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# Mechanism of the vasodilator effect of 12-*O*-methylcurine in rat aortic rings

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**Abstract**

The vasodilator effects of 12-*O*-methylcurine (OMC), a bisbenzylisoquinoline alkaloid isolated from *Chondrodendron platyphyllum* (Menispermaceae), and its respective mechanism of action were investigated in rat aorta. In either endothelium-intact or endothelium-denuded aortic rings, OMC induced concentration-dependent relaxation in vessels pre-contracted with 0.1 $\mu$M phenylephrine (IC50 = 63.2±8.8 $\mu$M and 73.9±5.3 $\mu$M, respectively), 100 $\mu$M 5-hydroxytryptamine (IC50 = 49.6±13 $\mu$M and 49.9±10 $\mu$M, respectively) and 50 mM KCl (IC50 = 19.9±6.8 $\mu$M and 21.1±4.5 $\mu$M, respectively). OMC also inhibited in a concentration-dependent and non-competitive manner the concentration–response curves induced by $CaCl_2$ in high $K^+$ (IC50 = 16.7±1.6 $\mu$M). In addition, OMC (100 $\mu$M) strongly inhibited phenylephrine-induced contractions dependent on calcium influx in the absence and presence of nifedipine (10 $\mu$M). In $Ca^{2+}$-free medium, the transient contractions induced by phenylephrine (0.1 $\mu$M) were strongly inhibited by OMC (100 $\mu$M), whereas those induced by caffeine (20 mM) were not altered. H-89 (1 $\mu$M) and Rp-8-pCPT-cGMPs (3 $\mu$M), selective inhibitors of protein kinase A and G, respectively, did not change the relaxant effect of OMC in aortic rings pre-contracted with phenylephrine. Finally, OMC induced a concentration-dependent relaxation (IC50 = 62.8±12.5 $\mu$M) of the sustained contractions induced by the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate in normal, but not in $Ca^{2+}$-free, solution. The above results suggest that OMC induces a vasodilator effect in rat aortic rings by a mechanism independent of the presence of functional endothelium and dependent on the influx of calcium ions through voltage- and receptor-operated calcium channels. Furthermore, it can also be suggested that the inhibition of calcium influx activated by protein kinase C is involved in the vasodilator effect of OMC.

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## Introduction

Calcium influx is important for the control of vascular tonus and its blockage is the mechanism involved in the antihypertensive effect of calcium-channel blockers, such as verapamil, nifedipine and nitrendipine (Baker 2000). Bisbenzylisoquinoline alkaloids (BBA) are known to block calcium influx through the L-type voltage-dependent and non-selective calcium channels (Felix et al 1992; Low et al 1996). 12-*O*-Methylcurine (OMC; Figure 1) is a BBA isolated from the root bark of *Chondrodendron platyphyllum*, a plant used in folk medicine for the treatment of fever associated with malaria and as an antispasmodic (Corrêa 1974), as previously described by Tang et al (1980). This alkaloid presents a non-depolarizing neuromuscular blocking action similar to that observed for tubocurarine, and

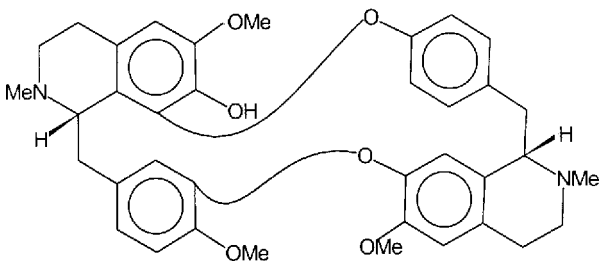


**Figure 1** Structure of 12-*O*-methylcurine.

weaker ganglionar blocking and histamine release actions (Tang et al 1980). In addition, OMC reduces the mean blood pressure, which was associated with the release of histamine (Tang et al 1980). Because of the lack of studies in the vascular system with this BBA, the present study was performed to investigate the effects of OMC and its respective mechanism of action in rat aortic rings.

## Materials and Methods

### Animals

The investigation conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and was approved by the ethics committee for animal experimentation of the Federal University of Minas Gerais (UFMG). Male Wistar rats were bred in our animal facilities at CEBIO (Center of Bioterism, UFMG).

### Experimental procedure

The aortic rings were mounted as described by Lemos et al (1999). Briefly, male Wistar rats (200–250 g) were killed by cervical dislocation and exsanguinated. The descending thoracic aorta was excised, free of fat and connective tissue, cut into rings of approximately 4–5 mm in length and set up in gassed (95% $O_2$ and 5% $CO_2$) Krebs–Henseleit solution of the following composition (mM): NaCl 110.8, KCl 5.9, $NaHCO_3$ 25.0, $MgSO_4$ 1.07, $CaCl_2$ 2.49, $NaH_2PO_4$ 2.33 and glucose 11.51. When necessary, the endothelium was removed by rubbing the intimal surface with a wooden stick. The tissues were maintained at 37°C under a tension of 1 g and equilibrated for a period of 1 h before initiating experimental protocols. During this period, the incubation medium was changed every 15 min. After the equilibration period, two contractile responses were evoked by submaximal concentrations of phenylephrine (0.1 $\mu$M) to elicit reproducible responses. The presence of functional endothelium was assessed by the ability of acetylcholine (1 $\mu$M) to induce greater than 50% relaxation of vessels pre-contracted with phenylephrine (0.1 $\mu$M). The absence of acetylcholine relaxant activity indicated the absence of a functional endothelium. OMC was added cumulatively during the tonic contractions induced by phenylephrine (0.1 $\mu$M), 5-hydroxytryptamine (5-HT; 100 $\mu$M) and KCl (50 mM) in tissues suspended in normal Krebs–Henseleit or 1 $\mu$M 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) in normal or $Ca^{2+}$-free (without $CaCl_2$ plus 1.0 mM EGTA) solution. The results are expressed as the percentage decrease in the maximal contraction induced by the contractile agents, considering 100% relaxation as the point when the basal line was reached. The 50% inhibitory concentration (IC50) values were calculated from concentration–response curves. The participation of cyclic AMP and cyclic GMP in the vasodilator effects of cumulative concentrations of OMC was investigated using selective inhibitors of protein kinase A and protein kinase G, such as H-89 (1 $\mu$M) and Rp-8-pCPT-cGMPs (3 $\mu$M), respectively, in aortic rings pre-contracted with phenylephrine (0.1 $\mu$M). In order to investigate the effect of OMC on the release of the intracellular $Ca^{2+}$, contractile responses of phenylephrine (0.1 mM) and caffeine (20 mM) were performed in $Ca^{2+}$-free solution (Freitas et al 1996). Phenylephrine and caffeine induced contractile responses that were considered as controls in the absence of OMC. The effect of OMC on calcium influx induced by phenylephrine (0.1 $\mu$M) was also investigated. The aortic rings were maintained in $Ca^{2+}$-free solution and stimulated with phenylephrine twice to deplete the intracellular $Ca^{2+}$ stores. After this, the aortic rings were stimulated with phenylephrine in normal Krebs–Henseleit solution and the contractions obtained were considered as controls. The above steps were repeated in rings pre-treated with OMC (100 $\mu$M) alone or in the presence of nifedipine (10 $\mu$M). The responses of the tissues were recorded using isometric transducers and a data acquisition system (WPI, Inc., USA).

### Drugs

Acetylcholine chloride, 5-HT, nifedipine, phenylephrine chloride, caffeine and TPA were purchased from Sigma (USA). H-89 and Rp-8-pCPT-cGMPs were purchased from Calbiochem (USA). OMC was isolated as described by Tang et al (1980), yielding approximately 0.1% (150 mg) from the total ethanolic extract (150 g). OMC was solubilized in a mixture of distilled water/chremophor as a $10^{-1}$ M solution and diluted to the

desired concentration with distilled water just before use. The final concentration of chremophor never exceeded 0.1%, which was without effect when exposed to control preparations. TPA was solubilized in ethanol as a store solution and diluted in distilled water for the desired concentrations. The other compounds were freely dissolved in water.

### Statistics

Results are expressed as the mean±s.e.m of five experiments. The Mann–Whitney test was used to compare differences found for the effect of OMC on sustained contractions induced by the agonists studied and also to compare the effect of OMC on transient contractions observed under different conditions as described above. One-way analysis of variance with Bonferroni's multiple comparison post-test was used to compare concentration–response curves with OMC obtained in aortic rings pre-contracted with TPA in normal and $Ca^{2+}$-free solutions. All statistical analyses were considered significant at a value of $P < 0.05$. IC50 values were calculated graphically from the individual concentration–response curves using a non-linear regression equation.

## Results

### Vasodilator effect of OMC in the rat aorta

In the presence of functional endothelium, OMC induced a concentration-dependent vasodilator effect in the rat aorta pre-contracted with phenylephrine (0.1 $\mu$M), 5-HT (100 $\mu$M) and KCl (50 mM), as illustrated by IC50 and $E_{max}$ values in Table 1. In the absence of functional endothelium, the vasodilator effect of OMC was not significantly different from that observed in endothelium-intact rings (Table 1).

**Table 1** Vasorelaxant effect of 12-*O*-methylcurine in rat aortic rings pre-contracted with different agonists.

| Drugs | Endothelium-intact | | Endothelium-denuded | |
|---|---|---|---|---|
| | IC50 ($\mu$M) | $E_{max}$ (%) | IC50 ($\mu$M) | $E_{max}$ (%) |
| Phenylephrine | 63.2±8.8 | 94.8±0.7 | 73.9±5.3 | 95.3±0.7 |
| 5-Hydroxytryptamine | 49.6±13.0 | 98.7±1.3 | 49.9±10.0 | 98.4±1.6 |
| KCl | 19.9±6.8* | 96.2±1.5 | 21.2±4.5* | 98.2±1.1 |

The results represent the mean±s.e.m of five experiments. *$P < 0.05$ compared with values obtained with phenylephrine and 5-hydroxytryptamine.

### Mechanisms involved in the vasodilator effect induced by OMC

*Calcium influx*

As the above results indicated the participation of voltage-operated calcium channels in the vasorelaxation induced by OCM, the effect of this alkaloid was investigated in the contractions induced by calcium in depolarized aortic rings (KCl 50 mM) in $Ca^{2+}$-free medium. Here, OMC induced a concentration-dependent and non-competitive inhibition of cumulative response curves induced by $CaCl_2$, with an IC50 value of 16.7±1.6 $\mu$M (Figure 2; n = 5). The IC50 value was similar to that observed in aortic rings pre-contracted with KCl (50 mM).

The effect of OMC (100 $\mu$M) was also investigated against the calcium-influx-dependent contractions in rat aortic rings stimulated with phenylephrine (0.1 $\mu$M) in the presence of nifedipine (10 $\mu$M). Nifedipine strongly inhibited the contractions induced by calcium influx (62.2±2.9% of inhibition; $P < 0.001$ compared with control, n = 5). Nevertheless, OMC was still able to induce a complementary reduction in the calcium-influx-dependent contractions (86.0±3.9% of inhibition; $P < 0.001$ compared with control without nifedipine, n = 5).

The effect of OMC was also investigated against contractions dependent on the capacitative calcium entry. For this, rat aortic rings were maintained in $Ca^{2+}$-free medium, the intracellular calcium stores were

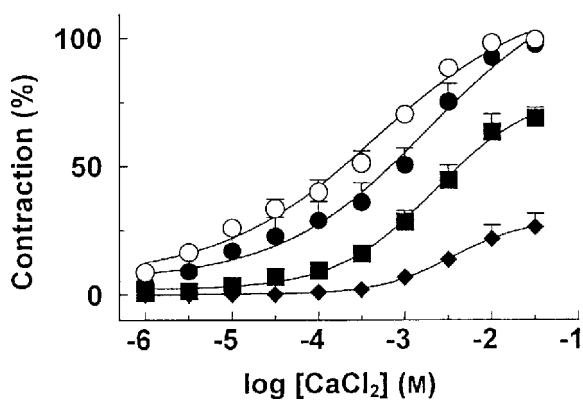


**Figure 2** Effect of 12-*O*-methylcurine on the concentration–response curves of $CaCl_2$ in rat aortic rings. The results represent the mean±s.e.m of five experiments. ○, Control; ●, 12-*O*-methylcurine 3 $\mu$M; □, 12-*O*-methylcurine 10 $\mu$M; ◆, 12-*O*-methylcurine 30 $\mu$M.

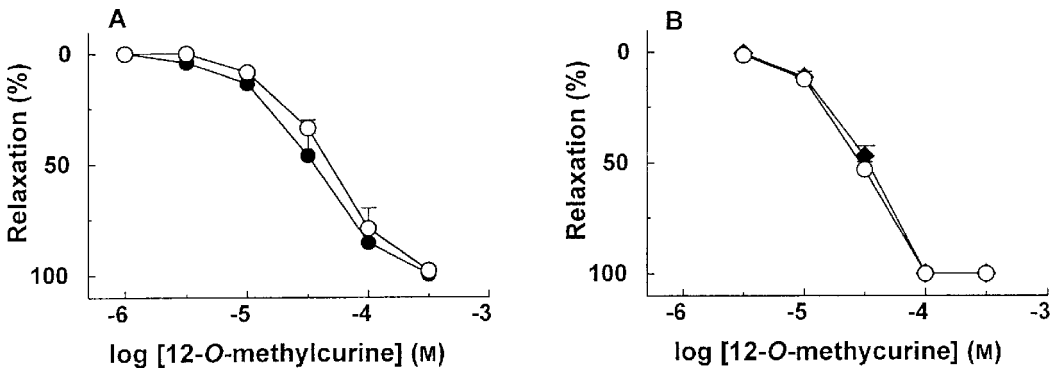


**Figure 3** Vasodilator effect of 12-*O*-methylcurine in the presence of 1 $\mu$M H-89 (A) and 3 $\mu$M Rp-8-pCPT-cGMPs (B) in rat aortic rings pre-contracted with 0.1 $\mu$M phenylephrine. The results represent the mean$\pm$s.e.m of five experiments. ○, Control; ●, 12-*O*-methylcurine+H-89; ◆, 12-*O*-methylcurine+Rp-8-pCPT-cGMPs.

depleted by repeated stimulation with phenylephrine (0.1 $\mu$M), and then pre-treated with OMC (100 $\mu$M) before administration of $CaCl_2$ (2.5 mM). We observed that OMC induced a dramatic reduction in the contraction induced by $CaCl_2$ (95.6$\pm$1.8% of inhibition; $P < 0.001$ compared with control, n = 5).

*Intracellular calcium stores*

To investigate the effect of OMC on the intracellular calcium stores, the rat aortic rings were maintained in $Ca^{2+}$-free medium and stimulated with phenylephrine (0.1 $\mu$M) or caffeine (20 mM). OMC (100 $\mu$M) significantly inhibited the transient contractions induced by phenylephrine (78.6$\pm$2.4% of inhibition; $P < 0.001$ compared with control, n = 5). However, no significant effect of OMC was observed in the transient contractions induced by caffeine (19.6$\pm$10.9% of inhibition; n = 5).

*Participation of protein kinases*

The involvement of proteins kinases A and G in the vasorelaxant effect induced by OMC was investigated in the presence of selective inhibitors, H-89 (1 $\mu$M) and Rp-8-pCPT-cGMPs (3 $\mu$M), respectively. These inhibitors did not modify the vasorelaxant effect of OMC (Figure 3A, B) in aortic rings pre-contracted with phenylephrine (0.1 $\mu$M). The IC50 values were 45.4$\pm$4.0 $\mu$M and 44.5$\pm$9.0 $\mu$M (control vs H-89, respectively; n = 5) and 29.7$\pm$1.8 $\mu$M and 33.0$\pm$2.3 $\mu$M (control vs Rp-8-pCPT-cGMPs, respectively; n = 5).

To investigate the involvement of protein kinase C, the vasodilator effect of OMC was investigated in rat aortic rings contracted with TPA (1 $\mu$M) in the presence and in the absence of extracellular calcium. TPA caused a sustained and slowly developing contraction in normal

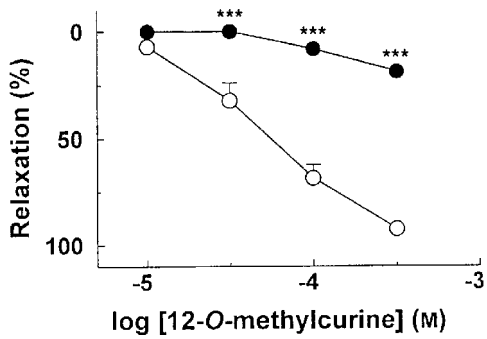


**Figure 4** Vasodilator effect of 12-*O*-methylcurine on rat aortic rings pre-contracted with 1 $\mu$M TPA in normal (○) or $Ca^{2+}$-free (●) solution. The results represent the mean$\pm$s.e.m of five experiments. ***$P < 0.001$ compared with values in normal solution.

(1.3$\pm$0.1 g) and $Ca^{2+}$-free solution (0.8$\pm$0.1 g). In $Ca^{2+}$-containing solution, OMC induced a concentration-dependent relaxation with an IC50 value of 62.8$\pm$12.5 $\mu$M (Figure 4; n = 5). In $Ca^{2+}$-free solution, the vasodilator effect of OMC was strongly inhibited (Figure 4; n = 5).

## Discussion

In the present work, we investigated the mechanism of the vasodilator effect of OMC in the rat aortic rings. The effect of this alkaloid was independent of the presence of a functional endothelium or activation of proteins kinase A and G. However, the effect of OMC was dependent on inhibition of the calcium influx through voltage-operated calcium channels and non-selective ion channels. The inhibition of contractions dependent on the release of intracellular calcium stores sensitive to inositol trisphosphate ($InsP_3$) is also involved

in the mechanism of the vasodilator effect of OMC. Our results also demonstrated the participation of protein kinase C-dependent mechanisms in the vasodilator effect induced by OMC.

OMC induces a concentration-dependent vasodilatation in rat aortic rings contracted with different agonists. The denudation of the endothelium did not change the vasodilator effect of OMC for any contractile agonist studied, suggesting that the relaxant or contractile factors derived from the endothelium did not participate in or alter the activity of OMC.

Among the contractile agonists studied, we observed that the effect of OMC was more potent in rings stimulated with KCl, in endothelium-containing and endothelium-denuded rings. It is well known that stimulation with KCl induces contraction through activation of voltage-operated calcium channels (Karaki et al 1997). Therefore, the above observation suggests that OMC induces its vasodilator effect, at least in part, by inhibition of these channels. To confirm this hypothesis, we formed concentration–response curves to $CaCl_2$ in the absence and in the presence of different concentrations of OMC. We observed that OMC induced a concentration-dependent and non-competitive inhibition of $CaCl_2$ concentration–response curves, with values of IC50 similar to those observed in KCl precontracted rings. Therefore, the inhibition of voltage-operated calcium channels seems to be involved in the vasodilator effect of OMC. This effect was also reported with other types of BBAs, such as warifteine (Freitas et al 1996) and tetrandrine (Liu et al 1995).

We also performed experiments in attempt to verify the participation of other calcium channels in the vasodilator effect of OMC. For this purpose, we observed the effect of OMC in contractions dependent on calcium influx in rings stimulated with phenylephrine in the presence of a maximal active concentration of nifedipine (Noguera & D'Ocon 1993). We observed that OMC was still able to induce an additional inhibition of contractile responses, suggesting that OMC could inhibit other types of calcium channels. We therefore investigated the effect of OMC in contractions induced by calcium entry in aortic rings where calcium stores were depleted. These contractile responses are believed to be induced by calcium entry through the capacitative model (Putney 1990). We observed that OMC strongly inhibited these contractions and thus OMC might act by inhibiting different types of calcium channels in addition to voltage-operated channels.

It is well known that protein kinases modulate the activity of ion channels. Protein kinase A and G have modulatory effects in calcium channels present in vascular smooth muscle cells (Xiong & Sperelakis 1995). To evaluate the participation of these protein kinases in the vasodilator effect of OMC, we formed OMC concentration–response curves in the presence of H-89 and Rp-8-PCPT-cGMPs, selective inhibitors of protein kinase A and G, respectively (Chijiwa et al 1990; Smolenski et al 1998). None of these inhibitors altered the OMC concentration–response curves. Therefore, proteins kinase A and G seem not to be involved in the vasodilator effect of OMC nor, consequently, in the inhibitory effect of OMC in contractions mediated by calcium influx.

As it has been described that different types of alkaloids inhibit the contractions dependent on the release of calcium ions from their intracellular stores, we investigated the effect of OMC under two different experimental conditions. In one experiment, we investigated the effect of OMC in contractions induced by phenylephrine in aortic rings maintained in $Ca^{2+}$-free medium, which are believed to occur owing to the release of calcium from $InsP_3$-sensitive stores (Noguera & D'Ocon 1992). OMC dramatically inhibited these contractions, suggesting that OMC may inhibit the mechanisms involved in the release of calcium from stores sensitive to $InsP_3$. In the other experiment, we investigated the effect of OMC on contractions induced by caffeine, which is believed to occur owing to the release of calcium from ryanodine-sensitive stores (Watanabe et al 1992). OMC was not able to inhibit caffeine-induced contractions, suggesting that OMC did not affect the mechanisms involved in calcium release from ryanodine-sensitive stores.

Finally, we investigated the relationship between the activation of protein kinase C-dependent mechanisms and the vasodilator effect of OMC. The relevant role of protein kinase C in the control of the maintenance of the sustained contraction is well reported in the literature (Karaki et al 1997). Protein kinase C activates the mechanisms involved in the opening of calcium channels, increases the sensitivity of contractile proteins to calcium and reduces the reuptake and efflux of calcium from the cytoplasm (Karaki et al 1997). For these reasons we contracted aortic rings with TPA, a protein kinase C activator (Sato et al 1992), and formed concentration–response curves to OMC in rings maintained in the presence or absence of extracellular calcium. OMC induced a concentration-dependent vasodilatation in aortic rings contracted with TPA in normal salt solution. However, in $Ca^{2+}$-free solution, the vasodilator effect of OMC was dramatically reduced. Because there was a strong difference between vasodilator effects for OMC when tissues were stimulated

in normal or $Ca^{2+}$-free solution, it is possible that calcium entry controlled by protein kinase C rather than $Ca^{2+}$ sensitivity of contractile proteins could be inhibited by OMC. As different isoforms of protein kinase C are involved in contractions induced by phorbol esters in the presence ($\alpha$- and $\delta$-isoforms) and in the absence ($\epsilon$- and $\xi$-isoforms) of extracellular calcium (Karaki et al 1997), further studies are necessary to verify if the mechanism involved in the effect of OMC is related to its interaction with a specific isoform of PKC.

The results indicate that OMC is a vasodilator alkaloid that acts through inhibition of voltage-operated and non-selective calcium channels and by inhibition of contractile mechanisms dependent on the release of calcium from stores sensitive to $InsP_3$. Finally, the results also suggest the inhibition of calcium influx induced by activation of protein kinase C as one of the mechanisms involved in the vasodilator effect of OMC.

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