was assessed by its constrictor response to 10 mmol/L $[Ca^{2+}]_{ex}$. The $[Ca^{2+}]_i$ was monitored as fluorescence intensity and was normalized to the basal fluorescent emission prior to the beginning of each experiment. The fluorescence and diameter values taken for statistical analysis represent averages of the $[Ca^{2+}]_i$-fluorescence and the vascular diameter over 30 sec beginning 30 sec after the onset of stimulation. We carefully validated the $[Ca^{2+}]_i$ -measurements with the non-ratiometric Ca^{2+}-dye fluo-4 by (i) excluding artefacts due to vessel diameter changes (evaluated by $[Ca^{2+}]_{ex}$-versus ET-1 dose-response curves), (ii) excluding significant differences in the magnitude of $[Ca^{2+}]_i$ -changes between preparations, and (iii) assessing the same optimal dye-loading conditions in each experiment [9,17].

Affinity constants (K_{DB}) and concentrations that cause a half-maximal inhibition (IC_{50}) were determined in cumulative experiments and averaged after logarithmic transformation (pK_{DB} and pIC_{50}) as previously described [17].

The Ca^{2+} sensitivity of the contractile apparatus was determined by a correlation of $[Ca^{2+}]_i$ and the vascular diameter as described previously [9]. Changes in $[Ca^{2+}]_i$ were induced by changes in $[Ca^{2+}]_{ex}$. Stepwise increases in $[Ca^{2+}]_{ex}$ from 0 to 1, 3 and 10 mmol/L caused increases in $[Ca^{2+}]_i$ and decreases in the vascular diameter. Correlations were found to be linear ($r > 0.95$) within the measured range (the relation between calcium concentration and tension gets sigmoidal if the $[Ca^{2+}]_{ex}$ and thus the $[Ca^{2+}]_i$ is further increased [9]). Slopes were quantified in the arbitrary unit µm/Ca^{2+}, where µm represent the change in the vascular diameter and Ca^{2+} represents the normalized change in the cytosolic Ca^{2+} concentration. Linear slopes were obtained to compare the Ca^{2+} sensitivity within paired experiments. In each vessel segment, the Ca^{2+} sensitivity was assessed under control and under experimental conditions.

Experiments of dbcAMP induced dilations were bracketed by Ca^{2+} free manoeuvres that were performed to induce a maximal vasodilation and reduce the $[Ca^{2+}]_i$ to a minimal level. Vascular diameter or fluorescence intensity in the absence of Ca^{2+} was considered as baseline. The magnitude of the vascular tone or fluorescence intensity was determined as the difference between the recorded value and the baseline value. Measurements were averaged over a period of 1 min to average vasomotion. DbcAMP-induced effects were normalized to the magnitude of the vascular tone or fluorescence intensity during 1 min immediately prior to the application of dbcAMP.

Statistical analysis

All results are expressed as average ± SEM of n experiments with n representing the number of vessel segments. The significance of changes in the vascular diameter and of changes in the Ca^{2+} sensitivity were determined using Student's paired *t*-test. Differences were considered to be significant at error probabilities less than 0.05 ($P < 0.05$).

Results

This report is based on recordings of 50 vessels from 34 animals. The average vascular diameter was 65 ± 1 µm.

ET-1-induced constriction is reversed via Rho-kinase inhibitors

ET-1 (10 nmol/L) induced a transient increase in $[Ca^{2+}]_i$, a strong and long-lasting vasoconstriction and a robust increase in the vasomotion of the gerbil spiral modiolar artery (Fig. 1A). The $[Ca^{2+}]_i$ returned to almost resting levels after the transient increase, while the constriction was maintained. The ET-1-induced vasoconstriction was not readily reversible upon removal of ET-1 from the perfusate. The constriction and the increased vasomotion were observed without a significant change for at least 20 minutes after removal of ET-1 from the superfusate (*data not shown*). Note that ET-1-induced a transient $[Ca^{2+}]_i$ increase and a sustained vasoconstriction while exposure to 10 mmol/l Ca^{2+} induced an increase in $[Ca^{2+}]_i$ and a parallel vasoconstriction.

We tested the potency of different Rho-kinase inhibitors reversing ET-1-induced constriction. Figure 1B shows an original recording of an ET-1-induced constriction which is antagonized by increasing concentrations of fasudil. Fasudil mediated vasodilation were induced without significantly altering $[Ca^{2+}]_i$-levels. The Rho-kinase inhibitors Y-27632, fasudil and hydroxy-fasudil reversed ET-1-induced constriction (10 nmol/L) in a dose-dependent manner (Figure 1C). The $1C_{50}$ for Y-27632-, fasudil- and hydroxy-fasudil- mediated reversion of constriction was 3 µmol/L (pIC_{50} = 5.50 ± 0.31; n = 6), 15 µmol/L (pIC_{50} = 4.71 ± 0.13; n = 7) and 111 µmol/L (pIC_{50} = 3.95 ± 0.24; n = 6), respectively. The Ca^{2+} sensitivity of the contractile apparatus was assessed as linear slopes obtained from correlations of $[Ca^{2+}]_i$ and vascular diameter. ET-1 (100 pmol/L) increased the Ca^{2+} sensitivity (-36 ± 9 versus -62 ± 13 µm/Ca^{2+}, n = 8; Figure 1D), fasudil (3 µmol/L) prevented the ET-1-induced increase in the Ca^{2+} sensitivity (-17 ± 3 versus -16 ± 2 µm/Ca^{2+}, n = 8, Figure 1E). Taken together, these observations demonstrate that ET-1-induced constriction in the SMA is maintained by a Rho-kinase-mediated increase of the Ca^{2+} sensitivity of the contractile apparatus, which can be effectively reversed by Rho-kinase inhibition.

Exogenous, cell-permeable cAMP (dbcAMP) reverses ET-1-induced constriction and Ca^{2+}sensitization

DbcAMP induced dose-dependant decreases in $[Ca^{2+}]_i$ and reversal of constriction induced by 1 nmol/L ET-1