

# Vasorelaxation Effects of 2-Chloroethanol and Chloroacetaldehyde in the Isolated Rat Aortic Rings

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2-Chloroethanol (2-CE) is a commonly used solvent in industry; unfortunately, severe hypotension is one of main toxic signs during intoxication. Calcium ion modulation is considered to be an important role of vasorelaxation. The aim of this study is to evaluate either 2-CE or its main metabolite, chloroacetaldehyde (CAA), possible cause of hypotension, by using isolated rat aortic rings. Results revealed that 2-CE caused a weakly relaxation in the phenylephrine (PE) pre-induced endothelium-intact aortic rings. However, its metabolite, CAA induced vasorelaxation and showed dose dependency in endothelium-intact and -denuded aortic rings. The half inhibitory concentration (IC<sub>50</sub>) of 2-CE exceeded 50 mM; meanwhile, the IC<sub>50</sub> values of CAA in the endothelium-intact and -denuded aortic rings were 3.3 and 2.7 mM, respectively. The CAA-induced relaxation could be significantly attenuated by adding calcium (CaCl<sub>2</sub>) and various Ca<sup>2+</sup> channel blockers, dantrolene, nifedipine, and NiCl<sub>2</sub>. Nifedipine presents the most strong inhibition effect among the calcium blockers. In conclusion, it is suggested that the hypotension effect of 2-CE intoxicated cases may be mainly mediated by its metabolite CAA, and calcium channels are partially involved inducing the vasorelaxation.

**Key words** — 2-chloroethanol, chloroacetaldehyde, relaxation, calcium channel blocker, rat aorta ring

## INTRODUCTION

2-Chloroethanol (2-CE) (CAS 107-07-3) is commonly applied as a solvent in industry. It is also an intermediate product in the synthesis of various chemical compounds. Acute poisoning cases of 2-CE by ingestion of film cement have been reported.<sup>1)</sup> In Taiwan, some grape farmers use 2-CE to cultivate grapevines as it hastens sprouting. For this, accidental acute poisonings or fatalities of 2-CE in farmers have been reported.<sup>2)</sup> In fatal cases of 2-CE, patients show hypotension, metabolic acidosis, coma, respiratory failure and death after acute exposure.<sup>2,3)</sup> The 2-CE also

causes neurotoxicity, characterized by lethargy, unresponsiveness to touch and erratic movements that is mediated by its metabolite chloroacetaldehyde (CAA).<sup>4)</sup> Another possible mechanism of CAA's toxicity in hepatocytes involves protein-thiol depletion, and mitochondrial damage, which causes adenosine triphosphate (ATP) decrease and calcium cycling.<sup>5)</sup> Additionally, the membrane proteins can actively transport Ca<sup>2+</sup> out of the cytosol through the ATP-dependent Ca<sup>2+</sup> pumps in the plasma membrane and the sarcoplasmic reticulum (SR).<sup>6)</sup> Based on the structure relationship of CAA, the direct vasorelaxant action of acetaldehyde (AA), one of the metabolites of alcohol, on vascular smooth muscles is believed to play a significant role in the ethanol-induced peripheral vasodilation, hypotension and cardiovascular collapse that occur in the alcohol-abuse reaction.<sup>7)</sup>

Ca<sup>2+</sup> is well known to be important in regulat-

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ing vascular tone. A rise in free  $\text{Ca}^{2+}$  concentration, which is caused by  $\text{Ca}^{2+}$  entry through ion channels in the plasma membrane and by the release of  $\text{Ca}^{2+}$  from the SR via ryanodine receptors and inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) receptors, initiates the contraction of vascular smooth muscle.<sup>8)</sup> Extracellular  $\text{Ca}^{2+}$  enters the intracellular space mostly through voltage-dependent  $\text{Ca}^{2+}$  channels (VDCCs) and hormone-mediated receptor-operated  $\text{Ca}^{2+}$  channels (ROCCs).<sup>9,10)</sup> L-type voltage-operated  $\text{Ca}^{2+}$  channels (VOCCs) antagonist nifedipine and store-operated  $\text{Ca}^{2+}$  channels (SOCCs) antagonist  $\text{NiCl}_2$  are also found to block calcium channel.<sup>11)</sup> We hypothesized that the vascular effect of CAA may involve the mobilization of  $\text{Ca}^{2+}$  in the hypotension toxicity. However, the effects of 2-CE, and the metabolite of CAA on hypotension remain unclear. The aim of this study is to examine the vasorelaxation effect of 2-CE and CAA by using the isolated rat aortic rings. Furthermore, relaxation change of calcium ions blockers in the 2-CE and CAA treatments are also examined.

## MATERIALS AND METHODS

**Chemicals**— 2-CE, CAA, phenylephrine (PE), acetylcholine (ACh), ethylene glycol bis( $\beta$ -aminoethyl ether)- $N,N,N',N'$ -tetraacetic acid (EGTA), 2-aminoethyl diphenylborinate (2-APB), dantrolene, nifedipine and  $\text{NiCl}_2$  were purchased from Sigma Co. (Saint Louis, MO, U.S.A.).

**Animals**— Sprague-Dawley rats, male, weighing 250–300 g, were obtained from the BioLASCO Taiwan CO., Ltd. (I-Lan, Taiwan, China). The animals were housed in cages with ample access to food (Lab Diet<sup>®</sup> 5001 Rodent diet, PMI<sup>®</sup> Nutrition International, LLC, St. Louis, MO, U.S.A.), and were provided with deionized reverse osmosis water *ad libitum*. The animals were kept at 20–25°C and 40–70% relative humidity under a 12 hr light on/off cycle. The experiment was performed according to the criteria for the use and care of experimental animals, set out in the “A Guidebook for the Care and Use of Laboratory Animals.”<sup>12)</sup>

**Preparation and Treatment of Aortic Rings**— The rat aortic rings were isolated according to the method of den Hartiz *et al.*<sup>13)</sup> and our previous method.<sup>14)</sup> Rats were anesthetized by an intraperitoneal injection of urethane (0.6 g/kg) combined with chlorohydrate (0.4 g/kg), and blood was exsanguinated from the abdominal aorta. The tho-

racic aorta was carefully removed. Fat and tissue were dissected in normal Krebs solution (NaCl 118.5 mM, KCl 4.8 mM,  $\text{MgSO}_4$  1.2 mM,  $\text{KH}_2\text{PO}_4$  1.2 mM,  $\text{NaHCO}_3$  25 mM, glucose 11.1 mM,  $\text{CaCl}_2$  2.5 mM, pH 7.4). The aorta was cut into rings with diameters of approximately 5–8 mm in a 20 ml organ bath, and constantly gassed with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  at 37°C. Two “L” type stainless steel hooks were then inserted into the aortic lumen, with one fixed to the bottom of the organ bath and the other connected to a force transducer. The aortic rings were equilibrated in Krebs solution and maintained under the optimal tension of 1 g for 30 min during which period the Krebs solution was changed three times before the experimental procedures were performed. Aortic ring contraction was recorded, isometrically using a force displacement transducer (Grass SD9 stimulator, Quincy, MA, U.S.A.) that was connected to a PowerLab recorder (ADInstruments, Bella Vista, NSW, Australia). To prepare the denuded aorta rings, the endothelium was removed by rubbing with cotton, and the absence of ACh induced relaxation was regarded as indication of successful denudation.<sup>15)</sup>

**Experiment for Effects of 2-CE and CAA in Aortic Rings of Rats**— For the relaxation study, aorta rings were pre-contracted using PE (5  $\mu\text{M}$ ) for 15 min, before 2-CE (1, 3, 10, 25 and 50 mM) was added to the endothelium-intact aortic rings. Concentrations of 0.1, 0.5, 1, 10 mM CAA was individually incubated in either the endothelium-intact or endothelium-denuded aortic rings for 60 min. The percentages of PE-induced relaxation of 2-CE and CAA were calculated after incubation.

**Experiment for Effects of CAA-induced Relaxation with/without Calcium**— Endothelium-denuded aortic rings were incubated with either Krebs solution or a  $\text{Ca}^{2+}$ -free (which contained 50  $\mu\text{M}$  EGTA) modified Krebs solution, and were pre-contracted using PE (5  $\mu\text{M}$ ). Subsequently, CAA (3 mM) was added for 10 min before  $\text{CaCl}_2$  (5 mM) was added last. The CAA relaxation of aortic rings was measured for 60 min and PE-induced was expressed as a percentage of the PE-induced maximal contraction.<sup>16,17)</sup>

**Experiment for  $\text{Ca}^{2+}$  Channel Blockers in CAA Relaxed Aortic Ring of Rats**— The aortic rings of the rats were pre-incubated with various  $\text{Ca}^{2+}$  channel blockers: 2-APB (an  $\text{IP}_3$  receptor blocker, 75  $\mu\text{M}$ ), dantrolene (a ryanodine receptor blocker, 50  $\mu\text{M}$ ), nifedipine (a L-type blocker, 1  $\mu\text{M}$ ) and  $\text{NiCl}_2$  (non-selective  $\text{Ca}^{2+}$  channel blocker, 1 mM),

in standard Krebs solution, or  $\text{Ca}^{2+}$  free EGTA Krebs solution for 30 min. The resting tension of endothelium-intact aortic rings was initially set at 3 g. Then, the aortic rings were treated with PE ( $5\ \mu\text{M}$ ) for 15 min to induce contraction. Finally, CAA ( $3.0\ \text{mM}$ ) was added into the organ bath, and the remaining tension of the rings was measured for 60 min.

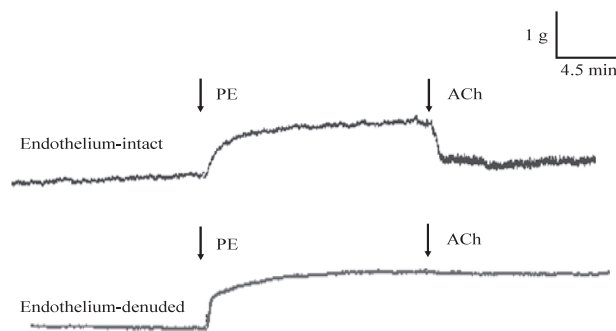
In order to evaluate the calcium channels blocker role of CAA. Aortic rings were incubated with CAA ( $3\ \text{mM}$ ) for 30 min. The resting tension of the endothelium-intact and endothelium-denuded aortic rings was initially set to 3 g. The aortic rings were pre-contracted using PE ( $5\ \mu\text{M}$ ), and then incubated with  $\text{Ca}^{2+}$  channel blockers 2-APB ( $75\ \mu\text{M}$ ) or nifedipine ( $1\ \mu\text{M}$ ). The final contraction responses to PE in the aortic rings were measured after 60 min. The relaxation tensions were measured following an initial tension of 3 g and a PE pre-constructed initial tension, and compared with 2-APB and nifedipine caused relaxation.

**Statistical Analysis** — The half inhibitory concentration ( $\text{IC}_{50}$ ) was calculated using Excel, MicroSoft. Data are expressed as mean  $\pm$  standard deviation. Statistical differences were evaluated by the Student *t*-test. Differences were regarded as significant at  $p < 0.05$ .

## RESULTS AND DISCUSSION

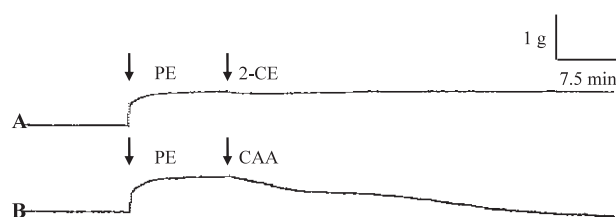
### 2-CE and CAA Relaxed Aortic Rings

Rat aortic rings pre-contracted with PE ( $5\ \mu\text{M}$ ) for 15 min, following by ACh ( $3\ \mu\text{M}$ ) treatment for verification of the function of the vascular endothelium. As shown in Fig. 1, the endothelium-intact function of aortic ring were verified by the relaxation rate of more than 60% (our result of undamaged endothelium relaxed was  $74.8 \pm 13.2\%$ ), while the endothelium-denuded was confirmed by the inability of aortic rings to relax in response to ACh.<sup>15)</sup> In this study, 2-CE caused a weakly relaxation in the PE pre-induced endothelium-intact aortic rings. However, its metabolite, CAA-induced vasorelaxation in a dose dependent manner in both endothelium-intact and -denuded aortic rings (Fig. 2). The 2-CE and CAA affected vasorelaxation are shown in Table 1. The  $\text{IC}_{50}$  of 2-CE in endothelium-intact aortic ring relaxation is higher than 50 mM. The  $\text{IC}_{50}$  values of CAA-induced relaxations in the endothelium-intact and endothelium-denuded aortic rings are 3.3 and



**Fig. 1.** Vasorelaxation Effect of PE Induced Contraction in Endothelium-intact and -denuded Rat Aortas

Aorta rings were precontracted with PE ( $5\ \mu\text{M}$ ) for 15 min and treated with ACh ( $3\ \mu\text{M}$ ) served as positive control.



**Fig. 2.** Effects of 2-CE and CAA in the Isolated Rat Aortic Rings

Chart-photograph of 10 mM 2-CE and 10 mM CAA induced relaxation in endothelium-intact aortic rings were recorded for 60 min after pre-contracting with PE ( $5\ \mu\text{M}$ ) for 15 min.

2.7 mM, respectively. In the clinics, 2-CE intoxicated patients commonly presented with marked hypotension.<sup>2)</sup> One of the known 2-CE metabolites in liver<sup>4)</sup> and in blood<sup>18)</sup> is CAA. It was also known that high doses of ifosfamide (IF) and cyclophosphamide (CP) are associated with severe but generally reversible cardiotoxicity in human. One of their metabolite CAA is commonly evoked to explain the adverse effects of both IF and CP.<sup>19–21)</sup> A previous study has shown that 0.02 ml/kg CAA could cause hypotension, respiration failure and always accompanies neuromuscular contraction in rabbits.<sup>22)</sup> The toxic effect of 2-CE leading to hypotension could be ascribed to its metabolite of CAA action in our finding.

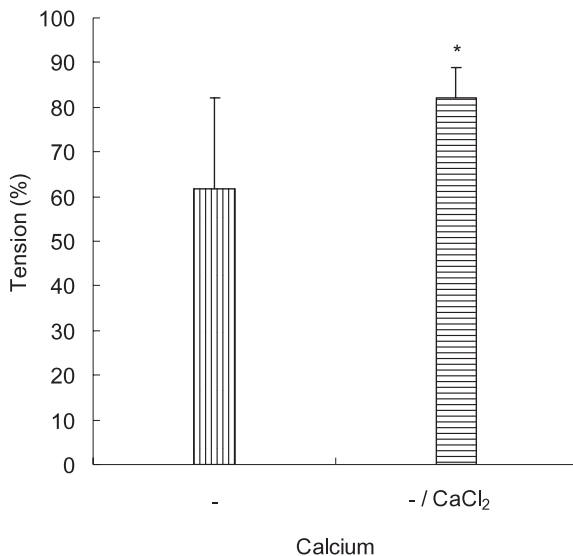
### $\text{CaCl}_2$ Attenuation on CAA Relaxed Aortic Rings

In a  $\text{Ca}^{2+}$ -free condition, the CAA-induced relaxation effect showed no difference between endothelium-intact and endothelium-denuded aortic rings. The tensions of CAA-induced relaxation in the endothelium-denuded aortic rings' tension rate were 61.7% of the control (Fig. 3). Addi-

**Table 1.** Relaxation Effects of 2-Chloroethanol and Chloroacetaldehyde in the Isolated Rat Aortic Rings

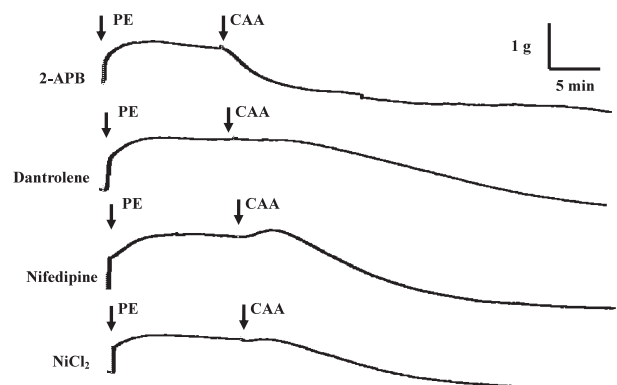
Chemical	Concentration (mM)	Relaxation (% of PE pre-contracted) <sup>a)</sup>	
		Intact aortic ring	Denuded aortic ring
2-CE	1	0.6 ± 1.0	–
	3	9.0 ± 4.2*	–
	10	11.5 ± 4.0*	–
	25	27.0 ± 5.5*	–
	50	34.5 ± 1.1*	–
CAA	0.05	0.5 ± 0.8	–
	0.1	11.5 ± 3.5*	0
	0.5	47.4 ± 8.1*	25.1 ± 8.6*
	1	52.9 ± 0.3* <sup>#</sup>	44.8 ± 6.2*
	10	100.0* <sup>#</sup>	100.0*
CAA	IC <sub>50</sub> (mM)	3.3	2.7

–: not done. a): The percentage of relaxation of PE-induced contraction by 2-CE or CAA were calculated at the end of 60 min incubation. Data are expressed as mean ± S.D. ( $n = 3-4$ ). \* $p < 0.05$  vs. control, <sup>#</sup> $p < 0.05$  vs. 2-CE.

**Fig. 3.** Effects of Calcium on CAA-induced Relaxation in the Endothelium-denuded Rat Aortic Rings

Incubation of CAA either with or without Ca<sup>2+</sup> solution was followed by provision of CaCl<sub>2</sub> that reduced relaxation in the endothelium-denuded aorta rings. Relaxation (%) of PE-induced contraction by CAA was recorded at the end of 60 min incubation. Data are expressed as mean ± S.D. ( $n = 4$ ) \* $p < 0.05$  vs. calcium free group.

tion of CaCl<sub>2</sub> to the Ca<sup>2+</sup>-free condition after CAA could significantly reverse the inhibited tension rate (82.4%) in the endothelium-denuded aortic rings (\* $p < 0.05$ , Fig. 3). It is suggested that the Ca<sup>2+</sup> ion acts as a trigger of contraction in vascular smooth muscles.<sup>8)</sup> Calcium ion influxes intracellular through the plasma membrane, and refluxes from SR to cause vascular contraction.<sup>9,23,24)</sup> Our results

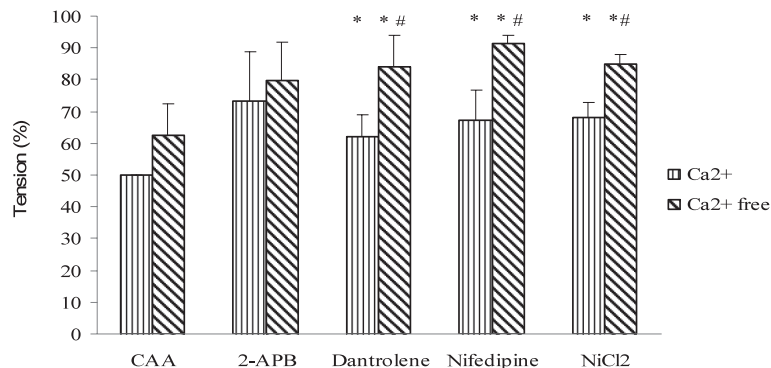
**Fig. 4.** Chart-photographs of Calcium and Calcium Channel Blockers on CAA-induced Relaxation of Endothelium-intact Rat Aortic Rings

Aortic rings were pre-incubated with various calcium channel blockers, 2-APB (75 μM), dantrolene (50 μM), nifedipine (1 μM) and NiCl<sub>2</sub> (1 mM) for 30 min before CAA was added to relax PE (5 μM) pre-contracted rat aortic rings for 60 min.

showed that CAA-induced vasorelaxation in the rat aortic rings might involve calcium mobilization in smooth muscle cells.

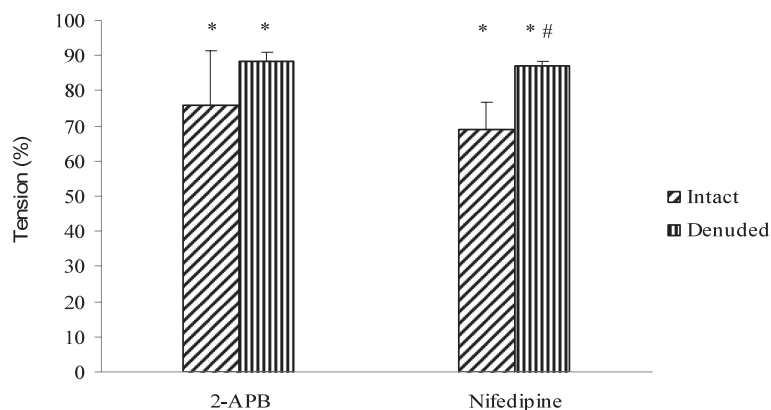
### Ca<sup>2+</sup> Channel Blockers Reduce CAA-induced Vasorelaxation

Chart-photographs of CAA-induced vasorelaxation of aortic rings after pre-incubation with various Ca<sup>2+</sup> channel blockers are shown in Fig. 4. The levels of remained tensions rate (%) in the aortic rings pre-incubated with 2-APB was 73.3%, and 81.4% in Ca<sup>2+</sup>-free media (Fig. 5). Calcium channel



**Fig. 5.** Inhibited Effects of Calcium Channel Blockers on CAA-induced Relaxation of Endothelium-intact Rat Aortic Rings

Aortic rings were pre-incubated with various calcium channel blockers, 2-APB (75  $\mu$ M), dantrolene (50  $\mu$ M), nifedipine (1  $\mu$ M) and NiCl<sub>2</sub> (1 mM) for 30 min, and then pre-contracted with PE (5  $\mu$ M), then treated with CAA (3 mM) for 60 min. Data are expressed as mean  $\pm$  S.D. ( $n = 4-6$ ) \* $p < 0.05$  vs. control, # $p < 0.05$  calcium free group vs. normal Krebs solution group.



**Fig. 6.** Inhibited Effects of CAA on Calcium Channel Blockers-induced Relaxation in the Functional Endothelium Aortic Rings

CAA were pre-incubated on endothelium-intact and -denuded aortic rings for 30 min, and then pre-contracted with PE (5  $\mu$ M), then treated with 2-APB (75  $\mu$ M) or nifedipine (1  $\mu$ M) for 60 min. Data are expressed as mean  $\pm$  S.D. ( $n = 4-6$ ) \* $p < 0.05$  vs. control, # $p < 0.05$  denuded vs. intact group.

blocker 2-APB is known to inhibit the IP<sub>3</sub>-induced release of Ca<sup>2+</sup> from mouse cerebellar membrane in a concentration-dependent manner without affecting IP<sub>3</sub> binding.<sup>25</sup>) In this study, aortic rings were pre-incubated with 2-APB, and the vasorelaxation caused by CAA were not significantly affected, either in Krebs solution or Ca<sup>2+</sup>-free conditions. Tirapelli<sup>26</sup>) has found that IP<sub>3</sub> causes intracellular Ca<sup>2+</sup> release after PE treatment, which does not contribute to the response of alcohol-treated aortic rings. These may undergo PE binding to the IP<sub>3</sub> receptor channel and release Ca<sup>2+</sup> from SR and cause the aortic rings to contract.<sup>27</sup>) These reasons might explain why PE does not cause contraction after 2-APB incubation under Ca<sup>2+</sup>-free conditions.

Ca<sup>2+</sup> channels open in the SR membrane via IP<sub>3</sub> receptors and ryanodine receptors.<sup>9,10</sup>) Dantrolene is an agent that inhibits the release of intra-

cellular Ca<sup>2+</sup> via ryanodine receptors.<sup>24</sup>) The tension of dantrolene treatment was 62.1% (\* $p < 0.05$  vs. CAA alone), and 83.9% in Ca<sup>2+</sup>-free conditions (\* $p < 0.05$  vs. CAA, # $p < 0.05$  vs. Krebs normal solutions, Fig. 5). Our results indicated that CAA-induced vasorelaxation might be involved in the inhibition of Ca<sup>2+</sup> release from intracellular stores via ryanodine receptors, and possibly inhibit Ca<sup>2+</sup> influx. On the other hand, the tension of nifedipine was 67.4% (\* $p < 0.05$  vs. CAA alone), and 91.5% in Ca<sup>2+</sup>-free conditions (\* $p < 0.05$  vs. CAA, # $p < 0.05$  vs. Krebs normal solutions, Fig. 5), the Ca<sup>2+</sup> channel blocker nifedipine had most effective inhibition of the CAA-induced relaxation, especially in a Ca<sup>2+</sup>-free medium. L-type plasma membrane Ca<sup>2+</sup> release channel in the SR is a response to a change in the electric potential across the plasma membrane.<sup>6</sup>)

In smooth muscle, other L-type  $\text{Ca}^{2+}$  channels participate in the extracellular  $\text{Ca}^{2+}$  cycle by controlling the  $\text{Ca}^{2+}$  that binds to and directly activates the contractile apparatus.<sup>11)</sup> The results obtained using L-type VOCCs antagonist, nifedipine significantly reduced the relaxation of aortic rings induced by CAA, both in Krebs solution and under  $\text{Ca}^{2+}$ -free conditions. The tension of the SOCCs antagonist,  $\text{NiCl}_2$  was 68.3% (\* $p < 0.05$  vs. CAA alone), and 84.9% in  $\text{Ca}^{2+}$ -free conditions (\* $p < 0.05$  vs. CAA, # $p < 0.05$  vs. Krebs normal solutions). These results showed that CAA-induced relaxation may involve the inhibition of the release of  $\text{Ca}^{2+}$  from a plasma membrane.<sup>6)</sup>

On the other hand, aortic rings were pre-incubated with CAA and then provided with 2-APB or nifedipine. Results showed that 2-APB tension caused in the endothelium-intact and the endothelium-denuded aortic rings was 76.0 and 88.3%, respectively (\* $p < 0.05$ , vs. CAA alone, Fig. 6). The effect of nifedipine was 69.1 and 86.9%, respectively (\* $p < 0.05$ , vs. CAA alone, # $p < 0.05$  vs. endothelium-intact, Fig. 6). These findings suggested that CAA-induced vasorelaxation was related to the inhibition of SR  $\text{Ca}^{2+}$  channels in smooth muscle. The antagonistic effect of CAA on the release of intracellular  $\text{Ca}^{2+}$  channel in smooth muscle might be related to hypotension due to 2-CE intoxication.

Nitric oxide (NO) is found to stimulate the uptake of cytosolic  $\text{Ca}^{2+}$  via the SR  $\text{Ca}^{2+}$  ATPase, therefore relaxing vascular smooth muscle by lowering intracellular free  $\text{Ca}^{2+}$ . NO reacts with superoxide to form peroxynitrite, which in turn caused glutathione to bind to SR  $\text{Ca}^{2+}$  ATPase cysteine thiols.<sup>28)</sup> Chloroacetaldehyde-caused vasorelaxation might cause the lowering of the concentration of intracellular free  $\text{Ca}^{2+}$  and provides one of the reasons for 2-CE caused hypotension in patients. However, the possible roles of NO involvement in CAA-caused vasorelaxation are still unclear.

In summary, 2-CE itself caused weak relaxation in the rat aortic rings. However, its metabolite, CAA, induced significant relaxation both in endothelium-intact and -denuded aortic rings. The CAA caused vasorelaxation might be related to acute intoxication of 2-CE, and the calcium channels might be involved in the initiation of CAA-induced vasorelaxation. However, the underlying mechanisms of the  $\text{Ca}^{2+}$  exchange remain unclear and warrant further study.

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