Mechanisms of Vasoconstriction Induced in Frog Vascular Smooth Muscle by MD1, a New Biotechnological Agent

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Abstract. The mechanism of action of MD1, a new biotechnological radiotherapeutic agent of plant origin, on smooth muscle contraction was investigated. Contraction induced by MD1 consists of an initial dose-dependent transient phasic response (PR) (with $ED_{50} = 0.11 \pm 0.02\%$, $n = 4$, the time of rise 15 s, onset of decay about 30 s) followed by a tonic contraction (TC) (at 10% MD1 only) with a maximum in 20 min ($0.78 \pm 0.07 \times$ the maximum contraction induced by 110 mmol/l KCl, $n = 8$, (TC/PR) $= 0.94 \pm 0.11$, $n = 5$). If the vessel was washed out during this maximum, the maximum tension was maintained for up to 2 h ("long-lasting" tension) and was abolished after perfusion with Ca^{2+}-free EGTA solution (E) or H7 (0.05 mmol/l) solution. With solution E being applied 30 min before 10% MD1-induced contraction, TC was reduced by $92 \pm 3\%$ ($n = 4$) in contrast to PR being reduced by $48 \pm 5\%$ ($n = 5$). During TC, calcium appeared to penetrate into cells through receptor operated channels, since tension neither depended on verapamil (0.05 mmol/l) nor cobalt (up to 10 mmol/l). In solution H7 (with 10 min pretreatment), PR and TC were almost completely inhibited. It is proposed that MD1 activates the C-kinase branch of the calcium messenger system.

Key words: Smooth muscle contraction — Calcium — Protein kinase C — Biotechnological agent

Introduction

MD1 has been shown to possess radioprotective property both *in vitro* and *in vivo*. The advantage of using MD1 is that it is effective when administered after irradiation (Sobol et al. 1992; 1994a). Additionally, MD1 has immunomodulatory and bactericidal properties, especially at 10% concentration (Sobol et al. 1994b).

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On the other hand this agent is effective in cardiac diseases such as myocarditis and aneurysm It can be used as a prophylactic agent against cerebral thrombosis and infarction

This study was undertaken to investigate the effect of MD1 on smooth muscle contraction to elucidate the mechanisms of MD1 action at the cellular level It is proposed that MD1 is a natural activator of protein kinase C (Sobol 1994)

Materials and Methods

Preparation

Male frogs *Rana temporaria* were used in the study The cava posterior vein was dissected under an operation microscope freed of connective tissue and rings about 1 0 mm in width and 0 8 mm in diameter were cut

Recording of mechanical activity

The rings were suspended between rigid L shaped steel prongs (total weight 1 mg) in organ bath (0 6 ml) containing physiological saline solution (PSS) One of the steel prongs was connected to a force tensotransducer (Sobol 1991) for recording of isometric tension whereas the other prong was connected to an immobile hook The rings were equilibrated for 60 min under a preload of 2 mN After the equilibration period passive tension was reduced to 0 3 0 1 mN The output was recorded on a 622 01 Endim recorder

Solutions

PSS was composed of (in mmol/l) 110 NaCl 2 5 KCl 1 $MgCl_2$ 2 5 $CaCl_2$ 0 75 NaH_2PO_4 0 5 $NaHCO_3$ pH 7 4 To keep pH unchanged in 10% MD1 PSS 30 mmol/l NaCl was replaced by an equimolar amount of NaOH High KCl solution was obtained by equimolar substitution of 107 5 mmol/l NaCl for KCl Ca^{++} free solution contained 1 mmol/l K_2EGTA and no $CaCl_2$ All experiments were carried out at 20°C

Drugs

EGTA was purchased from BDH Chemicals Ltd England H7 was from Sigma MD1 is a polycomponent liquid (pH 2 3) produced by a biotechnological method The production of MD1 is a high-tech process and it has been described in more detail in the patent application (Sobol et al 1992) Briefly plants berries and vegetables such as dandelion nettle carrot cabbage beet and others are washed chopped very finely and put into a tank (volume 50l) in appropriate proportions Various metals (in mmol/l) $Co(NO_3)_2$ 0 82 Na_2SeO_3 0 46 Cr_2O_3 0 53 $MnSO_4$ 0 46 $CuSO_4$ 0 56 and pepsin (0 1 g per 1 kg of product) are added to the mixture To cause fermentation a special bacterial ferment containing *Lactobacterium bulgaricum* 0 1% *Lactobacterium helveticum* 0 1% *Lactobacterium casei* 0 1% *Streptobacterium casei* 0 1% and *Penicillium candidum* 0 01% is introduced Fermentation takes about 7 10 days and at the end of this period the mixture is tested filtered and can be applied to the skin or taken orally

Experimental procedure

After the equilibration period three contractions were induced by high KCl solution to check the viability of vein rings A maximum of 30 min high KCl-induced contraction was used as a control Maximum amplitude of contraction was attained within 3 7 min

and all subsequent contractile responses were expressed as percentages of this contraction maximum, except for cumulative curves which were constructed based on cumulative addition of MD1 to the organ bath in increasing doses. Only one complete set of cumulative additions was done with each preparation. The ED_{50} value is the dose required to produce 50% of maximum response.

Statistics

Unless otherwise indicated, the results are expressed as the mean ± the standard error of the mean, and were analyzed for significance using Student's t-test. A value of $p < 0.05$ was considered to be statistically significant.

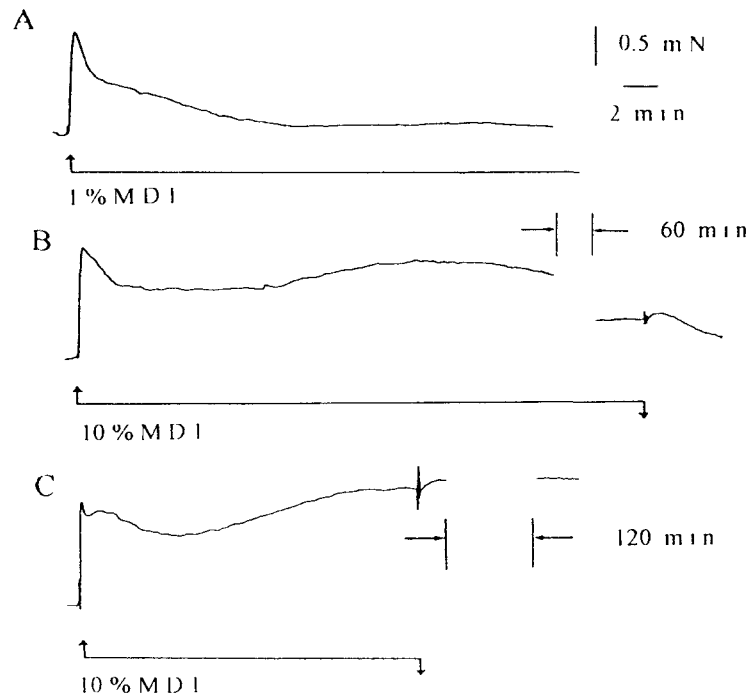

Figure 1. MD1-induced contractions. Recording of isometric force from frog vein in response to 1% (*A*) and 10% MD1 washed out 90 min (*B*) or 18 min (*C*) after onset of contraction.

Results

Effects of MD1 on mechanical activity of the cava posterior vein

The tensions obtained with 1% and 10% MD1 in cava posterior vein of the frog are shown in Fig. 1. Firstly, MD1 caused a concentration-dependent transient or phasic

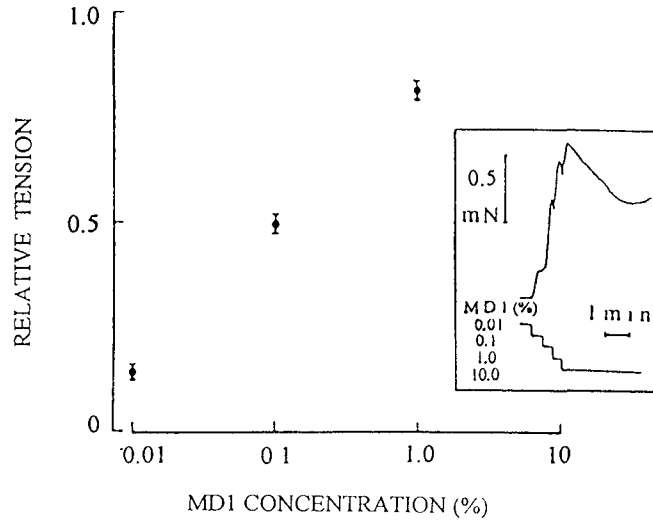

Figure 2. Concentration-response curve for MD1 in cava posterior vein. Maximum response to 10% MD1 was taken as 1.0. Each point is the mean of four experiments ± S.E.M. (vertical lines). *Inset*: Experimental cumulative curve.

contraction. This contraction reached a maximum within 15 s and then decreased in about 30 s. The threshold dose was 0.005% and ED_{50} was $0.11 \pm 0.02\%$ ($n = 4$) (Fig. 2). Secondly, at 10% concentration only, MD1 caused a sustained or tonic contraction with a maximum at 20 min followed by a reduction of tension by $63 \pm 3\%$ ($n = 4$) to steady-state level (at 60 min) (Fig. 1*B*). This level of tension remained unchanged during the following 90 min ($n = 3$). However, if the vessel was washed out during this maximum, then the maximum tension would be maintained for up to 2 h, even in normal constantly perfused PSS (Fig. 1*C*, $n = 5$). This so-called "long-lasting" tension depended on external Ca^{2+}, and was reduced by $87 \pm 3\%$ ($n = 4$) after perfusion with Ca^{2+}-free EGTA solution or by $112 \pm 4\%$ ($n = 3$) with H7 (0.05 mmol/l) solution.

The time dependences of tension induced by 1%, 10% MD1 and high-KCl are shown in Fig. 3. All contractions are expressed as percentages of the maximum KCl (110 mmol/l) control response (see Materials and Methods).

It should be noted that desensitization of MD1-induced contraction was observed. The degree of MD1-induced desensitization depended upon the concentration of MD1 employed in the incubation medium and the duration of incubation. When the vein was incubated for 15 min with 1% MD1, the second phasic contraction was reduced by $56 \pm 5\%$ ($n = 4$) and it took more than 4 hours to recover to 90% of the initial state. Moreover, with the reduction of phasic contraction both

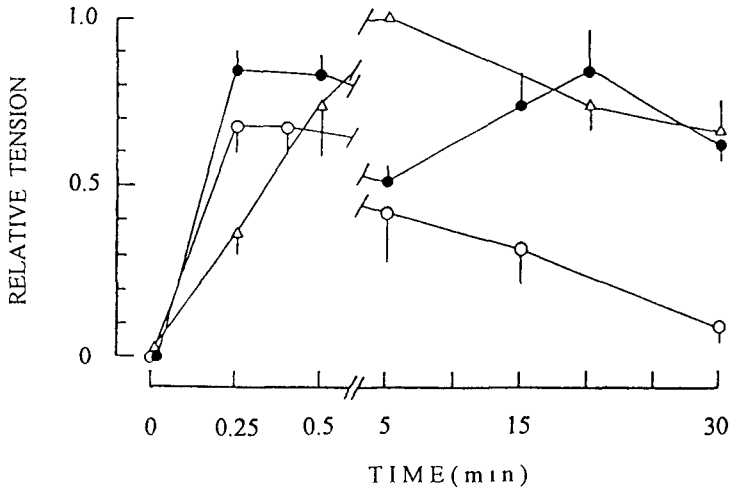

Figure 3. Time course of tension induced by 1% (open circles, $n = 5$); 10% MD1 (filled circles, $n = 8$) and control high-KCl (110 mmol/l) (open triangles, $n = 10$), respectively. Isometric force was related to the maximum contraction in control high-KCl solution (110 mmol/l). Vertical lines represent S.D.M.

the value and the velocity of the development of tonic response induced by 10% MD1 were also reduced (not shown).

A pretreatment for 3 min with 1% MD1 was sufficient to potentiate subsequent contractile response to KCl (without MD1) at steady-state level (at 30 min) by $23 \pm 5\%$ ($n = 5$), $p < 0.01$. Additional experiments are necessary to define whether this potentiation is linked to the change of sensitivity of the contractile apparatus to Ca^{2+}, as it was noted for 12-*o*-tetradecanoylphorbol-13-acetate (TPA) (Wakabayashi et al. 1988), or with other intracellular processes.

Effects of verapamil, cobalt, H7 and Ca^{2+}-free solution on 10% MD1-induced contraction

Because of desensitization it was impossible to get two similar 10% MD1-induced contractions. Therefore, two 10% MD1-induced contractions, related to control high-KCl contraction, of two rings cut from the same vein with or without verapamil were compared. Verapamil (0.05 mmol/l) was added to the bath 20 min before the 10% MD1-induced contraction. In five experiments neither phasic (0.82 ± 0.04 with and 0.86 ± 0.06 without verapamil, $n = 5$) nor tonic responses, (0.89 ± 0.09 with and 0.94 ± 0.07 without verapamil, $n = 5$) of MD1-induced contractions related to high-KCl, were significantly different ($p > 0.05$).

Cobalt, an inorganic blocker, was added to the bath 18–20 min after 10% MD1

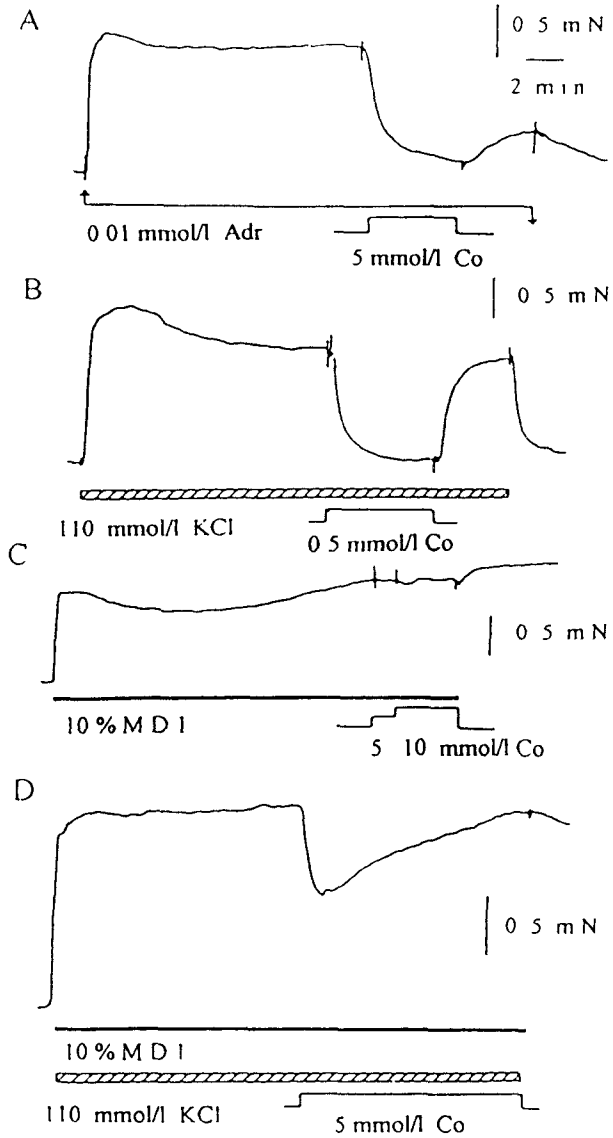

Figure 4. Effects of cobalt on tonic responses induced by adrenaline (0 01 mmol/l), high KCl (110 mmol/l) and 10% MD1 Effects of 5 mmol/l (*A*), 0 5 mmol/l (*B*), 5 and 10 mmol/l (*C*) cobalt on adrenaline, high KCl and 10% MD1-induced contractions, respectively *D* Effect of 5 mmol/l cobalt on a simultaneous high KCl (110 mmol/l) and 10% MD1-induced contraction

application, i.e at the maximum of tonic response Very high concentrations of cobalt (10 mmol/l) did not affect the tonic response maximum (Fig 4*C*). Simi-

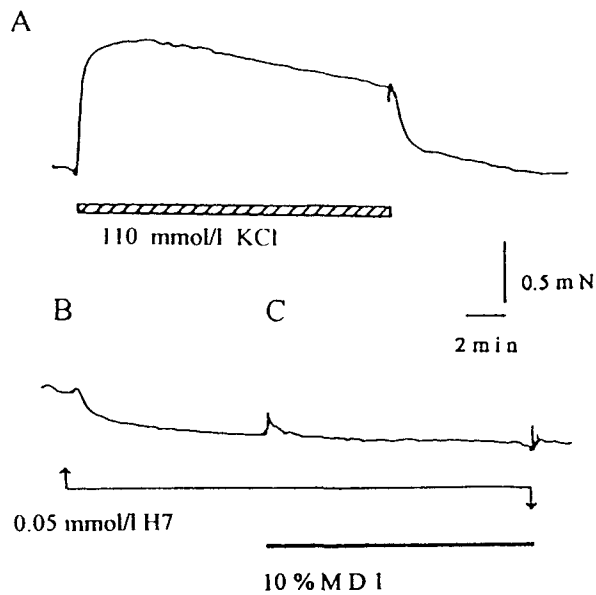

Figure 5. Recording of isometric force in frog vein in response to 10% MD1 in the presence of 0.05 mmol/l H7. Response to 110 mmol/l KCl is shown for comparison (*A*). First arrow, addition of 0.05 mmol/l H7 (*B*), the vein was rinsed for 10 min and then 10% MD1 was added (*C*).

lar results were observed in four experiments. On the other hand, 0.5–5 mmol/l concentrations of cobalt were sufficient to abolish both adrenaline (0.01 mmol/l) (Fig. 4*A*) and KCl (110 mmol/l) induced tonic contractions (Fig. 4*B*). These results suggest that the tonic 10% MD1-induced response is likely to be directly linked to receptor-operated channels (ROCs). An interesting effect was observed when cobalt was added during simultaneous KCl (110 mmol/l) and 10% MD1-induced contraction. A temporary reduction of tension occurred followed by recovery to the initial level (Fig. 4*D*). The possibility cannot be ruled out that after the blockade of voltage-operated channels (VOCs) by cobalt, Ca^{2+} begins penetrating into cells through ROCs to support tonic contraction.

To demonstrate the protein kinase C mediation of MD1-induced contraction, H7 an inhibitor of protein kinase C (Hidaka et al. 1984) was used. Fig. 5 shows the inhibitory effect of high concentrations of H7 (0.05 mmol/l) on 10% MD1-induced contraction. In H7 solution, with 10 min pretreatment, phasic and tonic responses were almost completely inhibited, whereas in Ca^{2+}-free EGTA solution there was only a two-fold reduction in phasic response (see below).

The effects of 10% MD1 in Ca^{2+}-free solution containing 1 mmol/l EGTA were

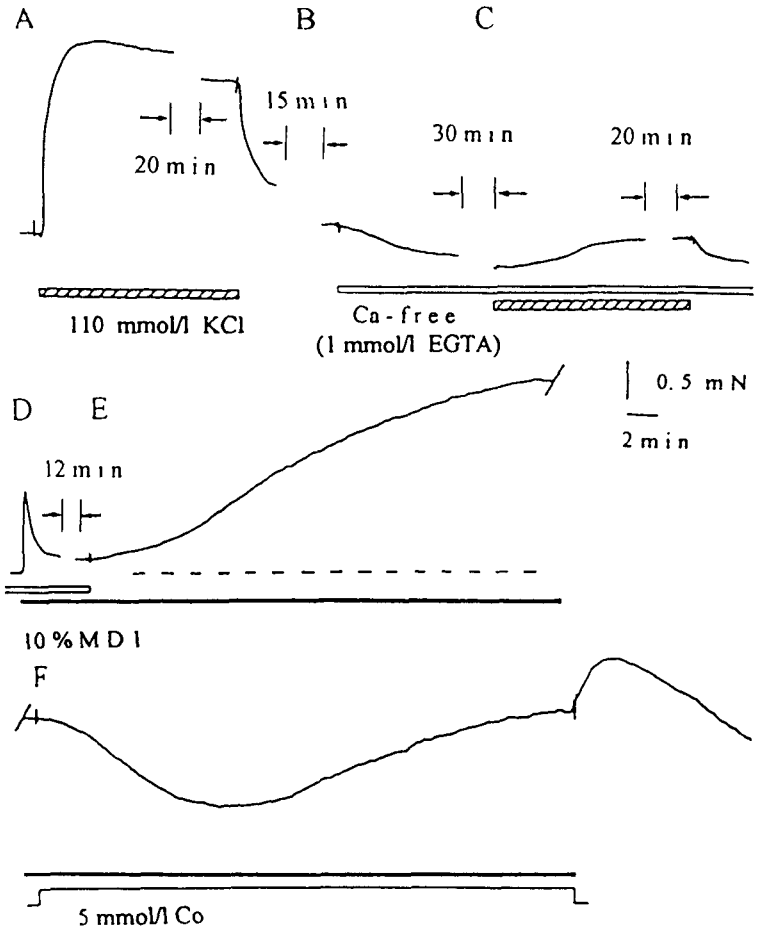

Figure 6. Effects of change in extracellular Ca^{2+} and cobalt on KCl and MD1-induced contraction in frog vein. *A*: A typical example of high-KCl (110 mmol/l)-induced control contraction in normal PSS. *B*: The tissue was rinsed in Ca^{2+}-free (1 mmol/l EGTA) solution for 40 min. *C*: Soaking in Ca^{2+}-free (1 mmol/l EGTA), low response to high KCl (110 mmol/l) test. *D*: Ca^{2+}-free (1 mmol/l EGTA), 10% MD1 applied. The dotted line represents the resting tension level. *E*: When tension dropped to a steady-state level, normal PSS (2.5 mmol/l Ca^{2+}) containing 10% MD1 was applied. *F*: At the maximum of tonic contraction, 28 min after perfusion with Ca^{2+}, 5 mmol/l cobalt was applied. Resting tension is not shown.

studied. Frog vein rings were equilibrated in this solution for 60 min before the addition of 10% MD1. The effects of successively applied 110 mmol/l KCl and 10% MD1 on mechanical responses in Ca^{2+}-free solution containing 1 mmol/l EGTA

are shown in Fig. 6. Under these conditions contraction induced by high-KCl was almost completely abolished (Fig. 6*C*'), whereas 10% MD1 produced phasic contraction amounting to 52 ± 4% (n = 4) of its response in normal PSS. The reduction of phasic contraction appeared to be connected with the exhausting of intracellular Ca^{2+}. The phasic contraction lasted for about 15 s followed by a reduction of 80±4% (n = 4) to steady-state level (Fig. 7*D*). Under these conditions, the tonic contraction was 8 ± 3% (n = 4) of its response in normal PSS. In three experiments, the substitution of Ca^{2+}-free EGTA (1 mmol/l) solution for normal PSS with 10% MD1, at 16 min after the onset of 10% MD1-induced contraction, resulted in the development of tonic response (Fig. 6*E*). When cobalt was added at the maximum of the tonic response, a temporary reduction of tension was observed (Fig. 6*F*, n = 2). The latter effect may be accounted for by the fact that the contractile responses of vascular tissues to Ca^{2+} repletion are primarily due to Ca^{2+} entry through the VOCs, possibly because of membrane destabilization of tissue after an equilibration period in Ca^{2+}-free EGTA solution (Guan et al. 1988). Therefore, after the blockade of VOCs by cobalt, ROCs are opened as it appears to take place in normal PSS with calcium during tonic contraction induced by 10% MD1.

Discussion

In frog vein, MD1 induced both phasic and tonic contractions, the latter only at 10%. The phasic contraction was due to an initial transient release of Ca^{2+} from intracellular pools, representing the initial phase of cellular response (Bolton 1979; Rasmussen and Barrett 1984; Nishizuka 1986; Taylor 1987; van Breemen and Saida 1989). Ca^{2+}-release is necessary for MLCK activation and C-kinase association with the plasma membrane that supports tonic contraction (Rasmussen et al. 1987). To support tonic contraction a sustained entry of Ca^{2+} through the plasma membrane is also necessary (Taylor 1987).

The main feature causing clear separation of the two phases in the mechanical reaction of MD1-induced contraction appears to be due to the fact that MD1 does not activate VOCs, because the tension depended neither on verapamil (0.05 mmol/l) nor cobalt (up to 10 mmol/l), and Ca^{2+} entered through ROCs only. Therefore, it appears to be the delay of Ca^{2+} in entering the cell from extracellular space to support tonic contraction which leads to the splitting of mechanical reaction induced by 10% MD1 into two phases, phasic and tonic.

Interestingly, under appropriate conditions MD1-induced tonic response was considerable. The appearance of such a "long-lasting" tension may be explained by protein kinase C activation. This enzyme is stimulated by MD1 in such a way that the vessel does not need further activation and only depends on calcium concentration in the submembrane domain of the plasma membrane (Rasmussen

et al. 1987). Supporting the latter suggestion is the fact that after complete relaxation of the vessel in Ca^{2+}-free EGTA (1 mmol/l) solution, addition of Ca^{2+} to the solution promoted a certain increase in tension (not shown). Additional confirmation of protein kinase C activation is that in H7 solution (0.05 mmol/l) MD1-induced contraction was almost completely inhibited. The "long-lasting" tension was also abolished after perfusion with H7 (0.05 mmol/l) solution.

It is interesting to note that this "long-lasting" tension with constitutively activated protein kinase C might be connected with the problem of cellular memory (Burgoyne 1989).

On the other hand, under long term incubation with 10% MD1, the maximum tonic contraction was reduced by 63±3% to steady-state level, and no "long-lasting" tension developed. This self-inhibition appears to be caused by down-regulation by protein kinase C (Nishizuka 1986; Berridge 1987). An analogous dual effect was observed in a phorbol ester (TPA), which directly activates protein kinase C (Castagna et al. 1982), on potassium-induced contraction in the mesenteric artery of the rabbit (Itoh et al. 1986).

It should be noted that when MD1 activates the blood vessel as mentioned above, inositol 1,4,5-trisphosphate (IP_3), a product of phosphatidylinositide 4,5-*bis*-phosphate (PIP_2), breakdown-induced Ca^{2+}-release seems to take place (Nishizuka 1986; Berridge 1987; Berridge and Irvine 1989). IP_3 is formed within several seconds and is quickly utilized (Nishizuka 1986; van Breemen and Saida 1989), which correlates well with the development time of phasic contraction of the cava posterior vein (15 s) followed by a reduction of this contraction within 30 s. Further experiments are necessary to determine PIP_2 breakdown.

In summary, in the cava posterior vein, MD1 produced initial phasic contraction and, at 10% concentration, also tonic contraction. The phasic component was due to Ca^{2+}-release from intracellular stores. Tonic contraction depended on external Ca^{2+} entering the cell probably through ROC only, and contractile response appeared to be due to protein kinase C activation. The main feature of MD1-induced contraction, unlike other known vasoconstrictors, is that during the tonic phase the activation of protein kinase C occurred in such a way that C-kinase did not need to be permanently activated. The contraction was maintained in the absence of MD1 and only depended on external Ca^{2+}.

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Final version accepted June 2 1995