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EXPERIMENTAL STUDY

The role of calcium entry on the relaxation response of rho-kinase inhibitor in rabbit renal artery

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Abstract: This study was performed to clarify the role of extracellular and intracellular Ca^{2+} on rho-kinase enzyme inhibition-induced relaxation in rabbit renal arteries. The response to rho-kinase inhibitor (Y-27632) was studied in isolated renal artery segments precontracted with phenylephrine in the presence of voltage-gated calcium channel blocker nifedipine and in the absence of intracellular or extracellular Ca^{2+}. Cumulative addition of rho-kinase inhibitor Y-27632 (10^{-8}–10^{-5} M) produced a concentration-dependent relaxation in renal artery rings precontracted with phenylephrine. Preincubation with nifedipine (1μM) resulted in a significant increase in relaxation response to rho-kinase inhibitor Y-27632 compared with preincubation with DMSO; the solvent of nifedipine. The maximal relaxation to Y-27632 in renal arteries precontracted with phenylephrine was significantly increased in the Ca-free Krebs containing 100 μmol/l ethylene glycol tetraacetic acid (EGTA) but after depletion of intracellular stores with 20 mmol/l caffeine and 1mmol/l EGTA in Ca^{2+} free Krebs there was no significant difference between the relaxation to Y-27632 from control response in 2.5 mmol/l Ca^{2+} Krebs in the renal artery. These results suggest the involvement of extracellular Ca and L-type voltage-operated Ca^{2+} channels in phenylephrine-induced rho-kinase activation *(Fig. 3, Ref. 20)*. Full Text in PDF *www.elis.sk.*
Key words: rho-kinase, calcium, rabbit, renal artery.

An achievement of vascular smooth muscle contraction, activation of subcellular system that mobilizes Ca^{2+} from extracellular and intracellular stores is required. Enrichment of free Ca^{2+} concentration increases the myosin light chain (MLC) kinase activity and MLC phosphorylation (1). It is also known that there is a second smooth muscle contractile mechanism that is defined as Ca^{2+} sensitization. Ca^{2+} sensitization of smooth muscle is composed of receptor stimulation which causes a decrease in MLC phosphatase activity mediated through the actions of protein kinase C. Rho-kinase is involved in vascular smooth muscle contraction (2).

Previous studies demonstrated that after stimulation of RhoA with receptor agonists including angiotensin-II, endothelin-I, and phenylephrine, the induced rho-kinase activation increases the Ca^{2+} sensitivity which results in myosin light-chain phosphatase inhibition and contraction (3-5) However it was also shown that Ca^{2+}-dependent rho-kinase activation also plays a role in smooth muscle contraction. Shabir et al have reported that Rho/Rho-kinase pathway targets Ca^{2+} entry through voltage-gated Ca^{2+} channels to enhance Ca^{2+} transients and contraction in rat and guinea-pig uretral smooth muscle (6).

There are distinct vasoconstriction mechanisms playing roles in renal artery vascular smooth muscle regulation (7). Rho-kinase enzyme activation involves agonist-induced vasocontriction in rat renal artery (8) by Ca^{2+} sensitization (9). In renal artery, the initial phase of phenylephrine-induced contraction is due to inositol triphosphate (IP3)-sensitive sarcoplasmic reticulum channels, while maintenance is supplied by L-type voltage-gated Ca^{2+} channels and receptor-operated Ca^{2+} channels (10). In various smooth muscles Rho/Rho-kinase inhibits phenylephrine-induced contraction (11, 12). Furthermore, the involvement of voltage-gated Ca^{2+} channels in rho-kinase enzyme inhibition in renal artery precontracted with phenylephrine is currently unknown.

Thus, this study was designed to determine the role of extracellular and intracellular Ca^{2+} on rho-kinase enzyme inhibition-induced relaxation in isolated rabbit renal arteries.

Methods

Animals and tissue preparation

The study protocol was approved by Dokuz Eylul University Ethical Committee on Care and Use of Laboratory Animals. A total of 7 adult New Zealand White male rabbits weighing 2.5±0.5 kg (3 months old) were purchased from Ege University of Animal research center and used in the study. Rabbits were housed identically in individual cages in an air-conditioned room (temperature 23±2 ºC, 12-h light/dark cycle). All animals were fed *ad libitum* with standard plain diet and were allowed free access to water. Animals were killed by intravenous injection of thiopental anesthesia (60 mg/kg). The left renal artery was excised and cleaned of excess fat and connective tissue. The ring segments (2–3mm in length) were prepared and placed between stainless steel hooks and

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mounted in a 10-ml organ bath chamber in Krebs-Ringer buffer of composition (mM) as follows: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 8.3, pH 7.4, aerated with 95 % O_2 and 5 % CO_2. The rings were equilibrated for 1h, washed every 15 minutes at +37 ºC with a resting tension of 1.5 g. The force of contraction was measured with isometric tension transducer (MAY transducer), and isometric contractions were recorded continuously with an amplifier system (MP30 Biopac systems Inc., Santa Barbara California, U.S.A.) on a computer by using the Biopac computer program.

Experimental protocols

Acetylcholine (Ach)-induced (10^{-6} M) relaxation after KCl (45 mM) precontraction was used to test the endothelial integrity. The depolarizing potassium solution had the same composition as the Krebs' buffer used except for NaCl that had been replaced with an equimolar amount of KCl. The cumulative concentration-response curves were determined for phenylephrine (10^{-6}–10^{-4} M) in order to calculate EC_{80} value. Rho-kinase enzyme inhibitor Y-27632-induced relaxation responses were expressed as percentage relaxation of constrictor response to phenylephrine.

Relaxation responses of rho-kinase inhibitor Y-27632 in rabbit renal artery: After precontraction of renal artery rings with EC_{80} of phenylephrine (10^{-5} M), rho-kinase inhibitor Y-27632 was added cumulatively (10^{-8}–10^{-5} M).

Relaxation responses of rho-kinase inhibitor Y-27632 in absence and presence of Ca^{2+} channel inhibitor nifedipine in rabbit renal artery. In group one, arterial rings were incubated with DMSO; as a vehicle of nifedipine; 15 min before phenylephrine contraction and cumulative relaxation responses to rho-kinase inhibitor, Y-27632 (10^{-8}–10^{-5}M) was determined. In group two, in order to study the role of voltage-dependent Ca^{2+} channel blockers on Y-27632-induced relaxations, arterial rings were incubated with Ca^{2+} channel blocker nifedipine (1 μM) (13), 15 min before the phenylephrine contraction.

Relaxation responses of rho-kinase inhibitor Y-27632 after depletion of intracellular Ca^{2+} stores in rabbit renal artery: The contribution of intracellular Ca^{2+} to relaxation induced by cumulative application of Y-27632 was assessed in renal arteries in Ca^{2+}-free Krebs solution containing 100μmol/L ethylene glycol tetraacetic acid (EGTA). The contribution of extracellular Ca^{2+} to relaxation response of Y-27632 was evaluated after depletion of intracellular Ca^{2+} stores. Depletion of intracellular Ca^{2+} stores was obtained by incubation of tissues with 20 mmol/l caffeine and 1mmol/l EGTA in Ca^{2+} free Krebs for 10 minutes. Then bath solutions were replaced with normal Krebs solutions and Y-27632 was added cumulatively after precontraction with EC_{80} of phenylephrine (13).

Drugs

The following compound of nifedipine was dissolved in Dimethyl sulfoxide (DMSO). The other compounds as acetylcholine chloride, phenylephrine, caffeine, EGTA were purchased from Sigma Chemical Co. and dissolved in distilled water, while rho-kinase inhibitor Y-27632 (Calbiochem) was dissolved in cold water(+4 °C).

Statistical analysis

Data are expressed as mean±SEM, with n referring to the number of strips used in each group. The sensitivity of artery to the cumulative relaxation response is presented as pD_2 values, which are calculated as the negative log of a dose required to produce the half-maximal response. The results of cumulative concentration response curves were analyzed by using Student's t-test and one way analysis of variance (ANOVA) followed by Tukey's test. A level of $p<0.05$ was considered statistically significant.

Results

Relaxation of renal artery with rho-kinase inhibitor Y-27632 precontracted with phenylephrine

Phenylephrine (10^{-6}–10^{-4} M) caused a dose-dependent contraction with an EC_{80} value (10^{-5}M) in isolated rabbit renal artery. Cumulative addition of Y-27632 (10^{-8}–10^{-5} M) produced a concentration-dependent relaxation in renal artery rings precontracted with EC_{80} dose of phenylephrine. The maximal relaxation responses and pD_2 values for Y-27632 were 46.5±4.9 % and 5.6±0.1 %, respectively.

Effect of Ca^{2+} channel blocker nifedipine on relaxation response to Y-27632

The effect of extracellular Ca^{2+} via voltage-gated L-type Ca^{2+} channels on response to Y-27632 was examined by the use of nifedipine. Preincubation with nifedipine (1μM) resulted in a significant increase in relaxation response to rho-kinase inhibitor Y-27632 (78.5±2.1 %), compared with the treatment with DMSO, solvent of nifedipine (57.2±3.6 %) and 2.5mmol/l Ca^{2+} Krebs (46.5±4.9 %) (p=0.001) (Fig. 1).

Effects of extra- and intracellular sources of Ca2+ on relaxation response to Y-27632

The contributions of extra and intracellular sources of Ca^{2+} to relaxation response induced by Y-27632 are shown in Figure 2. Relaxation response to Y-27632 in 2.5 mmol/l Ca^{2+} Krebs was used as control in renal artery. The maximal relaxation to Y-27632 (46.5±4.9 %) in renal arteries precontracted with phenylephrine

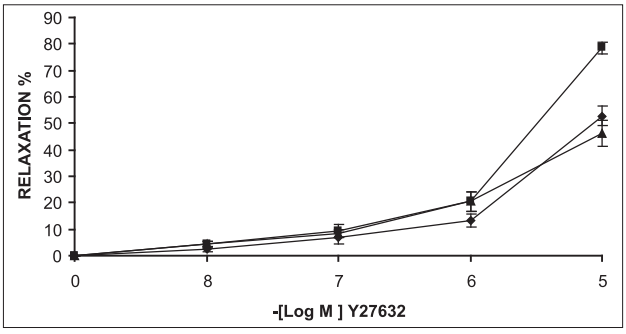

Fig. 1. Rho-kinase inhibitor, Y-27632-induced relaxation in rabbit renal artery in presence of Ca channel blocker of nifedipine (n: 6, ■), DMSO (n: 7, ♦) (solvent of nifedipine) and in 2.5 mmol/LCa Krebs (n: 8, ●). Data are mean±SEM and are expressed as percentage of maximal contraction to phenylephrine. * p< 0.001.

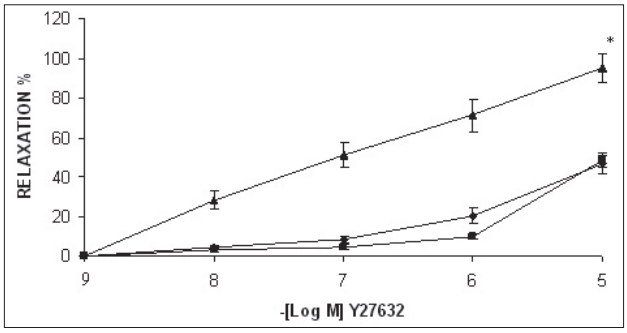

Fig. 2. Cumulative dose response curve for Y-27632 in 2.5 mmol/LCa Krebs (n: 8, ♦), after depletion of intracellular Ca stores (n: 7 ,■), in Ca free Krebs (n: 6 ▲) in rabbit renal artery. Data are expressed as mean±SEM. p<0.0001.

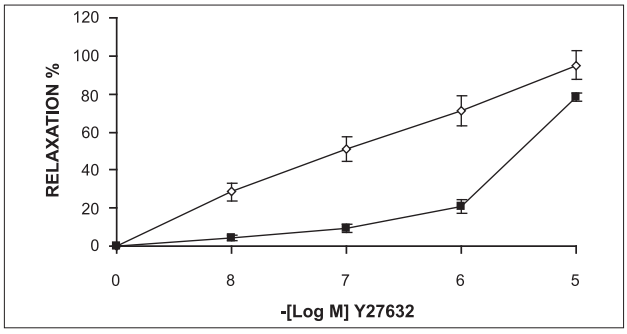

Fig. 3. Cumulative dose response curve for Y-27632 in presence of nifedipine (n: 6, ♦) and in Ca free Krebs (n: 6, ■), in rabbit renal artery. Data are expressed as mean±SEM. p>0.05.

was significantly increased in Ca^{2+}-free Krebs containing 100 µmol/l EGTA (95.1±7.4 %) but after depletion of intracellular stores with 20 mmol/l caffeine and 1mmol/l EGTA in Ca^{2+} free Krebs (48.7±3.9 %) there was no significant difference between the relaxation to Y-27632 from control response in 2.5 mmol/l Ca^{2+} Krebs in renal artery (Fig. 2). The pD_2 values of Y-27632 were 5.6±0.1 in 2.5 mmol/l Ca^{2+} Krebs, 4.4±0.3 in Ca^{2+}-free Krebs containing 100 µmol/l EGTA and 20 mmol/l caffeine and 6.5±0.2 in 1 mmol/l EGTA in Ca^{2+} free Krebs, and significantly different in all groups ($p<0.0001$)

As shown in the Figure 3, the maximal relaxation response of Y-27632 in presence of 1 mmol/l EGTA in Ca^{2+} free Krebs was higher than when voltage-gated Ca entry was blocked with nifedipine. Although a difference has been observed between groups it was not significant ($p=0.056$) (Fig. 3). Also pD_2 values of Y-27632 were significantly higher in the presence of 1mmol/l EGTA in Ca^{2+} free Krebs (6.49±0.2) than in that of nifedipine (4.49±0.2) ($p\leq0.001$).

Discussion

The present results indicate that Ca^{2+}-dependent mechanisms have a role in rho-kinase inhibitor-induced relaxation responses in rabbit renal artery precontracted with phenylephrine.

Rho-kinase inhibitor causes relaxation in vascular and other smooth muscles precontracted with different constrictors. It was reported that the rho-kinase inhibitor caused 55 % relaxation responses in the mouse anococcygeus muscle while 90 % in rabbit aorta (14). Also, Ark et al reported more than 75 % relaxation response of rho-kinase inhibitor Y-27632 in rat renal artery precontracted with ouabain (15). In our study we showed that rho-kinase inhibitor diminished 46 % of contraction induced by phenylephrine. According to these reports we suggested that activation of Rho/Rho-kinase pathway with different agonists in distinct smooth muscles caused different rates of relaxation responses.

Until now it has been known that a Rho/ Rho-kinase pathway-induced contraction is mediated by Ca^{2+} sensitization. However, there are some studies that revealed the relationship between Ca mobilization and Rho/rho-kinase pathway in different smooth muscles. A recent study has reported that Rho/Rho-kinase pathway affects agonist-induced Ca^{2+} mobilization. In this study it is well established that receptor-operated Ca^{2+} influx is inhibited by rho-kinase inhibition in tracheal smooth muscle (16). In another study, Shabir et al have shown that the rho-kinase activity without agonists modulates the myogenic contraction in both Ca^{2+}-dependent and independent mechanisms in ureteral smooth muscle (6). In another perspective it was reported that the involvement of Ca^{2+} entry induces the rho-kinase activation (17).

It has been shown that agonist-induced activation of Rho/Rho-kinase pathway causes contraction via Ca^{2+} sensitization in renal artery (18). It is also known that the increase in Ca^{2+} evoked by phenylephrine in renal artery originates from inositol triphosphate due to the release of Ca^{2+} from sarcoplasmic reticulum, capacitative Ca^{2+} entry intracellular store (19) and entry of Ca^{2+} through voltage-dependent Ca^{2+} channels (20). In our study we hypothesized the role of Ca^{2+} mobilization in Rho/Rho-kinase-induced contraction. According to our results, Y-27632 inhibited the contraction induced by phenylephrine in a concentration-dependent manner in an absence of extracellular Ca^{2+}. Also inhibiting the Ca^{2+} influx through voltage-gated Ca^{2+} channel attenuated the rho-kinase inhibition-induced relaxation. Our data are consistent with Ca^{2+} influx through voltage-operated channels affecting the rho-kinase activation in vascular smooth muscle (17). However, there is an increase in relaxation response induced by rho-kinase inhibitor in the presence of total withdrawal of Ca^{2+} compared to inhibition of Ca^{2+} entry through L-type Ca^{2+} channels. Nevertheless, the increase is not clearly significant at all concentrations (Fig 3). These results indicate that in addition to Ca^{2+} influx through voltage-operated channels, other Ca^{2+} influx pathways may also affect the inhibition of rho-kinase enzyme in renal artery. On the other hand, similarly to Shabir et al data, our results show that the depletion of sarcoplasmic reticulum Ca^{2+} stores did not contribute to the effects of rho-kinase inhibition (16).

In conclusion, we have determined the involvement of extracellular Ca^{2+} and L- type voltage-operated Ca^{2+} channels in phenylephrine-induced Rho-kinase activation that in addition to its role in Ca^{2+} sensitization of the contractile proteins in renal artery there is a relationship between Ca^{2+}mobilization and rho-kinase activation. The distinct role of Rho/Rho-kinase pathways in phenylephrine-induced contraction in renal artery may be attributable to a different Ca^{2+} entry in renal artery smooth muscle.

Therefore, a better understanding of intracellular mechanisms of contraction and relaxation of renal artery is of fundamental interest to improve the urological treatment.

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