Inhibition of protein kinase C-mediated contraction by Rho kinase inhibitor fasudil in rabbit aorta

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Abstract Protein kinase C (PKC) activation by a phorbol ester increases myosin light chain (MLC_{20}) phosphorylation through inhibition of MLC phosphatase (MLCP) and enhances contraction of vascular smooth muscle. We investigated whether Rho kinase, which is known to inhibit MLCP, is involved in the MLC_{20} phosphorylation caused by a phorbol ester, 12-deoxyphorbol 13-isobutyrate (DPB), in rabbit aortas.

DPB (1 μM) increased MLC_{20} phosphorylation and tension. The Rho kinase inhibitor fasudil (10 μM) inhibited the DPB-induced contraction and decreased the MLC_{20} phosphorylation at Ser19, a site phosphorylated by MLC kinase, although it did not affect the phosphorylation of total MLC_{20}. Rinsing a 65.4 mM KCl-contracted aorta with Ca^{2+}-free, EGTA solution caused rapid dephosphorylation of MLC_{20} and relaxation. When DPB was present in the rinsing solution, the MLC_{20} dephosphorylation and the relaxation were inhibited. In this protocol, Ro31-8220 (10 μM), a PKC inhibitor, suppressed the phosphorylation of total MLC_{20} and Ser19 induced by DPB. Fasudil also inhibited the Ser19 phosphorylation to a degree similar to Ro31-8220 and accelerated relaxation, which was less than the relaxation caused by Ro31-8220. The phospholipase A_2 inhibitor ONO-RS-082 (5 μM) inhibited the DPB-induced Ser19 phosphorylation but only transiently decreased the tension, suggesting the involvement of arachidonic acid in the phosphorylation but also the existence of a MLC_{20} phosphorylation-independent mechanism. When fasudil was combined with ONO-RS-082, fasudil exerted additional inhibition of the tension without further inhibition of the Ser19 phosphorylation. DPB phosphorylated the 130 kDa myosin binding subunit of MLCP and fasudil inhibited the phosphorylation.

These data suggest that the inhibition by fasudil of DPB-induced contraction and phosphorylation of MLC_{20} at the MLC kinase-targeted site is a result of inhibition of Rho kinase. Thus, the PKC-dependent Ca^{2+}-sensitization of vascular smooth muscle involves Rho kinase. A MLC_{20}

phosphorylation-independent mechanism is also involved in the Ca^{2+}-sensitization.

Key words Fasudil · Rho kinase · Protein kinase C · Myosin light chain phosphorylation · Phosphatase · Phorbol ester · Arachidonic acid · Vascular smooth muscle

Introduction

Protein kinase C (PKC) activation causes or enhances a contraction of vascular smooth muscle even when a rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is absent (Horowitz et al. 1996; Walsh et al. 1996). One mechanism for such Ca^{2+}-sensitization is related to an increase in the level of 20 kDa myosin light chain (MLC_{20}) phosphorylation, which is a primary determinant of contraction. An increase in MLC_{20} phosphorylation at a given $[Ca^{2+}]_i$ can occur through the inhibition of myosin light chain phosphatase (MLCP) or through the direct phosphorylation of MLC_{20} at Ser1, Ser2 or Thr9, although phosphorylation at these sites does not contribute to tension development but rather could lead to inhibition (Nishikawa et al. 1984; Ikebe et al. 1987; Sutton and Haeberle 1990). In the inhibition of MLCP by PKC, the involvement of arachidonic acid, which is released by phospholipase A_2 following PKC activation (Gong et al. 1992; 1995; Miura et al. 1997), or CPI-17, which inhibits MLCP when phosphorylated by PKC (Li et al. 1998; Kitazawa et al. 2000), has been suggested. Another pathway may be related to regulatory proteins associated with actin filaments. Caldesmon or calponin, which binds to actin and regulates actin-activated myosin Mg^{2+}-ATPase, loses its inhibitory action on the ATPase, when phosphorylated by PKC, and enhances contraction (Sobue and Sellers 1991; Barany et al. 1992; Rokolya et al. 1996; Earley et al. 1998; Je et al. 2001).

The other kinase responsible for Ca^{2+}-sensitization is Rho-associated kinase (Rho kinase, Kimura et al. 1996). Rho kinase phosphorylates 130 KDa (or 110 kDa) of the myosin binding subunit (MBS) of MLCP (Kimura et al. 1996), and this phosphorylation decreases the enzyme activity to dephosphorylate MLC_{20}. Rho can be activated when its GDP-bound form is converted to a GTP-bound form by Rho-guanine nucleotide exchange factor upon activation of certain types of receptor (Hart et al. 1998; Kozasa et al. 1998; Nakamura et al. 2003). Therefore, it is generally accepted that Rho kinase participates in the Ca^{2+}-sensitization following receptor activation (Seasholtz et al. 1999; Somlyo and Somlyo 2000).

PKC-dependent and Rho kinase-dependent Ca^{2+} sensitization may not

be independent of each other. For example, CPI-17 can be phosphorylated by Rho kinase (Kitazawa et al. 2000; Koyama et al. 2000) and, conversely, arachidonic acid can activate Rho kinase (Fu et al. 1998; Feng et al. 1999). Therefore, it is possible that the mechanisms downstream of PKC and Rho kinase converge at the vicinity of MLCP. However, it has also been shown that PKC-dependent MLC_{20} phosphorylation was insensitive to the Rho kinase inhibitor Y-27632 (Fu et al. 1998). Thus, it is unclear whether a signal from the PKC-dependent pathway is transmitted to Rho kinase to modify the latter activity in intact smooth muscle. This study was undertaken to clarify whether Rho kinase is involved in the contraction of rabbit aorta following PKC activation by a phorbol ester. We used 12-deoxyphorbol 13-isobutyrate (DPB) because this phorbol ester has advantage over other phorbol esters in that it scarcely increases $[Ca^{2+}]_i$ (Sato et al. 1992; Miura et al. 1997).

Methods

Preparations. The following experimentation was approved by the Animal Care and Use Committee at Miyazaki University Faculty of Agriculture. Thoracic aorta was isolated from male Japanese White rabbits weighing 2-2.2 kg anesthetized with pentobarbital Na (45 mg/kg i.v.). The aorta was cleaned of fat and connective tissues, and the endothelium was removed by gently rubbing the intimal surface with a cotton swab. An aortic ring 4-5 mm wide was cut open into a rectangular strip.

Tension experiments. The strip was suspended in an organ bath containing 5 ml physiological saline solution (PSS) containing (mM) NaCl 136.8, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 11.9 and glucose 5.5 (pH was 7.3-7.4 when saturated with 95% O_2 and 5% CO_2 at 37˚C). After equilibration for 1 h under a resting tension of 1 g, the preparation was contracted with 65.4 mM KCl solution, in which NaCl in PSS was substituted with equimolar KCl, until

stable responses were obtained. Then, the muscle was subjected to experimental protocols.

Phosphorylation of MLC_{20}. The phosphorylation of total MLC_{20} or MLC_{20} at Ser19 was measured as described previously (Miura et al. 1997). Briefly, a rested or a contracted muscle was quickly removed from the organ bath, taking care not to stretch it, and frozen by immersion in acetone containing 10% trichloroacetic acid (TCA) and 5 mM dithiothreitol (DTT) cooled in dry ice/acetone. It took about 3 sec until the preparation was frozen and this demounting did not change the MLC_{20} phosphorylation level (Ito et al. 2003). A frozen sample was cut into pieces with fine scissors, and proteins were extracted with 8 M urea and 10 mM DTT. For the determination of phosphorylation of total MLC_{20} (including phosphorylation at Ser1, Ser2, Thr9, Thr18 and Ser19), the proteins were electrophoresed in polyacrylamide gels containing 15% acrylamide, 0.75% bisacrylamide and 40% glycerol (glycerol-PAGE). Then the proteins were transferred onto a nitrocellulose membrane, and the membrane was incubated overnight with 10 μg/ml of anti-MLC_{20} IgG prepared as described previously (Seto et al. 1990). Then, peroxidase-linked anti-rabbit IgG was applied to the membrane as a secondary antibody. The regions of MLC_{20} were visualized using the enhanced chemiluminescence method (ECL, Amersham Biosciences, Tokyo, Japan). Densitometry of the immunoblots and quantification of the absorbance peaks were determined with a densitometer (Densitron, Jokoo, Tokyo, Japan). The extent of MLC_{20} phosphorylation is expressed as the percent of total MLC_{20}.

To determine the phosphorylation of MLC_{20} at Ser19, a one-fifth volume of sampling buffer containing 12.5% SDS and 25% β-mercaptoethanol in 50 mM Tris-buffer was added to the solubilized samples. Samples in an equal amount were subjected to two sets of SDS-PAGE (4-15% acrylamide gradient gel) and electrophoresed at 40 mA for 3 h. The proteins were electrophoretically transferred onto two membranes in methanol/Tris

solution. After blotting, anti-MLC_{20} IgG was applied to one membrane, and anti-phospho Ser19 MLC_{20}-IgG (Sakurada et al. 1994) to the other membrane. Then, peroxidase-labeled anti-rabbit IgG or anti-mouse IgG was applied to the respective membrane. The extent of phosphorylation at Ser19 in the tested preparation was expressed as the relative value of the Ser19 phosphorylation in the rested preparations.

Phosphorylation of MBS. A polyclonal antibody (antibody pMBS-T654) against MBS phosphorylated at Thr654 was raised in New Zealand White rabbits, using a synthetic peptide corresponding to residues 648-660 (RQSRRSTQGVTLTC) of 130 kDa MBS containing phosphorylated Thr654 (chicken sequence) as antigen, as previously described (Hartshorne et al. 1998). Characterization of the pMBS-T654 antibody has been previously established (Ito et al. 2003).

Samples were prepared as described in "*Phosphorylation of MLC_{20}*". The proteins were separated on 8% SDS-PAGE, and then electrotransferred onto two nitrocellulose membranes. One membrane was probed with anti-MBS antibody (Nagumo et al. 2000) and the other with anti-phospho Thr654 MBS (pMBS-T654) antibody. The ratio of the density of a phosphorylated MBS band to a MBS band in the resting state was referred to as 1.

Drugs. 12-Deoxyphorbol 13-isobutyrate (DPB, Funakoshi, Tokyo, Japan), fasudil (HA-1077, Asahi Chemical Industry, Tokyo, Japan), Y-27632 (R-[+]-*trans*-4-[1-aminoethyl]-N-[4-pyridyl]-cyclohexanecarboxamide dihydrochloride monohydrate, Welfide Corp, Osaka Japan), ONO-RS-082 (2-[p-amylcinnamoyl]amino-4-chlorobenzoic acid, Ono Pharmaceutical Co., Osaka, Japan) and Ro31-8220 (bisindolylmaleimide IX, Sigma-RBI, St. Louis, MO, USA) were used.

Statistics. Data are expressed as mean ± SEM. Significance was tested by the Student's t-test for a single comparison. For a multiple comparison, one-way analysis of variance followed by appropriate *post hoc* test (Newman-Keuls) was performed. Significance was considered at the level of $P < 0.05$.

Results

Effects of Rho-kinase inhibitors on the DPB-induced contraction and MLC_{20} phosphorylation in normal medium

In the normal PSS (2.5 mM Ca^{2+}), DPB induced a slowly developing contraction, which attained a plateau level at 20-30 min. At 30 min after addition of DPB, the tension level was 84.6 ± 5.0 % of the 65.4 mM KCl-induced contraction and the total MLC_{20} phosphorylation increased from 9.8 ± 1.2 % under the resting condition to 29.1 ± 8.6 % (n=6, Fig. 1). As previously reported (Asano et al. 1995; Miura et al. 1997), DPB scarcely increased $[Ca^{2+}]_i$ so that this phosphorylation occurred independently of $[Ca^{2+}]_i$. The addition of fasudil (10 μM) at 30 min of DPB application decreased the tension by 42.3 ± 9.2 % (n=6) in 30 min. When MLC_{20} phosphorylation was measured 30 min after the addition of fasudil, the total MLC_{20} phosphorylation did not significantly decrease (30.7 ± 6.2 % in DPB alone group vs. 27.4 ± 7.1 % in fasudil-treated group, Fig. 1). However, fasudil significantly inhibited the phosphorylation at Ser19 of MLC_{20}, a site phosphorylated by MLC kinase (Fig. 2). Another Rho kinase inhibitor, Y-27632 (1 μM), also inhibited the phosphorylation at Ser19 of MLC_{20} and the contraction (Fig. 2).

Inhibition by DPB of MLC_{20} dephosphorylation

When a muscle in which MLC_{20} is highly phosphorylated by KCl is rinsed with Ca^{2+}-free, EGTA (1 mM) solution, the muscle tension and the MLC_{20} phosphorylation rapidly decrease as a consequence of a relative increase in the phosphatase activity over the MLC kinase activity. As previously reported (Miura et al. 1997), when DPB was added to activate PKC simultaneously with removal of external Ca^{2+}, the dephosphorylation was inhibited so that the MLC_{20} phosphorylation and the tension were sustained higher than the resting level. Figure 3 shows such a case. In this protocol, the PKC inhibitor Ro31-8220 (10 μM), which was applied 60 min before changing to Ca^{2+}-free, EGTA solution and continuously present in the solution, significantly inhibited the phosphorylation of either total MLC_{20} or Ser19, and accelerated the relaxation. Ro-31-8220 at this concentration scarcely affected the KCl-induced contraction (93.8 ± 5.7% of the response in the absence of Ro31-8220, n = 7).

With the same protocol as Fig. 3, fasudil (10 μM), which was applied when the 65.4 mM KCl-solution was replaced with Ca^{2+}-free, EGTA solution containing DPB, partially decreased the tension and suppressed the Ser19 phosphorylation of MLC_{20} (Fig. 4).

Combined effects of ONO-RS-082 and fasudil

It has been reported that phorbol esters activate phospholipase A_2 and release arachidonic acid and that arachidonic acid inhibits the dephosphorylation of MLC_{20} (Gong et al. 1992, 1995; Gailly et al. 1997; Fu et al. 1998). In the previous paper (Miura et al. 1997), ONO-RS-082, a phospholipase A_2 inhibitor, antagonized the DPB-induced inhibition of MLC_{20} dephosphorylation, suggesting that ONO-RS-082 could be used to detect the involvement of

arachidonic acid-dependent mechanism. Confirming the previous data (Miura et al. 1997), ONO-RS-082 (5 μM), which was added 20 min before 65.4 mM KCl inhibited the phosphorylation of MLC_{20} at Ser19. However, the inhibition of tension by ONO-RS-082 was transient since at later times tension rose again. In order to know whether the ONO-RS-082-sensitive mechanism (arachidonic acid-dependent mechanism) and the fasudil-sensitive mechanism (possibly Rho kinase-dependent mechanism) independently contribute to the inhibition of MLC_{20} dephosphorylation or whether the two mechanisms share a common pathway, we tested the effect of a combination of ONO-RS-082 (5 μM) and fasudil (10 μM) on the DPB-induced contraction and Ser19 phosphorylation of MLC_{20}. Contraction that remained in Ca^{2+}-free, EGTA solution containing DPB was more greatly inhibited by a combination of fasudil and ONO-RS-082 than by a single use of fasudil or ONO-RS-082 (Fig. 5). However, fasudil did not show any additional inhibition of the Ser19 phosphorylation of MLC_{20} produced by ONO-RS-082 (Fig. 5).

Phosphorylation of MBS

We have confirmed the specificity of the antibody against MBS phosphorylated at Thr654 (pMBS-T654, Ito et al. 2003). Thirty minutes after the application of DPB (3 μM) the phosphorylation of MBS at Thr654 was significantly increased. Fasudil (10 μM) lowered the level of phosphorylation to below that in the resting state (Fig. 6).

DPB-induced contraction and MLC_{20} phosphorylation in Ca^{2+}-free medium

DPB could induce a contraction without a rise in $[Ca^{2+}]_i$ (Asano et al. 1995; Miura et al. 1997). To see whether the DPB-induced contraction in the

preparations having very low $[Ca^{2+}]_i$ accompanied an increase in the MLC_{20} phosphorylation, we observed the effect of DPB (1 μM) in Ca^{2+}-free PSS containing 1 mM EGTA. To prevent the influence of intracellular Ca^{2+}, intracellular stores were depleted of Ca^{2+} by repeated applications of ryanodine (3 μM) and phenylephrine (10 μM) in the Ca^{2+}-free, EGTA PSS. In the Ca^{2+}-depleted muscles, DPB induced a sustained contraction, which was 49.1 ± 7.8 % (n=6) of the 65.4 mM KCl-induced contraction (Fig. 7). Namely, the magnitude of DPB-induced contraction in Ca^{2+}-depleted muscles was about 50-60 % of that elicited in normal PSS. The MLC_{20} phosphorylation in the resting state decreased from 8.9 ± 2.5 % to 1.5 ± 0.3 % (n=6, $P<0.05$) after Ca^{2+} derivation. DPB increased the MLC_{20} phosphorylation to 5.2 ± 1.2 % but it was still lower than the level in the resting state in normal PSS.

Fasudil (10 μM), which was treated 1 h before DPB, significantly inhibited the DPB-induced contraction in Ca^{2+}-depleted muscles. We did not measure the MLC_{20} phosphorylation in the presence of fasudil, because the DPB-induced MLC_{20} phosphorylation did not exceed the level in the resting state in Ca^{2+}-containing PSS (Fig. 7).

Discussion

In this study we measured the MLC_{20} phosphorylation in two ways; one is determination of total MLC_{20} phosphorylation including phosphorylation at Ser1, Ser2, Thr9, Thr18 or Ser19 and the other is determination of Ser19 phosphorylation. PKC directly phosphorylates MLC_{20} at Ser1, Ser2 and Thr9, but the phosphorylation at these sites does not contribute to tension development (Ikebe and Hartshorne 1985; Ikebe et al. 1987). In a previous paper (Miura et al. 1997) we reported that the DPB-induced total MLC_{20} phosphorylation did not parallel the tension development and suggested that a

major fraction of the MLC_{20} phosphorylation occurs at sites not related to tension development. Ser19 of MLC_{20} is phosphorylated by MLC kinase but not by PKC and the phosphorylation at this site contributes to force development (Sutton and Haeberle 1990; Masuo et al. 1994). In fact, the phosphorylation at Ser19 of MLC_{20} was correlated to a change in tension during the DPB-induced contraction (Miura et al. 1997). Therefore we have to focus attention on the MLC_{20} phosphorylation at Ser19 when a relationship between PKC activation and tension development is concerned.

In this study, fasudil inhibited the phosphorylation of MLC_{20} at Ser19 but not the total MLC_{20} phosphorylation induced by DPB. In contrast, the PKC inhibitor Ro31-8220 inhibited not only the Ser19 phosphorylation of MLC_{20} but also the total MLC_{20} phosphorylation. If the inhibition of MLC_{20} phosphorylation by fasudil was due to the inhibition of PKC, fasudil should have inhibited the phosphorylation of total MLC_{20}, as seen in the case of Ro31-8220. The inability of fasudil to inhibit the phosphorylation of total MLC_{20} indicates that fasudil had no inhibitory effect or only a weak effect on PKC in this experiment and that the fasudil-induced inhibition of MLC_{20} phosphorylation at Ser19 is not a result of the inhibition of PKC. It is clear from the present data that the inhibition of DPB-induced contraction by Rho kinase inhibitors is at least partly due to the inhibition of MLC_{20} phosphorylation at Ser19, an MLC kinase-sensitive site. Fasudil could inhibit the MLC_{20} phosphorylation through inhibition of MLC kinase, since at a high concentration it inhibits MLC kinase *in vitro* (Seto et al. 1991). However, fasudil (10 μM) decreased the Ser19 phosphorylation that remained 15 min after the Ca^{2+} removal in the presence of DPB (Fig. 4). Since MLC kinase was inactive at this point, the inhibition of phosphorylation by fasudil could not be due to inhibition of MLC kinase. Therefore, the involvement of inhibition of MLC kinase may be small, if any, in the effect of 10 μM fasudil.

The level of MLC_{20} phosphorylation is determined by a balance between phosphorylation by MLC kinase and dephosphorylation by MLCP.

Therefore, another possibility for a decrease in MLC_{20} phosphorylation at Ser19 by fasudil or Y-27632 is the acceleration of dephosphorylation. The effect of fasudil on the MLC_{20} dephosphorylation process was seen in the experiment shown in Fig. 4. In this protocol, DPB inhibited the dephosphorylation of Ser19 when the KCl-contracted muscle was rinsed with Ca^{2+}-free, EGTA solution and fasudil canceled the inhibitory action of DPB on the Ser19 dephosphorylation. So far, several factors are known to inhibit MLCP. First, when CPI-17 is activated upon phosphorylation by PKC, it inhibits MLCP (Li et al. 1998; Kitazawa et al. 1999). Second, arachidonic acid can inhibit the enzyme (Gong et al. 1992; 1995). Third, Rho kinase inhibits MLCP by phosphorylating the MBS of MLCP (Kimura et al. 1996; Hartshorne et al. 1998). When anti-phosphoMBS antibody (pMBS-T654) was used to detect the phosphorylation of MBS, DPB significantly increased the phosphorylation at Thr654 of MBS, a site to be phosphorylated by Rho kinase, and fasudil decreased it. Therefore, the data strongly suggest that DPB phosphorylated the MBS of MLCP through Rho kinase and fasudil decreased the phosphorylation as a result of the inhibition of Rho kinase. Similar~~ly~~ to our idea, Kandabashi et al. (2000) reported that PKC is located upstream on Rho kinase in the mechanism of interleukin β-induced porcine coronary artery spasm.

Next question is why PKC activation leads to the phosphorylation of MLCP in a Rho kinase-dependent manner. ONO-RS-082, a phospholipase A_2 inhibitor, has been shown to inhibit release of arachidonic acid in rabbit femoral arteries (Gailly et al. 1997). In this and the previous studies (Miura et al. 1997), ONO-RS-082 inhibited the Ser19 phosphorylation of MLC_{20} in the presence of DPB. This suggests that arachidonic acid is involved in the increase in Ser19 phosphorylation. The interaction of PKC and arachidonic acid is rather complicated, in the sense that PKC activates phospholipase A_2 to release arachidonic acid (Gong et al. 1995) and arachidonic acid in turn activates an isoform of PKC (probably PKCζ, Gailly et al. 1997). The

sensitivity of PKC-dependent Ser19 phosphorylation to a phospholipase A_2 inhibitor indicates that arachidonic acid is at least partly involved in the phosphorylation. Some studies have shown that arachidonic acid can activate Rho kinase (Fu et al. 1998; Feng et al. 1999; Araki et al. 2001). Therefore, it is possible that arachidonic acid activated Rho kinase, and the kinase in turn inhibited MLCP. When fasudil was combined with ONO-RS-082, the inhibition of Ser19 phosphorylation was not greater than the inhibition by ONO-RS-082 alone. This suggests that an increase in Ser19 phosphorylation induced by DPB is exclusively dependent on arachidonic acid. However, so far we can not exclude a possibility that ONO-RS-082 has an action other than inhibition of phospholipase A_2 leading to inhibition of MLC_{20} phosphorylation or a contraction. Further experiment is required to confirm the involvement of arachidonic acid.

In contrast to our data, Fu et al. (1998) showed the data that a contraction induced by 20 μM phorbol 12,13-dibutyrate (PDBu) in permeabilized rabbit pulmonary arteries was resistant to Y-27632. It is possible that at a high concentration of phorbol ester a Rho kinase-independent mechanism masks a Rho-kinase-dependent one. Alternatively, arachidonic acid released from membrane phospholipid in permeabilized cells may diffuse differently from intact cells. In fact, the authors raised a question why arachidonic acid released by PDBu did not have the Ca^{2+}-seisitizing effect.

When fasudil or Y-27632 decreased the Ser19 phosphorylation of MLC_{20} to the resting level, the tension was still over the resting level (Figs. 2 and 4), while Ro31-8220 suppressed the tension (Fig. 3). This suggests that an increase in MLC_{20} phosphorylation is not the sole mechanism responsible for the DPB-induced contraction. In the experiment shown in Fig. 5, ONO-RS-082 transiently decreased the tension during the first 5 min but the tension began to elevate thereafter, whereas it inhibited the Ser19 phosphorylation at 15 min. These findings indicate that the later

development of tension was independent of MLC_{20} phosphorylation. When fasudil was simultaneously added with ONO-RS-082, this later tension development did not appear. Since the inhibition of Ser19 phosphorylation of MLC_{20} seems to be achieved completely by ONO-RS-082 and fasudil, the additional inhibition of contraction by fasudil may be through a mechanism that is not related to MLC_{20} phosphorylation. This MLC_{20} phosphorylation-independent mechanism seems to be partially sensitive to fasudil and ONO-RS-082 since the inhibition of contraction by a combination of fasudil and ONO-RS-082 was greater than a single use of each drug (Fig. 5).

In Ca^{2+}-free, EGTA solution DPB caused a slowly developing contraction with a slight increase in MLC_{20} phosphorylation (Fig. 7). Since the DPB-induced MLC_{20} phosphorylation in Ca^{2+}-depleted muscles was less than the level under the resting condition in Ca^{2+}-containing PSS, the phosphorylation might not contribute to the tension development. This also suggests the existence of a MLC_{20} phosphorylation-independent component in the DPB-induced contraction. This component may be related to an actin binding protein, calponin or caldesmon. Calponin is associated with thin filaments and inhibits the actin-activated Mg^{2+}-ATPase activity (Winder and Walsh 1990; Itoh et al. 1995) and the phosphorylation of calponin reverses its inhibitory action on the ATPase (Winder and Walsh 1990; Rokolya et al. 1996). It has been shown that PDBu phosphorylated calponin and fasudil inhibited the phosphorylation (Nagumo et al. 1998). Therefore, it is possible that calponin is a candidate that is phosphorylated by DPB and plays a predominant role in the contraction appeared in Ca^{2+}-depleted muscles. In summary, PKC-mediated Ca^{2+}-sensitization of vascular smooth muscle contraction includes at least two mechanisms; a Rho kinase-dependent MLC_{20} phosphorylation and a MLC_{20} phosphorylation-independent mechanism. The latter mechanism is partially sensitive to fasudil. It has to be clarified what factors are involved in the MLC_{20} phosphorylation-independent mechanism.

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Legends to figures

Fig. 1 Effects of fasudil on the DPB-induced contraction and MLC_{20} phosphorylation in rabbit aortas. Fasudil (10 μM) was added 30 min after the application of DPB (1 μM). (**a**) The change in tension. The abscissa represents the time after the addition of DPB. The ordinate represents the tension relative to the maximum contraction to 65.4 mM KCl. * $P<0.05$ (vs. DPB alone) (**b**) Phosphorylation of total MLC_{20} (MLC-P) measured in the resting state, 30 min after the addition of DPB and 30 min after the addition of fasudil (60 min after DPB). N.S.: not significant. Data are mean ± SEM (n=6).

Fig. 2 Effects of fasudil and Y-27632 on the DPB-induced contraction and phosphorylation at Ser19 of MLC_{20} in rabbit aortas. (**a**) Changes in tension following the addition of fasudil (10 μM) or Y-27632 (1 μM). Thirty minutes after the addition of DPB fasudil or Y-27632 was applied (the tension development for the first 30 min after DPB was omitted from the figure). The level reached after 30 min application of DPB is expressed as 100% in the

ordinate. (**b**) Phosphorylation at Ser19 of MLC_{20} (Ser19-P). Upper panels are examples of bands probed with anti-phosphoSer19 antibody. Both were sampled 60 min after the addition of DPB (30 min after fasudil or Y-27632). In the lower panels phosphorylation is expressed as a value relative to the level in the resting state. Data are mean ± SEM (*n*=4).

Fig. 3 Inhibition by DPB of dephosphorylation of MLC_{20} and relaxation in Ca^{2+}-free, EGTA solution, and the effect of Ro31-8220. (**a**) The change in tension. After the KCl (65.4 mM)-induced contraction attained a peak (5 min), the external medium was switched to Ca^{2+}-free solution containing 1 mM EGTA and 1 μM DPB. When present, Ro31-8220 (10 μM) was applied 1 h before switching the medium. Ro31-8220 did not affect the KCl-induced contraction. The ordinate represents the relative value of maximum contraction to 65.4 mM KCl in normal medium. Data are mean ± SEM (*n*=6). * Significantly different from DPB alone ($P<0.05$). (**b**) Phosphorylation of total MLC_{20} (MLC-P) measured in the resting state, at the peak of KCl-induced contraction, 15 min after the addition of DPB in the absence (DPB) or presence of Ro31-8220. MLC-P is expressed as a percentage of phosphorylated MLC_{20} of the sum of phosphorylated and unphosphorylated MLC_{20} (*n* = 4). (**c**) Phosphorylation at Ser19 of MLC_{20} (Ser19-P) measured in the resting state and 15 min after the addition of DPB in the absence or presence of Ro31-8220. Ser19-P is expressed as a percentage of phosphorylation in the resting state. Data are mean ± SEM (*n* = 4). * Significantly different from DPB alone ($P<0.05$). † Significantly different from the level in the resting state ($P<0.05$).

Fig. 4 Effects of fasudil on the DPB-induced inhibition of dephosphorylation at Ser19 of MLC_{20} and relaxation in Ca^{2+}-free, EGTA solution. (**a**) The change in tension. After KCl (65.4 mM)-induced contraction attained the peak (5 min), the external medium was switched to

Ca^{2+}-free solution containing 1 mM EGTA and 1 μM DPB. When present, fasudil (10 μM) was applied simultaneously with DPB. Data are mean ± SEM (n = 6). (**b**) Ser19 phosphorylation (Ser19-P) of MLC_{20} measured in the resting state (numbered as 1 in **a**), at the peak of KCl-induced contraction (numbered as 2 in **a**), 15 min after the addition of DPB in the absence or presence of fasudil (numbered as 3 in **a**). Ser19-P is expressed as a percentage of phosphorylation in the resting state. Data are mean ± SEM (n = 4). * Significantly different from DPB alone ($P<0.05$). † Significantly different from the level in the resting state ($P<0.05$).

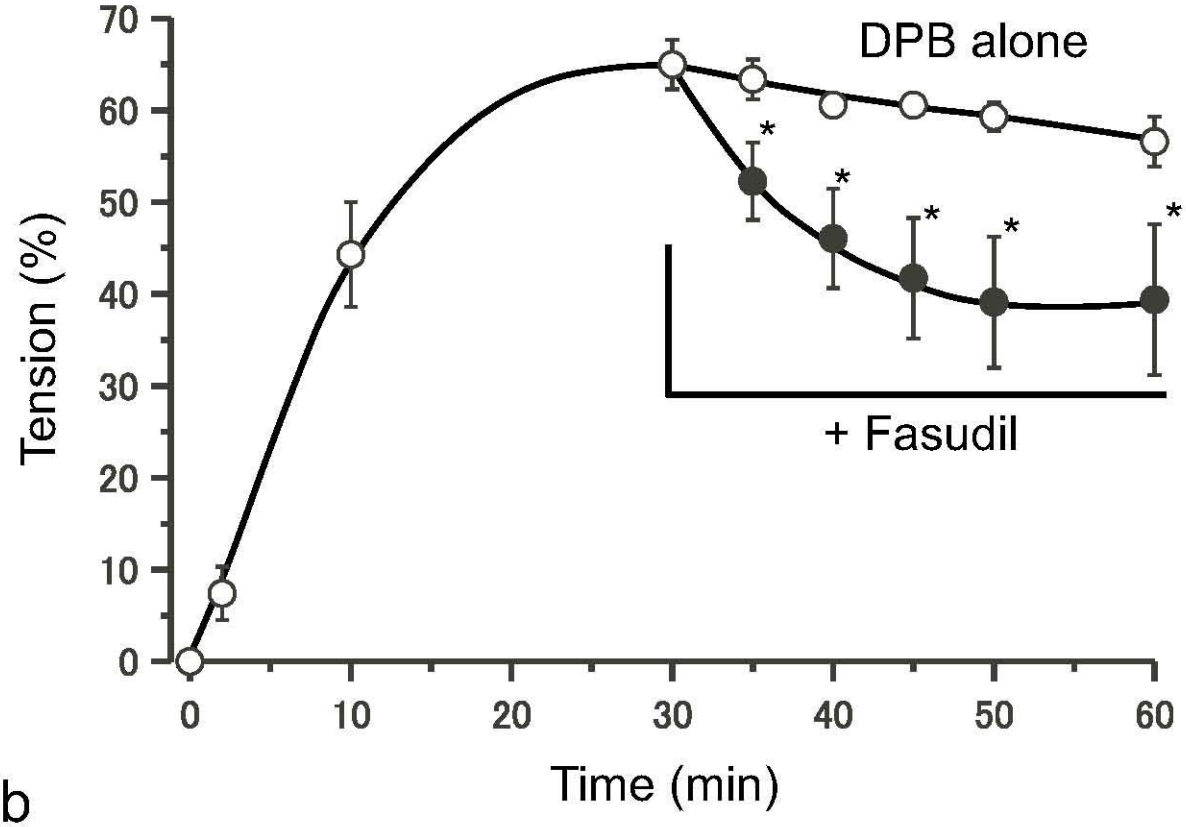
Fig. 5 Effects of a combination of fasudil and ONO-RS-082 on the DPB-induced inhibition of MLC_{20} dephosphorylation and relaxation in Ca^{2+}-free, EGTA solution. (**a**) The change in tension. After KCl (65.4 mM)-induced contraction attained the peak (5 min), the external medium was switched to Ca^{2+}-free solution containing 1 mM EGTA and 1 μM DPB. ONO-RS-082 (5 μM) was applied 20 min before the addition of 65.4 mM KCl. ONO-RS-082 did not affect the KCl-induced contraction. Fasudil (10 μM) was applied simultaneously with DPB. Open circles; DPB alone. Open squares; DPB + ONO-RS-082. Filled squares; DPB + ONO-RS-082 + fasudil. Data are mean ± SEM (n = 6). * Significantly different from DPB alone ($P<0.05$). § Significantly different from DPB + ONO-RS-082 ($P<0.05$). (**b**) Phosphorylation at Ser19 of MLC_{20} (Ser19-P) measured 15 min after the addition of DPB in the absence or presence of ONO-RS-082 and fasudil. Ser19-P is expressed as a percentage of phosphorylation in the resting state. * Significantly different from DPB alone ($P<0.05$). N.S.: not significant.

Fig. 6 DPB-induced contraction and phosphorylation of MBS, and the inhibition by fasudil. After the DPB (3 μM)-induced contraction reached a steady state (30 min), fasudil (10 μM) was added. Tension and MBS phosphorylation were measured 30 min after the addition of fasudil (mean ± SEM, n = 4). Open columns are tension, represented as a value relative to the maximal contraction to 65.4 mM KCl, which was observed before

challenge by DPB. Closed columns are MBS phosphorylation, which is expressed as the relative value of the phosphorylation in the resting state. † Significantly different from the resting level ($P < 0.05$). * Significantly different from DPB alone ($P < 0.05$).

Fig. 7 Contraction and MLC_{20} phosphorylation caused by DPB in Ca^{2+}-free, EGTA solution. After changing the medium to Ca^{2+}-free, EGTA (1 mM) solution, the sarcoplasmic reticulum was depleted of Ca^{2+} by repeated application of ryanodine (3 µM) and phenylephrine (10 µM). Then, DPB (1 µM) was applied. (**a**) Time course of tension development. Open circles; DPB alone. Closed circles; in the presence of fasudil (10 µM), which was applied 1 h before DPB. (**b**) Total MLC20 phosphorylation measured in the resting state in normal PSS or in Ca^{2+}-free, EGTA PSS, and at 30 min after addition of DPB in Ca^{2+}-free, EGTA PSS. MLC_{20} phosphorylation was measured with glycerol PAGE and probing with anti-MLC_{20} antibody. Phosphorylation data in DPB-treated muscles were obtained 30 min after DPB. Data are mean ± SEM (n=6).

a


DPB alone
+ Fasudil
Tension (%)
Time (min)
0
10
20
30
40
50
60
70
*

b

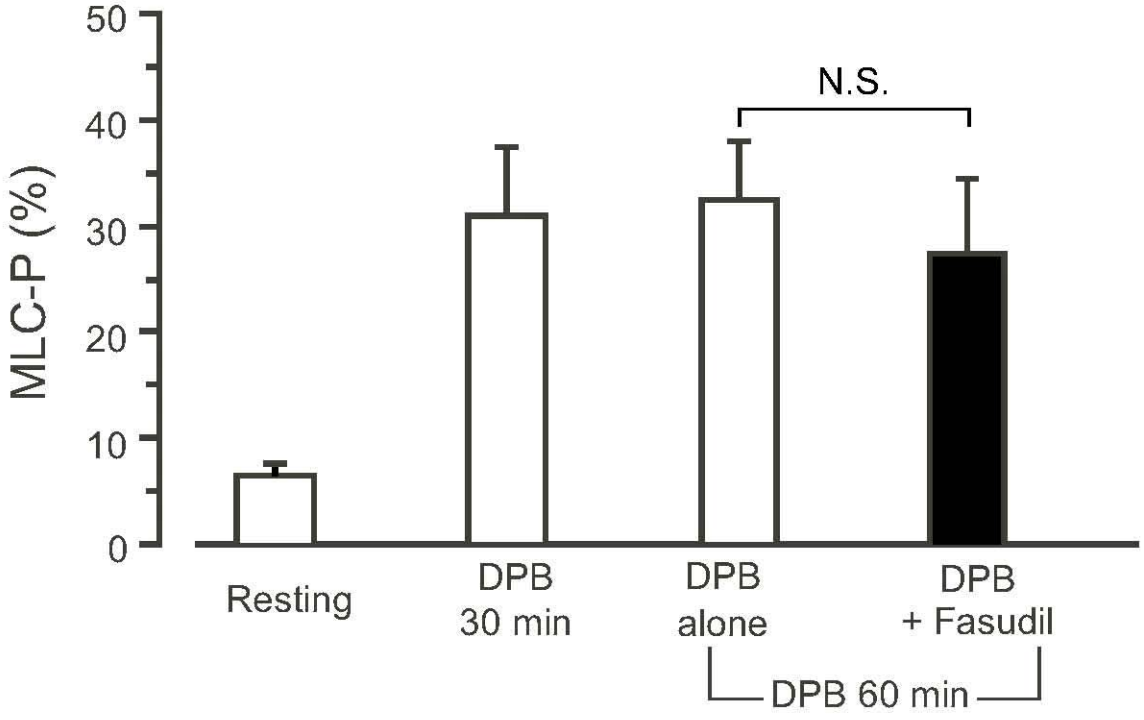

MLC-P (%)
N.S.
Resting
DPB
30 min
DPB
alone
DPB
+ Fasudil
DPB 60 min
0
10
20
30
40
50

Fig.1

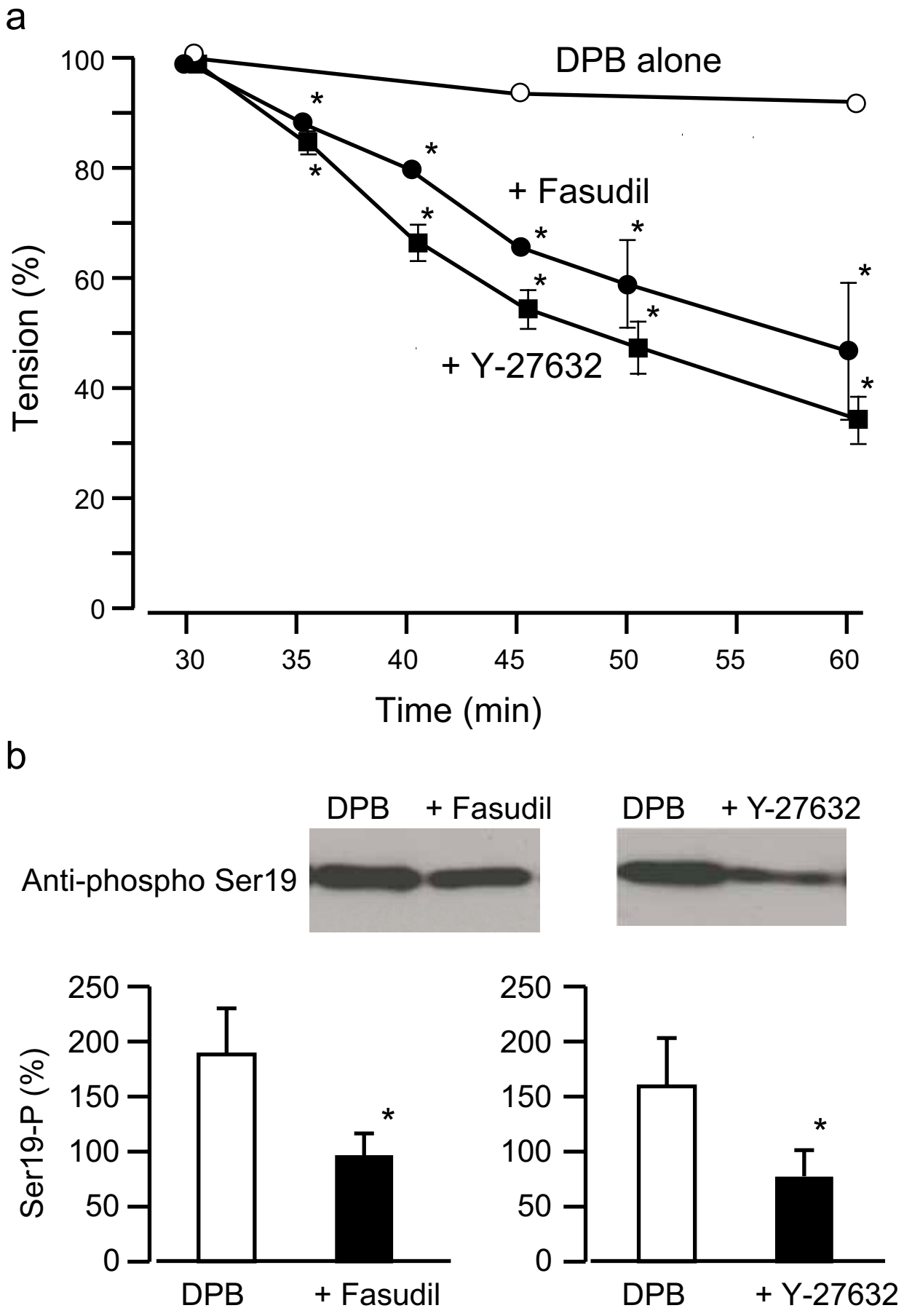

a
DPB alone
+ Fasudil
+ Y-27632
Tension (%)
Time (min)
b
DPB
+ Fasudil
DPB
+ Y-27632
Anti-phospho Ser19
Ser19-P (%)
DPB
+ Fasudil
DPB
+ Y-27632

Fig. 2

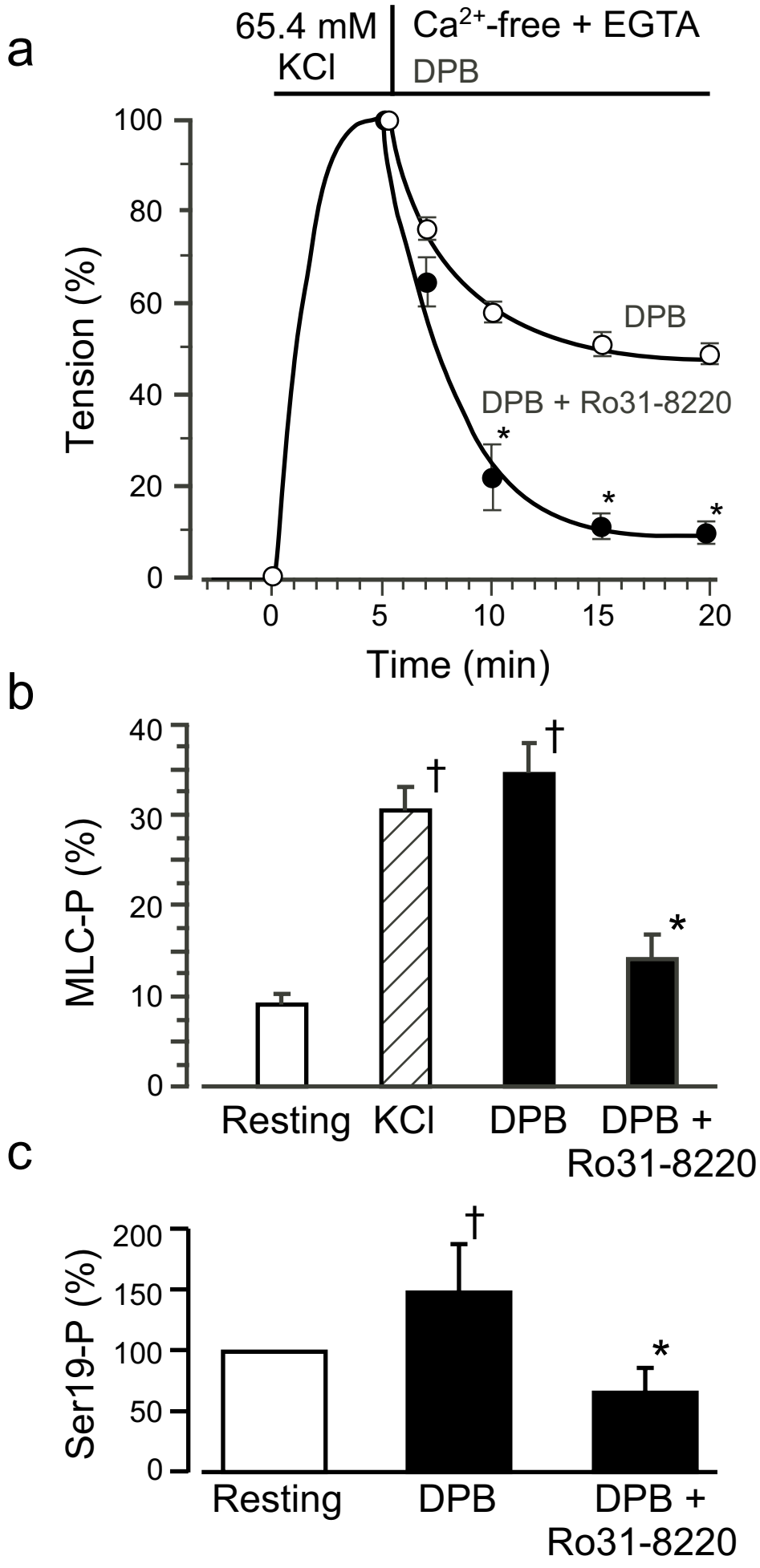

a
65.4 mM KCl
Ca2+-free + EGTA
DPB
Tension (%)
100
80
60
40
20
0
DPB
DPB + Ro31-8220
*
*
*
0
5
10
15
20
Time (min)
b
MLC-P (%)
40
30
20
10
0
†
†
*
Resting
KCl
DPB
DPB + Ro31-8220
c
Ser19-P (%)
200
150
100
50
0
†
*
Resting
DPB
DPB + Ro31-8220

Fig. 3

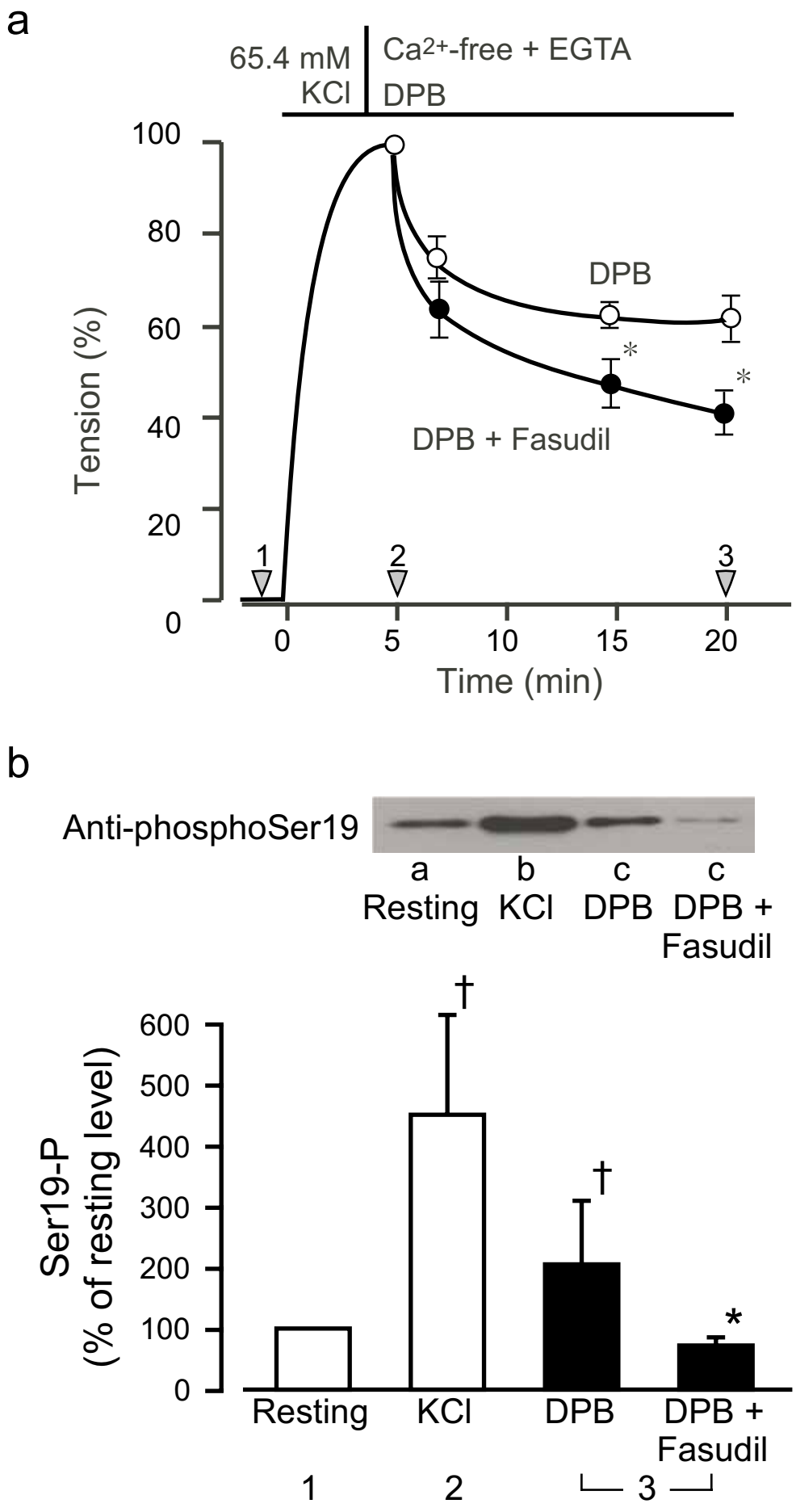

a
65.4 mM KCl
Ca2+-free + EGTA
DPB
Tension (%)
100
80
60
40
20
0
DPB
DPB + Fasudil
*
*
1
2
3
0
5
10
15
20
Time (min)
b
Anti-phosphoSer19
a
b
c
c
Resting
KCl
DPB
DPB + Fasudil
Ser19-P
(% of resting level)
600
500
400
300
200
100
0
†
†
*
Resting
KCl
DPB
DPB + Fasudil
1
2
3

Fig. 4

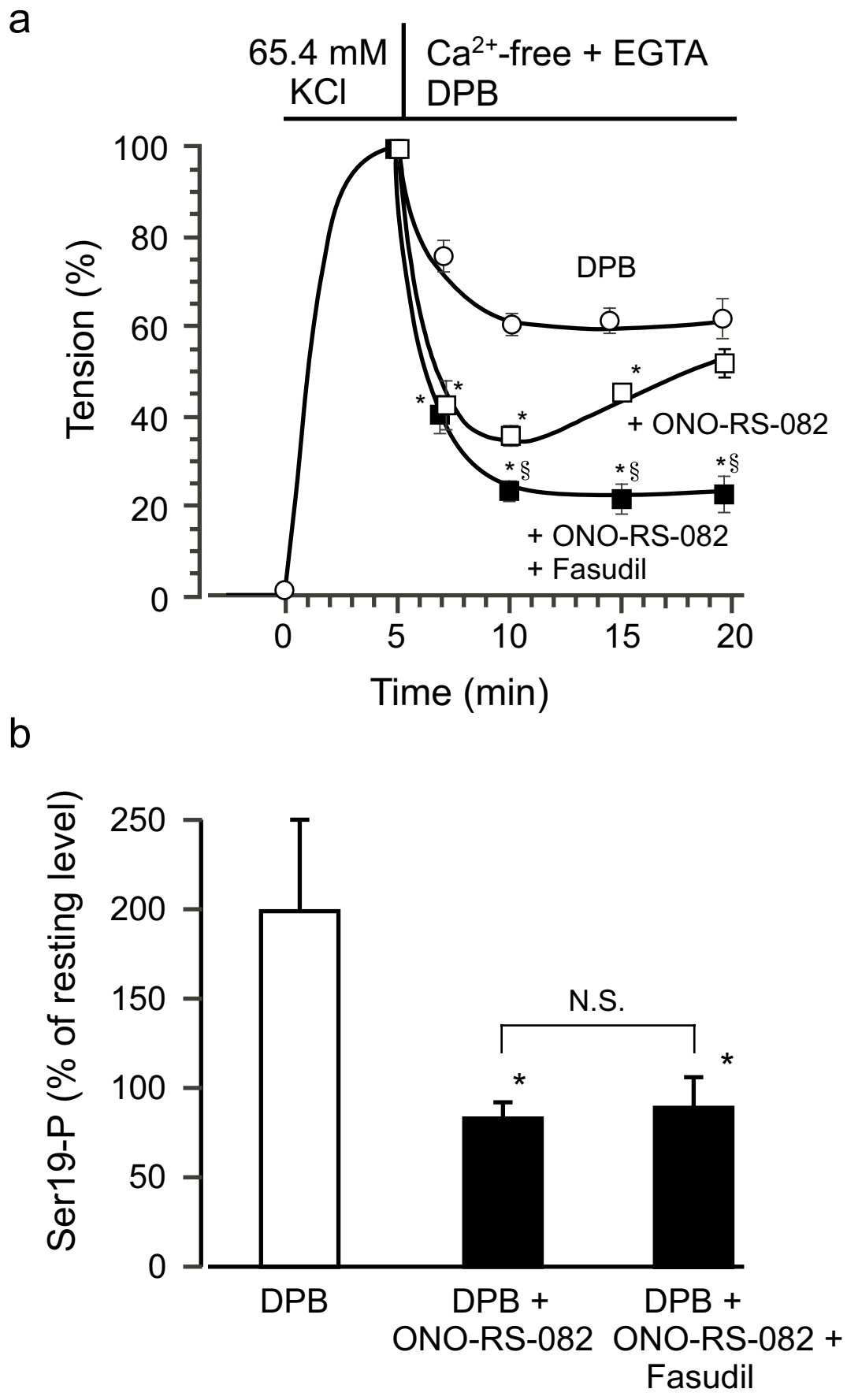

a
65.4 mM KCl
Ca2+-free + EGTA DPB
Tension (%)
Time (min)
DPB
+ ONO-RS-082
+ ONO-RS-082 + Fasudil
b
Ser19-P (% of resting level)
N.S.
DPB
DPB + ONO-RS-082
DPB + ONO-RS-082 + Fasudil

Fig. 5

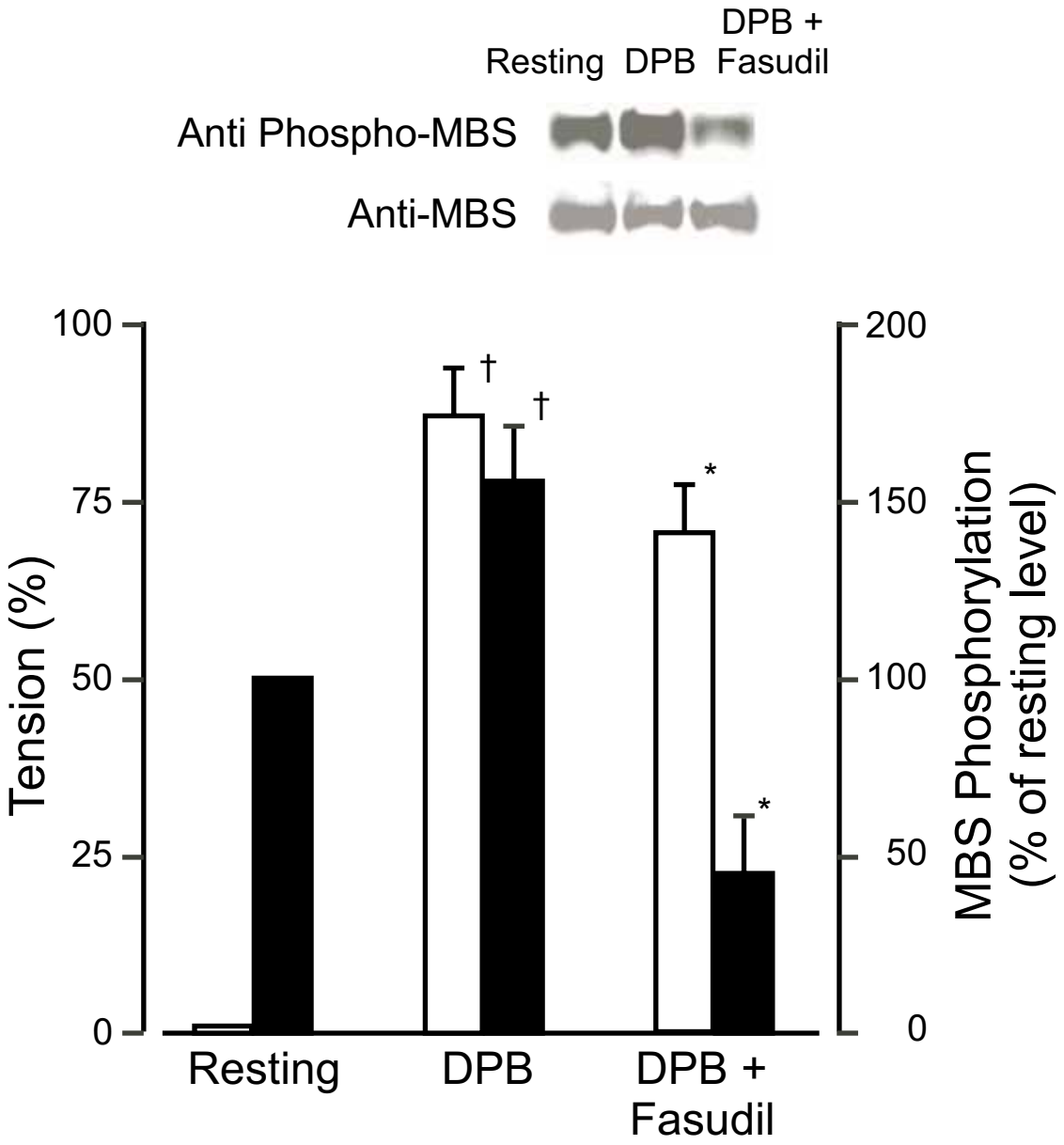

Resting
DPB
DPB +
Fasudil
Anti Phospho-MBS
Anti-MBS
100
75
50
25
0
Tension (%)
200
150
100
50
0
MBS Phosphorylation
(% of resting level)
†
†
*
*
Resting
DPB
DPB +
Fasudil

Fig. 6