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ROCK ACTIVITY ON THE TEMPERATURE-DEPENDENT RESPONSES OF CALF CARDIAC VEIN

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ABSTRACT

At present very little is known about the role of Rho-kinase (ROCK) activity in the effects of temperature on vascular reactivity. To evaluate the role of ROCK activity in the cooling (28 °C)- and warming (41 °C)-induced effects on vascular reactivity, isolated calf cardiac vein rings were studied. Rings obtained from calf hearts were suspended in organ baths containing 25 ml of Krebs-Henseleit solution, maintained at 37 °C, continuously gassed with 95% O_2-5% CO_2. At the end of the resting period, the preparations were contracted with carbachol (10^{-6} M), serotonin (5-HT, 10^{-6} M), U46619 (10^{-7} M), $PGF_{2\alpha}$ (10^{-6} M), or endothelin-1 (ET-1, 10^{-8} M) at 37 °C in different preparations. The same protocol was repeated at 28 °C and 41 °C after the preparations were allowed to equilibrate at this temperature for 60 min. In order to analyze the role of ROCK activity in the cooling- and warming-induced vascular response, each contractile agent was applied in the presence of fasudil (10^{-6} M) or Y-27632 (10^{-6} M). Carbachol, 5-HT, U46619, $PGF_{2\alpha}$ and ET-1 produced reproducible contractions. Cooling decreased the contractions to carbachol, 5-HT, U46619, $PGF_{2\alpha}$ and ET-1 and warming enhanced significantly. Treatment with fasudil and Y-27632 at both 28 and 41 °C decreased the responses to carbachol, 5-HT, U46619, $PGF_{2\alpha}$ and ET-1. The results of this study suggests a role for ROCK signalling in the temperature-induced changes of calf cardiac vein.

Keywords: Cardiovascular activity, Hypothermia, Rho kinase, Hyperthermia.

INTRODUCTION

Temperature can influence vascular smooth muscle cell function and alter the reactivity of the vessels. Moderate hypothermia; cooling to 25-31°C, has been shown to have a variable influence on the vascular sensitivity to various drugs and endogenous substances in different parts of the vascular system of animals [1, 2]. Most of the previous studies examining the effect of temperature on smooth muscle responses to vasoactive agents have focused on the cutaneous vessels and information about non-cutaneous vessels is rather limited. Furthermore, in contrast to the effect of cooling on smooth muscle reactivity to contractile agents, the influence of physiologically relevant increases in temperature above 37°C on vascular reactivity to vasoactive agents is unclear because of limited information and disparate results. For example, there are reports about norepinephrine-induced contractions that elevated temperature increases [3], do not modify [4] or decrease [5] the vasoconstriction to this agent. Moreover, Padilla [6] reported that in rabbit femoral artery, warming increased the vasoconstrictor response to potassium, ET-1, and norepinephrine, and the characteristics of this increase varied depending on the vasoconstrictor used. Recently, we studied the effects of different temperatures on the smooth muscle responses in a variety of vessels from different species and observed that the contractile responses were temperature-dependent [7, 8].

The Rho-kinase (Rho-associated coiled-coil-containing protein kinase, ROCK) signaling pathway is substantially involved in vascular contraction induced by G-protein coupled receptors. Various vasoactive agonists like angiotensin II, endothelin-1 and thromboxane A2 activate G-protein coupled receptors and lead not only to an increase in intracellular Ca^{2+} via G-proteins of the Gq/G11-family but also activate G-proteins of the G12/G13-family that stimulate the Rho/Rho-kinase signalling pathway, thereby activating ROCK. In many preclinical models of cardiovascular diseases, including vasospasm, arteriosclerosis, hypertension, pulmonary hypertension, stroke, ischemia–reperfusion injury, and

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heart failure, ROCK inhibitors have shown a remarkable efficacy in reducing vascular smooth muscle cell hypercontraction, endothelial dysfunction, inflammatory cell recruitment, vascular remodeling, and cardiac remodeling [9]. Some of the commonly known ROCK inhibitors include Y-27632 and fasudil. Fasudil is an isoquinoline derivative that also inhibits ROCK by competing with ATP for the kinase active site. Y-27632 is a synthetic pyridine derivative that inhibits ROCK by competing with ATP for the kinase active site [10]. It is well known that ROCK signaling pathway plays an important role in a variety of cardiovascular diseases [11]. Blocking this pathway can reduce the vascular tone and protect myocardial I/R injury. However, the pharmacological action of Rho kinase inhibition on smooth muscle contractility during cooling/warming has not yet been studied.

The aim of the present study was to determine the role of ROCK activity on the cooling and warming-induced responses of calf cardiac vein. For this study, the calf cardiac vein was selected because it is a noncutaneous vessel and is easily accessible and endothelium layer was removed mechanically, excluding endothelial nitric oxide synthesis. Therefore, ROCK inhibitors fasudil and Y27632 were used in preparations constricted with carbachol, serotonin, U46619, $PGF_{2\alpha}$, or endothelin-1 during cooling and warming.

MATERIALS AND METHODS

Tissue preparations

Calf hearts were obtained from a slaughterhouse and were immediately placed in Krebs-Henseleit solution. Segments of the great cardiac vein were removed and cut into rings 2.5 mm in length. The endothelial layer of each preparation was mechanically removed from the vascular rings. Each ring was mounted in 25 ml organ baths containing Krebs-Henseleit Solution (KHS), aerated with 95 % O_2 and 5% CO_2. KHS was composed of (mM): NaCl 119, KCl 4.70, $MgSO_4$ 1.50, KH_2PO_4 1.20, $CaCl_2$ 2.50, $NaHCO_3$ 25, Glucose 11. The absence of endothelium was confirmed by the lack of relaxation in response to acetylcholine (ACh, 10^{-9}-10^{-4} M) in rings pre-constricted with 5-HT (10^{-6} M).

Changes in isometric tension were recorded by a force-displacement transducer (BIOPAC MP36, Santa Barbara, California, USA) connected through amplifiers to a ITBS08 Integrated Tissue Bath System (Commat, Ankara, Turkey). The tissues were allowed to equilibrate for 60 mins under a resting tension of 1 g with repeated washing every 15 min.

Experimental design

After the stabilization period, first, cardiac vein preparations were contracted with one of the constrictor agents at 37°C, mentioned below. Tested constrictors were: carbachol (10^{-6} M), serotonin (5-HT, 10^{-6} M), U46619 (10^{-7} M), $PGF_{2\alpha}$ (10^{-6} M), and endothelin-1 (ET-1, 10^{-8} M). After the contraction had reached steady state, the preparations were washed and allowed to reestablish resting tension before being cooled. When preparations stabilized (30 min), the bath temperature was decreased to 28 °C. Preparations were allowed to equilibrate at this temperature for 1 h before a second dose-response curve was determined with the same contractile agent. In order to analyse the role of ROCK pathway in the cooling-induced vascular response, the curve to same contractile agent was determined in the presence of fasudil (10^{-6} M) or Y-27632 (10^{-6} M) in the same preparation. This drug was added to the bath 20 min before beginning the experiment. This experimental protocol was conducted in seperate groups of preparations contracted with all mentioned agents, respectively.

In another series of experiments, the effects of warming were investigated. At first, cardiac vein preparations were contracted with one of the constrictor agents mentioned. After the contraction had reached steady state, the preparations were washed and allowed to reestablish resting tension before being warmed. When preparations stabilized (30 min), the bath temperature was increased to 41 °C. Preparations were allowed to equilibrate at this temperature for 1 h before a second dose-response curve was determined with the same contractile agent. In order to analyse the role of ROCK pathway in the warming-induced vascular response, the curve to same contractile agent was determined in the presence of fasudil (10^{-6} M) or Y-27632 (10^{-6} M) in the same preparation. This drug was added to the bath 20 min before beginning the experiment. This experimental protocol was conducted in seperate groups of preparations contracted with all mentioned agents, respectively.

STATISTICAL ANALYSIS

The peak height contractions elicited by each agonist were compared at 37 and 28 or 41 °C and also in the absence or presence of the inhibitors at each temperature. Values were expressed as percentages. Statistical comparisons were performed using paired or unpaired t-test as appropriate and significance was accepted at $p<0.05$.

Drugs

Carbachol, 5-HT, U46619, $PGF_{2\alpha}$, ET-1, fasudil and Y-27632 were used in the study. All drugs were obtained from Sigma, ST. Louis, MO, USA. Carbachol, 5-HT, $PGF_{2\alpha}$, ET-1, fasudil and Y27632 were dissolved in distilled water. Stock solutions of U46619 were made in ethanol. The final concentration of ethanol in the bath in each case was always ≤ 0.2%. All drugs used in functional studies were freshly prepared on the day of the experiment. Ethanol (vehicle) was added to the control preparation and did not display a contractile effect on the

preparation.

RESULTS

In calf cardiac vein, carbachol (10^{-6} M), 5-HT (10^{-6} M), U46619 (10^{-7} M), $PGF_{2\alpha}$ (10^{-6} M) and ET-1 (10^{-8} M) produced reproducible contractions. Figure 1,2,3,4 and 5 show the effects of carbachol, 5-HT, U46619, $PGF_{2\alpha}$ and ET-1 on endothelium-denuded calf cardiac vein at 37, 28 (cooling), and 28 °C in the presence of fasudil and Y-27632. During cooling, the responses to carbachol, 5-HT, U46619, $PGF_{2\alpha}$ and ET-1 were significantly lower ($p<0.05$) than at 37°C. At 28°C, the maximum tension generated in response to carbachol 5-HT, U46619, $PGF_{2\alpha}$, and ET-1 were 3.5 ± 54.2%, 4.3 ± 58.6%, 3.6 ± 54.7%, 2.7 ± 30.3%, 4.3 ± 70.2% of control (37°C), respectively. Treatment with Y-27632 and fasudil, significantly ($p<0.05$) decreased the contractile responses to carbachol, 5-HT, U46619, $PGF_{2\alpha}$ and ET-1 during cooling. The maximum tension generated in response to carbachol 5-HT, U46619, $PGF_{2\alpha}$ and ET-1 in the presence of Y-27632 were 2.3 ± 37.8%, 2.4 ± 34.4%, 2.8 ± 32.5%, 3.3 ± 57.0%, 3.2 ± 55.8% and in the presence of fasudil were 3.8 ± 45.1%, 3.3 ± 65.6%, 3.4 ± 61.1%, 3.9 ± 71.6% and 3.8 ± 46% of control (28°C), respectively.

Figure 1-5 also show the effects of carbachol, 5-HT, U46619, $PGF_{2\alpha}$ and ET-1 on endothelium-denuded calf cardiac vein at 37, 41 (warming), and 41°C in the presence of fasudil and Y-27632. During warming, the responses to carbachol, 5-HT, U46619, $PGF_{2\alpha}$ and ET-1 were significantly higher ($p<0.05$) than at 37°C. At 41 °C, the maximum tension generated in response to carbachol 5-HT, U46619, $PGF_{2\alpha}$ and ET-1 were 4.5 ± 166.9%, 5.3 ± 170.4%, 4.6 ± 164.5%, 4.7 ± 249.7%, 5.7±229.3% of control (37°C), respectively. Treatment with fasudil and Y-27632 significantly ($p<0.05$) decreased the contractile responses to only carbachol and 5-HT, during warming. The maximum tension generated in response to carbachol 5-HT, U46619, $PGF_{2\alpha}$ and ET-1 in the presence of Y-27632 were 3.3 ± 53.4%, 3.4 ± 36.6%, 3.8 ± 62.9%, 3.3 ± 44.0%, 3.8 ± 60.0% and in the presence of fasudil were 3.2±33.8%, 3.6±36.2%, 3.2±49.5%, 3.0 ± 41.0% and 3.2 ± 80% of control (41°C), respectively.

Fig 1. Responses to carbachol (10^{-6} M) in the presence of Y27632 and fasudil. Each value is derived from six experiments.

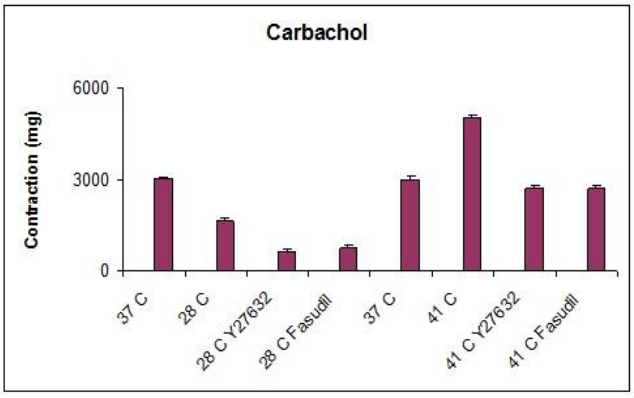


Fig 2. Responses to 5-HT (10^{-6} M) in the presence of Y27632 and fasudil. Each value is derived from six experiments

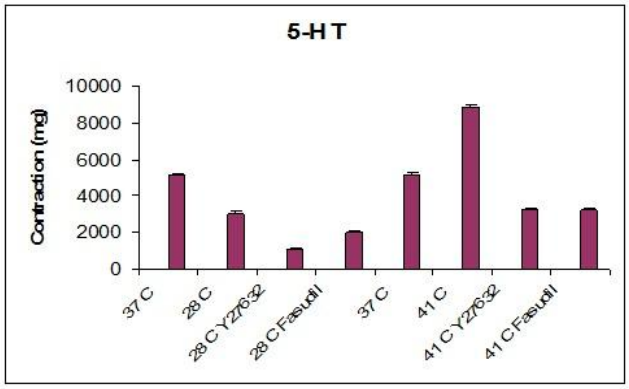


Fig 3. Responses to U46619 (10^{-7} M) in the presence of Y27632 and fasudil. Each value is derived from six experiments

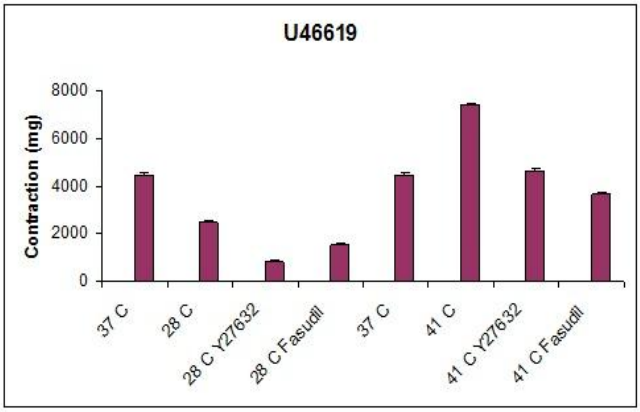


Fig 4. Responses to $PGF_{2\alpha}$ (10^{-6} M) in the presence of Y27632 and fasudil. Each value is derived from six experiments.

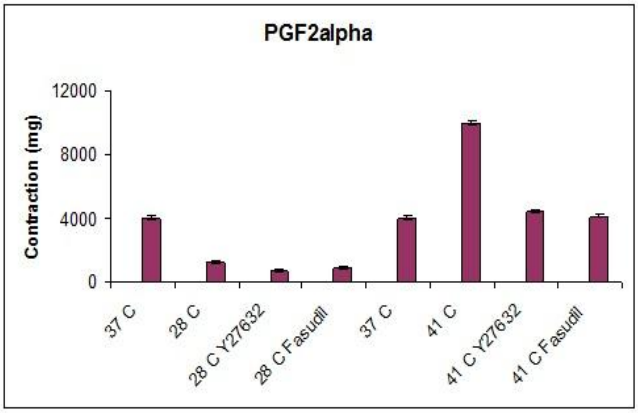
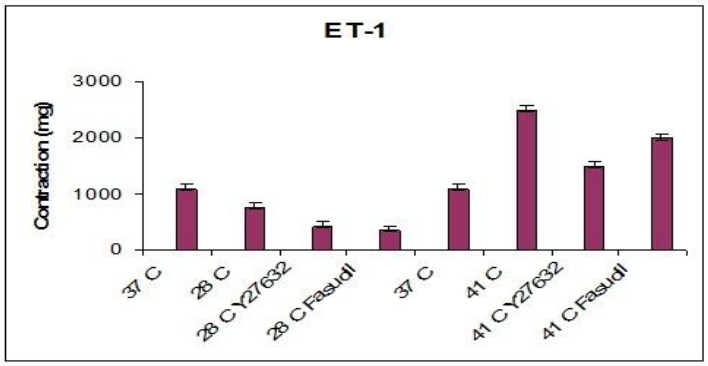


Fig 5. Responses to ET-1 (10^{-8} M) in the presence of Y27632 and fasudil. Each value is derived from six experiments.



DISCUSSION

In the present work, we studied the effects of ROCK signalling during cooling (28°C) and warming (41°C) on carbachol, 5-HT, U46619, $PGF_{2\alpha}$ and ET-1-induced contractions of isolated calf cardiac vein. The cardiac vein is an easily accessible noncutaneous blood vessel and the role of ROCK signalling pathway, that mediates vascular smooth muscle contraction, on cooling and warming-induced effects of this vessel has not been studied before. The temperature utilized in this study; 28˚C, for cooling was considered to be "moderate cooling" and 41˚C, "moderate warming" temperature accordingly to previous studies [12]. Since vascular tone is regulated via various vasoactive substances synthesized by vascular endothelial cells, and ROCK can regulate the production of nitric oxide [13], we used endothelium-removed cardiac vein preparations in this study.

Our results indicate that at 37 °C, carbachol, 5-HT, U46619, $PGF_{2\alpha}$ and ET-1-induced reproducible contractions in cardiac vein. Compared with the control responses, cooling decreased and warming increased these contractions. This suggests that cooling and warming could nonspecifically effect the contractions of cardiac vein when it is activated by different types of vasoactive stimuli. In smooth muscle, contractile agonists are known not only to increase Ca^{2+} influx but also to increase Ca^{2+}- sensitivity. Recently, there is increasing evidence that activation of the ROCK pathway leads to Ca^{2+}- sensitization through inhibition of phosphatase and increase in phosphorylated myosin light chain [14]. Rho is a member of the Ras family of proteins, which regulates the organization of actin cytoskeleton and mitogenic signalling in response to extracellular signals [15]. Fasudil is a selective ROCK inhibitor, competing with ATP for the binding to the kinase [16]. Y-27632 has been shown to be a specific inhibitor of ROCK and is, therefore, a useful tool to study the physiological role of ROCK [17]. Fasudil and Y-27632, have been used in previous studies to assess ROCK activity in vitro and in vivo [18]. In this study, the use of ROCK inhibitors, fasudil or Y-27632 resulted in a clear reduction in the contractions of all agonists during cooling and warming. This observation indicates that the mechanism of carbachol, 5-HT, U46619, $PGF_{2\alpha}$ and ET-1-induced contraction in calf cardiac vein during cooling and warming involves the ROCK signaling pathway. The present findings that the two inhibitors reduced contractions caused by muscarinic, serotonergic, TP receptor, $PGF_{2\alpha}$-receptor and ET-1-receptor activation, reinforce the conclusion that Rho kinase is the common pathway activated by those receptors in vascular smooth muscle. Similarly [19] that, in rat aorta, ROCK inhibitors reduced endothelium-independent contractions of preparations, in confirmation of previous observations in isolated blood vessels [20, 21]. The investigators also reported that the relative inhibitory effect of the ROCK inhibitors varied depending on agonist used. Thus, the concentration-response curve to U46619 and prostaglandin $F_{2\alpha}$ were shifted to the right, whereas the contraction to phenylephrine was abolished. However, in this study, ROCK inhibitors reduced the contractions of all agonists used similarly. To our knowledge, we report the first demonstration of the presence of ROCK in cardiac vein smooth muscle cells, and of the relaxant effect of a ROCK inhibitor. Furthermore, [22] demonstrate that the ROCK signaling pathway plays an important role in the cold-induced augmentation of α_2-adrenergic receptor activity in mouse tail artery a cutaneous artery. Similarly, [23] that in rat detrusor ROCK and extracellular Ca^{2+} are important for the cooling responses. The current findings are in agreement with these previous reports. To our knowledge, this is the first study to investigate whether the ROCK signaling pathway plays a role in cold/warm induced modulation of a non-cutaneous vessel.

In conclusion, the mechanism of the inhibitory effects of cooling and the stimulatory effects of warming in noncutaneous vessels is uncertain, but the finding in this study suggests a role for ROCK signalling in the temperature-induced changes of calf cardiac vein.

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CONFLICT OF INTEREST:
The authors declare that they have no conflict of interest.

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