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The Vasorelaxant Effects of *Anaxagorea luzonensis* A. Grey in the Rat Aorta

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Abstract: The aim of the present research was to study vasorelaxant effects of dichloromethane extract of *Anaxagorea luzonensis* (CH_2Cl_2-AL) and its underlying mechanisms. CH_2Cl_2-AL (1-300 μg mL^{-1}) induced concentration-dependent vasorelaxations which were reduced by endothelial denudation, 300 μM N^G-nitro-L-arginine methyl ester (L-NAME) and a combination of 10 μM indomethacin and 300 μM L-NAME, but not indomethacin alone. Raising the extracellular KCl concentration to 60 mM inhibited vasorelaxant responses to CH_2Cl_2-AL in both endothelium-intact and -denuded rings. Moreover, the responses to CH_2Cl_2-AL were inhibited by 30 μM barium chloride, 2 μM clotrimazole, 10 μM glibenclamide and 10 μM 1-[(2-chlorophenyl) diphenylmethyl]-1H-pyrazole (TRAM-34), but not 1 mM 4-aminopyridine. Pre-incubation with CH_2Cl_2-AL (1-100 μg mL^{-1}) inhibited contractions induced by $CaCl_2$ in a Ca^{2+}-free, high KCl buffer. The present findings demonstrate, in the rat isolated aorta, that vasorelaxant responses to CH_2Cl_2-AL are, in part, mediated via the endothelium and NO-dependent pathways. Moreover, activation of K_{IR}, K_{Ca}, K_{ATP} channels seems to play a role in CH_2Cl_2-AL-induced responses. Interestingly, Inhibition of extracellular Ca^{2+} influx is largely involved in the action of CH_2Cl_2-AL. The present study provides scientific evidence to support the use of CH_2Cl_2-AL as a vasodilator agent.

Key words: *Anaxagorea luzonensis*, endothelium, nitric oxide, K^+ channels, Ca^{2+} influx, vasorelaxation

INTRODUCTION

Anaxagorea luzonensis A. Grey (AL) belongs to Annonaceae family, a pantropical angiosperm family (Scharaschkin and Doyle, 2005, 2006). Anaxagorea is the only genus of Annonaceae with distribution in the Southeast Asia and America (Scharaschkin and Doyle, 2005). In Thailand, AL has been known as Kam-lang-wua-thalueng. It is a shrub with light green, smooth, oblong leaves, 7-15 cm long and 3-5 cm wide. The flowers are solitary opposed to the leaves of about 2 cm in diameter with a short peduncle. Sepals are ovate and obtuse. Petals are ovate and oblong. Stamens are all similar and fertile. There are 2-4 oblong ovaries. Carpels are about 4 cm spatulate, obtuse, obliquely mucronate, glabrous and rather rugose. Heartwood is reddish-brown, hard, with a little of the whitish bark (Hooker, 1875; Na Songkhla, 1982).

The heartwood of AL has been widely used in Thai traditional medicine as a health promoting herb. The extract of AL has several pharmacological effects including blood tonic, antihistamine, antioxidant and antihypertensive agents (Mokkhasmit *et al*., 1971; Gonda *et al*., 2000; Takada and Yokoyama, 2000; Panriansaen *et al*., 2008).

Chemical investigations have demonstrated that the extract of the heartwoods of AL contains flavones, flavonones, flavonols and xanthones (Kitaoka *et al*., 1998; Gonda *et al*., 2000; Takada and Yokoyama, 2000). For example, 20 natural compounds are isolated from the bark of AL including one new flavonoid, 3, 5, 7, 4'-tetrahydroxy-2'-methoxyflavone and five new xanthones, 1, 3, 6-trihydroxy-5-methoxy-4-prenylxanthone, 1, 3, 5-trihydroxy-6-methoxy-2-prenylxanthone, 1, 3, 5-trihydroxy-4-(3-hydroxy-3-methylbutyl) xanthone, 1, 3, 6-trihydroxy-4-prenylxanthone, 3, 6-dihydroxy-1, 5-dimethoxyxanthone together with seven known xanthones, 1, 3, 5-trihydroxy-4-prenylxanthone, 1, 3, 5-trihydroxy-2-prenylxanthone, 1, 3, 6-trihydroxy-5-methoxyxanthone, genistein (1, 3, 7-trihydroxyxanthone), 1, 3, 5-trihydroxyxanthone, 3, 5-dihydroxy-1-methoxyxanthone and 1, 3, 5, 6-tetrahydroxy xanthone and seven known flavonoids, biochanin A,

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chrysin, 3'-methylorobol, orobol, taxifolin, kaempferol and quercetin. Almost all flavonoids and 1, 3, 5, 6-tetrahydroxy xanthone exhibit antioxidant activity toward α,α-diphenyl-β-picryhydrazyl radical (Gonda *et al*., 2000). In addition, 3, 5, 7, 4'-tetrahydroxy-2'-methoxyflavone isolated from AL has antioxidant effect on autoxidation of epinephrine hydrochloride (Takada and Yokoyama, 2000). A previous study has reported that 8-isopentenylnaringenin in the extract exerts estrogenic activity greater than genistein, a standard compound (Kitaoka *et al*., 1998).

In other plants, methoxyflavones and flavonoids induce vasorelaxation in isolated blood vessels (Ajay *et al*., 2003; Morello *et al*., 2006; Tep-Areenan *et al*., 2010; Tep-Areenan and Sawasdee, 2010). However, there is no pharmacological evidence about the vasorelaxant effects of AL which is rich in flavones and flavonoids. The present study aimed to investigate the vasorelaxant effect of the dichloromethane extract of AL on vascular tone and its underlying mechanisms in the isolated rat aorta.

MATERIALS AND METHODS

Extraction of *Anaxagorea luzonensis* A. Grey (AL): The dried and chopped heartwood (5 kg) of AL was extracted four times with methanol using soxhlet extractor. After filtration, the methanolic extract was concentrated under reduced pressure and was then partitioned with dichloromethane (CH_2Cl_2), yielding about 10.5 g of CH_2Cl_2-AL after evaporation.

Tissue preparation: In 2009, Experiments were performed using aorta obtained from male Wistar rats (300-350 g) purchased from National Laboratory Animal Center, Mahidol University, Thailand. Rats were housed in standard environmental condition (25°C) under 12 h light/dark cycles and fed with standard laboratory rat chow and tap water *ad libitum*. All experimental procedures were reviewed and approved by the Animal Research Ethics Committee of the Faculty of Medicine, Srinakharinwirot University.

Male Wistar rats were anaesthetized with Zolitil 50 mg kg^{-1} (tiletamine chloridrate and zolazepan chloridrate) into quadriceps muscle and killed by cervical dislocation. Following a thoracotomy, the thoracic aorta was carefully removed, cleaned of fat and connective tissue and cut into 5 mm ring segments. Each ring was transferred to a jacketed organ bath filled with 20 mL of modified Krebs-Henseleit solution, composed of (mM) NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2, D-glucose 10, that was maintained at 37°C and bubbled continuously with 95 % O_2 and 5% CO_2 mixture. The solution in the organ bath was exchanged every 15 min for 1 h. The rings were mounted between two triangular stainless steel hooks that were passed through the lumen and stretched to an optimal passive tension of about 1 g and maintained at this tension for 1 h. The upper hook was connected to a isometric force transducer (MLT 0210, New South Wales, Australia) and changes in isometric force were recorded on a MacLab recording system (AD instruments, New South Wales, Australia).

Experimental protocol: Following a 1 h equilibration period, methoxamine (10-60 μM) was used to increase tone by approximately 1 g. Once a stable contraction was estabilshed, CH_2Cl_2-AL (1-300 μg mL^{-1}) was added cumulatively. To explore the mechanisms involved in vasorelaxation induced by CH_2Cl_2-AL, aortic rings were incubated with various inhibitors added to the organ bath before methoxamine was added to increase tone. In vehicle-control experiments, dimethyl sulphoxide (DMSO) alone was added cumulatively in the same volumes as those used in the experiments with CH_2Cl_2-AL.

To examine the contribution of the endothelium in vasorelaxant responses to CH_2Cl_2-AL, the endothelium was mechanically removed by gently rubbing the luminal surface with a cocktail stick. Removal of the endothelium was demonstrated by vasorelaxation to 10 μM carbachol being less than 10% of the induced tone. To investigate the involvement of vasodilator prostanoids via the cyclooxygenase (COX) pathway and Nitric Oxide (NO) in vasorelaxation to CH_2Cl_2-AL, aortic rings were treated with indomethacin (10 μM), a COX inhibitor and N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM), an inhibitor of endothelial nitric oxide synthase, respectively.

To investigate the potential involvement of K^+ channels in vasorelaxation to CH_2Cl_2-AL, aortic rings were pre-contracted with a high extracellular concentration of KCl (60 mM), which was prepared by replacing an equimolar concentration of NaCl with KCl (Tep-Areenan *et al*., 2003). These experiments were also performed in endothelium-denuded rings to determine any involvement of Endothelium-Derived Relaxing Factors (EDRFs) in the vasorelaxant effects of CH_2Cl_2-AL on activation of K^+ channels. To characterized the types of K^+ channels involved in vasorelaxation to CH_2Cl_2-AL, concentration-response curves to CH_2Cl_2-AL were constructed after incubation with 4-aminopyridine (4-AP, 1 mM), a voltage-gated K^+ (K_V) channel inhibitor, glibenclamide (10 μM), an ATP-sensitive (K_{ATP}) inhibitor, or barium chloride ($BaCl_2$, 30 μM), an inward-rectifier (K_{IR}) channel inhibitor, clotrimazole (2 μM), an intermediate-conductance calcium-activated potassium (IK_{Ca}) channel inhibitor and 1-[(2-chlorophenyl)diphenylmethyl]-1H-

pyrazole (TRAM-34, 10 μM), a specific inhibitor of IK_{Ca} channels.

To examine the vascular effect of CH_2Cl_2-AL on extracellular Ca^{2+} influx, concentration-response curve to $CaCl_2$ (10 μM-30 mM) were constructed in the presence and absence of CH_2Cl_2-AL (1, 10 and 100 μg mL^{-1}) for 30 min. Aortic rings were first allowed to equilibrate at 1 g tension in a Ca^{2+}-free Krebs solution and then the rings were bathed with Ca^{2+}-free, high KCl (100 mM) Krebs solution. In vehicle-control experiments, DMSO was added in the same volume as that used in the experiments with CH_2Cl_2-AL.

Data and statistical analysis: The concentration of vasorelaxant giving half-maximal relaxation (EC_{50}) and maximal responses (R_{max}) were obtained from the concentration-response curve fitted to a sigmoidal logistic equation using the GraphPad Prism package described by Tep-Areenan *et al*. (2003). R_{max} and pEC_{50} values (negative logarithm of the EC_{50}) were compared by analysis of variance (ANOVA) with statistically significant differences between groups being determined by Bonferroni's post-hoc test. These were expressed as Mean±SEM. The results were considered statistically significant when p value was less than 0.05. The number of animals in each group is represented by n.

Chemicals: All drugs and chemicals were purchased from Sigma Chemical Company (St. Louis, Missouri, USA), but zoletil was purchased from Virbac (Carros Cedex, France). Indomethacin was dissolved in ethanol. CH_2Cl_2-AL and glibenclamide were dissolved in DMSO. 4-AP, $BaCl_2$ and TRAM-34 were dissolved in distilled water. The remaining drugs were dissolved in the Krebs solution. All drugs were made up on the day of the experiment.

RESULTS

The effects of endothelial denudation, indomethacin and L-NAME on vasorelaxation to CH_2Cl_2-AL in rat aortic rings: CH_2Cl_2-AL (1-300 μg mL^{-1}) caused vasorelaxation in a concentration-dependent manner (pEC_{50} = 4.87±0.08 with R_{max} = 118±4%, n = 6, Fig. 1). Removal of the endothelium significantly ($p<0.05$) reduced the effects of CH_2Cl_2-AL (R_{max}: control = 118±4%, n = 6; denuded = 96.1±7.5%, n = 6, Fig. 1). Similarly, vasorelaxant responses to CH_2Cl_2-AL were significantly ($p<0.05$) reduced after pretreatment with a combination of indomethacin plus L-NAME, which were not different from L-NAME alone (R_{max}: L-NAME alone = 99.3±5.5%, n = 6; indomethacin plus L-NAME = 95.3±8.6%, n = 6, Fig. 1).

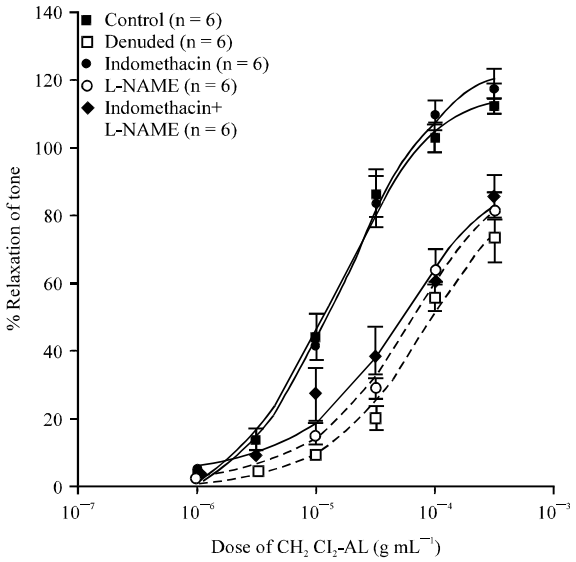

Fig. 1: Effects of removal of the endothelium (denuded), indomethacin (10 μM), N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) and a combination of indomethabin and L-NAME on vasorelaxation to CH_2Cl_2-AL in aortic rings precontracted with methoxamine. Data were shown as Mean±SEM

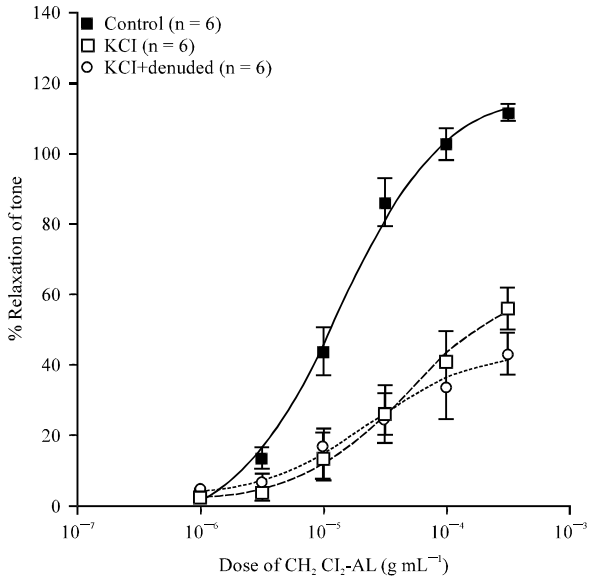

Fig. 2: Effects of a high concentration of extracellular K^+ (60 mM KCl) on vasorelaxation to CH_2Cl_2-AL in endothelium-intact and -denuded aortic rings precontracted with methoxamine. Data were shown as Mean±SEM

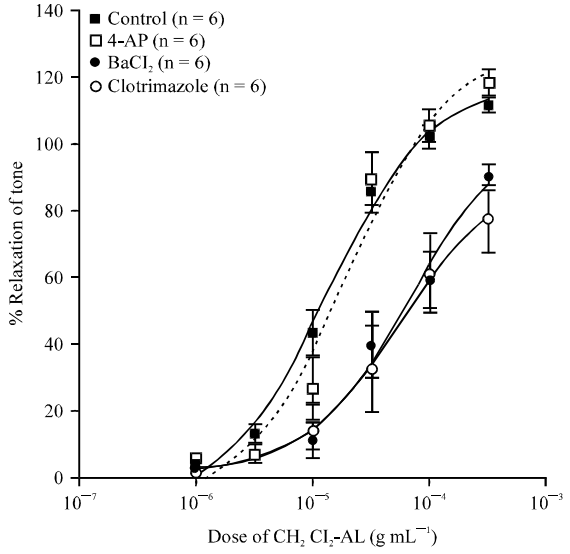

Fig. 3: Effects of 4-aminopyridine (4-AP, 1 mM), barium chloride ($BaCl_2$, 10 μM) and clotrimazole (2 μM) on vasorelaxation to CH_2Cl_2-AL in aortic rings precontracted with methoxamine. Data were shown as Mean±SEM

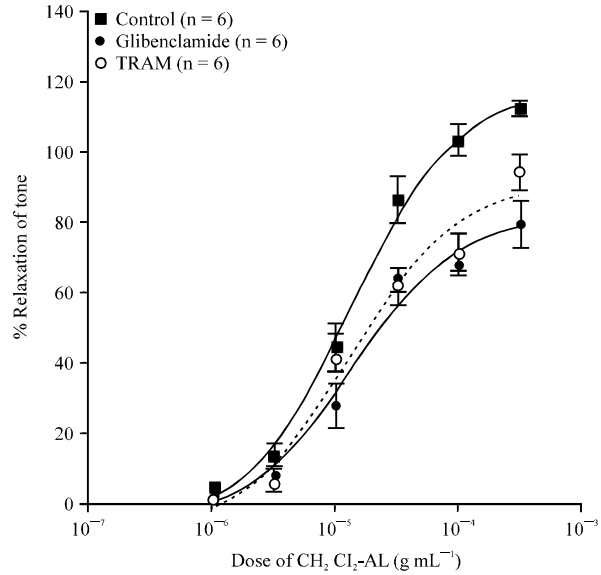

Fig. 4: Effects of glibenclamide (10 μM) and 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34, 10 μM) on vasorelaxation to CH_2Cl_2-AL in aortic rings precontracted with methoxamine. Data were shown as Mean±SEM

The effects of high extracellular potassium and potassium channel inhibitors on vasorelaxation to CH_2Cl_2-AL: Raising extracellular K^+ concentration (60 mM KCl) significantly ($p<0.001$) inhibited vasorelaxation induced by CH_2Cl_2-AL in endothelium-intact and-denuded rings (R_{max}: 60 mM KCl = 63.1±9.1%, n = 6; 60 mM KCl with denuded = 44.4±6.1%, n = 6, Fig. 2). However, there was no significant difference between two groups.

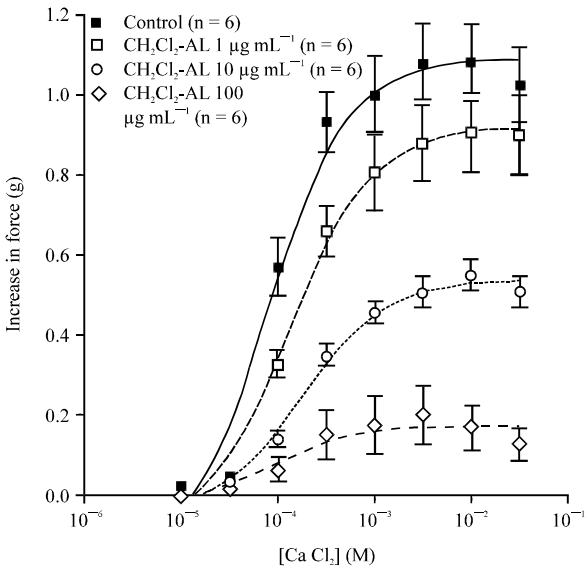

Fig. 5: Effects of CH_2Cl_2-AL on $CaCl_2$-induced contraction in aortic rings depolarized by 100 mM KCl. Data were shown as Mean±SEM

In intact endothelium, vasorelaxant effects of CH_2Cl_2-AL were significantly ($p<0.05$) inhibited by either $BaCl_2$, or clotrimazole, but not 4-AP (R_{max}: $BaCl_2$ = 101±5%, n = 6; clotrimazole = 91.7±10.8%, n = 6, Fig. 3). Moreover, pretreatment with glibenclamide or TRAM-34 significantly ($p<0.001$) inhibited vasorelaxation induced by CH_2Cl_2-AL (R_{max}: glibenclamide = 82.5±4.35%, n = 6; TRAM-34 = 91.0±4.7%, n = 6, Fig. 4).

The effects of CH_2Cl_2-AL on $CaCl_2$-induced contraction in rat aortic rings: $CaCl_2$ (10 μM-30 mM) elicited concentration-dependent contraction of KCl (100 mM) depolarized rings in Ca^{2+}-free medium. CH_2Cl_2-AL concentration-dependently reduced contractions induced by $CaCl_2$ (R_{max}: control = 1.09±0.04 g, n = 6; 1 μg mL^{-1} CH_2Cl_2-AL = 0.92±0.04 g, n = 6; 10 μg mL^{-1} CH_2Cl_2-AL = 0.54±0.01 g, n = 6; 100 μg mL^{-1} CH_2Cl_2-AL = 0.17±0.02 g, n = 6, Fig. 5).

DISCUSSION

The present study has demonstrated, for the first time, about the vasorelaxant effects of CH_2Cl_2-AL and mechanisms involved in its action. CH_2Cl_2-AL causes

vasorelaxation in the isolated rat aorta, which is partly due to endothelium-derived NO. Activation of K^+ channels and inhibition of Ca^{2+} influx are largely involved in the vasorelaxant effects of CH_2Cl_2-AL.

Previous studies have reported that the extract of *Anaxagorea luzonensis* A.Gray contains several flavones and flavonoids (Kitaoka *et al*., 1998; Gonda *et al*., 2000). Therefore, vasorelaxant responses of rat aortic rings to CH_2Cl_2-AL may be caused by the effects of these compounds. The present findings are in agreement with several earlier investigations with plant extract showing that flavonoids exert a vasorelaxant property (Herrera *et al*., 1996; Ajay *et al*., 2003; Gilani *et al*., 2006; Morello *et al*., 2006). Recently, studies by our group have shown that the ethanolic extract of *Kaempferia parviflora* and its methoxyflavones cause vasorelaxation in the rat isolated aorta (Tep-Areenan *et al*., 2010; Tep-Areenan and Sawasdee, 2010).

The vascular endothelium plays an essential role in regulating vascular tone via synthesis and release Endothelium-Derived Relaxing Factors (EDRFs), such as NO and prostacyclin (Lokhande *et al*., 2006; Vanhoutte, 2009; Vanhoutte *et al*., 2009). The present experiments show that relaxations induced by CH_2Cl_2-AL were inhibited by removal of the endothelium. Moreover, the effects of CH_2Cl_2-AL were inhibited by L-NAME, a NOS inhibitor, but not inhibition of a COX pathway by indomethacin. These results indicate the participation of endothelium-derived NO in the effects of CH_2Cl_2-AL. The present study also found that pretreatment with L-NAME plus indomethacin showed no further inhibition of responses to CH_2Cl_2-AL than those observed with L-NAME alone. Therefore, these results indicate that the relaxant responses to CH_2Cl_2-AL involve NO, but not vasodilator prostanoids via the COX pathway. Although, previous studies have demonstrated that vasorelaxant effects of some flavones and flavonoids involve the COX pathway (Ajay *et al*., 2003; Uydes-Dogan, *et al*., 2005), our CH_2Cl_2-AL constituents may be different. Further studies would be needed to identify the active ingredients in our extract.

Several types of K^+ channels are located in vascular smooth muscle cells, including K_{ATP}, K_{Ca}, K_V, and K_{IR} channels. Opening of K^+ channels in the cell membrane of vascular smooth muscle cells increases K^+ efflux, causing hyperpolarization, which closure of voltage-gated Ca^{2+} channels and subsequently vasorelaxation (Jackson, 2000; Sobey, 2001). To investigate the involvement of K^+ channels in vasorelaxant effects of CH_2Cl_2-AL, a high concentration of KCl was used to increase vascular tone. It was found that 60 mM KCl inhibited the effects of CH_2Cl_2-AL in both endothelium-intact and denuded aortic rings. However, there was no further inhibition of responses to CH_2Cl_2-AL in denuded rings, compared to intact rings. Taken together, it is suggested that opening of K^+ channels on smooth muscle cells by CH_2Cl_2-AL is unlikely due to EDRFs.

In order to investigate the contribution of exact types of K^+ channels involved in vasorelaxant effects of CH_2Cl_2-AL, we used different types of K^+ channel inhibitors. It was found that vasorelaxation induced by CH_2Cl_2-AL were inhibited by barium chloride, a K_{IR} channel inhibitor, glibenclamide, a K_{ATP} channel inhibitor, clotrimazole, an intermediate-conductance K_{Ca} channels, TRAM-34, a specific inhibitor of intermediate-conductance K_{Ca} (IK_{Ca}) channels. Conversely, 4-aminopyridine, a K_V channel inhibitor, did not affect vascular responses to CH_2Cl_2-AL. These results suggest that vasorelaxant responses to CH_2Cl_2-AL are mediated by increasing K^+ efflux, at least in part, through K_{IR}, K_{ATP}, IK_{Ca} channels.

Another aspect examined in the present study was whether vasorelaxant responses to inhibition of extracellular Ca^{2+} influx involved. We found that contractile responses of rat aortic rings to $CaCl_2$ in Ca^{2+}-free medium containing KCl were inhibited by CH_2Cl_2-AL. These findings support the notion that CH_2Cl_2-AL can block Ca^{2+} influx from the extracellular space. In addition, in the presence of CH_2Cl_2-AL, there were rightward shifts in concentration-response curves for $CaCl_2$ with reduction in the R_{max} values, thus suggesting that CH_2Cl_2-AL acts as a non-competitive calcium antagonist. Clinically, Ca^{2+} antagonist are potentially used to treat hypertension (Ishimitsu *et al*., 2009; Stokes, 2009; Adatia and Shekerdemian, 2010). Concerning the vasorelaxant effects of CH_2Cl_2-AL, it is likely to suggest that the extract of *Anaxagorea luzonensis* A. Grey may acts as antihypertensive agent.

CONCLUSION

From our data, it is possible to conclude that CH_2Cl_2-AL exerts its vasorelaxant effects by acting on multiple sites of actions. Vascular responses to CH_2Cl_2-AL are partly mediated by endothelium-dependent NO. Additionally, inhibition of Ca^{2+} entry and activation of K^+ channels are required for the vasorelaxant effects of CH_2Cl_2-AL. Further chemical and pharmacological experiments are required to isolate and identify the active constituents responsible for this vascular effect and to investigate the *in vivo* effect of GL extract.

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