells,\(^3\,4\,5\) which in endothelial cells was associated with activation of myosin phosphatase.\(^5\) Unfortunately, it was not possible to obtain sufficient material from the collagen-rich tail arteries to determine the activity of Rho. However, inhibition of ROK by Y-27632 dephosphorylated \( \text{MYPT1} \) to a similar extent as urocortin or the PKA activator, whereas urocortin did not further enhance the relaxing effect of Y-27632. These results are compatible with a mechanism that leads to inhibition of Rho/ROK signaling by urocortin. Our results also show that in the permeabilized mouse tail artery ROK is at least partially active in the absence of an agonist. The mechanism by which Rho/ROK is activated under this condition is not yet clear. It is possible that activation of purinergic receptors\(^6\) by the ATP-containing incubation solutions leads to activation of Rho. A \( \text{Ca}^{2+} \)-dependent activation\(^6\) appears less likely because removal of \( \text{Ca}^{2+} \) by EGTA does not lead to dephosphorylation of \( \text{MYPT1} \). It should also be noted that neither urocortin nor Y-27632 completely dephosphorylated \( \text{MYPT1} \), suggesting that \( \text{MYPT1} \) is phosphorylated by additional kinases.\(^3\)

\( \text{MYPT1} \) can be phosphorylated by PKA and PKG at \( \text{Ser695} \), which has no effect on \( \text{SMPP-1M} \) activity but interferes with subsequent phosphorylation of \( \text{MYPT1}^{\text{Thr96}} \) and vice versa.\(^3\) This mechanism could also account for the decrease in \( \text{MYPT1} \) phosphorylation observed when urocortin was added before activation with \( \text{pCa} \) 4.3 and also possibly when urocortin was added to submaximally precontracted arteries, provided that there is a phosphorylation turnover of \( \text{MYPT1}^{\text{Thr96}} \). Our results indicate that a PKA-mediated activation of \( \text{PP-2A} \)\(^26\) does not appear to be functional in this preparation. They do suggest that a type 1 \( \text{MYPT1} \) phosphatase is active. Thus, if \( \text{Thr96} \) is dephosphorylated by this phosphatase, PKA could phosphorylate \( \text{Ser695} \), which then would prevent dephosphorylation of \( \text{Thr96} \). Future experiments must show whether urocortin acts through inactivation of the Rho/ROK pathway or through these interactive phosphorylation sites or both.

\( \text{SMPP-1M} \) is bound to myosin, and, hence, its activity may have a functional spatial restriction that is not present with proteins in solution, ie, in muscle lysates. Because of this, we determined the rate of \( \text{MLC}_{20} \) phosphorylation in the permeabilized arteries under conditions of full \( \text{MLCK} \) inhibition as a surrogate of \( \text{SMPP-1M} \) activity.\(^1\) This rate was increased \( \approx \)2.2-fold in the presence of urocortin, which compares to the 1.4-fold increase in permeabilized rabbit femoral arteries treated with 8-bromo-cGMP\(^19\) and the 3- to 4-fold increase in the activity of \( \text{SMPP-1M} \) determined in lysates of sodium nitroprusside-stimulated carotid arteries.\(^18\) The \( \approx \)3-fold slower rate of relaxation, which was also accelerated by urocortin, suggests that tension decline like in other types of smooth muscle\(^27\) is rate limited by cross-bridge detachment.

Although it is assumed that phosphorylating reactions are rapidly inactivated by EGTA and ML-9,\(^24\) we cannot exclude the possibility that the rate of dephosphorylation may be confounded by kinase reactions. This possibility must be considered because urocortin via cAMP might decrease the activity of MLCK.\(^13\) However, if urocortin decreased the MLCK activity, the measured, ie, apparent rate constant of dephosphorylation of \( \text{MLC}_{20} \), would be lower in the presence of urocortin rather than higher as observed. This is because it is determined by the sum \( \left( k_1 + k_2 \right) \) of the rate constants of phosphorlyation \( \left( k_1 \right) \) and dephosphorylation \( \left( k_2 \right) \) reactions.\(^3\)

Furthermore, the \( Q_{10} \) value of the rate of relaxation of \( \approx 4.4 \) is much higher than that of MLCK activity \( \left( Q_{10} = 1.7\right)^{37} \) and is closer to the \( Q_{10} \) of 5.1 for \( \text{SMPP-1M} \) activity.\(^37\) Finally, the rate of contraction under conditions of fully inhibited phosphatase was not decreased by urocortin, which would be the case if MLCK activity were decreased.\(^19\,23\) We, therefore, propose that urocortin through activation of PKA increases the activity of \( \text{SMPP-1M} \). Because \( \text{MLC}_{20} \) phosphorylation is proportional to \( k_1/\left( k_1 + k_2 \right) \), a decrease in steady-state \( \text{MLC}_{20} \) phosphorylation and, hence, tension will ensue.

Our study is limited because the effects of urocortin on \( \text{SMPP-1M} \) activity were assessed with the maximum effective concentration (100 nmol/L) with regard to vasorelaxation and increased cAMP levels,\(^12\) which is \( \approx 10\) to 100-fold higher than reported pD\(_2\) values.\(^4\) Plasma levels in humans ranged from 14 pmol/L\(^7\) to \( \approx 1 \) nmol/L.\(^38\) Following systemic application in sheep, the blood pressure–lowering effects were associated with plasma levels of \( \approx 7.5 \) mmol/L.\(^38\) We cannot eliminate the possibility that vasorelaxation at lower concentrations of urocortin involves a different mechanism, but the dose-response relation for \( \text{Ca}^{2+} \)-desensitization and \( \text{MYPT1} \) dephosphorylation in permeabilized arteries (cf, supplemental Figures I and II) are similar to the relaxation of intact arteries in vitro (our study and Schilling et al\(^4\)). Therefore, we propose that cAMP-dependent activation of \( \text{SMPP-1M} \) is an important factor contributing to urocortin-mediated vasorelaxation. Because of the limited specificity of the inhibitor and activator of PKA, this cannot be ascribed with certainty to activation of PKA. It is possible that urocortin/cAMP disinhibits \( \text{SMPP-1M} \) PKA independently, eg, by cross-activation of PKG,\(^39\) an unlikely possibility because cAMP-induced relaxation was not attenuated in PKG\(^{-/-}\) mice,\(^40\) or perhaps by novel cAMP targets (cf, legend of supplemental Figure III and references therein). Thus, future investigations must unravel the precise mechanism that lead to urocortin/cAMP-induced dephosphorylation of \( \text{MYPT1} \) and must also show whether \( \text{MYPT1} \) dephosphorylation acts in conjunction with dephosphorylation of CPI-17, which, when phosphorylated, inhibits \( \text{SMPP-1M} \).\(^19\) PKA-mediated phosphorylation of telokin, an endogenous putative activator of \( \text{SMPP-1M} \),\(^17\) may be of minor importance because of its low expression in tonic smooth muscle.

Our study adds further evidence to the notion that \( \text{SMPP-1M} \) activity in addition to [\( \text{Ca}^{2+} \)]\(_i\) is critical for regulating vascular tone. Its activity is determined by the
balance of activating signals, ie, NO/cGMP\textsuperscript{16,18,19} and agonist/cAMP (this study), and inhibitory signals originating from vasoconstrictors.\textsuperscript{13} Evidence is increasing that this balance is disturbed under certain pathological conditions.\textsuperscript{22-29,41} Prostacyclin, currently among the best drugs to treat primary pulmonary hypertension,\textsuperscript{42} elevates cAMP levels. Hence, its beneficial effect may, in part, be caused by dis-inhibition of SMPP-1M and normalization of this balance. Understanding the mechanisms that regulate SMPP-1M in vivo will likely result in new therapeutic options.

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References

11. Lubomirov L, Gagov H, Petkova-Kirova P, Duridanova D, Kalentchuk M. Kneese is gratefully acknowledged.


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Urocortin-Induced Decrease in Ca^{2+}-Sensitivity of Contraction in Mouse Tail Arteries is Attributable to cAMP-dependent Dephosphorylation of MYPT1 and Activation of Myosin Light Chain Phosphatase

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Expanded Materials and Methods

Dissection and mounting of intact vessels. Male HimOF 1 or C57/BL6NCR mice were treated by procedures approved by the local Animal Care and Use Committee (Regierungspräsidium Köln). The animals were sacrificed by cervical dislocation and the ventral tail artery was isolated. Arteries (2 mm in length) were mounted in a wire-myograph (model 300A or 610A, JP Trading, Denmark) in physiological salt solution, PSS (see solutions), which was continuously bubbled with 100% oxygen. Isometric tension was recorded with the program Myodaq (JP Trading, Denmark). To mechanically remove the endothelium a wire was gently moved through the lumen of the vessel, a procedure that abolished acetylcholine induced relaxations. The vessels were stretched radially to their optimal lumen diameter corresponding to 90% of the passive diameter of the vessel at 100 mmHg\(^1\) and were allowed to stabilize for 15 minutes. Thereafter, the reactivity of the vessel was tested with two applications of a solution containing 10 µM noradrenaline\(^2\) and the intactness of endothelium was tested by application of 10 µM acetylcholine on top of a constriction induced by 1 µM noradrenaline. The potassium-rich solution was made by an equimolar replacement of sodium. To eliminate adrenergic stimulation during high potassium preconstriction, vessel preparations were pretreated with 2 µM propranolol and 5 µM phentolamine for 15 min. All drugs were applied directly into the experimental chamber. Experiments were performed at 37°C.

Tension measurement of permeabilized vessels. Permeabilization with α-toxin was performed with minor modifications as described previously\(^3\). In brief, a 1.8 – 2.0 mm
long piece of a mouse tail artery was incubated for 5 minutes in Ca^{2+}-free PSS containing 2 mM EGTA. Thereafter, the artery was placed for 10 minutes in relaxing solution (see solutions). Permeabilization of the artery was performed in 50 µl relaxing solution containing 3000 U *Staphylococcus aureus* α-toxin for 30 minutes. The permeabilized vessels were then mounted in the wire-myograph and stretched to their optimal lumen diameter in relaxing solution as described above. To deplete the sarcoplasmic reticulum of Ca^{2+} the vessels were treated with 10 µM A 23187\textsuperscript{4}. For some experiments the arteries were permeabilized with β-escin as in\textsuperscript{5}. If not indicated otherwise the vessels were submaximally activated at pCa 6.1 for 15 min and then either urocortin, the specific activator of PKA, Sp-5,6-DCI-cBIMPS, the inhibitor of ROK, Y-27632 or vehicle (control preparations) were added for another 15 min. Relaxation was expressed as percentage of the Ca^{2+}-activated force prior to addition of the compound. In experiments in which the response to urocortin was blocked by either the CRF-receptor inhibitors 9-41 CRF and antisauvagine-30 or the PKA inhibitor, Rp-8-CPT-cAMPS, the compounds were added to relaxing solution 15 min prior to stimulation with pCa 6.1 solution and were then continuously present. At the end of the experiments vessels were stimulated maximally with pCa 4.3. If not indicated otherwise, all experiments were carried out at room temperature (22 – 23 °C).

*Measurement of MYPT1 phosphorylation.* Intact and permeabilized vessels of identical dimensions were mounted on wires and subjected to the same experimental procedure as for force measurements. At the desired time points intact arteries were shock frozen and pulverized in liquid nitrogen and proteins were dissolved in SDS-buffer
(intact arteries) or immersed in an acetone/dry ice slurry and homogenized in SDS-buffer
(permeabilized arteries). The lysates (15 µl/lane) were subjected to 12.5 (intact arteries)
or 6 % (permeabilized arteries) SDS-PAGE. To ascertain that the antibodies were used
within the linear range, the samples were 2- or 3-times exponentially diluted in SDS-
buffer in the initial experiments and loaded as same final volume (15 µl/lane). The
proteins were transferred to nitrocellulose and probed with monoclonal site and phospho-
specific antibodies against either the Thr696 (dilution 1:10000), or the Thr850 (dilution
1:10000) phosphorylated form of MYPT1 or a polyclonal antibody against total MYPT1
(dilution 1:10000). Immunoreactive protein bands were detected with enhanced
chemiluminescence (SuperSignal® West Dura, Pierce) and quantified by densitometrical
scanning of the chemiluminograms using Phoretix software (Biostep). To correct for
slight variations in protein loading the Ponceau Red-stained membranes were also
scanned and the optical densities of the MYPT1 immunoreactive bands were expressed
relative to the intensity of the caldesmon band. Phosphorylation was expressed as the
ratio of the corrected signals from the respective phospho-form of MYPT1 and
unphosphorylated MYPT1.

**Measurement of MLC$_{20}^{\text{Ser19}}$ phosphorylation.** Vessels were treated as described
above and the protein lysates were separated by 15% SDS-PAGE. Samples were also
diluted exponentially to ascertain that the signal was within the linear range. The proteins
were transferred to nitrocellulose membranes and probed with a phospho-specific
monoclonal antibody against Serin19 of MLC20 (dilution 1:5000) or a polyclonal
antibody against total MLC\textsubscript{20} (dilution 1: 10000). Immunoreactivity was detected with enhanced chemiluminescence and the signal was evaluated as above.

\textit{Determination of the rates of MLC\textsubscript{20}^{Ser19} dephosphorylation and relaxation under conditions of inhibited MLCK activity.} Following a maximal contraction (pCa 4.3) relaxation was initiated by incubation in relaxing solution containing 10 mM EGTA and the MLCK inhibitor, ML-9 (200 μM). To reduce non-specific effects the concentration of ML-9 was lowered to 20 μM in phosphorylation experiments. At the desired time points the preparations were shock frozen in an acetone/dry ice slurry to determine MLC\textsubscript{20}^{Ser19} phosphorylation as described above. In urocortin (100 nM), Sp-5,6-DCI-cBIMPS (100 μM) or 8-Br-cAMP (300 μM) treated arteries, the substances were added to relaxing solution prior to stimulation with pCa 4.3 solution for 20 min and were then continuously present. The time course of relaxation was fitted as in\textsuperscript{6} by a function consisting of linear and an exponential term:

\[
Y = A + S \times t + B \quad \text{for } t < t_{\text{LIN}} \quad \text{and}
\]
\[
Y = (A + S \times t_{\text{LIN}}) \exp(-k_{\text{EXP}}(t - t_{\text{LIN}})) + B \quad \text{for } t > t_{\text{LIN}}
\]

where \(Y\) is the force, \(A\) is the amplitude of overall force decay, \(S\) is the slope and \(t_{\text{LIN}}\) is the time of the initial linear force decay, respectively and \(k_{\text{EXP}}\) is the rate constant of the exponential force decay. In this approach, the linear phase was treated as if it were the initial part of an exponential force decay that would theoretically extrapolate to the final force level with a rate constant of \(\approx k_{\text{LIN}}\). Accordingly \(k_{\text{LIN}}\) was calculated by \(k_{\text{LIN}} = -S/A\).
Time course of contraction under conditions of inhibited myosin phosphatase activity. The rate of contraction was determined in α-toxin permeabilized mouse tail arteries with minor modifications as described previously\(^7\) in the presence of 10 μM microcystin-LR which completely inhibits MLCP activity\(^8\). Vessels were isolated and mounted in the myograph as described above. The relaxed vessels were incubated with urocortin (which was then continuously present) in relaxing solution for 20 min followed by washing 3 – 4 times in rigor solution to prevent Ca\(^{2+}\)-independent contractions before 10 μM microcystin-LR was added for another 10 minutes. Thereafter, contraction was elicited with pCa 6.95 in ATP-containing contraction solution in the continued presence of microcystin-LR

**Solutions:**

*Physiological salt solution (PSS) (in mM):* 118 NaCl, 5 KCl, 1.2 NaH\(_2\)PO\(_4\), 1.2 MgCl\(_2\), 1.6 CaCl\(_2\), 10 Glucose, and 24 HEPES, pH 7.4 at 37°C.

*Relaxing solution (in mM):* 20 imidazole, 7.5 Na\(_2\)ATP, 10 EGTA, 10 Mg-acetate, 10 creatinephosphate, 31.25 potassium-methanesulfonate, 5 NaN\(_3\), 0.01 GTP, 0.001 leupeptin, 2 DTT at a pH of 7.00 adjusted with CaCl\(_2\)-free KOH.

*Contraction solution* had the same composition as relaxing solution except that it contained 10 mM CaCl\(_2\). To obtain intermediate concentrations of free Ca\(^{2+}\) relaxing and contracting solutions were mixed in the appropriate ratio as described before\(^7\). Free [Ca\(^{2+}\)] was calculated as in\(^9\). The ionic strength of the relaxing, contracting and rigor solutions was adjusted with potassium-methanesulphonate to 150 mM.
Rigor solution had the same composition as relaxing solution except that it contained no ATP, no creatine phosphate and 3 mM Mg-acetate.

SDS (Laemmli)-buffer (in mM): Tris-HCl 50, urea 4000, dithiotreitol 2, leupeptin 0.001, phenylmethanesulfonyl fluoride (PMSF) 1, aprotinin 20 µg/ml, sodium dodecysulfate (SDS) 1%.

Statistics:

Results are expressed as means±SEM. Statistical significance was evaluated with the unpaired Student’s t-test with p<0.05 being considered significant.

Materials and chemicals: Rat urocortin, Staphylococcus aureus α-toxin, antisauvegine-30, 8-Br-cAMP, propranolol, phenolamine, noradrenaline, ML-9 and microcystin-LR were purchased from Sigma, A 23187 from Tocris and 9-41 CRF from RBI. The Rho-kinase inhibitor Y-27632 was generous gift from Welfride Company (Osaka, Japan). The PKA-inhibitor, Rp-8-CPT-cAMPS and the PKA-activator, Sp-5,6-DCI-cBIMPS, were purchased from Biolog, Bremen, Germany. The PKA-inhibitory peptide (5-24) was purchased from Calbiochem. The phosphospecific antibodies against MYPT1^{Thr696} were from M. Ito (Mie University, Japan) or purchased from Upstate. The phospho-specific monoclonal antibody against MYPT1^{Thr850} was from Upstate. The polyclonal antibody against MYPT1 was from D. Hartshorne (University of Arizona, USA). The phospho-specific, monoclonal antibody against phospho-Ser19 of the MLC_{20} was from Rockland and the polyclonal antibody against total MLC_{20} from Sigma. SuperSignal® West Dura was from Pierce.
Online Figure 1:
Concentration-response relation of urocortin (UCN-1) on tension in α-toxin-permeabilized mouse tail arteries.

Submaximal contractions were elicited by pCa 6.1 which after an initial peak declined to a plateau force. Addition of cumulatively increasing concentrations of urocortin at the plateau of the submaximal contraction relaxed the preparations at constant [Ca^{2+}] (a) while the vehicle had no effect (b). Maximal force was not affected (p= 0.39; n =5)
indicating that urocortin decreased myofilament Ca$^{2+}$-sensitivity. Submaximal force before addition of urocortin was not different between groups (p= 0.85; n =5). (c) Summary of results. Dose-response curve to urocortin was fitted by a sigmoidal function 
\[ E = E_0 + \frac{(E_{\text{max}} - E_0)}{1 + 10^{((\log EC_{50} - \log C)n_h)}}; \]
E is the effect of urocortin; C is the concentration; \( n_h \) is the Hill-slope of the curve) giving a pD$_2$ 8.54±0.19 and E$_{\text{max}}$ of 64±7 % (n =5). The values are given in means ±SEM and \( n \) indicates the number of independent experiments.
Online Figure 2:

Effect of urocortin (UCN-1) on MYPT1^{Thr696} and MYPT1^{Thr850} phosphorylation in submaximally activated α-toxin-permeabilized mouse tail arteries.

The vessels were submaximally activated with pCa 6.1 for 15 min and then treated with a single application of urocortin at the indicated concentrations, or vehicle (time control; tc) for another 15 min. At this time point the preparations were shock frozen and processed for gel electrophoresis as described in the “Methods section”. The upper part of the gel was used for Western blotting with phosphospecific antibodies against MYPT1^{Thr696} and MYPT1^{Thr850}. The lower part was coomassie stained and the actin band was used for correcting slight variations in protein loading. Representative western blots showing the concentration-dependent reduction in p-MYPT1^{Thr696} - (a) and p-
MYPT1$^{\text{Thr}850}$ immunoreactivity. (b, d) Summarized data: The optical densities of the chemiluminescence signal was divided by that of the actin signal and the values of the urocrortin treated samples were expressed as percentage of the mean MYPT1/actin ratio of the time controls. Bars represent mean ±SEM of n = 4 independent experiments.
Online Figure 3:

Urocortin-induced relaxation at constant pCa 6.38 is inhibited by the peptide inhibitor of PKA, PKAi peptide (5-24).

In α-toxin permeabilized arteries, relaxation induced by urocortin was completely inhibited by Rp-8-CPT-cAMPS. Because Rp-8-CPT-cAMPS also binds to EPAC (exchange protein directly activated by cAMP)\(^\text{10}\) and because this protein has been suggested to be involved in a number of cAMP-regulated processes such as cell migration and adhesion\(^\text{11}\), we tested whether the more specific inhibitor of PKA, PKAi peptide (5-24) also inhibits urocortin-induced relaxation. Because α-toxin permeabilized vessels are not permeable to the inhibitory peptide, arteries were permeabilized with β-
escin. Following permeabilization the preparations were submaximally activated with pCa 6.38. Once force approached the plateau, urocortin (100 nM UCN-1) was added which induced a partial relaxation. Relaxation was significantly attenuated by 10 µM of the PKA inhibitory peptide. In paired time control experiments (vehicle) but not in the PKAi treated vessels, force slowly increased further. Thus, it is possible that unlike Rp-8-CPT-cAMPS, the PKAi peptide does not completely block Ca^{2+}-desensitization. Original tracings are shown in (a, b, c) and (d) summarizes the results. Bars represent mean ±SEM of n = 3 independent experiments (* p < 0.05, ** p < 0.01).

These results are in support of the hypothesis that at least part of the urocortin-induced Ca^{2+}-desensitization is mediated by activation of PKA. Future studies are needed to clarify whether and if so to what extent crossactivation of PKG or activation of new PKA independent cAMP-singalling pathways^{11,12} contributes to cAMP-mediated disinhibition of SMPP-1M.

REFERENCES


2. Lubomirov L, Gagov H, Petkova-Kirova P, Duridanova D, Kalentchuk VU, Schubert R. Urocortin relaxes rat tail arteries by a PKA-mediated reduction of the


