Endothelin-1–Induced Vasospasms of Spiral Modiolar Artery Are Mediated by Rho-Kinase–Induced Ca\(^{2+}\) Sensitization of Contractile Apparatus and Reversed by Calcitonin Gene–Related Peptide

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Background and Purpose—Vasospasms of the spiral modiolar artery may cause an ischemic stroke of the inner ear that manifests itself by a sudden hearing loss. Previously we have shown that endothelin-1 (ET-1) induces vasospasms of the spiral modiolar artery. Here we tested the hypotheses that ET-1–induced vasospasms are (1) reversible by ET\(_A\) receptor antagonists; (2) mediated by a Ca\(^{2+}\) sensitization of the contractile apparatus via a Rho-kinase–induced inhibition of myosin light chain phosphatase; and (3) reversible by the vasodilator calcitonin gene–related peptide (CGRP).

Methods—The Ca\(^{2+}\) sensitivity of the contractile apparatus was evaluated by correlation between the smooth muscle cell Ca\(^{2+}\) concentration and the vascular diameter, which were measured by microfluorometry with the fluorescent dye fluo-4 and videomicroscopy, respectively.

Results—ET-1–induced vasospasms were prevented but not reversed by the ET\(_A\) receptor antagonists BQ-123 and BMS-182874. The Ca\(^{2+}\) sensitivity of the contractile apparatus was increased by ET-1 and by inhibition of myosin light chain phosphatase with calyculin A and was decreased by CGRP. ET-1–induced vasospasms and Ca\(^{2+}\) sensitization were prevented and reversed by the Rho-kinase antagonist Y-27632 and by CGRP.

Conclusions—ET-1 induces vasospasms of the spiral modiolar artery via ET\(_A\) receptor–mediated activation of Rho-kinase, inhibition of myosin light chain phosphatase, and an increase in Ca\(^{2+}\) sensitivity, which is reversed by CGRP. The observation that vasospasms were reversed by Y-27632 but not by BQ-123 or BMS-182874 suggests that Rho-kinase, rather than the ET\(_A\) receptor, is the most promising pharmacological target for the treatment of ET-1–induced vasospasms, ischemic strokes, and sudden hearing loss. (Stroke. 2002;33:2965-2971.)

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that this peptide is localized in perivascular nerve vesicles of the SMA and induces vasodilations of this artery via CGRP receptors.\textsuperscript{15,16} CGRP might therefore act as a neurogenic regulator of cochlear blood flow and limit the effects of vasoconstrictors such as ET-1.

A number of studies in large and small arteries demonstrate that ET-1 exerts its effect primarily by an increase in [Ca\(^{2+}\)], which leads to an increase in myosin light chain (MLC\(_{20}\)) phosphorylation and consequent vasoconstriction.\textsuperscript{17–19} This mechanism seems not to play a predominant role in the SMA, where constrictions in response to ET-1 are maintained at resting [Ca\(^{2+}\)].\textsuperscript{20} Such “Ca\(^{2+}\)”-independent” vasoconstrictions have previously been studied in permeabilized vessels, where ET-1 was found to induce constrictions by increasing the phosphorylation state of MLC\(_{20}\) at constant [Ca\(^{2+}\)].\textsuperscript{21,22} Recently, it has been shown that ET-1–induced constrictions of basilar arteries resulted from an inhibition of the MLCP and a subsequent increase in MLC\(_{20}\) phosphorylation.\textsuperscript{23} In the latter study, ET-1–induced constrictions were inhibited partially with the nonselective Rho-kinase/protein kinase C inhibitor hydroxyfasudil, indicating a potential involvement of these kinases.

Much of our understanding of vascular signaling mechanisms originates from the investigation in large vessels and cell cultures. Regulatory mechanisms of large arteries do not necessarily apply to the microcirculation. Control of the microcirculation is especially important in sensory organs such as the cochlea or the retina, where blood flow depends on a single microvessel. Interestingly, the role of Rho-kinase in ET-1–induced vasospasms is unknown in the microcirculation. Given the relevance of vasospasms in the etiology of sudden hearing loss, the purpose of the present study was to assess the mechanisms of ET-1–induced vasospasms in the SMA and how vasospasms can be prevented or reversed.

**Materials and Methods**

### Drugs and Solutions

The physiological salt solution (PSS) contained (in mmol/L) 150 NaCl, 3.6 KCl, 1.0 MgCl\(_2\), 1.0 CaCl\(_2\), 5.0 HEPES, and 5.0 glucose (pH 7.4). Extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_o\)]) was raised to 3 and 10 mmol/L, by addition of CaCl\(_2\). When [Ca\(^{2+}\)\(_o\)] was raised to 30 and 50 mmol/L, the concentration of NaCl was reduced to 100 mmol/L. A maximal vasodilation was induced by the removal of extracellular Ca\(^{2+}\). The nominally Ca\(^{2+}\)-free solution contained (in mmol/L) 150 NaCl, 3.6 KCl, 1.0 MgCl\(_2\), 1.0 EGTA, 5.0 HEPES, and 5.0 glucose (pH 7.4). The fluorescent dye fluo-4-AM (Molecular Probes) was dissolved in anhydrous dimethyl sulfoxide and stored in 1-mmol/L aliquots. Y-27632 was kindly provided by Welfide. Calyculin A was obtained from Alomone, and BMS-182874 was obtained from Tocris Cookson. All other chemicals were obtained from Sigma.

### Preparation of SMA

Experiments were conducted on tissues isolated from Mongolian gerbils (Meriones unguiculatus) under a protocol that was approved by the Institutional Animal Care and Use Committee at Kansas State University. Gerbils were anesthetized with sodium pentobarbital (100 mg/kg IP) and decapitated. Temporal bones were removed, opened, and placed into a microdissection chamber containing PSS at 4°C. The SMA was isolated from the cochlea by microdissection as described previously.\textsuperscript{24} Briefly, the cochlea was opened. The bone surrounding the modiolus was carefully removed, and the SMA,
which is only loosely attached to the eighth cranial nerve, was isolated. Care was taken to not stretch the artery.

Localization of the Ca\textsuperscript{2+} Signal in the Vascular Wall

Segments of the SMA were loaded with fluo-4. Fluorescence was elicited by a 488-nm laser and detected by confocal laser-scanning microscopy (Carl Zeiss).

Simultaneous Measurement of Vascular Diameter and [Ca\textsuperscript{2+}]

The simultaneous measurement of vascular diameter and [Ca\textsuperscript{2+}], has been described previously.\textsuperscript{20} Briefly, the smooth muscle cells of vessel segments were loaded with the Ca\textsuperscript{2+} indicator dye fluo-4 by incubation in PSS containing 5 μmol/L fluo-4-AM for 35 minutes at 37°C. After loading, vessel segments were washed with PSS and maintained at 4°C before experimentation at 37°C. Vessel segments were transferred into a bath chamber mounted on the stage of an inverted microscope (Nikon). Fluorescence emitted by fluo-4 (518 to 542 nm) in response to excitation at 488 nm (Photon Technology International) was detected by a photon counter (Photon Technology International). For measurements of the vascular diameter, the vessel was illuminated at 605 to 615 nm, and the transmission image was recorded with a chilled charge-coupled device camera (Hamamatsu). The outer vascular diameter was measured by 2 video edge detectors (Crescent). Fluorescence and calibrated diameter signals were digitized and recorded simultaneously (Photon Technology International).

Experimental Protocols

Experiments were started 20 minutes after loading with fluo-4. Vessel segments were superfused at a rate of 9 mL/min. This flow rate corresponds to an exchange rate of 2 bath chamber volumes per second, given the bath chamber volume of 75 μL. On the start of the superfusion, the unpressurized artery develops a spontaneous vascular tone that is sensitive to removal of extracellular Ca\textsuperscript{2+} and

Figure 2. ET-1 and the MLCP inhibitor calyculin A increased the Ca\textsuperscript{2+} sensitivity of the contractile apparatus. The Ca\textsuperscript{2+} sensitivity was determined by correlation between [Ca\textsuperscript{2+}] and the vascular diameter. Changes in [Ca\textsuperscript{2+}] were used to induce changes in [Ca\textsuperscript{2+}] and the vascular diameter. A, Time control experiments were performed to test whether the Ca\textsuperscript{2+} sensitivity was stable over time. Averages and SEM of experiments that included 2 determinations of the Ca\textsuperscript{2+} sensitivity, each consisting of a series of changes in the [Ca\textsuperscript{2+}], (n=6), are shown. Measurements of [Ca\textsuperscript{2+}] were normalized (fluorescence values at times a and b were set to 1). B, Analysis of experiments shown in A. Note that the Ca\textsuperscript{2+} sensitivity of the contractile apparatus was stable over time. C, ET-1 (100 pmol/L) increased the Ca\textsuperscript{2+} sensitivity, as evident from the increased steepness of the correlation (n=8). D, Calyculin A (10 nmol/L) increased the Ca\textsuperscript{2+} sensitivity (n=8).
inhibition of L-type Ca\(^{2+}\) channels with nanomolar concentrations of nifedipine.\(^{20}\) The viability of each vessel was assessed by its constrictor response to 10 mmol/L [Ca\(^{2+}\)]. The [Ca\(^{2+}\)], was monitored as fluorescence intensity and was normalized to the basal fluorescent emission before the beginning of each experiment. The fluorescence and diameter values taken for statistical analysis represent averages of the [Ca\(^{2+}\)], fluorescence and the vascular diameter over 30 seconds beginning 30 seconds after the onset of stimulation. Affinity constants ([K\(_{i}\)]) and concentrations that cause a half-maximal inhibition (IC\(_{50}\)) were determined in cumulative experiments and averaged after logarithmic transformation (pK\(_{i}\)) as previously described.\(^{20}\) A paired experiment was designed to determine whether ET\(_{\alpha}\) antagonists not only prevent but also reverse ET-1-induced vasospasms (Figure 1B and 1C). The design included a high concentration of ET-1 (10 nmol/L), which was chosen to best illustrate differences between prevention and reversal. As expected, the peptide antagonist BQ-123 (1 \(\mu\)mol/L) prevented the transient [Ca\(^{2+}\)], increase and the development of vasospasms induced by 10 nmol/L ET-1. Unexpectedly, BQ-123 was unable to reverse vasospasms. Similar observations were made with the nonpeptide antagonist BMS-182874 (Figure 1C). BMS-182874 (10 \(\mu\)mol/L) prevented ET-1–induced vasospasms with a \(K_{DB}\) of 28 nmol/L (pK\(_{DB}\)=7.56±0.15; n=6) but was unable to reverse vasospasms (Figure 1C).

**ET-1 Increases Ca\(^{2+}\) Sensitivity of Contractile Apparatus**

The Ca\(^{2+}\) sensitivity of the contractile apparatus was assessed as linear slopes obtained from correlations of [Ca\(^{2+}\)], and the vascular diameter. Consecutive determinations of the Ca\(^{2+}\) sensitivity revealed no significant differences (≈36±4 versus −43±7 \(\mu\)mol/L; n=6) (Figure 2A and 2B). These control experiments demonstrate that the Ca\(^{2+}\) sensitivity is stable over time. The design of the experiment, which sought to determine whether ET-1 increases the Ca\(^{2+}\) sensitivity of the contractile apparatus, included a low concentration of ET-1 (100 \(\mu\)mol/L). This concentration was chosen to reveal whether the Ca\(^{2+}\) sensitization is physiologically relevant or merely a phenomenon limited to pharmacological doses of ET-1.

**Statistical Analysis**

All results are expressed as mean±SEM of n experiments, with n representing the number of vessel segments. The significance of changes in the vascular diameter and the significance of changes in Ca\(^{2+}\) sensitivity were determined with Student’s t test for paired data. Differences were considered significant at error probabilities <0.05 (P<0.05).

**Results**

This report is based on recordings of 79 vessels from 44 animals. The average vascular diameter was 62±1 \(\mu\)m (n=79). The assumption that measurements of [Ca\(^{2+}\)], originated from VSMC was verified by confocal microscopy (n=5) (Figure 1A).

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**Figure 3.** ET-1–induced vasospasms are maintained by a Rho-kinase–mediated increase in the Ca\(^{2+}\) sensitivity of the contractile apparatus. A, Effect of the Rho-kinase inhibitor 10 \(\mu\)mol/L Y-27632 on 10 nmol/L ET-1–induced vasospasms (averages and SEM; n=7). In the presence of Y-27632, ET-1–induced [Ca\(^{2+}\)], increases were not significantly different from control experiments (compare with Figure 1B). Note that ET-1–induced vasoconstrictions paralleled the [Ca\(^{2+}\)], [Ca\(^{2+}\)], and vascular diameter were normalized to the value before application of Y-27632 (values at time \(x\) were set to 1). B, Y-27632 (10 \(\mu\)mol/L) reversed 10 nmol/L ET-1–induced vasospasms (representative original recording). C, Summary of data shown in B (n=8). Corresponding sections of the experiment shown in B are marked (a, b, and c). D, Y-27632 (1 \(\mu\)mol/L) completely prevented the Ca\(^{2+}\) sensitization induced by 100 \(\mu\)mol/L ET-1 (n=6; compare with Figure 2C).
ET-1. Interestingly, the Ca\textsuperscript{2+} sensitivity was increased in the presence of 100 pmol/L ET-1 (−36±9 versus −62±13 μm/Ca\textsuperscript{2+}; n=8) (Figure 2C). Increases in [Ca\textsuperscript{2+}], were not different under control conditions and in the presence of ET-1, whereas constrictions were significantly enlarged in the presence of ET-1.

**Inhibition of MLCP Increases Ca\textsuperscript{2+} Sensitivity of Contractile Apparatus**

If ET-1 increases the Ca\textsuperscript{2+} sensitivity via inhibition of MLCP, pharmacological inhibition of MLCP would be expected to cause Ca\textsuperscript{2+} sensitization. Calyculin A (10 nmol/L), which has been shown to be a selective inhibitor of MLCP,\textsuperscript{26} caused a significant increase in the Ca\textsuperscript{2+} sensitivity (−18±3 versus −56±11 μm/Ca\textsuperscript{2+}; n=8) (Figure 1D). These observations support the hypothesis that ET-1 induces the Ca\textsuperscript{2+} sensitization via an inhibition of MLCP.

**Inhibition of Rho-Kinase Prevents and Reverses ET-1–Induced Vasospasms and Ca\textsuperscript{2+} Sensitization**

If ET-1 induces vasospasms via a Rho-kinase–mediated inhibition of MLCP, it would be expected that pharmacological inhibition of Rho-kinase is able to prevent and reverse vasospasms. Y-27632 at a concentration of 10 μmol/L has been shown to be a selective Rho-kinase inhibitor.\textsuperscript{27} Preincubation of vessel segments with 10 μmol/L Y-27632 did not prevent 10 nmol/L ET-1–induced intracellular Ca\textsuperscript{2+} mobilizations and “Ca\textsuperscript{2+}-dependent” constrictions but strongly prevented the “Ca\textsuperscript{2+}-independent” component of the ET-1–induced vasospasm (compare Figure 3A with Figure 1B). Furthermore, vasospasms in the continuous presence of 10 nmol/L ET-1 were reversed by Y-27632 with an IC\textsubscript{50} of 3 μmol/L (pIC\textsubscript{50}=5.50±0.31; n=6) (Figure 3B and 3C). These observations suggest that ET-1–induced vasospasms are mediated by Rho-kinase. If activation of Rho-kinase induces vasospasms via inhibition of MLCP and an increase in the Ca\textsuperscript{2+} sensitivity, it would be expected that Y-27632 prevents the ET-1–induced Ca\textsuperscript{2+}-sensitization. Indeed, the increase in the Ca\textsuperscript{2+} sensitivity that was observed in the presence of 100 pmol/L ET-1 was abolished in the presence of 1 μmol/L Y-27632 (−26±4 versus 30±4 μm/Ca\textsuperscript{2+}; n=6) (compare Figure 3D with Figure 2C). Taken together, these observations demonstrate that ET-1–induced vasospasms in the SMA are maintained by a Rho-kinase–mediated increase of the Ca\textsuperscript{2+} sensitivity of the contractile apparatus.

**Activation of CGRP Receptors Prevents and Reverses ET-1–Induced Vasospasms and Ca\textsuperscript{2+} Sensitization**

We have previously shown that CGRP is localized in perivascular nerves of the SMA and that CGRP causes a vasodilation of ET-1 preconstricted vessels.\textsuperscript{16} Given that CGRP is among the most potent vasodilators, it is conceivable that CGRP alters the Ca\textsuperscript{2+} sensitivity of the contractile apparatus. CGRP (100 nmol/L) induced a transient decrease in [Ca\textsuperscript{2+}], and a sustained vasodilation that persisted after CGRP had been removed from the perfusate. Removal of Ca\textsuperscript{2+} from the superfusate (0Ca) caused a [Ca\textsuperscript{2+}]i decrease and a parallel vasodilation. B, CGRP (10 nmol/L) decreased the Ca\textsuperscript{2+} sensitivity in vessels that were not preconstricted. C, CGRP (10 nmol/L) prevented the Ca\textsuperscript{2+} sensitization induced by 100 pmol/L ET-1 (n=6; compare with Figure 2C).
Endothelial cells release ET-1 under pathological conditions such as hypoxia, subarachnoidal hemorrhage, increased oxidized low-density lipoproteins, and shear stress. Rho-kinase appears to be the key mediator of ET-1–mediated constrictions. ET-1–induced Ca2+ mobilization in the SMA, however, appears to play a minor role.20 In the present study we show that the major mechanism of ET-1–induced vasospasms is an increase in the Ca2+ sensitivity of the contractile apparatus. This increase in the Ca2+ sensitivity appears to be mediated by a Rho-kinase–dependent inactivation of MLCP. This hypothesis is supported by 2 observations. First, inhibition of Rho-kinase with the selective Rho-kinase inhibitor Y-27632 abolished the ET-1–induced increase in the Ca2+ sensitivity. Second, inhibition of MLCP by the selective inhibitor calyculin A increased the Ca2+ sensitivity comparable to ET-1. It has been shown that the Rho-kinase–dependent inhibition of the MLCP results from phosphorylation of the myosin-binding subunit of the enzyme.6 It remains unclear whether Rho-kinase phosphorylates MLCP directly or activates a ZIP-like kinase downstream of Rho-kinase, which phosphorylates MLCP.29 The link between the ET4 receptor and Rho-kinase is currently unknown, although evidence from cultured aortic smooth muscle cells suggests that ET4 receptors activate Rho-kinase via G12/13 and the small G-protein Rhoc.60 The observation that inhibition of Rho-kinase prevented and reversed ET-1–induced vasospasms suggests that Rho-kinase is a promising pharmacological target for the treatment of vasospasms.

Clinical Outlook
Certain forms of sudden hearing loss are thought to be caused by vasospasms that lead to an ischemic stroke of the inner ear. ET-1 is a key factor in the development of vasospasms. Endothelial cells release ET-1 under pathological conditions such as hypoxia, subarachnoidal hemorrhage, increased oxidized low-density lipoproteins, and shear stress. Rho-kinase appears to be the key mediator of ET-1–mediated vasospasms. Recent studies acknowledge Rho-kinase as the primary mediator in the development of vasospasms of cerebral10 as well as of coronary arteries.7,8 In particular, the beneficial effect of fasudil in the treatment of cerebral vasospasms after subarachnoid hemorrhage is well established.23 We conclude that inhibition of Rho-kinase may be a promising pharmacological target for the treatment of ET-1–mediated sudden hearing loss, and therefore this study may provide the first step toward a rational pharmacotherapy of this disorder.

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