an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and/or by an increase in the Ca^{2+} sensitivity of the contractile apparatus [1,2]. One key mechanism enhancing Ca^{2+} sensitivity and thus vascular tone is Rho-kinase signalling, which results in inhibition of myosin light chain phosphatase [2,3]. Rho-kinase activation has been shown to cause vasospasm of coronary, cerebral and spiral modiolar arteries [4-9].

One of the strongest Rho-kinase activators described so far is the vasoconstrictor endothelin-1 (ET-1). The synthesis of ET-1 by endothelial cells is activated by physiological stimuli such as shear stress, insulin, thrombin and other vascular factors [10]. ET-1 and ET_A receptors play a fundamental role in the maintenance of basal vasomotor tone in resistance arteries [11]. The synthesis of ET-1 can be increased by hypoxia and elevated oxidized low-density lipoproteins [12,13] and has been implicated in the pathogenesis of a number of cerebrovascular disorders, including stroke, ischemia, and, in particular, cerebral vasospasm [14,15]. Thus, ET-1 possesses pathological potential in addition to its physiological functions. ET-1 is present in the SMA and induces strong, long-lasting constriction via ET_A-receptor-mediated Rho-kinase activation [9,16,17]. Taken together ET-1 is likely an endogenous regulator of inner ear microvascular tone.

We have previously shown that CGRP is able to reverse ET-1-induced constrictions in the SMA via an increase in vascular smooth muscle cAMP [18]. CGRP is present in perivascular nerves of the SMA and therefore is a potential endogenous vasodilator of the SMA. We propose, therefore, that reversal of ET-1-induced constriction is not necessarily limited to inhibition of ET-1-related mechanisms (e.g., Rho-kinase signalling).

These findings provide a clinical perspective for a new treatment of SSHL, because both Rho-kinase signalling and cAMP can be targeted via pharmacological agents. Therefore, we assessed the potency of clinically relevant Rho-kinase inhibitors and a cell-permeable analogue cAMP (dbcAMP) in terms of reversing ET-1-induced constriction and Ca^{2+}-sensitization in the SMA.

Methods

Drugs and solutions

The physiologic salt solution (PSS) contained (in mmol/L) 150 NaCl, 3.6 KC1, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 5.0 HEPES, and 5.0 glucose, pH 7.4. Extracellular Ca^{2+} concentration ($[Ca^{2+}]_{ex}$) was raised to 3 and 10 mmol/L by addition of $CaCl_2$. 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 KC1, 1.0 $MgCl_2$, 1.0 EGTA, 5.0 HEPES, and 5.0 glucose, pH = 7.4. Fluo-4-AM (Molecular Probes) was dissolved in anhydrous DMSO and stored in 1 mmol/L aliquots. Y-27632 was kindly provided by Welfide. Fasudil was obtained from Calbiochem. Fasudil (obtained from Tocris Cookson) was modified to hydroxyfasudil by Dr. Duy Hua, Dept of Chemistry, Kansas State University. All other chemicals were obtained from Sigma.

Preparation of the spiral modiolar artery (SMA)

Experiments were conducted on tissues isolated from gerbils 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 i.p.) and decapitated. Temporal bones were removed, opened and placed into a micro-dissection chamber containing PSS at 4°C. The SMA was isolated from the cochlea by micro-dissection as described previously [19]. 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.

Simultaneous measurement of vascular diameter and $[Ca^{2+}]_i$

The simultaneous measurement of vascular diameter and $[Ca^{2+}]_i$ has been described previously [17]. Briefly, the smooth muscle cells of vessel segments were loaded with the Ca^{2+} indicator dye fluo-4 by incubation in PSS containing 5 μmol/L fluo-4-AM for 35 min at 37°C. After loading, vessel segments were washed with PSS and maintained at 4°C for 20 minutes prior to 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 (518–542 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 605–615 nm and the transmission image was recorded with a chilled CCD camera (Hamamatsu). The outer vascular diameter was measured by two video edge detectors (Crescent). Fluorescence and calibrated diameter signals were digitized and recorded simultaneously (Photon Technology International).

Experimental protocols

Experiments were started 20 min after loading with fluo-4. Vessel segments were superfused at a rate of 9 ml/min with PSS. This flow rate corresponds to an exchange rate of 2 bath chambers volumes/sec, given the bath chamber volume of 75 μl. Upon start of the superfusion, the unpressurized artery develops a spontaneous vascular tone that is sensitive to removal of extracellular Ca^{2+} and inhibition of L-type Ca^{2+} channels with nanomolar concentrations of nifedipine [19]. The viability of each vessel