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.)*

**Key Words:** calcium signaling • cell communication • cochlea • regional blood flow • signal transduction • vasospasm
that this peptide is localized in perivascular nerve vesicles of the SMA and induces vasodilatations 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 \([\text{Ca}^{2+}]_{i}\), which leads to an increase in myosin light chain (MLC\textsubscript{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 \([\text{Ca}^{2+}]_{i}\).\textsuperscript{20} Such “\([\text{Ca}^{2+}]_{i}\)-independent” vasoconstrictions have previously been studied in permeabilized vessels, where ET-1 was found to induce constrictions by increasing the phosphorylation state of MLC\textsubscript{20} at constant \([\text{Ca}^{2+}]_{i}\).\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\textsubscript{20} phosphorylation.\textsuperscript{23} In the latter study, ET-1–induced constrictions were inhibited partially with the nonselective Rho-kinase/protein kinase C inhibitor hydroxysudfild, 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\textsubscript{2}, 1.0 CaCl\textsubscript{2}, 5.0 HEPES, and 5.0 glucose (pH 7.4). Extracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{o}) was raised to 3 and 10 mmol/L, by addition of CaCl\textsubscript{2}. When [Ca\textsuperscript{2+}] was raised to 30 and 50 mmol/L, the concentration of NaCl was reduced to 100 mmol/L. A maximal vasodilatation was induced by the removal of extracellular Ca\textsuperscript{2+}. The nominally Ca\textsuperscript{2+}-free solution contained (in mmol/L) 150 NaCl, 3.6 KCl, 1.0 MgCl\textsubscript{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 Ca²⁺ 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 [Ca²⁺]

The simultaneous measurement of vascular diameter and [Ca²⁺], has been described previously.²⁰ Briefly, the smooth muscle cells of vessel segments were loaded with the Ca²⁺ 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 (Photonic 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 Ca²⁺ and

Figure 2. ET-1 and the MLCP inhibitor calyculin A increased the Ca²⁺ sensitivity of the contractile apparatus. The Ca²⁺ sensitivity was determined by correlation between [Ca²⁺] and the vascular diameter. Changes in [Ca²⁺] were used to induce changes in [Ca²⁺] and the vascular diameter. A, Time control experiments were performed to test whether the Ca²⁺ sensitivity was stable over time. Averages and SEM of experiments that included 2 determinations of the Ca²⁺ sensitivity, each consisting of a series of changes in the [Ca²⁺], (n=6), are shown. Measurements of [Ca²⁺] were normalized (fluorescence values at times a and b were set to 1). B, Analysis of experiments shown in A. Note that the Ca²⁺ sensitivity of the contractile apparatus was stable over time. C, ET-1 (100 pmol/L) increased the Ca²⁺ sensitivity, as evident from the increased steepness of the correlation (n=8). D, Calyculin A (10 nmol/L) increased the Ca²⁺ sensitivity (n=8).
inhibition of L-type Ca²⁺ channels with nanomolar concentrations of nifedipine. The viability of each vessel was assessed by its constrictor response to 10 nmol/L [Ca²⁺]. The [Ca²⁺] 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 [Ca²⁺], fluorescence, and the vascular diameter over 30 seconds beginning 30 seconds after the onset of stimulation. Affinity constants (Kᵦ) and concentrations that cause a half-maximal inhibition (IC₅₀) were determined in cumulative experiments and averaged after logarithmic transformation (pKᵦ and pIC₅₀) as previously described.

The Ca²⁺ sensitivity of the contractile apparatus was determined by a correlation of [Ca²⁺], and the vascular diameter. Changes in [Ca²⁺], were induced by changes in [Ca²⁺]. Stepwise increases in [Ca²⁺], from 0 to 1, 3, and 10 nmol/L, caused increases in [Ca²⁺], and decreases in the vascular diameter. Correlations were found to be linear (r>0.95) at least within the measured range. Linear slopes were obtained to compare the Ca²⁺ sensitivity within paired experiments. Slopes were quantified in the arbitrary unit μm/Ca²⁺, where μm represent the change in the vascular diameter and Ca²⁺ represents the normalized change in the cytosolic Ca²⁺ concentration. In each vessel segment, the Ca²⁺ sensitivity was assessed under control and experimental conditions.

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 Ca²⁺ 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 μm (n=79). The assumption that measurements of [Ca²⁺], originated from VSMC was verified by confocal microscopy (n=5) (Figure 1A).

ET-1–Induced Vasospasms Are Prevented but Not Reversed by ET₄ Receptor Antagonists

We have previously shown that ET-1 induces transient [Ca²⁺], increases and vasospasms of the SMA with an EC₅₀ of 0.8 nmol/L and that vasospasms are mediated via ET₄ receptors and prevented by the ET₄ receptor antagonist BQ-123 (Kᵦ=24 nmol/L). A paired experiment was designed to determine whether ET₄ 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 μmol/L) prevented the transient [Ca²⁺], 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 μmol/L) prevented ET-1–induced vasospasms with a Kᵦ of 28 nmol/L (pKᵦ=7.56±0.15; n=6) but was unable to reverse vasospasms (Figure 1C).

ET-1 Increases Ca²⁺ Sensitivity of Contractile Apparatus

The Ca²⁺ sensitivity of the contractile apparatus was assessed as linear slopes obtained from correlations of [Ca²⁺], and the vascular diameter. Consecutive determinations of the Ca²⁺ sensitivity revealed no significant differences (~36±4 versus ~43±7 μm/Ca²⁺; n=6) (Figure 2A and 2B). These control experiments demonstrate that the Ca²⁺ sensitivity is stable over time. The design of the experiment, which sought to determine whether ET-1 increases the Ca²⁺ sensitivity of the contractile apparatus, included a low concentration of ET-1 (100 pmol/L). This concentration was chosen to reveal whether the Ca²⁺ sensitization is physiologically relevant or merely a phenomenon limited to pharmacological doses of
ET-1. Interestingly, the Ca²⁺ sensitivity was increased in the presence of 100 pmol/L ET-1 (−36±9 versus −62±13 μm/Ca²⁺; n=8) (Figure 2C). Increases in [Ca²⁺], 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 Ca²⁺ Sensitivity of Contractile Apparatus
If ET-1 increases the Ca²⁺ sensitivity via inhibition of MLCP, pharmacological inhibition of MLCP would be expected to cause Ca²⁺ sensitization. Calyculin A (10 nmol/L), which has been shown to be a selective inhibitor of MLCP, caused a significant increase in the Ca²⁺ sensitivity (−18±3 versus −56±11 μm/Ca²⁺; n=8) (Figure 1D). These observations support the hypothesis that ET-1 induces the Ca²⁺ sensitization via an inhibition of MLCP.

Inhibition of Rho-Kinase Prevents and Reverses ET-1–Induced Vasospasms and Ca²⁺ 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. Preincubation of vessel segments with 10 μmol/L Y-27632 did not prevent 10 nmol/L ET-1–induced intracellular Ca²⁺ mobilizations and “Ca²⁺-dependent” constrictions but strongly prevented the “Ca²⁺-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 IC₅₀ of 3 μmol/L (pIC₅₀=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 Ca²⁺ sensitivity, it would be expected that Y-27632 prevents the ET-1–induced Ca²⁺-sensitization. Indeed, the increase in the Ca²⁺ 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/L Ca²⁺; 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 Ca²⁺ sensitivity of the contractile apparatus.

Activation of CGRP Receptors Prevents and Reverses ET-1–Induced Vasospasms and Ca²⁺ 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. Given that CGRP is among the most potent vasodilators, it is conceivable that CGRP alters the Ca²⁺ sensitivity of the contractile apparatus. CGRP (100 nmol/L) induced a transient decrease of [Ca²⁺], and a sustained vasodilation that persisted after CGRP had been removed from the perfusate. Removal of Ca²⁺ from the superfusate (0Ca) caused a [Ca²⁺] decrease and a parallel vasodilation. CGRP (10 nmol/L) decreased the Ca²⁺ sensitivity in vessels that were not preconstricted. CGRP (10 nmol/L) prevented the Ca²⁺ sensitization induced by 100 pmol/L ET-1 (n=6; compare with Figure 2C).

Figure 4. CGRP decreased the Ca²⁺ sensitivity of the contractile apparatus and thereby reversed ET-1–induced vasospasms. A, CGRP (100 nmol/L) reversed vasospasms induced by 1 nmol/L ET-1. Simultaneous measurements of [Ca²⁺] and vascular diameter (averages and SEM; n=7) are shown. The [Ca²⁺] and vascular diameter were normalized to the values before application of ET-1 (values at time x were set to 1). Note that CGRP induced a transient decrease of the [Ca²⁺], and a sustained vasodilation that persisted after CGRP had been removed from the perfusate. Removal of Ca²⁺ from the superfusate (0Ca) caused a [Ca²⁺] decrease and a parallel vasodilation. B, CGRP (10 nmol/L) decreased the Ca²⁺ sensitivity in vessels that were not preconstricted. C, CGRP (10 nmol/L) prevented the Ca²⁺ sensitization induced by 100 pmol/L ET-1 (n=6; compare with Figure 2C).
(−18±2 versus −17±4 μm/Ca2++; n=6) (Figure 4B). These observations support the hypothesis that CGRP and ET-1 can interact as functional antagonists at the level of the Ca2+ sensitivity of the contractile apparatus.

Discussion

The major findings of this study suggest that ET-1–induced vasospasms are (1) prevented but not reversed by ETA receptor antagonists; (2) mediated via an activation of Rho-kinase, an inhibition of MLCP, and an increase in the Ca2+ sensitivity of the contractile apparatus; and (3) reversed by CGRP, which decreases the Ca2+ sensitivity of the contractile apparatus and can act on the level of Ca2+ sensitivity as functional antagonist of ET-1.

Activation of ETα receptors by ET-1 induces vasospasms of the SMA.20 Interestingly, antagonism of ETα receptors with the structurally unrelated antagonists BQ-123 and BMS-182874 is able to prevent but not to reverse ET-1–induced vasospasms. This observation could be explained by each of 3 hypotheses. First, ETα receptor activation triggers a signaling cascade without depending on continuous receptor occupancy. Second, ET-1 binds irreversibly to the receptor, whereas BQ-123 and BMS-182874 are competitive antagonists. Third, ET-1–occupied ETα receptors signal continuously but are internalized and thereby inaccessible to the antagonists. Such a mechanism is likely because it has been observed in other preparations.28 The fact that antagonists such as BQ-123 and BMS-182874 cannot reverse vasospasms indicates that ETα receptors may not be the most promising pharmacological target for the release of ET-1–induced vasospasms.

In general, ET-1–induced constrictions have been found to be elicited by different Ca2+-mobilizing mechanisms, including Ca2+ release from intracellular Ca2+ stores via a phospholipase C–mediated activation of IP3 receptors and activation of L-type and nonselective Ca2+ channels.17–19 Ca2+ mobilization has been considered to be the main mechanism of ET-1–induced 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 ETα receptor and Rho-kinase is currently unknown, although evidence from cultured aortic smooth muscle cells suggests that ETα receptors activate Rho-kinase via G12/13 and the small G-protein RhoA.30 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.

The vasodilator peptide CGRP is present in perivascular nerves of the gerbil SMA and mediates a vasodilation via CGRP receptors.16 CGRP receptors signal in most vessels via increase in the cytosolic cAMP concentration, stimulation of protein kinase A, activation of K+ channels, hyperpolarization of the membrane potential, closure of voltage-gated L-type Ca2+ channels, and decrease of [Ca2+]i.31,32 K+ channels that mediate CGRP-induced vasodilations in the SMA include ATP-dependent (KATP) and Ca2+-activated (BK) K+ channels.16 In the present study we show that a major mechanism of CGRP-induced vasodilations is a decrease in the Ca2+ sensitivity of the contractile apparatus. Three observations support this conclusion. First, CGRP-induced dilations were sustained and exceeded the presence of the agonist, while the decrease in [Ca2+]i was only transient. Second, CGRP decreased the Ca2+ sensitivity in vessels that were not preconstricted. Third, CGRP prevented the ET-1–induced increase in Ca2+ sensitivity. Taken together, the observations suggest that CGRP-mediated vasodilations are initiated by an activation of K+ channels and a decrease in [Ca2+]i, and that the vasodilations are maintained by a decrease in Ca2+ sensitivity. CGRP-mediated changes in Ca2+ sensitivity have been observed thus far in only one other preparation, namely, in intramural coronary arteries.32 The finding that ET-1 increases the Ca2+ sensitivity whereas CGRP decreases the Ca2+ sensitivity of the contractile apparatus suggests that CGRP can act on the level of Ca2+ sensitivity as a functional antagonist of ET-1.

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 cerebral as well as of coronary arteries.7,8 Our study suggests that Rho-kinase, rather than the ETα receptor, is the most promising pharmacological target for the treatment of ET-1–induced vasospasms in general and sudden hearing loss in particular. The key role of Rho-kinase in vasospasms is supported by the results of clinical trials in which the Rho-kinase inhibitor hydroxyfasudil successfully reversed vasospasms of coronary and cerebral arteries.7,8 In particular, the beneficial effect of fasudil in the treatment of cerebral vasospasms after subarachnoidal hemorrhage is well established.33 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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