Vasorelaxing Action of Rutaecarpine: Effects of Rutaecarpine on Calcium Channel Activities in Vascular Endothelial and Smooth Muscle Cells¹

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Received for publication January 28, 1999 This paper is available online at http://www.jpet.org

ABSTRACT

Rutaecarpine (Rut) has been shown to induce hypotension and vasorelaxation. In vitro studies indicated that the vasorelaxant effect of Rut was largely endothelium-dependent. We previously reported that Rut increased intracellular Ca²⁺ concentrations ([Ca²⁺]i) in cultured rat endothelial cells (ECs) and decreased [Ca²⁺]i in cultured rat vascular smooth muscle (VSMCs) cells. The present results showed that the hypotensive effect of Rut (10–100 μg/kg i.v.) was significantly blocked by the nitric oxide synthase inhibitor Nω-nitro-L-arginine. In aortic rings, Rut (0.1–3.0 μM)-induced vasorelaxation was inhibited by Nω-nitro-L-arginine and hydroquinone but not by antagonists of the various K⁺ channels. Thus, Rut was suggested to regulate Ca²⁺ influx and at 1.0 μM increased cyclic GMP (cGMP) production in endothelium-intact rings and to a lesser extent in endothelium-denuded rings. In whole-cell patch-clamp recording, nonvoltage-dependent Ca²⁺ channels were recorded in ECs and Rut (0.1, 1.0 μM) inhibited significantly the L-type voltage-dependent Ca²⁺ channels. In ECs, Rut (0.1, 1.0, 10.0 μM) increased nitric oxide release in a Ca²⁺-dependent manner. Taken together, the results suggested that Rut lowered blood pressure by mainly activating the endothelial Ca²⁺-nitric oxide-cGMP pathway to reduce smooth muscle tone. Although the contribution seemed to be minor in nature, inhibition of contractile response in VSMCs, as evidenced by inhibition of Ca²⁺ currents, was also involved. Potassium channels, on the other hand, had no apparent roles.

Rutaecarpine (Rut) is a quinazolinocarboline alkaloid isolated from a well known Chinese herbal drug Wu-Chu-Yu, the dried, unripe fruit of Evodia rutaecarpa (Juss) Benth. Pharmaceutical preparations based on Wu-Chu-Yu have been widely used in China for hundreds of years to treat gastrointestinal disorders, dysmenorrhea (Chang and But, 1986), and hypertension (Tang and Eisenbrand, 1992). The reported biological effects of Rut include diuresis, perspiration (Kametani, 1977), uterotonic action (King et al., 1980), improvement in cerebral functions (Yamahara et al., 1988), vasorelaxation (Chiou et al., 1994), and antinociception (Matuda et al., 1997). Rut also inhibits KCN-induced anoxia (Yamahara et al., 1989), specific 2,3,7,8-tetrachlorodibenzo-p-dioxin binding (Gillner et al., 1989), cytochrome P-450 isozyme (Rannug et al., 1992), and platelet aggregation (Sheu et al., 1996).

In a previous study (Wang et al., 1996), we showed that Rut lowered blood pressure in anesthetized rats and inhibited the tension development induced by norepinephrine (NE) in rings from rat aortae mainly in an endothelium-dependent manner, although the vasorelaxation effect persisted in endothelium-denuded aorta, albeit to a much reduced extent. Using the [Ca²⁺]i detecting fura-2/AM (1-[2-(5-carboxyoxazol-2-yl)-6-aminoenzoburan-5-oxyl]-2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N,N-tetraacetic acid pentaacetoxymethyl ester) technique, Rut was shown in the same study to sustain an increase in [Ca²⁺]i in cultured endothelial cells (ECs) through activation of Ca²⁺ influx, possibly via Ca²⁺ release from intracellular stores in vascular smooth muscle cells (VSMCs), suggesting the inhibition of Ca²⁺ channels. Thus, Rut was suggested to regulate Ca²⁺ chan-

ABBREVIATIONS: Rut, rutaecarpine; EC, endothelial cell; VSMC, vascular smooth muscle cell; NO, nitric oxide; l-NNA, Nω-nitro-L-arginine; NE, norepinephrine; VDCC, voltage-dependent Ca²⁺ channel; KCa, Ca²⁺-dependent K⁺; KV, voltage-dependent K⁺; MAP, mean arterial pressure; FCS, fetal calf serum; HBSS, Hanks’ balanced salt solution; ROCC, receptor-operated Ca²⁺ channel; I-V, current voltage.

Accepted for publication January 28, 1999
nels in ECs and VSMCs in opposing manners in achieving vasorelaxation. As the definition of Rut’s actions on Ca\(^{2+}\) fluxes had been based on indirect evidence, in the present study, characterization of the biochemical mechanisms of Rut was augmented by direct assessment of changes in Ca\(^{2+}\) channel activities in single cultured rat aortic ECs and VSMCs using the whole-cell version of the patch-clamp technique.

Nitric oxide (NO) generation has been implicated in the vasorelaxant effect of Rut (Chiu et al., 1994). To determine whether NO is involved in Rut-induced decrease in mean arterial pressure (MAP) and vasorelaxation, we investigated the influence of NO-nitro-l-arginine (l-L-NNa), an NO synthase inhibitor (Wang et al., 1993), and hydroquinone, an NO inactivator (Moncada et al., 1986), on the effects of Rut in vivo and/or in vitro. Because the functional NO synthase in the endothelium is believed to be constitutive and Ca\(^{2+}\)-dependent, we also measured Rut-induced NO production in ECs under both normal Ca\(^{2+}\) and Ca\(^{2+}\)-free conditions by a sensitive chemiluminescence technique. Evidence indicates that the NO-induced vasorelaxation is a result of cGMP production (Rapport and Murad, 1983). To determine whether the Rut-induced vasorelaxation was because of an increase in cGMP, the levels of cGMP were monitored.

Increases in [Ca\(^{2+}\)]\(_i\) could also trigger the release of a K\(^+\) channel-activating endothelium-derived hyperpolarizing factor from the ECs (Chen and Suzuki, 1990). In addition, one possible mechanism of NO or cGMP-induced vasorelaxation is the activation of Ca\(^{2+}\)-dependent K\(^+\) (K\(_{ca}\)) channels (Arrer et al., 1994; Bolotina et al., 1994). Moreover, it is now well established that the opening of K\(^+\) channels serves to hyperpolarize the cell membrane and also interacts with Ca\(^{2+}\) channels in the VSMC plasma membrane as well as intracellular Ca\(^{2+}\) release, leading to relaxation of vascular smooth muscle (Kühberger et al., 1993). To assess whether the vasorelaxing effect of Rut was mediated by K\(^+\) channels, 4-aminopyridine, a specific inhibitor of voltage-dependent K\(^+\) (K\(_v\)) channels (Okabe et al., 1987), apamin, a specific blocker of the small conductance K\(_{ca}\) channels (Nakao et al., 1986), charybdotoxin, a specific blocker of the large conductance K\(_{ca}\) channels (Gimenez-Gallego et al., 1988), and glibenclamide, a specific inhibitor of ATP-sensitive K\(^+\) channels (Quast and Cook, 1989) were used to define the possible mechanisms of action.

The results obtained show that the activation of the endothelial Ca\(^{2+}\)-NO-cGMP cascade could largely account for the hypotensive and vasorelaxant actions of Rut. A minor direct Ca\(^{2+}\) influx inhibition, through both voltage-dependent Ca\(^{2+}\) channels (VDCCs) and receptor-operated Ca\(^{2+}\) channels (ROCCs), in VSMCs also seemed to be involved. In contrast, K\(^+\) channels seemed not to be involved.

**Materials and Methods**

**Rats**

Adult male Sprague-Dawley rats, weighing 200 to 250 g (Taconic Farms, Germantown, NY), were used. The rats were allowed to acclimate in environmentally controlled quarters with temperature maintained at 20–22°C, relative humidity 55%, and lighting with 12:12-h light/dark cycles. Standard laboratory chow (Purina Mills, Richmond, IN) and drinking water were provided ad libitum.

**Blood Pressure Measurement**

Rats were anesthetized i.p. with 50 mg/kg sodium pentobarbital and placed on a heating pad to maintain body temperature at 37°C. The right femoral artery was cannulated for the recording of arterial blood pressure with a Gould model 3400S polygraph (Gould, Valley View, OH) via a P23XL pressure transducer (Viggo-Spectramed, Oxnard, CA). Polyethylene catheters (PE-50; Clay Adams, Parsippany, NJ) were also inserted into both femoral veins for the administration of drugs. One venous catheter was used for bolus i.v. injection of Rut (10, 30, or 100 µg/kg). The second venous catheter was used for continuous infusion of saline or saline containing l-L-NNa (2.22 mg/kg/min) at a rate of 0.5 ml/min for 30 min before the administration of Rut and throughout the entire duration (45 min) of the experiment. Dose-dependent MAP changes in response to bolus i.v. injection of Rut was determined. The changes in MAP from nine rats given Rut in the presence of l-L-NNa were compared with those from another nine rats that received Rut at the same dose but without l-L-NNa.

**In Vitro Vascular Tension Study**

Another group of rats was sacrificed by decapitation. Sections of the thoracic aortae between the aortic arch and the diaphragm were excised carefully and fixed isometrically in organ chambers (15 ml) containing a modified Krebs’ solution: 120 mM NaCl, 4.5 mM KCl, 2.5 mM CaCl\(_2\), 1 mM MgSO\(_4\), 27 mM NaHCO\(_3\), 1 mM KH\(_2\)PO\(_4\), and 10 mM glucose maintained at 37°C and through which a mixture of 95% O\(_2\), 5% CO\(_2\) was bubbled. The details of the preparation procedure have been described previously (Wang et al., 1996). Briefly, aortic rings of 3 to 4 mm in length were equilibrated under passive tension of 1.0 g for 60 min. During this time, the tissues were washed every 15 min. After equilibration, the aortic rings were stabilized with a near maximal contraction induced by phenylephrine (0.3 µM). After the rings achieved a stable contractile tension, acetylcholine (1 µM) was added to the baths to assess endothelial integrity. In some preparations, the intima was gently frayed with a cotton swab to disrupt the endothelium. The absence of acetylcholine-induced relaxation indicated that the vessels were denuded successfully.

**Effects of Inhibitors on the Rut-Induced Vasorelaxation**

For the evaluation of relaxation, Rut (0.1–3.0 µM) was added in a cumulative manner during the tonic phase of contraction (considered as 100%) induced by phenylephrine (0.3 µM) in both endothelium-intact and -denuded aortic rings. Construction of concentration-response curves for Rut was based on percent of relaxation of the phenylephrine-induced contraction. To determine the contribution of endothelium-derived NO in the vasorelaxing effect of Rut, the endothelium-intact preparations were preincubated with l-L-NNa (100 µM) or hydroquinone (10 µM) for 10 min before phenylephrine-induced contraction. Then, the cumulative doses of Rut (0.1–3.0 µM) were applied during the sustained phase of contraction. To determine the contribution of K\(^+\) channels in the vasorelaxing effect of Rut, another group of endothelium-intact preparations was preincubated with various K\(^+\) channel blockers such as 4-aminopyridine (5 mM), apamin (0.1 µM), charybdotoxin (0.1 µM), or glibenclamide (1 µM) for an appropriate period (10–20 min) before phenylephrine-induced contraction. The concentration-response curves of Rut (0.1–3.0 µM) in the presence of various blockers were constructed as described above. To obtain similar control tension generation in the presence or absence of blocker, 0.1 or 3.0 µM phenylephrine, respectively, was used to induce contraction. The effects of the various blockers were studied by comparing the degrees of vasorelaxation induced by Rut in the absence and presence of those blockers. The dosages of the blockers used have been reported to be adequate to produce the necessary NO and K\(^+\) channel blockade.
Effects of Extracellular Ca\textsuperscript{2+} on Rut\textquotesingle s Modulation of NE-Induced Contraction

To delineate the inhibition of extracellular Ca\textsuperscript{2+} influx involved in Rut-induced relaxation, after equilibration, the experiments were carried out in Ca\textsuperscript{2+}-free Krebs\textquotesingle s solution. Addition of NE (0.1 \textmu M) induced a transient vasocontraction, which was dependent on intracellular stored Ca\textsuperscript{2+} release (Suematsu et al., 1984). As the NE-induced contraction reached a steady state, cumulative concentrations of Ca\textsuperscript{2+} (0.1–3.0 \textmu M) were applied to permit the observation of the effects of a step-wise increase in extracellular Ca\textsuperscript{2+} concentration. The contraction by NE under such conditions was therefore the result of Ca\textsuperscript{2+} influx from extracellular space. After washing and equilibration for 30 min, the experiment was repeated in the presence of Rut (0.1, 1.0 \textmu M) or vehicle for 5 min. With the maximal tension attained by vehicle at Ca\textsuperscript{2+} (3.0 \textmu M) being considered as 100\%, concentration-response curves for the added Ca\textsuperscript{2+} were constructed and compared in endothelium-intact and -denuded aortic rings.

cGMP Measurement

The method was essentially the same as reported previously (Huang et al., 1995). Aortic rings were isolated as described above. The tissues were incubated in Krebs\textquotesingle s solution with Rut (1.0 \textmu M) or vehicle at 37°C for 10 min. The cyclic nucleotide phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (10 \textmu M) was added during the last 5 min of the incubation period. The reaction was stopped by immersing the tissue in liquid nitrogen and storing at −80°C up to the time of thawing in chilled 6\% trichloroacetic acid. The thawed tissues were homogenized and centrifuged at 10,000 g for 5 min. The supernatant fractions were extracted four times with 5 volumes of ethanol and stored at −20°C. The cGMP levels were assayed for cGMP content by radioimmunoassay (radioimmunoassay supernatant fractions were extracted four times with 5 volumes of ethanol and stored at −20°C). The thawed tissues were homogenized and centrifuged at 10,000 g for 5 min. The supernatant fractions were extracted four times with 5 volumes of ethanol and stored at −20°C. The thawed tissues were homogenized and centrifuged at 10,000 g for 5 min. The supernatant fractions were extracted four times with 5 volumes of ethanol and stored at −20°C.

In Vitro Whole-Cell Patch-Clamp Recording

Cell Culture. Rat aortic ECs were prepared and identified as previously described (Wang et al., 1996). The standard maintenance growth medium (minimum essential medium) was supplemented with 10\% fetal calf serum (FCS) and antibiotics. Single VSMCs were isolated by collagenase-elastase dissociation from the rat thoracic aorta by using previously published procedures developed in our laboratory (Wang et al., 1996). The cells were grown in Dulbecco\textquotesingle s modified Eagle\textquotesingle s medium supplemented with 10\% FCS and antibiotics. All growth was carried out in a humidified incubator equilibrated with 5\% CO\textsubscript{2} atmosphere at 37°C. ECs exhibited typical cobblestone appearance and expressed factor VIII antigen. Examination by phase-contrast microscopy showed that VSMCs formed a uniform layer of cells with few cell gaps. ECs were identified as a monolayer of cells with a uniform layer of cells with a cobblestone appearance.

Electrophysiology. Ca\textsuperscript{2+} channel activity was determined in single ECs and VSMCs by the whole-cell version of the patch-clamp technique (Hamill et al., 1981). Whole-cell Ca\textsuperscript{2+} current measurements were carried out using an Axopatch-2C patch clamp amplifier (Axon Instruments, Foster City, CA). Patch pipettes were pulled and fire polished from borosilicate glass capillaries (o.d., 1.2 mm; i.d., 0.9 mm; FHC, Brunswick, ME) with a wall thickness of 0.3 mm. The tip diameter was approximately 1 \textmu m with a resistance range of 4 from 8 MΩ when filled with pipette solution. Further application of suction via a tube attached to the pipette holder permitted the formation of a gigaseal and the harvesting of a patch membrane. In all experiments, Ba\textsuperscript{2+} was used as the charge carrier. Because the inward Ba\textsuperscript{2+} currents were small and the series resistance was less than 0.1 ohms, series-resistance compensation was not usually used. The currents were monitored using a digital oscilloscope (Nicolet Instrument Corp., Madison, WI) and filtered at 1 kHz with a low pass filter (Axon Instruments). The software pCLAMP and a labmaster interface (Axon Instruments) were used for the generation of test pulses and storage and analysis of data. Leakage and capacitive currents were subtracted during analysis. Simultaneously slow records were taken on an SC 284 chart recorder (Gould, Valley View, OH). All recording was done at room temperature (20–22°C).

Effects of Rut on Ca\textsuperscript{2+} Channel Activity in ECs

To generate current-voltage (I-V) curves, the Ba\textsuperscript{2+} current through the Ca\textsuperscript{2+} channels was elicited by depolarizing the VSMC from a test pulse of −30 mV to more positive test potentials at a frequency of 0.1 Hz. The duration of the depolarizing test pulses was 250 ms at intervals of 5 s. Peak currents were used to construct the I-V relationships. Only cells showing stable channel activity for at least 5 min were used to test the effect of Rut. The I-V relationships were measured repeatedly for 5 min after the addition of Rut (10.0 \textmu M) or vehicle in the medium. At the end, Bay K 8644 (5 \textmu M), a Ca\textsuperscript{2+} slow channel agonist, and nifedipine (10 \textmu M), a dihydropyridine Ca\textsuperscript{2+} channel blocker, were added to confirm that the inward currents recorded were carried almost exclusively by L-type Ca\textsuperscript{2+} channels.

NO Measurement

ECs were grown in 35-mm² dishes. Upon reaching confluence in about 4 days, the medium was changed to HBSS with L-arginine (100 \textmu M) and added CaCl\textsubscript{2} (to 2.5 mM). The changing over to HBSS was necessary because it provided the least interference in the assay. However, additional Ca\textsuperscript{2+} was required to make the final concentra-
tion comparable with that in normal Krebs’ solution. The cells were then equilibrated for 60 min at 37°C. Aliquots of the supernatant were collected for analysis of nitrite by chemiluminescence (considered as 100%). Rut (1.0, 10.0 μM) or vehicle was then added for 10 min to stimulate NO release. The cell supernatants were also collected for analysis of nitrite. To study whether Rut-induced NO release was dependent on extracellular Ca²⁺, similar experiments were also carried out in Ca²⁺-free HBSS containing EGTA (2 mM). Samples (100 μl) containing nitrite were measured by adding a reducing agent (1% KI in glacial acetic acid) to the purge vessel to convert nitrite to NO, which was then carried by a flow of helium to the NO analyzer (model 280; Sievers Research Inc., Boulder, CO). Nitrite concentrations were calculated by comparison with standard solution of sodium nitrite. The number of cells in each dish was counted immediately by the 0.4% trypan blue exclusion method. The results, normalized to amount (picomoles) per 10⁶ cells, were expressed as percentage of changes with basal NO content being 100%.

**Drugs**

The following drugs were used: Rut (mw 287.31) was isolated and purified by the National Research Institute of Chinese Medicine (Taipei, Taiwan, Republic of China) (Lin et al., 1991); acetylcholine, 4-aminopyridine, FCS, hydroquinone, 3-isobutyl-1-methylxanthine, NE, nifedipine, tNNA, phenylephrine, and sodium nitrite were purchased from Sigma Chemical Co. (St. Louis, MO); apamin and glibenclamide were purchased from RBI Research Biochemicals Co. (Natick, MA); Bay K 8644 was obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA); charybdoxin was purchased from Alomone Labs (Jerusalem, Israel); and Dulbecco’s modified Eagle’s medium and minimum essential medium were purchased from Life Technologies Inc. (Gaithersburg, MD). Apamin was dissolved in 0.05 M acetic acid. Bay K 8644, glibenclamide, and nifedipine were dissolved in absolute ethanol. Charybdoxin was dissolved in Krebs’ buffer. Other drugs were dissolved in distilled water and kept at −20°C. Except Rut, which was dissolved in dimethyl sulfoxide and ethanol mixture (2:8) to make 0.1 to 10 mM stock solutions. The final concentration of the vehicle in the solution did not exceed 0.1%, which had no effects on vascular tension, cGMP levels, magnitude/kinesis of the inward current of ECs and VSMCs, or NO production.

**Statistical Analysis**

The data are presented as mean ± S.E., and n represents the number of experiments. In most cases, it was the peak inward current that was used for displays. Statistical analysis was made by Student’s unpaired t test when applicable, P values less than 0.05 were considered to be significant.

**Results**

**Antagonistic Effect of tNNA on the Rut-Induced Hypotension.** A 30-min infusion period of tNNA elevated MAP from the baseline level of 104 ± 4 mm Hg progressively to 138 ± 4 mm Hg. This elevated MAP was sustained throughout the entire period of the experiment. Normal saline infusion did not significantly change the baseline MAP of 103 ± 4 mm Hg. As Rut was dissolved in a mixture of dimethyl sulfoxide and ethanol (2:8), the vehicle was first tested for possible effects on MAP. The decrease in MAP of 8 ± 3 mm Hg was statistically not significant. Consistent with a previous report (Wang et al., 1996), i.v. injection of Rut in anesthetized rats induced a decrease in MAP in a dose-dependent manner (Fig. 1). tNNA infusion significantly attenuated the MAP-lowering effects of the higher doses of Rut (Fig. 1).

**Effects of Inhibitors on the Rut-Induced Vasorelaxation.** As previously reported (Wang et al., 1996), Fig. 2 shows that Rut (0.1–3.0 μM) caused a concentration-dependent relaxation in phenylephrine-induced contraction in endothelium-intact rat thoracic aorta. Arterial rings were precontracted with phenylephrine, and the change in tension is expressed as a percentage of the active tension originally generated by phenylephrine. The tensions developed in the absence of Rut and agents in endothelium-intact and -denuded rings were 1384.0 ± 70.0 and 1656.1 ± 69.7 mg, respectively. Values are mean ± S.E.; n = 7 to 8 in each group. * and **, statistically significant difference (P < .05) between the normal saline and the tNNA-treated group.

**Effects of Extracellular Ca²⁺ on Rut’s Modulation of NE-Induced Contraction.** In Ca²⁺-free Krebs’ solution,
dependent on extracellular Ca\(^{2+}\) was treated with Rut at 0.1 or 1.0 mM in endothelium-intact rat thoracic aorta. Arterial rings were precontracted with phenylephrine, and the change in tension is expressed as a percentage of the active tension originally generated by phenylephrine. The tensions developed in the absence of Rut and agents in endothelium-intact rings was 1364.0 \pm 70.0 mg. Values are mean \pm S.E.; \(n = 7\) to 8 in each group.

NE induced only a transient contraction, and the intracellular Ca\(^{2+}\) store was readily depleted. By adding Ca\(^{2+}\) (0.1–3.0 mM) back to the bathing buffer, NE induced a sustained contraction at levels that depended on the concentration of the external Ca\(^{2+}\), suggesting that Ca\(^{2+}\) influx was essential for the NE-induced contraction. Figure 4 shows that treatment with NE (0.1 \(\mu\)M) induced a Ca\(^{2+}\)-dependent contraction in endothelium-intact aortic rings. When the aortic ring was treated with Rut at 0.1 or 1.0 \(\mu\)M 5 min before NE, the NE-induced contraction was attenuated, suggesting that Ca\(^{2+}\) influx was probably involved. To determine the role of the endothelium in Rut-induced inhibition of Ca\(^{2+}\), similar experiments were conducted on endothelium-denuded aortic rings. Treatment with Rut at 1.0 \(\mu\)M but not at 0.1 \(\mu\)M inhibited the contraction caused by NE in buffers containing different concentrations of Ca\(^{2+}\) (Fig. 5).

Fig. 3. Effects of 4-aminopyridine (5 mM, □), apamin (0.1 \(\mu\)M, ◆), charybdotoxin (0.1 \(\mu\)M, △), and glibenclamide (1 \(\mu\)M, ▽) treatments on Rut (0.1–3.0 \(\mu\)M)-induced relaxation in endothelium-intact (●) rat thoracic aorta. Arterial rings were precontracted with phenylephrine, and the change in tension is expressed as a percentage of the active tension originally generated by phenylephrine. The tensions developed in the absence of Rut and agents in endothelium-intact rings was 1364.0 \pm 70.0 mg. Values are mean \pm S.E.; \(n = 7\) to 8 in each group.

Effects of Rut on cGMP. The basal cGMP levels in endothelium-intact and -denuded aortic rings were 1.31 \pm 0.17 and 0.32 \pm 0.04 pmol/mg, respectively (Fig. 6). In endothelium-intact tissues, treatment with Rut (1.0 \(\mu\)M) for 10 min significantly increased it 3- to 4-fold to 5.60 \pm 0.38 pmol/mg, whereas vehicle treatment resulted in an insignificant increase to 1.53 \pm 0.39 pmol/mg. In endothelium-denuded preparations, Rut induced a small but significant accumulation of cGMP, whereas vehicle treatment had insignificant effects (0.70 \pm 0.22 versus 0.30 \pm 0.05 pmol/mg protein).

Effects of Rut on Ca\(^{2+}\) Channel Activity in ECs. With Ba\(^{2+}\) as the permeable extracellular ion, the inward current averaged 5.60 \pm 2.40 pA. The concentrations of Rut chosen were 0.1 and 1.0 \(\mu\)M as they appeared to be in the linear section of the concentration-relaxation curve, the relaxation induced by 3.0 \(\mu\)M being considered maximal (Fig. 2). Figure 7 shows a typical recording of inward currents during the application of Rut (0.1, 1.0 \(\mu\)M) or vehicle in three different preparations, Rut induced a small but significant accumulation of cGMP, whereas vehicle treatment had insignificant effects (0.70 \pm 0.22 versus 0.30 \pm 0.05 pmol/mg protein).

Fig. 4. Inhibitory effect of Rut (0.1 and 1.0 \(\mu\)M) on the contraction, dependent on extracellular Ca\(^{2+}\) influx, induced by NE (0.1 \(\mu\)M) in Ca\(^{2+}\)-free Krebs’ solution of endothelium-intact rat thoracic aortic rings from Sprague-Dawley rats. Aorta was preincubated with vehicle (●) or Rut (0.1 \(\mu\)M, ▲; 1.0 \(\mu\)M, ■) at 37°C for 5 min; then, cumulative concentrations of Ca\(^{2+}\) (0.1–3.0 mM) were used to trigger the contraction. The mean maximum contractile responses induced by Ca\(^{2+}\) (3.0 mM) in the absence of Rut in endothelium-intact rings was 444.8 \pm 29.1 mg. Values are mean \pm S.E.; \(n = 10\) to 12 in each group. * and **, statistically significant difference \((P < .05 and P < .01, respectively)\) between the vehicle and the Rut-treated group.

Fig. 5. Inhibitory effect of Rut (0.1 and 1.0 \(\mu\)M) on the contraction, dependent on extracellular Ca\(^{2+}\) influx, induced by NE (0.1 \(\mu\)M) in Ca\(^{2+}\)-free Krebs’ solution of endothelium-denuded rat thoracic aortic rings from Sprague-Dawley rats. Aorta was preincubated with vehicle (○) or Rut (0.1 \(\mu\)M, △; 1.0 \(\mu\)M, □) at 37°C for 5 min; then, cumulative concentrations of Ca\(^{2+}\) (0.1–3.0 mM) were used to trigger the contraction. The mean maximum contractile responses induced by Ca\(^{2+}\) (3.0 mM) in the absence of Rut in endothelium-denuded rings was 684.6 \pm 32.8 mg. Values are mean \pm S.E.; \(n = 10\) to 12 in each group. **Statistically significant difference \((P < .01)\) between the vehicle and the Rut-treated group.

Fig. 6. Rut (1.0 \(\mu\)M) increased cGMP accumulation in isolated endothelium-intact (●) and -denuded (○) aortae. Basal cGMP contents in endothelium-intact and -denuded aortic rings were 1.31 \pm 0.17 and 0.32 \pm 0.04 pmol/mg protein, respectively. The amounts of cGMP generated in response to vehicle in both endothelium-intact and -denuded rings were not significantly different. Values are mean \pm S.E.; \(n = 12\) in each group. * and ***, statistically significant difference \((P < .05 and P < .001, respectively)\) between the vehicle and the Rut-treated group.
The concentration dependence of the peak amplitude of the Ca\(^{2+}\) current is plotted in Fig. 8. When the hyperpolarized ECs was treated with Rut (0.1 \(\mu\)M), a long-lasting inward current of up to 25 pA was observed, whereas vehicle alone had insignificant effect on the current (4.48 ± 1.04 pA). The current reached a peak after about 10 s, remained at a steady current of 17.20 ± 6.88 pA for 19 s, and then returned to the holding potential. Rut (1.0 \(\mu\)M) significantly increased the amplitude and caused a sustained increase in Ca\(^{2+}\) currents to 34.72 ± 5.60 pA and then declined over a period of 2 to 3 min in the continuing presence of Rut. The average duration of the Ca\(^{2+}\) currents, measured midway between the holding potential and peak negativity, was 35 s with Rut (1.0 \(\mu\)M).

**Effects of Rut on Ca\(^{2+}\) Channel Activity in VSMCs.**

VSMCs were depolarized from -30 to 60 mV with the ramp protocol to investigate the channel openings. Ba\(^{2+}\) currents through long-lasting L-type Ca\(^{2+}\) channels were observed in VSMCs. During a 5-min application of vehicle alone, no significant changes (−0.5 ± 1.2%) in the kinetics and I-V relationship of L-channel current occurred (not shown). Figure 9 shows that a 5-min application of Rut (10 \(\mu\)M) reduced the L-type current to below the immediately preceding current measured in vehicle-treated specimen. The decrease in the magnitude of L-channel currents induced by Rut was evident within 2 to 3 min and reached a steady-state level within 5 min. Figure 10 summarizes the results from several experiments; the maximal reduction caused by Rut was 12.31 ± 3.30%.

**Effects of Rut on NO Production.** The average basal NO content in cultured ECs was 65.6 ± 5.6 pmol/10\(^6\) cells. Rut (1.0 and 10.0 \(\mu\)M) significantly stimulated NO production in ECs in HBSS containing Ca\(^{2+}\) in a concentration-dependent manner (Fig. 11). The vehicle itself did not significantly alter the basal NO in ECs. Rut in Ca\(^{2+}\)-free, EGTA-added HBSS had no effect on NO production (Fig. 11).

**Discussion**

Previous studies have indicated that Rut exerts hypotensive effects in the rat and dilates isolated aortic ring. The
actions are largely endothelial dependent and likely involve Ca²⁺ fluxes, mobilization, and NO release (Wang et al., 1996). Using patch-clamping techniques and cultured cells, the present study focused on Rut’s cellular actions in ECs and VSMCs.

With K⁺ and Na⁺ channels blocked, whole-cell patch-clamp studies in isolated cultured ECs indicated Rut elicited macroscopic ionic currents. Such Ca²⁺ currents, induced by 0.1 and 1.0 μM Rut, were relevant to the concentration-dependent vasorelaxing effects of Rut on endothelium-intact aortic rings. These inward Ca²⁺ current measurements also corroborated previous spectrofluorometric observations (Wang et al., 1996), indicating that the augmentation in inward Ca²⁺ currents correlated with exaggerated Ca²⁺ entry. Taken together, these observations suggested that Rut modulated Ca²⁺ fluxes directly through some Rut-sensitive mechanisms in the Ca²⁺ channels. However, there was a conspicuous temporal discrepancy between Rut-induced membrane activation, as reflected by the rise in Ca²⁺ currents, and increase in [Ca²⁺]. Several possibilities might help to explain such a temporal lapse. First, patch-clamp measurements represent only changes in the Ca²⁺ currents per se. The rapid development of the Rut-induced inward Ca²⁺ currents might reflect the opening of the Ca²⁺ channel, whereas the subsequent decay might reflect the kinetics of drug and action site interactions and inner kinetics of Ca²⁺ channels themselves. Second, Ca²⁺ channel opening may just trigger off a cascade of intracellular events leading eventually to increase in [Ca²⁺], a process that may take much longer to complete. Finally, a finite time is needed for the penetration of Rut from the edge of the confluent ECs to those cells in the optical recording field used in the fura-2/AM studies.

The fact that the NO inhibitors l-NNA and hydroquinone readily blocked Rut-induced endothelium-dependent relaxation in the rat aorta suggested that NO was likely the mediator responsible. Examination using a sensitive chemiluminescence NO detection method in this study indicated that Rut increased NO production in a Ca²⁺-dependent manner. Together, with a concomitant elevation in cGMP production, these observations were consistent with the notion that a rise in [Ca²⁺], led to enhancement of the NO-cGMP vasorelaxing axis.

Patch-clamping studies in isolated VMSCs indicated again Rut directly inhibited Ca²⁺-generated currents in the L-type VDCCs, the predominant Ca²⁺ channels in VMSCs. However, Rut also inhibited NE-induced contraction, suggesting that it also attenuated Ca²⁺ influx through ROCCs. Thus, it seems that suppression of both VDCCs and ROCCs in VMSCs are involved. Therefore, in addition to inhibition of Ca²⁺ release from intracellular stores previously reported (Wang et al., 1996), it seems that Rut can also suppress membrane VDCCs and ROCCs in VMSCs.

The possibility of activation of K⁺ channels was examined with several types of antagonists including 4-aminopyridine, apamin, charybdotoxin, and glibenclamide. The failure of any of these antagonists to inhibit Rut’s actions precluded the involvement of K⁺ channels.

Figure 12 summarizes the major findings of this study. In essence, the proposed Rut-induced vasodilatory and hypotensive mechanisms consisted of major endothelial Ca²⁺-NO-cGMP actions as well as minor direct inhibition of membrane Ca²⁺ channel and intracellular Ca²⁺ release in VMSCs.

Acknowledgments

We thank Dr. Andrew Yau-Chik Shum of the Department and Institute of Pharmacology, The National Yang-Ming University, and Dr. Alan Maydwell for proofreading and editing the manuscript.

References


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Fig. 11. Rut (1.0 and 10.0 μM) increased NO production in cultured ECs isolated from Sprague-Dawley rat thoracic aorta in HBSS containing Ca²⁺ (○). Rut did not significantly alter the NO content in Ca²⁺-free plus EGTA HBSS (2 mM; □). Basal NO content in the ECs was 65.6 ± 5.6 pmol/10⁶ cells. The amounts of NO generated in response to vehicle in both Ca²⁺-containing and Ca²⁺-free plus EGTA (2 mM) were not significantly different. Values are mean ± S.E.; n = 9 to 11 in each group. * and **, statistically significant difference (P < .05 and P < .01, respectively) between vehicle and Rut-treated group.

Fig. 12. Proposed mechanism for Rut-induced vasorelaxation. Rut acts on both ECs and VSMCs directly, but to a lesser extent in the latter. In the ECs, Rut activates non-VDCCs and increases [Ca²⁺]i by inhibiting Ca²⁺ influx, leading to increased NO production, which then diffuses across to neighboring VSMCs to induce cGMP formation. In VSMCs, Rut reduces [Ca²⁺]i, by inhibiting Ca²⁺ entry, from both L-type Ca²⁺ channels and perhaps ROCCs, and stored Ca²⁺ release. Both mechanisms can produce vasorelaxation and therefore contribute to a decrease in blood pressure.


