

Research Article

***Cinnamomi ramulus* Ethanol Extract Exerts Vasorelaxation through Inhibition of Ca^{2+} Influx and Ca^{2+} Release in Rat Aorta**

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Contraction of vascular smooth muscle cells depends on the induction of cytosolic calcium ion (Ca^{2+}) due to either Ca^{2+} influx through voltage-gated Ca^{2+} channels or to receptor-mediated Ca^{2+} release from the sarcoplasmic reticulum. The present study investigated the vasorelaxation effect of *Cinnamomi ramulus* ethanol extract (CRE) and the possible mechanisms in rat aorta. CRE (0.1 mg/mL) relaxed vasoconstriction induced by phenylephrine (PE; 1 μM) and angiotensin II (5 μM). Preincubation with CRE significantly reduced the rat aortic contraction by addition of CaCl_2 in Ca^{2+} -free Krebs solution and FPL64176 (10 μM). Pretreatment with nifedipine (100 μM) or verapamil (1 μM) significantly reduced the CRE-mediated vasorelaxation of PE-induced vascular contraction. In addition, CRE also relaxed the vascular contraction caused by m-3M3FBS (5 $\mu\text{g}/\text{mL}$), but U73122 (10 μM) significantly inhibited the vasorelaxation of PE precontracted aortic rings. Furthermore, CRE significantly reduced the magnitude of PE- and caffeine (30 mM)-induced transient contraction. In vascular strips, CRE downregulated the expression levels of phosphorylated PLC and phosphoinositide 3-kinase elevated by PE or m-3M3FBS. These results suggest that CRE relaxes vascular smooth muscle through the inhibition of both Ca^{2+} influx via L-type Ca^{2+} channel and inositol triphosphate-induced Ca^{2+} release from the sarcoplasmic reticulum.

1. Introduction

Increasing cytosolic calcium ion (Ca^{2+}) concentration is essential for the contraction of smooth muscle cells. The increase results from the influx of Ca^{2+} through the plasma membrane and release of Ca^{2+} from intracellular stores, mainly the sarcoplasmic reticulum (SR) [1–4].

Phenylephrine (PE) or angiotensin II (Ang II) induces receptor-coupled G protein-induced phosphoinositide 3-kinase (PI3K) and phospholipase C (PLC) activation, resulting in Ca^{2+} -dependent vasoconstriction in smooth muscle cells [5–10]. PI3K activity facilitates the production of 3-phosphorylated phosphoinositides such as phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P₂), and phosphatidylinositol (3,4,5)-triphosphate (PI(3,4,5)P₃) [11, 12]. Among these, PI(3,4,5)P₃ stimulates the L-type Ca^{2+} channel (12), a voltage-dependent Ca^{2+} channel that plays an important role in the regulation of vascular tone [5, 12, 13].

Activated PLC is an effector in the stimulation of Ca^{2+} release from the endoplasmic reticulum (ER) or the SR [14–16]. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol (3, 4, 5)-triphosphate (IP₃) [14]. The latter is released as a soluble structure into the cytosol, where it binds to IP₃ receptors in the SR [15, 17]. This binding process increases the cytosolic Ca^{2+} concentration and smooth muscle constriction [15, 16].

The herb *Cinnamomi ramulus* (CR) has traditionally been used in Asia and Europe to treat maladies involving blood circulation and inflammation. In one study, an aqueous extract of CR ameliorated sucrose-induced blood pressure elevation in spontaneously hypertensive rats [18]. Recently, we reported that CR ethanol extract (CRE) reduces vascular contraction through the inhibition of voltage-dependent Ca^{2+} channels [19]. However, the possible mechanisms of CRE were not elucidated. The present study explored the suggestion that the vasodilatory effect of CRE is related to Ca^{2+} -dependent mechanisms in rat aorta.

2. Materials and Methods

2.1. Materials. Male Sprague-Dawley rats weighing 320–350 g were used for all experiments. All animals were provided with food and water *ad libitum* and allowed to adapt to the experimental conditions (temperature, $21 \pm 2^\circ\text{C}$; humidity, 50–60%) for 1 week. Rabbit polyclonal antibodies against phosphorylated PLC (pPLC), β -actin, and anti-rabbit secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif, USA). PI3K/p85 antibody was purchased from Cell Signaling Technology (Beverly, Mass, USA). PE, Ang II, verapamil, nifedipine, FPL64176, 2, 4, 6-trimethyl-N-(meta-3-trifluoromethylphenyl)-benzenesulfonamide (m-3M3FBS), U73122 and caffeine were purchased from Sigma-Aldrich (St. Louis, Mo, USA). PE, Ang II, nifedipine, and verapamil were dissolved in distilled water. FPL64176, U73122, m-3M3FBS, and caffeine were prepared in dimethylsulfoxide. All drugs were diluted in Krebs solution in the organ bath.

2.2. Plant Material. CR (twigs of *Cinnamomum cassia* Blume) collected in China in November 2009 was purchased from Humanherb (Gyeongsan, Korea). The identity of the purchased material was verified by H.M. Shin (College of Oriental Medicine, Dongguk University, Gyeongju, Korea). A voucher specimen (CRE08) has been deposited in the College of Oriental Medicine, Dongguk University.

2.3. Preparation of CRE. Dried CR (100 g) was extracted with 500 mL of 70% ethanol by heating at 75°C for 3 h. The extract was filtered through Whatman filter paper (Whatman International, Maidstone, UK) to remove the insoluble materials. After filtration, the extracts were concentrated by rotary evaporation using a model VV2000 apparatus (Heidolph, Walpersdorfer, Germany) at a temperature of 75°C and then dried using a model FD8508S freeze dryer (Ilshin, Busan, Korea). The yield of dry matter from the extracts was approximately 2.1%. The material was stored at 4°C until use. The EC_{50} value of 0.1 mg/mL CRE was used in all experiments. In a previous research [20], cinnamaldehyde and coumarin were analyzed as main compounds of CRE by gas chromatography-mass spectrometry. Also, cinnamaldehyde was known as major active compound of CR for vasodilation, antitumor, and antifungal activity.

2.4. Preparation of Thoracic Aortic Rings. All procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee of Dongguk University. A previously described procedure [21] was employed with some modification. Briefly, rats were sacrificed and their thoracic aortas were immediately excised and immersed in ice-cold Krebs solution (115.0 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgCl_2 , 25.0 mM NaHCO_3 , 1.2 mM KH_2PO_4 , and 10.0 mM dextrose). The aortas were cleaned of all adherent connective tissue and cut into 3 mm long ring segments. Endothelium was removed from the internal surface of each segment by gentle rubbing with forceps.

2.5. Organ Bath Study. Tension was measured by a modification of a previously described procedure [22]. Briefly,

two stainless-steel triangles were inserted through each vessel ring. One triangle was anchored to a stationary support and the other was connected to a FT03 isometric force transducer (Grass, Quincy, Mass, USA). Each vessel ring was incubated in a water-jacketed organ bath (10 mL) that was maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2 . Each ring was stretched passively by imposing the optimal resting tension of approximately 2.0 g, which was maintained throughout the experiment. Each endothelium-free aortic ring was allowed to equilibrate in the organ bath for at least 50 min before the experiment involving the contractile response to $5 \mu\text{M}$ Ang II, $1 \mu\text{M}$ PE, $10 \mu\text{M}$ FPL64176, $5 \mu\text{g/mL}$ m-3M3FBS, or 30 mM caffeine. Endothelium-free rings were used because preliminary experiments (data not shown) established that CRE relaxes vascular constriction in an endothelium-independent manner. The denudation of endothelium was assessed by treating the rings with $1 \mu\text{M}$ acetylcholine. Isometric tension was recorded using a PowerLab/8SP computerized data acquisition system (ADInstruments, Castle Hill, NSW, Australia). The influence of CRE on extracellular Ca^{2+} influx was studied in Ca^{2+} -free Krebs solution. After equilibration of the ring in Ca^{2+} -free Krebs solution containing 60 mM KCl, cumulative doses of CaCl_2 were added (0.3, 0.6, 1, 1.5, 2.5, 5, and 10 mM, in order) with preincubation of CRE in organ bath. The CaCl_2 dose-dependent maximum constriction of the aortic ring with 60 mM KCl in Ca^{2+} -free Krebs solution was expressed as 100%. To determine the influence of CRE on Ca^{2+} influx through the L-type Ca^{2+} channel, aortic rings were pretreated with nifedipine or verapamil before PE contraction, and were preincubated with CRE before contraction by FPL64176. To investigate the inhibitory effect of CRE on intracellular Ca^{2+} release by PE in Ca^{2+} -free conditions, and by caffeine in normal Krebs solution, the transient contraction of CRE preincubated aortic rings was measured. To further investigate the relationship with the PLC pathway, aortic rings were constricted with m-3M3FBS, and were preincubated with U73122 prior to contraction by PE. When the constriction reached a plateau, CRE was added to the organ bath.

2.6. Preparation of Aorta Protein Extracts and Western Blot Analysis. A previously described protocol [22] was used for preparation of protein extract with some modifications. Briefly, endothelium-free aortic rings were contracted with $1 \mu\text{M}$ PE or $5 \mu\text{g/mL}$ m-3M3FBS, and then treated with CRE for 30 min. The aortic rings were quick frozen by immersion in acetone containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT) precooled to -80°C . When used, recovered samples were homogenized in buffer containing 320 mM sucrose, 50 mM Tris, 1 mM EDTA, 1% Triton X-100, 1 mM DTT, and the following protease inhibitors: leupeptin ($10 \mu\text{g/mL}$), trypsin ($10 \mu\text{g/mL}$), aprotinin ($2 \mu\text{g/mL}$), or phenylmethylsulphonyl fluoride ($100 \mu\text{g/mL}$). The protein samples were electrophoresed and the resolved proteins were transferred to a nitrocellulose membrane. The membrane was incubated with primary antibodies and then treated with horseradish peroxidase-conjugated anti-rabbit IgG as a secondary antibody. All

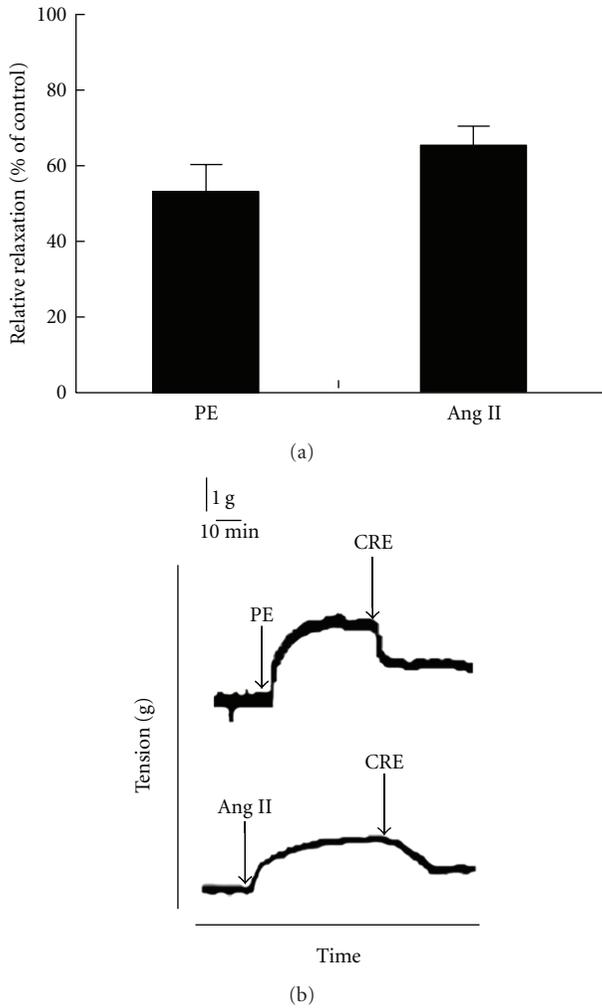


FIGURE 1: The vasodilative effect of CRE on PE- or Ang II-induced contraction in rat aorta. Rat aortic rings were contracted by PE or Ang II, and then treated with CRE (0.1 mg/mL). Results are expressed as relative percentage of the relaxation of CRE on PE- or Ang II-induced contraction. Each value is the average of three-way repeated measures. Data are shown as means \pm SD. CRE, *Cinnamomi ramulus* ethanol extract; PE, phenylephrine; Ang II, angiotensin II.

bands were detected using an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK).

2.7. Statistical Analyses. Each set of experiments was done at least three times and results are presented as the mean \pm SD. The statistical significance of differences between mean values was assessed with Student's *t*-test or ANOVA. Test values that resulted in $P < 0.05$ were considered as significant.

3. Results

3.1. Vasorelaxation Effect of CRE on PE- or Ang II-induced Constricted Aorta. Ang II increases the intracellular Ca^{2+} concentration in vascular smooth muscle cells through a sequence of events following activation of Ang II type 1

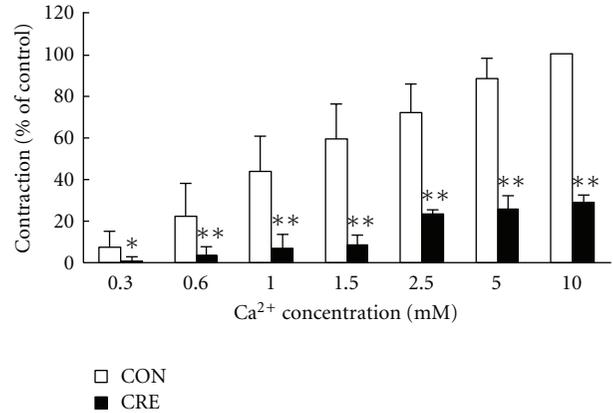


FIGURE 2: Effects of CRE on extracellular Ca^{2+} influx. White bars show the vasoconstriction effect by accumulative addition of $CaCl_2$ (0.3–10.0 mM, CON) and black bars (CRE) show inhibitory effects by CRE (0.1 mg/mL) pretreatment under Ca^{2+} -free isotonic depolarizing solution containing 60 mM KCl. Each value is the average of four-way repeated measures. Values are mean \pm SD. * $P < 0.05$, ** $P < 0.005$ as compared with CON of 10 mM $CaCl_2$.

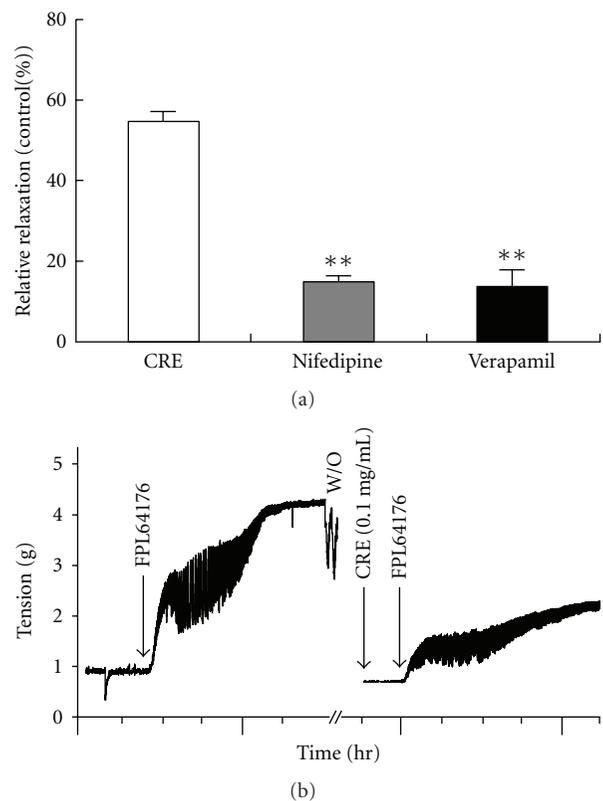


FIGURE 3: Change of vasodilative effect of CRE by Ca^{2+} channel inhibitor or activator. (a) Pretreatment of nifedipine or verapamil inhibited the vasodilative activity of CRE on PE-precontracted aortic rings. Results are expressed as percentage of the maximal tension induced by PE treatment. (b) CRE pretreatment inhibited the vasoconstriction of FPL64176. Representative trace of inhibitory effect of CRE is expressed as tension (g). Each experiment was tried at least three times. Data are shown as means \pm SD. ** $P < 0.005$.

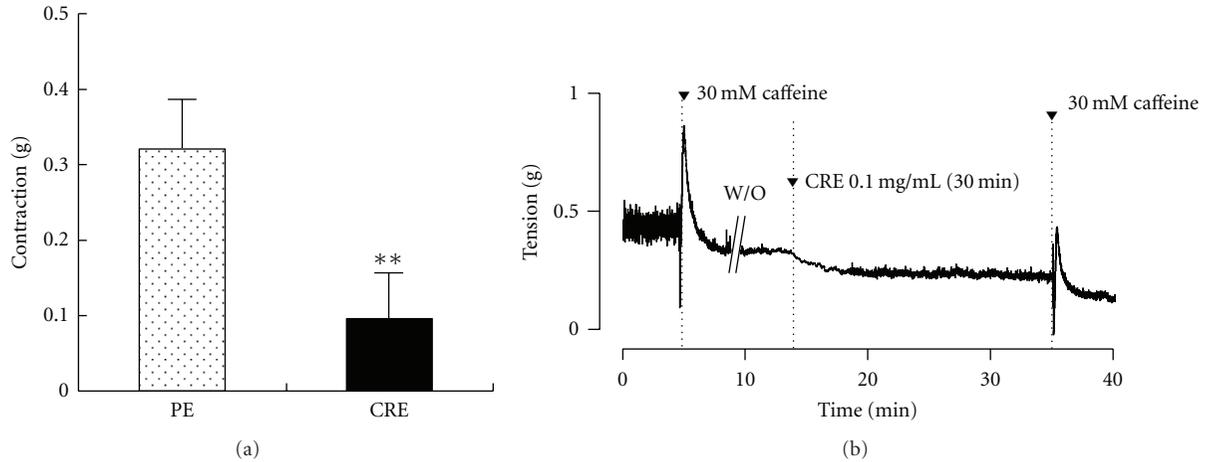


FIGURE 4: Effects of CRE on transient contraction by Ca^{2+} -release in sarcoplasmic reticulum (SR). CRE significantly reduced PE (a)- or caffeine (b)-induced vasoconstriction in Ca^{2+} -free solution or in normal Krebs solution, respectively. Values are shown as mean \pm SD, $**P < 0.005$ as compared with PE.

receptor and L-type calcium channels [6, 7]. PE- or Ang II-induced contraction was significantly dilated by $53.6 \pm 7.8\%$ and $66.0 \pm 5.4\%$, respectively, as compared to maximal tension (Figure 1), indicating that that CRE-mediated vasodilation may be related to decreased intracellular Ca^{2+} concentration.

3.2. Effects of CRE on Ca^{2+} Influx from the Extracellular Space. To determine the influence of CRE on Ca^{2+} influx, the change of contraction was measured by adding CaCl_2 in an accumulative manner (0.3, 0.6, 1, 1.5, 2.5, 5, and 10 mM, in order) before and after CRE pretreatment in Ca^{2+} -free Krebs solution containing 60 mM KCl. The vasoconstriction rate at the aforementioned CaCl_2 concentrations were $7.7 \pm 8.1\%$, $23 \pm 16\%$, $44 \pm 16.7\%$, $59 \pm 17\%$, $72 \pm 14\%$, $88.4 \pm 9.4\%$, and 100%, respectively, (the latter represents the maximum contraction value of the aortic ring, achieved at 10 mM CaCl_2). These contractions were significantly reduced by $1.9 \pm 2.2\%$, $5.8 \pm 4.1\%$, $8.3 \pm 6.3\%$, $11.4 \pm 4.4\%$, $22.3 \pm 1.9\%$, $24.8 \pm 6.2\%$, and $29.7 \pm 3.8\%$ (same respective CaCl_2 concentrations) with CRE-pretreatment (Figure 2).

3.3. Effect of CRE on Ca^{2+} Influx through L-type Ca^{2+} -Channels. To discern the effect of CRE on the L-type calcium channel, the influence of the L-type calcium channel blocker nifedipine (100 μM) or verapamil (1 μM), and the L-type calcium channel activator FPL64176 (10 μM) on vasorelaxation of CRE against PE-induced contraction of aortic rings was measured. Pretreatment of aortic rings with nifedipine or verapamil significantly inhibited the relaxant effect of CRE (Figure 3(a)). Previous studies have shown that FPL64176 increases extracellular Ca^{2+} entry, thereby enhancing the cytosolic Ca^{2+} concentration [23, 24]. Presently, FPL64176 induced contraction, which plateaued at 3.75 ± 0.22 g in 30 min, was inhibited by 2.4 ± 0.1 g with preincubation of CRE (Figure 3(b)).

3.4. Effect of CRE on Ca^{2+} Release from SR. To assess whether CRE is involved in Ca^{2+} release-mediated vasoconstriction

from intracellular stores, the transient contraction by PE or caffeine was examined in CRE preincubated aortic rings. Preincubation reduced the magnitude of contraction by PE from 0.32 ± 0.6 g to 0.1 ± 0.6 g (Figure 4(a)). The transient contraction induced by 30 mM caffeine was also reduced by CRE pretreatment (Figure 4(b)).

3.5. Effect of CRE on PLC Pathway. To evaluate whether the relaxant effect of CRE was involved in the PLC pathway, the PLC pathway inhibitor U73122 and activator m-3M3FBS were used. U73122 pretreatment significantly inhibited the relaxant effect of CRE on PE-induced contraction from $56.9 \pm 2.7\%$ to $8.5 \pm 1.3\%$ (Figure 5(a)). m-3M3FBS (5 $\mu\text{g}/\text{mL}$)-induced contraction was relaxed significantly with CRE treatment (Figure 5(b)).

3.6. Effect of CRE on the Expression Levels of PI3K and pPLC. PI3K is directly activated by G-protein for generation of PIP_3 , which eventually stimulates L-type calcium channels in vascular myocytes [5, 12]. PLC activation induces the generation of IP_3 and DAG. In turn, IP_3 stimulates intracellular Ca^{2+} release from the SR for vasoconstriction [15, 17]. Presently, PE and m-3M3FBS increased the expression levels of PI3K (4.5 ± 0.38 and 2.0 ± 0.32 , resp.) and pPLC (4.1 ± 0.30 and 2.5 ± 0.14 , resp.), which were significantly decreased by CRE. PE-induced PI3K expression was decreased by 2.8 ± 0.70 at 50 $\mu\text{g}/\text{mL}$ and by 0.2 ± 0.6 at 100 $\mu\text{g}/\text{mL}$. pPLC expression was also decreased by 2.0 ± 0.30 at 50 $\mu\text{g}/\text{mL}$ and by 1.8 ± 0.1 at 100 $\mu\text{g}/\text{mL}$ (Figure 6(a)). m-3M3FBS-induced PI3K expression was downregulated by 0.6 ± 0.1 at 50 $\mu\text{g}/\text{mL}$ and by 0.5 ± 0.2 at 100 $\mu\text{g}/\text{mL}$, and pPLC expression was decreased by 1.4 ± 0.1 at 50 $\mu\text{g}/\text{mL}$ and by 0.3 ± 0.1 at 100 $\mu\text{g}/\text{mL}$ (Figure 6(b)).

4. Discussion and Conclusions

Traditionally, *Cinnamomum cassia* has been used as a medicinal herb. Its bark and twig are known as *Cinnamomi cortex* (CC) and *Cinnamomi ramulus* (CR), respectively. CC

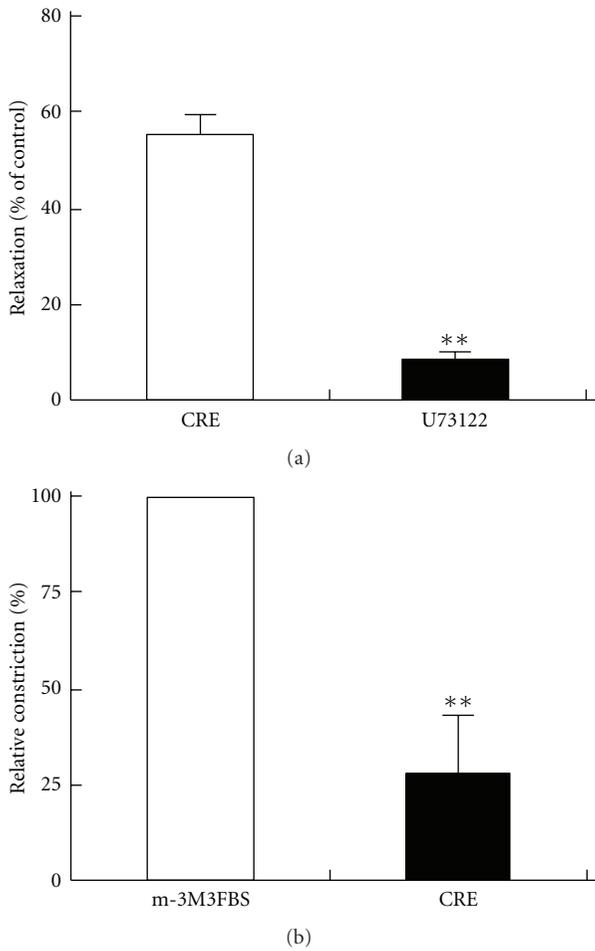


FIGURE 5: Vasorelaxant effects of CRE through PLC pathway. (a) U73122 (10 μ M) pretreatment reduced the vasorelaxation of CRE on PE-induced vasoconstriction. (b) CRE (0.1 mg/mL) relaxed m-3M3FBS (5 μ g/mL)-induced vasoconstriction. Each value is the average of three-way repeated measures. Data are shown as means \pm SD. ** $P < 0.005$.

inhibits *Helicobacter pylori* [25] and ameliorates sucrose-induced blood pressure elevation in spontaneously hypertensive rats [18]. Furthermore, CRE exerts an endothelium-independent vasodilatory response through inhibition of voltage-dependent Ca^{2+} channels [19]. However, the mechanism by which CRE exerts vasodilation remains to be elucidated. The present study investigated the vasodilatory effect of CRE resulting from the inhibition of both Ca^{2+} influx and release in rat aorta.

PE or Ang II stimulate PLC isoforms to generate IP_3 through the activation of G proteins, causing release of activator Ca^{2+} from SR [9, 10, 14–16]. Presently, CRE markedly and similarly relaxed aortic rings that were precontracted with PE or AngII. These results suggest that CRE-mediated vasodilation may be involved in the regulation of Ca^{2+} mobilization. To assess this, the regulation of Ca^{2+} influx and release was investigated. Firstly, whether CRE actually inhibits extracellular Ca^{2+} influx or not, we measured the

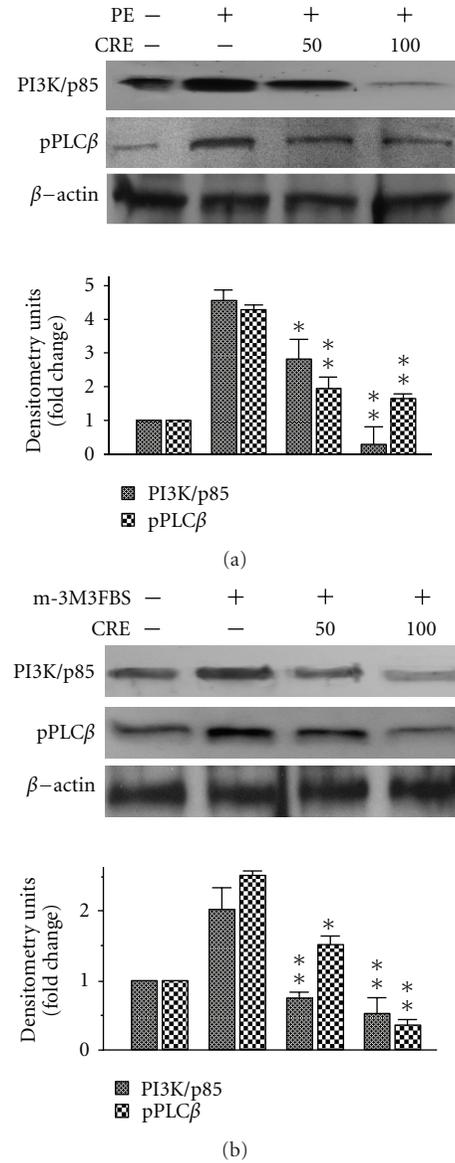


FIGURE 6: Effects of CRE on the expression levels of pPLC β and PI3K/p85 proteins in rat aortic strips. CRE downregulated the PE (a)-or m-3M3FBS (b)-induced overexpressions of pPLC β and PI3K/p85. All data are shown as mean \pm SD and indicate fold changes to normal untreated sample. * $P < 0.05$, ** $P < 0.005$ as compared to PE or m-3M3FBS.

tension of aortic rings by accumulative addition of $CaCl_2$ in Ca^{2+} -free Krebs solution containing 60 mM KCl. Preincubation with CRE significantly reduced rat aortic contraction by addition of $CaCl_2$, indicating inhibition of Ca^{2+} influx. Nifedipine (100 μ M) and verapamil (1 μ M) pretreatment inhibited the vasodilative effect of CRE on PE-induced constricted aortic rings from $54.9 \pm 4.1\%$ to $15.3 \pm 2.1\%$ and $13.9 \pm 5.2\%$, respectively. In addition, preincubation with CRE reduced the contraction of aortic ring by 10 μ M FPL64176. These results support the suggestion that the vasodilative effect of CRE is related to the inhibition of L-type calcium channel in the cell membrane.

The SR is the major source of Ca^{2+} release into the cytosol [1, 16, 17]. This Ca^{2+} release is induced by the IP_3 second messenger, which is generated by PLC activation [17]. Ca^{2+} release from the SR is considered to be the initial mechanism in agonists such as PE- and Ang II-induced vasoconstriction [6, 17]. PE-induced transient constriction is dependent on Ca^{2+} release from the SR through the IP_3 signal pathway in Ca^{2+} -free Krebs solution [26]; however, caffeine is dependent on Ca^{2+} -induced Ca^{2+} release from the SR [27, 28]. To demonstrate the effects of CRE on Ca^{2+} release from the SR, transient contractions induced by PE in Ca^{2+} -free Krebs solution and induced by caffeine in normal Krebs solution were investigated. CRE significantly reduced the magnitudes of transient contraction by PE and caffeine, suggesting CRE inhibits Ca^{2+} release from the SR by blocking the IP_3 -induced Ca^{2+} release and Ca^{2+} -induced Ca^{2+} release mechanisms.

Pretreatment with the PLC inhibitor U73122 significantly reduced the vasorelaxation of CRE on PE-induced vasoconstriction, and CRE relaxed m-3M3FBS-induced vasoconstriction. Additionally, we analyzed the expression levels of the intracellular signaling regulator proteins PI3K and PLC. PI3K generates various 3-phosphorylated phosphoinositides through activation by G-proteins, especially $\text{PI}(3,4,5)\text{P}_3$ stimulates the L-type Ca^{2+} channel that plays an important role in the regulation of vascular tone [8, 11, 12]. On the other hand, PLC formats the two potent second messengers IP_3 and DAG. Especially, IP_3 induces the activation of IP_3 receptor on the SR membrane, opening a calcium channel, resulting in the release of Ca^{2+} into the cytosol [14, 17]. Presently, PE- or m-3M3FBS-induced phosphorylation of PLC and upregulation of PI3K/p85 protein expression were inhibited by CRE (Figure 6). The collective data supports the idea that CRE dilates vascular contraction through the inhibition of both Ca^{2+} influx via the L-type Ca^{2+} channel and IP_3 -induced Ca^{2+} release from the SR.

In conclusion, the data supports the vasorelaxation of CRE through the inhibition of Ca^{2+} influx and Ca^{2+} release. Therefore, CRE may be useful as a drug for the treatment and prevention of high blood pressure associated with Ca^{2+} -dependent contraction of smooth muscle.

Acknowledgment

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