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# Mechanism of vasorelaxation of thoracic aorta caused by xanthone

Yu-Wen Cheng, Jaw-Jou Kang *

*Institute of Toxicology, College of Medicine, National Taiwan University, 1 Jen-Ai Rd, Section 1, Taipei, Taiwan*

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**Abstract**

The effect of xanthone on smooth muscle was studied in thoracic aorta isolated from rats. Xanthone relaxed the norepinephrine-induced contraction of rat thoracic aorta. This relaxing effect of xanthone persisted in endothelium-denuded aorta suggesting that the relaxation induced by xanthone is endothelium-independent. The norepinephrine and high-$K^+$-induced vasoconstriction was inhibited dose dependently in aorta pretreated with xanthone with $IC_{50}$ values of $60.26 \pm 8.43$ and $82.9 \pm 13.21$ μM, respectively. The inositol 1,4,5-trisphosphate formation induced by norepinephrine (3 μM) in rat aorta was not affected by xanthone (10–100 μM), suggesting that the vasorelaxant effect of xanthone was not exerted on the receptor. Xanthone concentration dependently inhibited the $^{45}Ca^{2+}$ influx induced by either norepinephrine or high-$K^+$, suggesting that xanthone might act as a blocker of both receptor-operated and voltage-dependent $Ca^{2+}$ channels. Furthermore, xanthone caused an increase in the level of intracellular cyclic adenosine 3′,5′-monophosphate (cAMP), but not cyclic guanosine 3′,5′-monophosphate (cGMP) content. These data suggested that the mechanism of xanthone-induced vasorelaxation might involve the increase of intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) content and block of $Ca^{2+}$ channels. © 1997 Elsevier Science B.V.

*Keywords:* Thoracic aorta; Vasorelaxation; Norepinephrine; cAMP; cGMP; $Ca^{2+}$ channel blocker

## 1. Introduction

Xanthone used in the preparation of xanthydrol and as ovicide for codling moth eggs is less effective as larvicide. It was also shown that xanthone is one of the predominant oxygenated polycyclic aromatic compounds found in the mutagenic fraction of diesel and gasoline engines exhaust particulate extract (Strandell et al., 1994). Derivatives of xanthone are widely distributed in plants among the *Gentianaceae* and *Guttiferae*. They show schistosomicidal activity, central nervous system stimulant effects, cardiotonic, antidepressant and hydrocholeretic effects (Shankaranarayan et al., 1979), antiallergic or bronchodilating activity (Lin et al., 1984) and inhibit cutaneous plasma extravasation (Wang et al., 1994).

Although much is known about the pharmacological actions of xanthone derivatives, few studies have been carried out regarding the pharmacological and toxicological actions of xanthone. Previously we have found that xanthone potentiated the twitch of mouse diaphragm and induced the release of $Ca^{2+}$ from the sarcoplasmic reticulum of skeletal muscle (Kang et al., 1996). In the present study, using isolated rat aorta, we have shown that xanthone behaves as a vasorelaxant, possibly through inhibition of $Ca^{2+}$ influx via receptor-operative and voltage-dependent $Ca^{2+}$ channels and through increasing the intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) content in smooth muscle of rat aorta.

## 2. Materials and methods

### 2.1. Chemicals

Xanthone, norepinephrine, acetylcholine, sodium nitroprusside, trichloroacetic acid, caffeine, ethylene-glycol-bis-(β-aminoethylene)-N,N,N′,N′-tetraacetic acid (EGTA), 3-isobutylmethyl-xanthine (IBMX), forskolin, Dowex-1 (100–200 mesh; X8 chloride) resin and myo-inositol were obtained from Sigma (St. Louis, MO, USA). Myo-[2-$^3$H]inositol, $^{45}Ca^{2+}$, cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP) EIA (enzyme immunoassay) kits were purchased from Amersham (Amersham, UK). When drugs were dissolved in dimethyl sulfoxide (DMSO), the final concentration of

* Corresponding author. Tel.: (886-2) 312-3456, ext. 8603; Fax: (886-2) 341-0217.

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DMSO in the bathing solution did not exceed 0.1%, which did not affect muscle contraction.

### 2.2. Preparation of rat aorta rings and tension recording

Male Wistar rats weighing 250–300 g were killed by decapitation and the thoracic aorta was removed carefully and fat and connective tissue were dissected away. The aorta was cut into rings about 5 mm in length in Krebs solution of the following composition (mM): NaCl, 118.2; KCl, 4.7; $CaCl_2$, 1.9; $MgSO_4$, 1.2; $KH_2PO_4$, 1.2; $NaHCO_3$, 25.0; and glucose, 11.7. The tissue bath solution was maintained at 37°C and bubbled with 95% $O_2$ + 5% $CO_2$. The aorta rings were equilibrated in the medium and maintained under an optimal tension of 1 g for 90 min, with three changes of the Krebs solution, before the experimental procedures were started. Contractions were recorded isometrically via a force-displacement transducer connected to a Gould polygraph (Model 2400). In denuded aorta, the endothelium was removed by rubbing with a cotton ball and the absence of acetylcholine-induced relaxation was taken as an indicator.

Normally, the norepinephrine-induced contraction was initiated in normal $Ca^{2+}$ Krebs (2.5 mM). For separation of the phasic and tonic contraction experiments, the phasic aorta contraction was first initiated with norepinephrine in $Ca^{2+}$-free Krebs (2.5 mM EGTA) and the tonic contraction was then induced by further addition of 3.5 mM $CaCl_2$.

The depolarization-induced contraction of rat aorta was studied in rings stabilized in high-$K^+$ (80 mM), $Ca^{2+}$-free Krebs solution and contraction was evoked by the addition of 3.5 mM $Ca^{2+}$. The high-$K^+$ solution was prepared by substituting an equimolar amount of KCl for NaCl.

### 2.3. Cyclic adenosine 3′,5′-monophosphate and cyclic guanosine 3′,5′-monophosphate assay of rat aorta

Rat aorta cyclic nucleotides were determined as described by Kauffman et al. (1987). After incubation with forskolin, sodium nitroprusside, 3-isobutylmethyl-xanthine, xanthone or DMSO for 2 min, the rat aortic rings were rapidly frozen in liquid nitrogen and stored at −70°C until homogenized in 0.5 ml of 10% trichloroacetic acid using a Potter glass/glass homogenizer. The homogenate was centrifuged at $10\,000 \times g$ for 5 min and the supernatant was removed and extracted 4 times with 3 volumes of ether. The cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP) content was then assayed using enzyme immunoassay kits. The precipitate was used for protein assay (Lowry et al., 1951). cAMP and cGMP levels were expressed as pmol/mg protein.

### 2.4. Measurement of [$^3$H]inositol phosphate

The procedure described by Hirata et al. (1990) was used to measure [$^3$H]inositol monophosphate formation in rat aorta. Rat thoracic aortas were exposed to Krebs solution containing 10 μCi/ml of [$^3$H]inositol for 3 h and gassed with 95% $O_2$/5% $CO_2$ mixture. The tissues were then transferred to tubes containing fresh Krebs solution containing DMSO or xanthone for 15 min, and saline or norepinephrine (3 μM) was added and incubated for a further 15 min. LiCl (10 mM) was added 5 min before norepinephrine to inhibit inositol monophosphatase (Borridge et al., 1982). The aortas were frozen in liquid nitrogen and homogenized in 1.3 ml of 10% trichloroacetic acid. After centrifugation, 1 ml of supernatant was collected and the trichloroacetic acid was removed by washing 4 times with 3 volumes of ether. Inositol monophosphate in the aqueous phases was analyzed by separation on a 1 ml volume Dowex-1 ion-exchange column according to method of Neylon and Summers (1987). The tissue pellets were resuspended in 1.0 M NaOH and boiled in 95°C for 1 h then assayed for protein according to the method of Lowry et al. (1951). The radioactivity levels of [$^3$H]inositol monophosphate were expressed as cpm/mg protein.

### 2.5. $^{45}Ca^{2+}$ measurement

$^{45}Ca^{2+}$ influx was measured in a manner similar to that described by Kaushik et al. (1980). Aortic rings were placed in test tubes containing Krebs solution with 2 μCi/ml $^{45}Ca^{2+}$ in the presence of DMSO (0.1%), verapamil (2 μM) or xanthone and incubated for 15 min. Norepinephrine (3 μM) or high-$K^+$ (80 mM) was then added and the incubation was continued for another 15 min. After the incubation period, the tissues were transferred to test tubes containing 2 ml of ice-cold $Ca^{2+}$-free Krebs solution with 2 mM EGTA for 40 min in order to remove extracellular $^{45}Ca^{2+}$. The tissues were then removed and dissolved in 37% perchloric acid (0.1 ml) at 75°C. The radioactivity was counted in a liquid scintillation counter (Backman Model 2200 CA).

### 2.6. Statistical analysis

The data are presented as the means ± S.E.M. for the number of experiments indicated. Statistical analysis was performed using the Student's *t*-test, and $P < 0.05$ was regarded as significantly different.

## 3. Results

### 3.1. Vasorelaxation induced by xanthone

The effects of xanthone were studied on norepinephrine-precontracted rat aorta. Norepinephrine induced a phasic contraction followed by a tonic contraction lasting for at least 15 min in both intact and denuded (endothelium-removed) aorta (Fig. 1a, upper traces). Intact but not de-

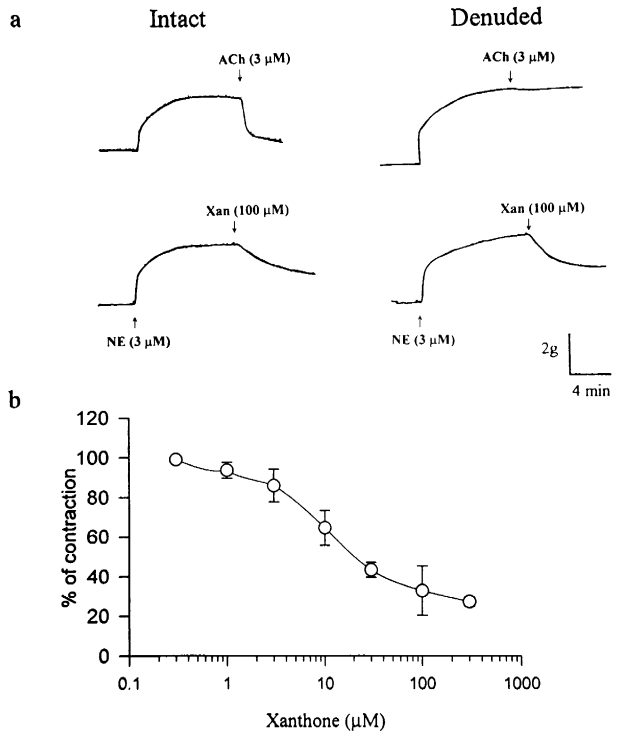


Fig. 1. (a) Relaxant effect of xanthone on norepinephrine-induced muscle contraction in intact and denuded aorta. DMSO (0.1%) or xanthone was added 10 min after the exposure of aorta to norepinephrine (NE 3 μM). Acetylcholine (ACh, 3 μM) was used to determine whether the aorta was intact or denuded. (b) Effects of xanthone on the contraction induced by norepinephrine (NE, 3 μM) in rat aorta. Rat aorta was pretreated with DMSO (0.1%, control), or xanthone (1, 3, 10, 30, 100 and 300 μM) at 37°C for 15 min in normal Krebs and norepinephrine was then added to trigger the contraction. Each point represents the mean ± S.E.M. of four determinations.

nuded aorta relaxed upon addition of acetylcholine suggesting that the acetylcholine-induced relaxation was endothelium-dependent. Xanthone (100 μM) caused a 47.37 ± 1.2 and 40.33 ± 2.3% relaxation of the norepinephrine-precontracted intact and denuded aorta, respectively (Fig. 1a, lower traces). The relaxation induced by xanthone was dose-dependent and endothelium-independent. Xanthone also dose dependently inhibited norepinephrine-induced contraction when added prior to the addition of norepinephrine (Fig. 1b) with an $IC_{50}$ value of 60.26 ± 8.43 μM ($n = 6$).

### 3.2. Xanthone inhibited both phasic and tonic contraction induced by norepinephrine

The phasic and tonic contractions were induced sequentially in order to examine the effect of xanthone on both phases of contraction. Norepinephrine induced a transient contraction in the $Ca^{2+}$-free medium (2.5 mM EGTA) while the tonic contraction was induced upon addition of 3.5 mM $Ca^{2+}$ (Fig. 2a). This suggests that the transient phasic contraction induced by norepinephrine was caused by the $Ca^{2+}$ released from an internal store, namely sarcoplasmic reticulum, and that the tonic contraction was induced by the influx of extracellular $Ca^{2+}$ through receptor-operated $Ca^{2+}$ channels. Pretreatment with 300 μM xanthone inhibited both phasic and tonic contractions induced by norepinephrine (Fig. 2b), 78.79 ± 5.14 and 38.59 ± 3.44%, respectively. The inhibition of phasic contraction was dose-dependent with an $IC_{50}$ value of 64.9 ± 6.23 μM. Higher concentrations of xanthone were not tested due to the solubility problem.

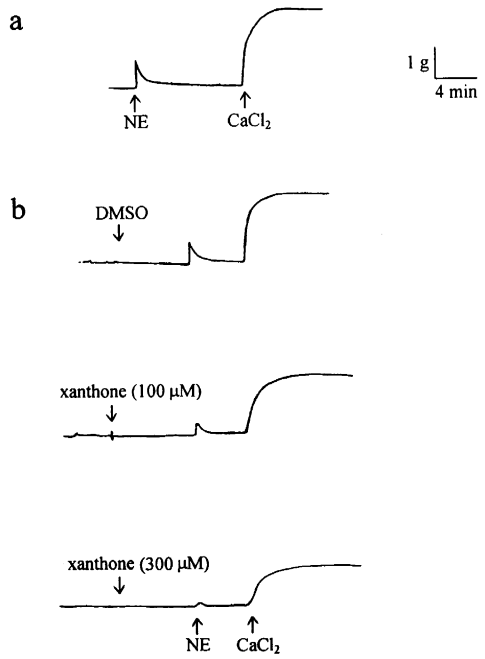


Fig. 2. Effects of xanthone on the phasic and tonic contractions induced by norepinephrine in rat aorta. DMSO (0.1%) or various concentrations of xanthone as indicated was added 15 min before the exposure of rat aorta to norepinephrine (3 μM) in $Ca^{2+}$-free Krebs containing 2.5 mM EGTA to obtain the phasic contraction. To obtain the tonic contraction, 3.5 mM $CaCl_2$ was added again according to procedures outlined in Section 2. The experiments were repeated at least 4 times.

### 3.3. Effect of xanthone on [$^3$H]inositol monophosphate formation

Norepinephrine, by activating phospholipase C, induced the formation of inositol 1,4,5-trisphosphate which further caused $Ca^{2+}$ release from the sarcoplasmic reticulum

Table 1
Effects of xanthone on [$^3$H]inositol monophosphate formation in rat aorta

| Treatment | [$^3$H]Inositol monophosphate (cpm/mg protein) |
|---|---|
| Resting | 866.21 ± 56.33 [a] (4) |
| Norepinephrine (3 μM) | 2803.33 ± 355.59 (5) |
| Xanthone (10 μM) + NE | 2191.21 ± 361.82 (5) |
| Xanthone (30 μM) + NE | 2791.49 ± 517.93 (5) |
| Xanthone (100 μM) + NE | 2537.55 ± 271.25 (5) |

[$^3$H]Inositol monophosphate formation of rat aorta was measured as described in Section 2. The results are expressed as the mean ± S.E.M. The numbers in parentheses indicate the $n$ values.
[a] Significant ($P < 0.001$) less than in tissues exposed to norepinephrine (NE).

through inositol 1,4,5-trisphosphate receptor channels (Ehrlich and Watras, 1988). The effect of xanthone on norepinephrine-induced inositol 1,4,5-trisphosphate formation, as judged by the formation of [$^3$H]inositol monophosphate in the presence of LiCl, was examined and the data are summarized in Table 1. Xanthone (10–100 μM) did not significantly affect [$^3$H]inositol monophosphate formation.

### 3.4. Effects of xanthone on high-$K^+$-induced contractions

Depolarization of aorta with high-$K^+$ (80 mM) induced a tonic contraction which could be inhibited by verapamil, an L-type calcium channel blocker, suggesting that the high-$K^+$-induced contraction was mainly caused by the influx of extracellular $Ca^{2+}$ through voltage-dependent $Ca^{2+}$ channels. Pretreatment with xanthone dose dependently inhibited the high-$K^+$-induced contraction with an $IC_{50}$ value of 82.93 ± 13.21 μM (Fig. 3).

### 3.5. Effect of xanthone on $^{45}Ca^{2+}$ uptake promoted by norepinephrine and high-$K^+$

Norepinephrine and high-$K^+$ promote the $Ca^{2+}$ influx through receptor-operated and voltage-dependent $Ca^{2+}$ channels, respectively (Murray and Kotlikoff, 1991). The $^{45}Ca^{2+}$ uptake measurement was used to assess the effects of xanthone and the data summarized in Table 2. As shown in Table 2, xanthone dose dependently inhibited both norepinephrine and high-$K^+$-induced $^{45}Ca^{2+}$ influx with 70.82 ± 4.12% and 65.04 ± 10.9% inhibition, respectively, at 100 μM. Verapamil (2 μM) almost completely inhibited the $^{45}Ca^{2+}$ influx induced by both agonists, norepinephrine and high-$K^+$.

### 3.6. Effect of xanthone on cyclic nucleotide formation

Foskolin and sodium nitroprusside relax the aorta through the activation of adenylate cyclase and guanylate cyclase, respectively (Gruetter et al., 1979; Ousterhout and Sperelakis, 1987). The effect of xanthone on both cyclase activities was examined by quantitation of cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP) contents and the results are summarized in Table 3. As expected, forskolin and sodium nitroprusside promoted cyclic adenosine 3′,5′-monophosphate and cyclic guanosine 3′,5′-monophosphate formation respectively. Xanthone (30–300 μM) dose dependently increased the formation of cyclic adenosine 3′,5′-monophosphate but not of cyclic guanosine 3′,5′-monophosphate. Co-treatment of xanthone with either forskolin or 3-isobutylmethyl-xanthine (IBMX) did not further increase the cyclic adenosine 3′,5′-monophosphate content.

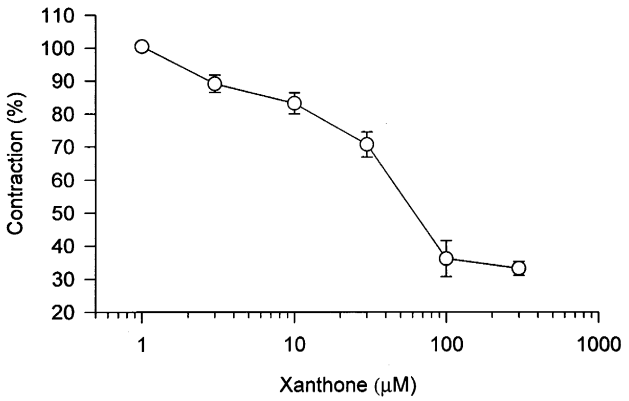


Fig. 3. Effects of xanthone on the contraction induced by high-$K^+$ in rat aorta. The effects of xanthone on depolarization-induced contraction were examined on rat aorta pretreated with DMSO (0.1%, control) or xanthone (1, 3, 10, 30, 100 and 300 μM) at 37°C for 15 min. Each point represent the mean ± S.E.M of eight determinations.

Table 2
Effect of xanthone on $^{45}Ca^{2+}$ influx induced in aorta by norepinephrine and high-$K^+$

| Treatment | % increase of $^{45}Ca^{2+}$ influx |
|---|---|
| Norepinephrine (3 μM) | 46.29 ± 2.78 |
| + xanthone (30 μM) | 18.32 ± 6.24 [a] |
| + xanthone (100 μM) | 13.95 ± 1.48 [a] |
| + verapamil (2 μM) | 1.70 ± 0.38 [b] |
| High potassium (80 mM) | 49.38 ± 4.17 |
| + xanthone (30 μM) | 24.78 ± 6.14 [a] |
| + xanthone (100 μM) | 15.90 ± 4.88 [a] |
| + verapamil (2 μM) | 1.50 ± 0.76 [b] |

The results are expressed as the mean ± S.E.M.
[a] $P < 0.05$.
[b] $P < 0.001$ was considered to be statistically significant according to the Student's *t*-test.

Table 3
Effects of xanthone, sodium nitroprusside, forskolin and 3-isobutylmethylxanthine (IBMX) on the cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP) content of isolated rat aorta

| | Cyclic AMP (pmol/mg protein) | Cyclic GMP (pmol/mg protein) |
|---|---|---|
| Control (DMSO) | 0.69 ± 0.39 | 0.16 ± 0.03 |
| Forskolin (10 μM) | 12.40 ± 1.50 [b] | n.d. |
| Sodium nitroprusside (10 μM) | n.d. | 1.76 ± 0.46 [a] |
| IBMX (100 μM) | 10.51 ± 2.86 [a] | 2.48 ± 0.75 [a] |
| Xanthone | | |
| 30 μM | 9.25 ± 0.74 [a] | 0.13 ± 0.02 |
| 100 μM | 10.28 ± 0.89 [a] | 0.15 ± 0.04 |
| 300 μM | 10.82 ± 0.83 [b] | 0.14 ± 0.03 |
| Forskolin + xanthone (100 μM) | 12.13 ± 2.04 [b] | n.d. |
| IBMX + xanthone (100 μM) | 13.99 ± 1.12 [b] | 2.69 ± 0.43 [b] |

The results are expressed as the mean ± S.E.M. n.d., not determined.
[a] $P < 0.05$.
[b] $P < 0.01$, was considered to be statistically significant according to the Student's *t*-test.

## 4. Discussion

In rat aorta, xanthone concentration dependently relaxed the contraction induced by an agonist, norepinephrine, and

by depolarization and high-$K^+$. The relaxant action of xanthone persisted in endothelium-denuded aorta, suggesting that the vasorelaxation caused by xanthone was endothelium-independent. Pretreatment with xanthone also dose dependently inhibited the contractile response to both norepinephrine and high-$K^+$.

It is widely accepted that the influx of external $Ca^{2+}$ through specific $Ca^{2+}$ channels or $Ca^{2+}$ release from internal stores plays an important role in excitation–contraction coupling of smooth muscle. By acting on specific membrane receptors, norepinephrine stimulates the formation of inositol 1,4,5-triphosphate, which binds to and opens specific inositol 1,4,5-triphosphate-receptor channels in the sarcoplasmic reticulum membrane (Ehrlich and Watras, 1988) and induces $Ca^{2+}$ influx through receptor-operated channel (Bohr, 1963; Hudgins and Weiss, 1968) causing phasic contraction and tonic contraction, respectively. On the other hand, the high-$K^+$-induced contraction of smooth muscle is the result of an increase in $Ca^{2+}$ influx through voltage-dependent $Ca^{2+}$ channels (Karaki and Weiss, 1979, 1984). In the present experiments, xanthone (1–300 μM) concentration dependently inhibited both the norepinephrine-induced tonic contraction (Fig. 2) and high-$K^+$-induced contractions (Fig. 3), suggesting that xanthone might act as a $Ca^{2+}$ channel blocker of both receptor-operated and voltage-dependent channels. The channel-blocking action of xanthone was further supported by the fact that $^{45}Ca^{2+}$ influx induced by norepinephrine and high-$K^+$ was also inhibited.

We have also found that the norepinephrine-induced phasic contraction, which is caused by $Ca^{2+}$ release from sarcoplasmic reticulum, induced by inositol 1,4,5-triphosphate, was also depressed by xanthone, suggesting that xanthone might either inhibit the formation of inositol 1,4,5-triphosphate or block the $Ca^{2+}$ release from sarcoplasmic reticulum. From Table 1, we know that xanthone, at concentrations that induced vasorelaxation, did not affect inositol 1,4,5-triphosphate formation, suggesting that the action of xanthone was not on the receptor level. However, we do not know, at this moment, whether xanthone will inhibit the $Ca^{2+}$ release from inositol 1,4,5-triphosphate receptor channel of sarcoplasmic reticulum. Xanthone has no effect on the caffeine-induced contraction induced by $Ca^{2+}$ release from a caffeine-sensitive $Ca^{2+}$ storage site (data not shown).

Other important mediators for relaxing vascular smooth muscle are cyclic nucleotides, such as cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP) (Murad, 1986). Cyclic adenosine 3′,5′-monophosphate activates protein kinase A and relaxes vascular smooth muscle either by causing phosphorylation of myosine light chain kinase (Silver and Disalvo, 1979; Adelstein and Eisenberg, 1980), or by decreasing intracellular $[Ca^{2+}]$ (Scheid et al., 1979; Velema et al., 1983; Suematsu et al., 1984; Kamm and Stull, 1985; Twort and van Breemen, 1989). Cyclic guanosine 3′,5′-monophosphate-activated protein kinase G inhibits $Ca^{2+}$ influx, augments $Ca^{2+}$ sequestration and decreases the sensitivity of contractile elements to $Ca^{2+}$ (Karaki et al., 1988). In the present experiments, xanthone concentration dependently increased cyclic adenosine 3′,5′-monophosphate formation in rat thoracic aorta with no effect on cyclic guanosine 3′,5′-monophosphate formation. The underlying cause for the elevation of cyclic adenosine 3′,5′-monophosphate content remains unclear. The formation and metabolism of cyclic adenosine 3′,5′-monophosphate are controlled by two molecules, namely adenylate cyclase and phosphodiesterase, respectively (Torphy and Undem, 1991; Nijkamp et al., 1992). Co-treatment with an adenylate cyclase activator, forskolin, or a phosphodiesterase inhibitor, 3-isobutylmethyl-xanthine (IBMX), did not augment the formation of cyclic adenosine 3′,5′-monophosphate induced by xanthone, suggesting that xanthone might affect both enzymes. However, further studies of direct effects of xanthone on both enzyme activities may help to understand the mechanism.

In conclusion, the results from this study suggest that the inhibitory effects of xanthone on contractile responses in rat thoracic aorta are due to elevation of the cyclic adenosine 3′,5′-monophosphate level in vascular smooth muscle and inhibition of $Ca^{2+}$ channels.

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