Essential role of Rho kinase in Ca^{2+}-sensitization of prostaglandin F2α-induced contraction of rabbit aortae

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Inhibition of dephosphorylation of the 20 kDa myosin light chain (MLC_{20}) is an important mechanism responsible for Ca^{2+}-sensitization of vascular smooth muscle contraction. We investigated whether this mechanism operates in prostaglandin F2α (PGF2α)-induced contraction of rabbit aortic smooth muscle and, if so, which of protein kinase C (PKC) or Rho kinase contributes to the inhibition of dephosphorylation. In normal medium, PGF2α (10 μM) increased phosphorylation of MLC_{20} and developed tension. Rho kinase inhibitors fasudil and hydroxyfasudil inhibited these changes, despite having no effect on a phorbol ester-induced MLC_{20} phosphorylation. After treatment with verapamil or chelation of external Ca^{2+} with EGTA, PGF2α increased the MLC_{20} phosphorylation and the tension without an increase in $[Ca^{2+}]_i$, which were both sensitive to fasudil and hydroxyfasudil. ML-9, a MLC kinase inhibitor, quickly reversed the KCl-induced MLC_{20} phosphorylation and contraction to the resting level. However, fractions of PGF2α-induced contraction and MLC_{20} phosphorylation were resistant to ML-9 but were sensitive to fasudil. Ro31-8220 (10 μM), a PKC inhibitor, did not affect the MLC_{20} phosphorylation and the tension caused by PGF2α, excluding the possibility of involvement of PKC in the PGF2α-induced MLC_{20} phosphorylation. PGF2α increased phosphorylation at Thr654 of the myosin binding subunit (MBS) of myosin phosphatase, which is a target of Rho kinase, and fasudil decreased the phosphorylation. These data suggest that the PGF2α-induced contraction is accompanied by the inhibition of MLC_{20} dephosphorylation through the MBS phosphorylation by Rho kinase, leading to Ca^{2+}-sensitization of contraction. Besides, an actin-associated mechanism may also be involved in the PGF2α-induced sensitization.

(Introduction)

A topic for this decade in vascular smooth muscle physiology and pathophysiology is "Ca^{2+}-sensitization" of contraction (Somlyo & Somlyo, 1994). Initially, this term was simply an expression for the enhancement of contraction at a given cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), but recently a molecular basis for this phenomenon has been provided. Ca^{2+}-sensitization refers to when phosphorylation of the 20 kDa myosin light chain (MLC_{20}), which is catalyzed by Ca^{2+}/calmodulin-dependent MLC kinase and is a primary determinant of contraction, is increased over the level expected from $[Ca^{2+}]_i$ (Horowitz *et al.* 1996). Alternatively, this term is also used when the developed tension is relatively high at a given level of MLC_{20} phosphorylation. The latter enhancement may be related to alterations in regulatory proteins on thin filaments (Katsuyama *et al.* 1992; Itoh *et al.* 1995; Je *et al*. 2001).

Ca^{2+}-sensitization resulting from an increase in MLC_{20} phosphorylation can occur either when the phosphatase (SMPP-1M) responsible for dephosphorylation of MLC_{20} is inhibited (Somlyo *et al.* 1989; Kitazawa *et al.* 1991) or when MLC_{20} is phosphorylated in a Ca^{2+}/calmodulin-independent manner (Kureishi *et al.* 1997; Weber *et al.* 1999). Reportedly, SMPP-1M is negatively regulated by several factors including small GTPase Rho-associated kinase (Rho kinase, Noda *et al.* 1995; Kimura *et al.* 1996), CPI-17 (Li *et al.* 1998) or arachidonic acid (Gong *et al.* 1992). Because the inhibition of phosphatase by arachidonic acid or CPI-17 is associated with the activation of protein kinase C (PKC) (Gong *et al.* 1992; Gailly *et al.* 1997; Li *et al.* 1998; Hartshorne *et al.* 1998) and Ca^{2+}-independent phosphorylation of MLC_{20} can also be caused by CPI-17 or Rho kinase (Kureishi *et al.* 1997; Li *et al.* 1998), PKC and Rho kinase are so far the two major determinants for the Ca^{2+}-sensitization in vascular smooth muscles.

When a receptor coupled to a heterotrimeric GTP binding protein is activated, Ca^{2+}-sensitization as well as Ca^{2+} mobilization occurs. If diacylglycerol, a product of phosphatidylinositol hydrolysis, increases to a level to sufficiently activate PKC, PKC-dependent Ca^{2+}-sensitization

should play a role in the enhancement of contraction. However, a role of PKC in receptor-mediated Ca^{2+}-sensitization is controversial, as some papers favor the role (Collins *et al.* 1992; Khalil & Morgan, 1992; Shimamoto *et al.* 1992; Parsons *et al.* 1996; Buus *et al.* 1998; Eto *et al.* 2001) but others do not (Singer *et al.* 1989; Hori *et al.* 1993; Jensen *et al.* 1996). On the other hand, Ca^{2+}-sensitization mediated by some types of receptor requires the presence of GTP (Nishimura *et al.* 1988; Kitazawa *et al.* 1991), and this requirement was explained by the involvement of Rho, which activates Rho kinase. The involvement of Rho kinase in receptor-mediated contractions has been claimed in some studies, in which *Botulinum* C3 exoenzyme, which ADP-ribosylates and inactivates Rho, was used (Hirata *et al.* 1992; Fujita *et al.* 1995) or Rho kinase inhibitors were used (Uehata *et al.* 1997; Nagumo *et al.* 2000).

Stimulation of Rho induces the inhibition of myosin phosphatase activity by stimulating Rho kinase-mediated phosphorylation of the 130 kDa myosin binding subunit (MBS) of SMPP1 in smooth muscle (Noda *et al.* 1995; Kimura *et al.* 1996; Nagumo *et al.* 2000). Some heterotrimeric GTP-binding proteins coupled to a receptor such as Gα12 or Gα13 can activate Rho through the action on the guanine nucleotide exchange factor (GEF) for Rho (Hart *et al.* 1998; Kozasa *et al.* 1998; Sakurada *et al.* 2001). Such a link indicates that Rho can be involved in receptor-mediated Ca^{2+}-sensitization. However, it is still unclear which of PKC and Rho kinase is more important for the Ca^{2+}-sensitization caused by the inhibition of MLC_{20} phosphatase following receptor activation or whether both kinases synergistically play a role to bring about the inhibition of the phosphatase. Moreover, involvement of Rho kinase has been proved in some types, but not all types of receptor-mediated contraction. Prostaglandin F2α (PGF2 α) is a unique Ca^{2+} sensitizer, because it causes diphosphorylation of MLC_{20} in rabbit aortic smooth muscles (Seto *et al.* 1990b). Since PGF2α is supposed to be involved in a delayed spastic contraction of the cerebral arteries after subarachnoid haemorrhage (Hagen *et al.* 1977; Chehrazi *et al.* 1989), PGF2α could be an important mediator of pathological vasospasm. In order to clarify the mechanism of PGF2α-induced Ca^{2+}-sensitization, we investigated the

following issues; 1) whether MLC_{20} dephosphorylation is inhibited during $PGF_{2\alpha}$ receptor-mediated contraction, 2) whether MBS is phosphorylated during the contraction, 3) which of PKC or Rho kinase is involved in the inhibition of dephosphorylation.

METHODS

Force measurement

The following experimentation was approved by the Animal Care and Use Committee at Miyazaki University Faculty of Agriculture. Adult male rabbits were anaesthetized with sodium pentobarbitone (45 mg kg^{-1}) and sacrificed by cervical dislocation. The thoracic aorta was isolated, the endothelium was removed by rubbing the intimal surface with a cotton swab, and a strip of 4-5 mm width and 7-8 mm length was made. The removal of endothelium was confirmed by abolition of relaxant response to acetylcholine (1 μM) in each strip. The strip was mounted in an organ bath containing 5 ml physiological saline solution (PSS) with the following composition (mM): NaCl 136.8, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 11.9 and glucose 5.5 (when saturated with 95% O_2 and 5% CO_2, pH was 7.3-7.4). A resting tension of 9.8 mN was applied throughout the experiment. Ca^{2+}-free, EGTA solution was made by omitting $CaCl_2$ from PSS and adding 1 mM EGTA. High KCl (65.4 mM) solution was made by substituting 60 mM NaCl in PSS with iso-osmolar KCl.

$[Ca^{2+}]_i$ measurement

For measurement of $[Ca^{2+}]_i$, the aortic strip was loaded with the Ca^{2+} indicator fura-PE3/AM (5 μM, TEF LABS, Austin, TX, U.S.A.) that had been sonicated with 0.02 % cremophore EL in PSS. Then, the strip was mounted in a bath constructed in a fluorimeter (CAF-100, JASCO, Tokyo, Japan). Fluorescence at 500 nm following alternate excitation at 340 nm (F_{340}) and 380 nm (F_{380}) was monitored together with tension. The ratio of F_{340}/F_{380} was taken as an index of $[Ca^{2+}]_i$.

Protein extraction for Western blotting

At an appropriate time during the tension experiments, a strip was quickly removed from the organ bath while taking care not to stretch it and immersed in dry-ice/acetone containing 10 % (v/v) trichloroacetic acid (TCA) and 5 mM dithiothreitol (DTT). It took about 3 sec until the preparation was frozen. This demounting did not affect the MLC_{20} phosphorylation level, since the phosphorylation level following this procedure in 65.4 mM KCl-contracted muscles was not different from when a mounted muscle was snap-frozen by dry-ice/acetone in the organ bath. Frozen tissues were rinsed with acetone containing 10 mM DTT to remove TCA and dried. The strip was cut into tiny pieces and proteins were extracted with 8 M urea and 10 mM DTT.

MLC_{20} phosphorylation

MLC_{20} phosphorylation was measured as described previously (Miura *et al.* 1997). Briefly, the proteins were subjected to glycerol-polyacrylamide gel electrophoresis (glycerol-PAGE). The proteins were electrotransferred from the gylcerol-PAGE gel onto a nitrocellulose membrane. The membrane was soaked in skim milk to prevent non-specific binding of antibody. Then, the membrane was incubated overnight with 10 μg ml^{-1} of polyclonal anti-MLC_{20} antibody, prepared as described elsewhere (Seto *et al.* 1990b) and thereafter exposed to peroxidase-labeled secondary antibody (anti-rabbit IgG, Amersham NA934). A band was visualized using an ECL kit (Amersham). A ratio of the sum of monophosphorylated and diphosphorylated forms to total MLC_{20} was calculated.

Preparation of antibodies against MBS phosphorylated at Thr654 and MBS

A polyclonal antibody (antibody pMBS-654) 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 (see Fig. 7), as previously described (Hartshorne *et al.* 1998). Antiserum was

purified through sequential affinity columns of immobilized phospho-MBS and non-phospho MBS peptides. A polyclonal antibody against MBS (antibody N-MBS) was raised using a synthetic peptide corresponding to the amino-terminal peptide (MKMADAKQKRNEC) of chicken MBS.

Preparation of recombinant MBS

The 3.8-kb cDNA encoding the entire coding region of MBS was cloned by hybridization screening of a pig aortic smooth muscle cDNA library, using the 480-bp rat MBS cDNA as a probe, and ligated into pAcHTL-C vector (PharMingen, San Diego, CA, USA) at an *Eco* RI site downstream of the polyhedrin promoter to produce pAcHTL-MBS. Sf9 cells were co-transfected with pAcHTL-MBS and Baculogold baculovirus DNA (PharMingen, San Diego, CA, USA) by the lipofection method, and the recombinant baculovirus encoding the MBS cDNA was recovered.

MBS phosphorylation

Characterization of the antibody against phosphorylated MBS (pMBS-654) was determined in cultured pig aortic smooth muscle cells between the 5th and the 15th passages and in the intact rabbit mesenteric artery. Aortic cells were permeabilized with β-escin (20 μM) as previously described (Noda *et al*. 1995; Nagumo *et al*. 2000).

Equal volumes of extracts from arteries or cells were subjected to two sets of sodium dodecyl sulphate (SDS)-PAGE. The proteins were transferred from the SDS-PAGE gel onto two nitrocellulose membranes in a buffer containing 20 mM Tris base (pH 7.5). One membrane was probed with the N-MBS antibody and the other with the pMBS-654 antibody using the ECL system. The extent of phosphorylation of MBS on Thr654 was normalized for the total expression levels of MBS.

Phosphorylation at Ser19 of MLC_{20}

Antibody against MLC_{20} phosphorylated at Ser19 was prepared as described previously (Sakurada *et al.* 1994). The proteins were electrophoresed as described in the above section and were transferred onto two nitrocellulose membranes. One membrane was probed with the monoclonal anti-phosphoSer19 antibody and the other with the anti-MLC_{20} antibody. Details of the method have been described previously (Miura *et al.* 1997).

Assay of protein kinase activity

Rho kinase was purified from bovine brain, according to the method of Matsui *et al.* (1996). The Ki value of fasudil and hydroxyfasudil for Rho kinase was determined, as described previously (Nagumo *et al.* 2000). For the immunoblot or autoradiography assay, recombinant MBS (35 μg ml^{-1}) was incubated with Rho kinase in a reaction mixture composed of 50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 100 μM ATP in the absence or presence of [γ-^{32}P]ATP at 37 °C.

MLC kinase and MLC_{20} were prepared from chicken gizzard as previously described (Walsh *et al*. 1983; Yoshida & Yagi 1988). Calmodulin was prepared from porcine brain, by the method of Yazawa *et al*. (1980). MLCK activity was determined by the method of Sakurada *et al*. (1994). PKC was prepared from porcine brain, by the method of Manenti *et al*. (1992). PKA was prepared from bovine heart by the method of Beavo *et al.* (191974). PKC and PKA activities were determined by using NRPK assay kit (MBL, Japan).

Drugs

Drugs used were fasudil (HA1077), hydroxyfasudil (HA1100, Asahi Chemical Co. Ltd., Tokyo, Japan), Y-27632 (Welfide Co. Ltd., Tokyo, Japan), ONO-RS-082 (Ono Pharmaceutical Co. Ltd, Osaka, Japan), Ro31-8220 (bisindoylmaleimide IX, Sigma-RBI, St. Louis. MO, USA) and verapamil (Eisai Co. Ltd., Tokyo, Japan).

Statistics

Data are expressed as mean ± standard error (S.E.M.). Significance was tested by the Student's *t*-test for a single comparison. For a multiple comparison, one-way analysis of variance followed by the Dunnet (comparison with a single control group) or Student-Newman-Keuls (comparison between all pairs) test was performed. Significance was considered at the level of $P < 0.05$.

RESULTS

Effects of fasudil and hydroxyfasudil on the KCl-, DPB- and PGF2α-induced contraction and MLC_{20} phosphorylation of rabbit aortae

In this study, we mainly used fasudil and hydroxyfasudil as Rho kinase inhibitors (Uehata *et al*. 1997; Swärd *et al*. 2000; Nagumo *et al*. 2000). Table 1 shows the Ki values of both compounds against Rho kinase, PKC, cyclic AMP-dependent protein kinase (PKA) and MLC kinase. Hydroxyfasudil is an active metabolite of fasudil produced in the liver and equally potent against Rho kinase but less potent than fasudil against MLC kinase and PKC. Therefore, if Rho kinase is involved in Ca^{2+}-sensitization caused by a constrictor, fasudil and hydroxyfasudil should inhibit the sensitization to a similar degree or, alternatively, if PKC is involved, fasudil should exhibit more potent inhibition than hydroxyfasudil. Based on these predictions, we compared the inhibitory action of fasudil and hydroxyfasudil on contractions mainly dependent on MLC kinase (KCl), PKC (DPB, a PKC activator) or receptor activation (PGF2α). Each compound was cumulatively added at an interval of 30 min during the sustained phase of KCl (65.4 mM)- DPB (1 μM)- or PGF2α (10 μM)-induced contraction. Both compounds dose-dependently decreased the tension elevated by each constrictor. The IC50 of both compounds is summarized in Table 2. Fasudil and hydroxyfasudil equally inhibited the PGF2α-induced contraction, whereas IC50 of hydroxyfasudil against the KCl- or the DPB-induced contraction was greater than IC50 of fasudil ($P < 0.05$, $n = 7$~8). Although IC50 of fasudil against the PGF2α-induced

contraction was not significantly different from IC50 against the DPB-induced one calculated from the cumulative dose study, the inhibition by fasudil on the DPB-induced contraction was significantly less than that on the PGF2α-induced one when a single dose of fasudil (10 μM) was applied (see Fig. 1). The order of potency of another Rho kinase inhibitor, Y-27632, was similar to that of fasudil (Table 2).

Next, we examined whether the inhibition of PGF2α- or DPB-induced contraction by fasudil and hydroxyfasudil was accompanied by inhibition of MLC_{20} phosphorylation (Fig. 1). After PGF2α (10 μM) or DPB (1 μM) induced a sustained contraction, fasudil or hydroxyfasudil was added. PGF2α increased the monophosphorylation level from 7.0 ± 0.6 % in the resting state to 34.7 ± 3.5 % and caused diphosphorylation of MLC_{20} (1.8 ± 0.8 %, n = 6) at 20 min. Diphosphorylation of MLC_{20} due to PGF2α was greater at earlier times and at later times the phosphorylation declined (Seto *et al.* 1990b; Seto *et al.* 1991) so in the later experiments we did not quantify the density of the band corresponding to diphosphorylation when the MLC_{20} phosphorylation was measured at later than 15 min after the application of PGF2α. Fasudil or hydroxyfasudil (10 μM) added during the sustained phase of PGF2α-induced contraction lowered the tension by 63.1 ± 6.1 % or 67.4 ± 5.0 %, respectively (n = 6). The decrease in tension by fasudil or hydroxyfasudil was accompanied by inhibition of the MLC_{20} phosphorylation. Diphosphorylation due to PGF2α was almost completely abolished by both compounds (Fig. 1A).

DPB (1 μM) also increased MLC_{20} phosphorylation, which attained a sustained phase at 5 min, whereas the tension gradually developed and attained a peak at 20-30 min (Miura *et al*. 1997). Fasudil or hydroxyfasudil (10 μM) did not affect the DPB-induced MLC_{20} phosphorylation measured at 30 min after DPB, although both compounds decreased the tension by 43.2 ± 12.6 % or 35.4 ± 6.9 %, respectively (n = 6, Fig. 1B).

Dependence of the PGF2α-induced contraction and MLC_{20} phosphorylation on Ca^{2+}

We examined how PGF2α mobilized Ca^{2+} and how the PGF2α-induced contraction depended on $[Ca^{2+}]_i$ in fura-PE3-loaded aortae. In normal PSS, PGF2α (10 μM) increased $[Ca^{2+}]_i$ in various fashions. Frequently observed patterns were that $[Ca^{2+}]_i$ rapidly increased following application of PGF2α and the level was sustained at a slightly depressed level (*n* = 10, Fig. 2) or $[Ca^{2+}]_i$ slowly increased and was sustained (*n* = 12). In rare cases, $[Ca^{2+}]_i$ rapidly increased to a peak within 1 min and then decreased to a sustained level of less than 50 % of the peak (*n* = 5). Irrespective of the pattern in $[Ca^{2+}]_i$, the developed tension persisted during the observation period (20-40 min) although the tension development was faster when the upstroke of $[Ca^{2+}]_i$ was rapid. On average, the maximal increase in $[Ca^{2+}]_i$ and contraction induced by PGF2α was 63.7 ± 4.6 % and 117.2 ± 6.9 % (n = 12), respectively, of those induced by 65.4 mM KCl. Verapamil (10 μM) slightly decreased $[Ca^{2+}]_i$ in the resting state and the subsequent addition of PGF2α only slightly increased $[Ca^{2+}]_i$ (6.6 ± 2.8 % of the response obtained in the presence of external Ca^{2+}, *n* = 8) but increased the tension to 73.6 ± 6.1 % (*n* =8) of the contraction obtained in normal PSS (Fig. 2). In a separate experiment, chelation of external Ca^{2+} with 4 mM EGTA lowered $[Ca^{2+}]_i$ to a greater degree than the case with verapamil. In this situation, PGF2α did not alter $[Ca^{2+}]_i$ but induced a contraction, which was 22.0 ± 3.0 % (*n* = 6) of that induced in normal PSS. Thus, it is likely that the PGF2α-induced Ca^{2+} mobilization depended on the Ca^{2+} entry through L-type Ca^{2+} channels in this artery. From these results it is evident that PGF2α can cause a sizeable contraction when an increase in $[Ca^{2+}]_i$ is absent or very small.

The contraction induced by PGF2α under the conditions where an increase in $[Ca^{2+}]_i$ was inhibited was accompanied by an increase in MLC_{20} phosphorylation (Fig. 2). In the presence of verapamil, PGF2α increased the MLC_{20} monophosphorylation (33.9 ± 6.1 % at 5 min after application of PGF2α) together with a slight diphosphorylation of MLC_{20} (0.4 ± 0.1 %, data not shown in Fig. 2B). Fasudil or hydroxyfasudil (10 μM), which had been pretreated 30 min before the application of PGF2α, abolished the diphosphorylation of MLC_{20} and inhibited both

the monophosphorylation and the contraction. In EGTA solution, PGF2α increased the MLC_{20} phosphorylation from 1.5 ± 0.3 % to 9.1 ± 1.5 % (*n* =12) without any detectable diphosphorylation (Fig. 2C). The MLC20 phosphorylation caused by PGF2α in the presence of EGTA was similar to that measured in normal PSS with no stimulation (8.9 ± 2.4 %). Fasudil and hydroxyfasudil decreased the phosphorylation to 3.4 ± 1.0 % and 3.0 ± 0.8 % (*n* =6), respectively, and inhibited the contraction.

There is a possibility about an increase in MLC_{20} phosphorylation due to PGF2α in muscles exposed to EGTA that PGF2α released Ca^{2+} from the sarcoplasmic reticulum (SR), but the increase in Ca^{2+} was restricted to a region around the contractile machinery so that it could not be detected as an averaged fura-PE3-Ca^{2+} signal. To more completely exclude the possible participation of Ca^{2+}, the SR was depleted of Ca^{2+} by application of ryanodine (3 μM) and phenylephrine (10 μM) during incubation in Ca^{2+}-free, EGTA (1 mM) solution for 30 min. In Ca^{2+}-depleted muscles, PGF2α (10 μM) increased the MLC_{20} phosphorylation and the tension. The MLC_{20} phosphorylation and the contraction caused by PGF2α (9.0 ± 2.5 % and 29.3 ± 7.8 %, respectively, *n* =6) were similar to those obtained under the condition where EGTA (4 mM) was added to the medium (Fig. 2). The similar degree of contraction and MLC_{20} phosphorylation under conditions where the SR had been depleted of Ca^{2+} or only external Ca^{2+} was chelated by EGTA suggests that PGF2α did not induce Ca^{2+} release from the SR. Fasudil (10 μM), which had been added 60 min before PGF2α, inhibited the increase in MLC_{20} phosphorylation and the tension caused by PGF2α under this condition (data not shown) .

Inhibition of MLC_{20} dephosphorylation during the PGF2α-induced contraction

Next, we examined how the relaxation and the MLC_{20} dephosphorylation took place when the MLC_{20} phosphorylation by MLC kinase was inhibited by a reduction in $[Ca^{2+}]_i$ during the PGF2α-induced contraction (Fig. 3). After a muscle was maximally contracted with 30 μM PGF2α in normal PSS, external Ca^{2+} was removed by switching the medium to Ca^{2+}-free, 1 mM

EGTA solution (5 min after PGF2α, Fig. 3). The tension decreased to about 30 % of the maximal contraction in 20 min and was sustained at that level. The MLC_{20} phosphorylation was maintained between 20-25 % during the sustained phase. When fasudil or hydroxyfasudil (10 μM) was present in Ca^{2+}-free, EGTA solution, the MLC_{20} phosphorylation and the tension were rapidly decreased (Fig. 3 left).

In a previous paper (Miura *et al.* 1997), we reported that DPB inhibited the dephosphorylation of MLC_{20}, and that the phospholipase A_2 inhibitor ONO-RS-082 antagonized the inhibition, suggesting that arachidonic acid was involved in the DPB-induced inhibition of dephosphorylation. Therefore, if arachidonic acid is involved in the inhibition of MLC_{20} dephosphorylation by PGF2α, ONO-RS-082 should antagonize the effect of PGF2α on the MLC_{20} phosphorylation. To test this possibility, ONO-RS-082 (5 μM) was applied 15 min before PGF2α. Pretreatment with ONO-RS-082 did not affect the MLC_{20} phosphorylation or the contraction induced by PGF2α in the same protocol, as shown in Fig. 3 (right).

To further examine the involvement of PKC, we compared the effects of the PKC inhibitor Ro31-8220 on the PGF2α- or DPB-induced inhibition of MLC_{20} dephosphorylation and relaxation. Since phorbol ester-induced MLC_{20} phosphorylation involves phosphorylation at sites directly phosphorylated by PKC (Ser1, Ser2) and at sites dependent on MLC kinase (Ser19, Thr18; Ikebe & Hartshorne, 1985; Ikebe *et al.* 1987; Miura *et al.* 1997) and if Rho-kinase is involved in the DPB-induced contraction, the phosphorylation should have increased at MLC kinase-dependent sites, we observed the Ser19 phosphorylation in this comparison. The protocol to examine the inhibition of dephosphorylation by PGF2α was the same as in Fig. 3. The protocol for DPB was that initially MLC_{20} was MLC kinase-dependently phosphorylated by KCl (65.4 mM) in normal PSS, then the muscle was rinsed with Ca^{2+}-free, EGTA (1 mM) PSS (5.4 mM K^+) to dephosphorylate MLC_{20}. DPB (1 μM), which was applied when the KCl-contracted muscle was rinsed with Ca^{2+}-free, EGTA (1 mM) PSS, inhibited the

dephosphorylation and maintained the tension at about 30-50 % of the maximum level (Miura *et al*. 1997). Ro31-8220 (10 μM), which was applied when the external Ca^{2+} was chelated with EGTA, did not affect the Ser19 phosphorylation of MLC_{20} or the tension caused by PGF2α, whereas it significantly inhibited these parameters caused by 1 μM DPB (Fig. 4).

Sensitivity to MLC kinase inhibitor of the MLC_{20} phosphorylation and the contraction induced by KCl, PGF2α or calyculin A

The MLC kinase inhibitor ML-9 (100 μM), which was added during the sustained phase of 65.4 mM KCl-induced contraction, rapidly decreased the MLC_{20} phosphorylation and the tension (Fig. 5). Fasudil (10 μM), which was added 15 min after ML-9, slightly accelerated the relaxation but did not accelerate the MLC_{20} dephosphorylation under this condition. When 100 μM ML-9 was applied during the PGF2α-induced contraction, it scarcely affected the MLC_{20} phosphorylation or the tension, so we used 200 μM ML-9 instead of 100 μM for the PGF2α-induced contraction. ML-9 (200 μM) partially inhibited the PGF2α-induced MLC_{20} phosphorylation and contraction. Subsequent addition of fasudil (10 μM) 15 min after ML-9 further decreased the MLC_{20} phosphorylation and the tension (Fig. 5). When the PKC inhibitor calphostin C (10 μM) was added instead of fasudil, it did not affect the time-course of change in tension (data not shown).

Calyculin A (300 nM), which inhibits the protein phosphatase type 1 and 2A (Hartshorne *et al.* 1989), gradually increased the MLC_{20} phosphorylation and the tension. At 30 min after addition of calyculin A, the MLC_{20} phosphorylation and the tension attained a steady state level. Addition of hydroxyfasudil (1-300 μM) at that time did not affect the MLC_{20} phosphorylation or the contraction. On the other hand, ML-9 at 100 μM significantly inhibited the contraction and at 300 μM it completely inhibited the MLC_{20} phosphorylation and the contraction (Fig. 6).

Phosphorylation by PGF2α of myosin binding subunit of phosphatase and the effects of

fasudil and hydroxyfasudil

Rho kinase phosphorylates MBS of the phosphatase SMPP-1M at Thr654, thereby inhibiting the phosphatase activity (Feng *et al.* 1999a). To determine whether PGF2α inhibits the MLC_{20} dephosphorylation through Rho kinase, the phosphorylation of MBS at Thr654 was measured using the antibody pMBS-T654, which recognizes MBS phosphorylated at T654.

At first, we characterized the specificity of the antibody pMBS-T654. The antibody recognized Rho kinase-phosphorylated recombinant 130 kDa MBS, but not non-phosphorylated MBS (compare lane 1 and lanes 2 and 3 of Fig. 7A). The antibody pMBS-T654-reactive band was completely quenched with excess T654-phospho-peptide (lane 4-6 in Fig. 7A). In contrast, two other phospho-peptides EKRRS(PO_3H_2)TGVSF and EKRRST(PO_3H_2)GVSF, which correspond to the amino acid 804-813 of chicken MBS and are similar in their core sequence to the T654-phosphopeptide, could not quench the pMBS-T654 antibody-reactive band (data not shown). Except for the amino acid sequences 804-813 in MBS, no other sequences homologous to the amino acid sequences 648-660 were found in MBS by a homology search. These results indicate that the antibody pMBS-T654 specifically recognizes MBS phosphorylated at Thr654.

Figure 7B shows the MBS phosphorylation probed with pMBS-T654 in intact rabbit mesenteric arteries. Only a single band corresponding to 130 kDa was detected between 47 and 200 kDa, indicating that the antibody does not react with a protein other than MBS. PGF2α (30 μM) increased the MBS phosphorylation at 1 min (lane 2) and slightly less at 2 min (lane 3) of application. Similar results were obtained with the carotid arteries (data not shown). In another preliminary study with β-escin permeabilized pig aortic cells, GTPγS (30 μM, 10 min) increased the MBS phosphorylation (Fig. 7C). Pretreatment with hydroxyfasudil (10 μM, 10 min) or C3 toxin (1 μg/ml, 15 min), which inactivates Rho, significantly decreased the GTPγS-induced MBS phosphorylation.

Using the antibody pMBS-T654 we tested whether PGF2α increased the phosphorylation of MBS in rabbit aortae. PGF2α significantly increased the phosphorylation at Thr654 of MBS 1 min after the application. At 15 min the MBS phosphorylation was less than the level at 1 min. Pretreatment with fasudil (10 μM) decreased the phosphorylation of MBS at 1 and 15 min (Fig. 8).

DISCUSSION

PGF2α-induced Ca^{2+}-sensitization

Ca^{2+}-senstization by PGF2α of vascular smooth muscle contraction has been observed by several groups (e.g. Bradley & Morgan, 1987; Balwierczak, 1991; Kurata *et al.* 1993) and this sensitization has been attributed to the enhancement of MLC_{20} phosphorylation (Suematsu *et al*. 1991; Hori *et al.* 1992; Seto *et al.* 1990a; Shin *et al*. 2002). The kinetics of MLC_{20} phosphorylation by PGF2α in rabbit aortae was analyzed by Seto *et al*. (1990b). Their paper reported that PGF2α increased MLC_{20} monophosphorylation, which was over the resting level during a 30-min observation period, and transient diphosphorylation at the initial stage, and suggested that the diphosphorylation contributed to acceleration of the rate of force generation. The diphosphorylation is specific for PGF2α since KCl or histamine did not cause the diphosphorylation (Seto *et al.* 1990b). Because the kinetic data of PGF2α-induced MLC_{20} phosphorylation in rabbit aortae are available from the paper by Seto *et al*., we did not measure the entire time course of MLC_{20} phosphorylation and did not quantify the diphosphorylation caused by PGF2α except for a few experiments. Here, we focused our attention on the mechanism of PGF2α-induced increase in MLC_{20} phosphorylation.

Verapamil or removal of extracellular Ca^{2+} inhibited a rise in $[Ca^{2+}]_i$ due to PGF2α. In the absence of external Ca^{2+}, the MLC_{20} phosphorylation and the tension development induced by PGF2α in muscles that had been depleted of Ca^{2+} in the SR were similar to those in muscles that had been treated with EGTA alone. This suggests that PGF2α does not employ Ca^{2+} released from the SR for MLC_{20} phosphorylation and contraction. Thus, it is likely that the PGF2α-induced increase in $[Ca^{2+}]_i$ exclusively depends on Ca^{2+} influx through L-type Ca^{2+} channels in rabbit aortae. This observation is consistent with the data from ferret aortae showing that PGF2α does not stimulate Ca^{2+} release from the intracellular Ca^{2+} stores (Bradley & Morgan, 1987). This is another unique property of PGF2α-induced contraction, since other receptor agonists usually utilize Ca^{2+} released from intracellular Ca^{2+} stores as well as Ca^{2+} that

enters from the extracellular space. However, this feature may not be shared by other types of vascular smooth muscle because PGF2α induces not only Ca^{2+} influx but also Ca^{2+} release from the SR in rat aortae (Kurata *et al.* 1993). PGF2α increased the MLC_{20} phosphorylation and the tension even when an increase in $[Ca^{2+}]_i$ was inhibited by verapamil or Ca^{2+}-free solution, although the extent of MLC_{20} phosphorylation under these conditions was smaller than that observed under the normal condition. Hence, PGF2α can enhance the MLC_{20} phosphorylation without a rise in $[Ca^{2+}]_i$ or with a very small increase in $[Ca^{2+}]_i$. The MLC_{20} phosphorylation and the tension due to PGF2α were higher in the presence of verapamil than after treatment with EGTA. This may be related to higher $[Ca^{2+}]_i$ in verapamil-treated muscles than in EGTA-treated ones, thereby causing the MLC kinase activity to be higher in the former muscles. Alternatively, we cannot exclude the possibility that in the presence of verapamil PGF2α still increased Ca^{2+} at a restricted region, which was not detected by the fluorescent dye, and that this increase partly contributes to an increase in MLC_{20} phosphorylation.

In this study, ML-9, which was added during the sustained contraction to PGF2α, only partially decreased the MLC_{20} phosphorylation and the contraction. Therefore, it seems that MLC kinase-dependent MLC_{20} phosphorylation does not play an exclusive role in maintenance of the sustained contraction. This is compatible with the data that PGF2α increased MLC_{20} phosphorylation in the situation where a rise in $[Ca^{2+}]_i$ was inhibited so that the MLC kinase activity was low (Fig. 2). If $[Ca^{2+}]_i$ in a contracted muscle is lowered, the MLC kinase activity would be decreased and the relative activity of phosphatase would increase, causing dephosphorylation of MLC_{20} and relaxation. When the PGF2α-contracted muscle was rinsed with Ca^{2+}-free, EGTA solution, the MLC_{20} phosphorylation and the tension did not easily return to the resting level (Fig. 3). This suggests that MLC_{20} dephosphorylation by the phosphatase was partially inhibited in the presence of PGF2α. The data by Shin *et al*. (2002) that PGF2α caused dissociation of catalytic subunit (PP1c) of myosin phosphatase in ferret portal vein cells supports the idea that PGF2α inhibited the dephosphorylation. It is thus likely that inhibition of

MLC_{20} dephosphorylation is responsible for the maintenance of MLC_{20} phosphorylation and tension during the sustained contraction to PGF2α.

Fasudil and hydroxyfasudil as protein kinase inhibitors

Fasudil and hydroxyfasudil are equally potent against Rho kinase, while fasudil is more potent than hydroxyfasudil against MLC kinase and PKC (Table 1, Sakurada *et al.* 1994; Nagumo *et al.* 1998; Nagumo *et al.* 2000). In the tension experiment, fasudil and hydroxyfasudil equally inhibited the PGF2α-induced contraction. Fasudil at 10 μM was less potent on the KCl- or the DPB-induced contraction than on the PGF2α-induced contraction, and hydroxyfasudil was a further weaker inhibitor on these contractions. The similar sensitivity to fasudil and hydroxyfasudil of the PGF2α-induced contraction indirectly suggests the involvement of a Rho kinase-dependent mechanism. Hydroxyfasudil up to 300 μM did not decrease the phosphatase inhibitor calyculin A-induced MLC_{20} phosphorylation and contraction, whereas ML-9 decreased these parameters. This indicates that the calyculin A-induced MLC_{20} phosphorylation was dependent on MLC kinase, which was partially active in the resting state. Although fasudil is about 4 times more potent than hydroxyfasudil on MLC kinase, the fact that 300 μM hydroxyfasudil did not affect the MLC_{20} phosphorylation induced by calyculin A suggests that fasudil is unlikely to exert an inhibitory effect on MLC kinase at the concentration used in this study (10 μM). Therefore, the target site of this compound, which is responsible for inhibition of the PGF2α-induced MLC_{20} phosphorylation, should be a kinase other than MLC kinase.

Rho kinase is responsible for the inhibition of phosphatase

If PGF2α produces diacylglycerol in an amount to sufficiently activate PKC, PKC could participate in the inhibition of myosin phosphatase through mechanisms including CPI-17 (Li *et al.* 1998) or arachidonic acid (Gong *et al.* 1992; Gong *et al.* 1995; Hartshorne *et al.* 1998; Feng *et al.* 1999b). For example, 5-hydroxytryptamine-induced Ca^{2+}-sensitization of rabbit mesenteric artery involved a PKC- and arachidonic acid-dependent mechanism because it was sensitive to

Ro31-8220, a non-selective PKC inhibitor, and quinacrine, a phospholipase A_2 inhibitor (Parsons *et al.* 1996). Likewise, histamine increased the phosphorylation of CPI-17, thereby causing Ca^{2+}-sensitization (Kitazawa *et al.* 2000; Eto *et al.* 2001). The report by Eto *et al.* (2001) showed that Ro31-8220 inhibited the phosphorylation of CPI-17, suggesting that the PKC isozyme responsible for the activation of CPI-17 is sensitive to Ro31-8220. Our previous paper (Miura *et al.* 1997) showed that activation of PKC by the phorbol ester DPB caused the inhibition of MLC_{20} dephosphorylation in rabbit aortae, which was partially dependent on arachidonic acid, since the phospholipase A_2 inhibitor ONO-RS-082 decreased the DPB-induced MLC_{20} phosphorylation and contraction. An increase in MLC_{20} phosphorylation at Ser19 by DPB was also inhibited by Ro31-8220 (Fig. 4). Therefore, if PKC plays a role in the PGF2α-induced Ca^{2+}-sensitization, the MLC_{20} phosphorylation should be sensitive to Ro31-8220 or ONO-RS-082 in rabbit aortae. In the present study, neither ONO-RS-082 nor Ro31-8220 affected the PGF2α-induced MLC_{20} phosphorylation and contraction (Fig. 3 and 4). These data suggest that PKC is not responsible for the PGF2α-enhanced MLC_{20} phosphorylation. In contrast, the paper by Katsuyama & Morgan (1993) showed that a PKC pseudosubstrate inhibitor effectively inhibited the 100 μM PGF2α-induced contraction of permeabilized ferret aortic cells clamped at pCa 7. It is possible that in the Katsuyama & Morgan's study that used a 10 times higher concentration of PGF2α, PKC might have participated in the contraction.

The Rho kinase inhibitors fasudil, hydroxyfasudil and Y-27632 inhibited the MLC_{20} phosphorylation caused by PGF2α. Since it is unlikely that fasudil or hydroxyfasudil inhibited contractions dependent on PKC or MLC kinase in this study, as discussed above, the most probable kinase that was inhibited by these inhibitors is Rho kinase. Feng *et al.* (1999a) reported that Rho kinase inhibited SMPP-1M by phosphorylating Thr695 of 133 kDa MBS, which corresponds to Thr654 of 130 kDa isoform (Hartshorne *et al.* 1998). To detect whether MBS is phosphorylated by PGF2α, we raised the antibody pMBS-T654 against recombinant phospho-MBS, which recognizes the phosphorylation at Thr654 of 130 kDa MBS. The identity

of the site that is phosphorylated by Rho kinase in MBS has been controversial. It was suggested that Rho kinase purified from the brain phosphorylated the same site (Thr654) as the site for endogenous kinase in gizzard myosin phosphatase (Hartshorne *et al.* 1998). In contrast, Kimura *et al*. (1996) reported that Rho kinase phosphorylated the C-terminal part of gizzard MBS (residues 753-1004) but not the N-terminal segment (residue 1-721) in an *in vitro* study. In this well-controlled immunoblotting experiment using the antibody pMBS-T654, we showed that Rho kinase phosphorylated Thr654 of full-length recombinant MBS (Fig. 7A). Furthermore, using permeabilized aortic smooth muscle cells, pMBS-T654 specifically detected a GTPγS-induced increase in phosphorylation of MBS, which was abolished by C3 toxin and the Rho kinase inhibitor (Fig. 7C). We conclude, therefore, that the Rho kinase-phosphorylated site includes Thr654 of MBS.

An endogenous inhibitor against MLC phosphatase other than Rho kinase is CPI-17 (Kimura *et al.* 1996; Li *et al.* 1998; Kitazawa *et al.* 2000). Since the activation of CPI-17 by PKC is unlikely to be involved in the PGF2α-induced increase in MLC_{20} phosphorylation, a candidate for the phosphorylation of MBS could be narrowed down to Rho kinase. The data showing that Thr654 of MBS was phosphorylated by PGF2α and fasudil inhibited the phosphorylation confirm this idea. Consistent with this, Shin *et al*. (2002) found that PGF2α transiently increased phosphorylation of MBS at Thr695 in ferret portal veins. Based on these discussions, we conclude that the PGF2α-induced enhancement of MLC_{20} phosphorylation is caused by the inhibition of phosphatase by Rho kinase. However, we cannot strictly exclude the possible involvement of CPI-17, because Rho kinase or protein kinase N, a downstream effector of Rho, can phosphorylate CPI-17, leading to the inhibition of the phosphatase (Hamaguchi *et al.* 2000; Kitazawa *et al.* 2000; Koyama *et al.* 2000).

The PGF2α-induced contraction involves an MLC_{20} phosphorylation-independent mechanism

In Ca^{2+}-free medium, PGF2α increased the MLC_{20} phosphorylation and the tension without an increase in $[Ca^{2+}]_i$. Rho kinase can directly phosphorylate MLC_{20} and cause contraction, independently of Ca^{2+} (Kureishi *et al.* 1997). Therefore, the MLC_{20} phosphorylation and the contraction induced by PGF2α in Ca^{2+}-depleted muscles could be due to Rho kinase. Alternatively, it is also possible that the inhibition of MLC_{20} dephosphorylation by Rho kinase increased the phosphorylation level despite the very low MLC kinase activity. Regardless, the MLC_{20} phosphorylation in these muscles (about 9 %) was similar to the level under the resting condition in Ca^{2+}-containing medium (8.9 ± 2.4 %), and thus it was not high enough to contribute to the tension development. Therefore, it is very likely that the PGF2α-induced contraction includes a mechanism independent of MLC_{20} phosphorylation.

A determinant other than MLC_{20} phosphorylation for smooth muscle contraction is the regulation of thin filaments by calponin or caldesmon, which binds to actin and inhibits actin-activated myosin Mg^{2+}-ATPase (Miki *et al.* 1992; Malmqvist *et al.* 1997; Earley *et al.* 1998; Je *et al.* 2001). When calponin or caldesmon is phosphorylated, these proteins lose the ability to inhibit the Mg^{2+}-ATPase (Winder & Walsh, 1990; Itoh *et al.* 1995; Horowitz *et al.* 1996). It has been reported that PGF2α increased calponin phosphorylation in porcine coronary arteries during the initial stage and that fasudil or hydroxyfasudil inhibited the phosphorylation with an ED50 value of 26 μM (Nagumo *et al.* 1998). Therefore, it is possible that PGF2α phosphorylated calponin and elevated the tension independently of MLC_{20} phosphorylation in Ca^{2+}-depleted muscles. PKC is known to phosphorylate calponin and caldesmon. Caldesmon can also be phosphorylated by p21-activated protein kinase (PAK), another Rho family GTPase (Van Eyk *et al.* 1998). However, it has not been determined whether Rho kinase can phosphorylate calponin or caldesmon. The sensitivity of PGF2α-induced contraction in Ca^{2+}-depleted muscles to fasudil indicates the possible involvement of PKC or Rho kinase. It should be clarified in a future study whether or not PGF2α phosphorylates calponin or caldesmon in Ca^{2+}-depleted muscles.

This study provided evidence that PGF2α activates Rho-kinase and also possibly an actin-associated sensitizing mechanism, both of which cause Ca^{2+}-sensitization. The latter mechanism also seems to be sensitive to fasudil. It should be determined in future studies what kinase is responsible for the mechanism.

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Table 1. Ki value of fasudil and hydroxyfasudil against Rho kinase, protein kinase C (PKC), cyclic AMP-dependent protein kinase (PKA) and myosin light chain kinase (MLCK).

	Rho kinase	PKC	PKA	MLCK
Fasudil	0.35 μM	3.3 μM	1.6 μM	36 μM
Hydroxyfasudil	0.56 μM	18 μM	2.5 μM	140 μM

Ki values of hydroxyfasudil are from the data by Seto *et al.* (1991).

Table 2. IC50 values of Rho kinase inhibitors for PGF2α-, KCl- or DPB-induced contractions in rabbit aortae.

	Constrictor		
	PGF2α (3 μM)	KCl (65.4 mM)	DPB (1 μM)
Fasudil	3.7 ± 1.4 μM*	22.1 ± 5.0 μM	4.6 ± 1.4 μM*
Hydroxyfasudil	4.0 ± 0.8 μM*§	48.8 ± 11.4 μM†	13.0 ± 3.0 μM*†
Y-27632	0.85 ± 0.29 μM*	2.8 ± 0.1 μM	1.23 ± 0.26 μM*

Each inhibitor was cumulatively added at an interval of 30 min on the sustained contraction due to PGF2α, KCl or DPB. Data are mean ± S.E.M. of 7-8 preparations. * $P < 0.05$ (vs. IC50 against the KCl-induced contraction). §$P<0.05$ (vs. IC50 against the DPB-induced contraction). † $P < 0.05$ (vs. the IC50 of fasudil). Comparison between IC50 of Y-27632 and that of fasudil or hydroxyfasudil is not shown.

Legends for figures

Figure 1. Effects of fasudil and hydroxyfasudil on the PGF2α- or DPB-induced contraction and MLC_{20} phosphorylation in rabbit aortae.

Fasudil (■) or hydroxyfasudil (OH-fasudil, ▲) at 10 μM was added 20 min after the application of 10 μM PGF2α (A) or 1 μM DPB (B). Left panels, a change in tension after the addition of fasudil or hydroxyfasudil. ○, agonist alone. Tension is expressed as a percentage of contraction just before the addition of fasudil or hydroxyfasudil. In the right panels, MLC_{20} phosphorylation (MLC-P) was observed 20 min (just before the addition of fasudil or hydroxyfasudil, denoted as **a** in the figure) or 50 min (denoted as **b**) after the addition of PGF2α or DPB, and is expressed as a percentage of phosphorylated MLC_{20} of total MLC_{20}. MLC_{20} phosphorylation in the resting state was 8-9 % in each case (data not shown). Since PGF2α caused diphosphorylation of MLC_{20}, monophosphorylation (MLC-P1, grey columns) and diphosphorylation (MLC-P2, black columns) are shown (A, right panel). Data are mean ± S.E.M (n = 5-6). * Significantly different from the time-matched control ($P < 0.05$).

Figure 2. Changes in $[Ca^{2+}]_i$, tension and MLC_{20} phosphorylation due to PGF2α under normal condition or the condition where an increase in $[Ca^{2+}]_i$ was inhibited.

A, examples of changes in $[Ca^{2+}]_i$ in normal PSS, in the presence of verapamil (10 μM) or EGTA (4 mM). For $[Ca^{2+}]_i$ and tension, 100 % represents a change induced by 65.4 mM KCl, which was obtained before the application of PGF2α. Verapamil or EGTA was applied 10 min before the addition of PGF2α (10 μM). B, summarized data of the PGF2α-induced contraction and MLC_{20} phosphorylation in the normal medium or in the presence of verapamil. Developed tension is expressed as % of the maximum contraction to 65.4 mM KCl. C, summarized data of the PGF2α-induced contraction and MLC20 phosphorylation in the normal medium or in the presence of EGTA. In B and C, MLC_{20} phosphorylation (MLC-P) was measured at 5 min after the addition of PGF2α (10 μM). When used, fasudil (10 μM) or hydroxyfasudil (OH-fasudil, 10

μM) was added 30 min before PGF2α. The protocol in B and C was the same as that shown in A but the experiments were performed in a different series. MLC_{20} phosphorylation under the resting condition in normal PSS was 8.9 ± 2.4 % (data not shown). Verap: verapamil.

Figure 3. Effects of fasudil, hydroxyfasudil or ONO-RS-082 on MLC_{20} phosphorylation and tension maintained in Ca^{2+}-free, EGTA solution in the presence of PGF2α.

A, change in tension. After PGF2α (10 μM)-induced contraction had reached a peak, the external medium was changed from normal PSS to Ca^{2+}-free, 1 mM EGTA solution (5 min in the figure). ○, PGF2α alone. When used, fasudil (△, 10 μM), hydroxyfasudil (□, 10 μM) was added at the time when the external Ca^{2+} was omitted (the left panel). ONO-RS-082 (●, 5 μM, the right panel) was applied 15 min before the addition of PGF2α. Data are expressed as a percentage of the maximal contraction to PGF2α in normal medium (n = 6-7). B, MLC_{20} phosphorylation (MLC-P) measured in the resting state (0 min), just before switching the medium to Ca^{2+}-free, EGTA solution (5 min), 5, 10 and 15 min after Ca^{2+} removal (10, 15 and 20 min after the addition of PGF2α). Open columns; controls. Hatched columns; fasudil-treated muscles. Filled columns; hydroxyfasudil-treated muscles. Grey columns; ONO-RS-082-treated muscles. * Significantly different from the level in the absence of fasudil or hydroxyfasudil ($P < 0.05$). § Significantly different from the resting level ($P < 0.05$).

Figure 4. Effects of the PKC inhibitor Ro31-8220 on phosphorylation of MLC_{20} at Ser19 and contraction induced by PGF2α and DPB.

A, the effects of Ro31-8220 on the PGF2α-induced inhibition of Ser19 dephosphorylation and relaxation. The protocol was the same as in Fig. 3. When the external medium was changed from normal PSS to Ca^{2+}-free, 1 mM EGTA solution, Ro31-8220 (5 μM) was simultaneously added to one group (filled columns) but not to the control group (open columns). B, the effects on the DPB-induced inhibition of Ser19 dephosphorylation and relaxation.. After 65.4 mM KCl

caused a maximal contraction (5 min), the external medium was switched to Ca^{2+}-free, 1 mM EGTA solution (5.4 mM K^{+}) containing 1 μM DPB. When present, Ro31-8220 (5 μM) was added simultaneously with DPB. Data were taken 15 min after the omission of external Ca^{2+}. The left panels represent a relative value of the 65.4 mM KCl-induced contraction, which was obtained before PGF2α. The right panels are Ser19 phosphorylation expressed as a value relative of the phosphorylation in the resting state. Data are mean ± S.E.M. (n = 4). * Significantly different from DPB alone ($P < 0.05$). N.S.: not significant.

Figure 5. Sensitivity of KCl- and PGF2α-induced contraction and MLC_{20} phosphorylation to ML-9 and fasudil.

After KCl (65.4 mM, left)- or PGF2α (10 μM, right)-induced contraction reached a respective peak (5 min), ML-9 (100 μM or 200 μM, respectively) was applied. Fasudil (10 μM) was added 15 min later. A, a change in tension in fasudil-treated (●) or untreated muscles (○). The ordinate represents the relative tension of maximum contraction to KCl or PGF2α, respectively. B, MLC_{20} phosphorylation (MLC-P) measured in the protocol shown in A. Data were sampled just before the addition of ML-9 (open columns, corresponding to **a** in A) and 30 min after the addition of ML-9 for KCl-induced contraction or 45 min after addition of ML-9 for PGF2α-induced contraction (corresponding to **b** in A). Hatched columns: in the absence of fasudil, filled columns: in the presence of fasudil. * Significantly different from the level before the addition of ML-9 ($P < 0.05$). § Significantly different from the level in the absence of fasudil ($P < 0.05$). n = 5-8.

Figure 6. Differential effects of hydroxyfasudil and ML-9 on the calyculin A-induced contraction and MLC_{20} phosphorylation.

Open columns: tension expressed as % of the maximum contraction due to 65.4 mM KCl, which was obtained before the addition of calyculin A. Filled columns: MLC_{20} phosphorylation

(MLC-P). At 30 min after the application of calyculin A (300 nM), hydroxyfasudil or ML-9 at each concentration was added. * Significantly different from the level in the absence of ML-9 ($P < 0.05$).

Figure 7. Characterization of the antibody pMBS-T654.

A, detection of Rho kinase-induced phosphorylation of MBS by pMBS-T654 *in vitro*. Recombinant MBS was phosphorylated by Rho kinase for 0 min (lanes 1 and 4), 10 min (lanes 2 and 5) or 20 min (lanes 3 and 6) and subjected to SDS-PAGE, followed by Western blotting with pMBS-T654 (the upper panel) or with the antibody N-MBS, which recognizes the N-terminus of MBS (the lower panel), in the absence (lanes 1-3) or presence (lanes 4-6) of phosphorylated antigen peptide (T654-phospho-peptide). B, PGF2α-induced phosphorylation at Thr654 of MBS in the rabbit carotid artery. Extracts of arteries before stimulation (lane 1) or 1 (lane 2) and 2 min (lane 3) after the stimulation with PGF2α (30 μM) were subjected to SDS-PAGE followed by Western blotting with pMBS-T654. C, GTPγS-induced phosphorylation at Thr654 of MBS (MBS-P) and its inhibition by hydroxyfasudil or C3 toxin in permeabilized pig aortic cells. Cells were treated with GTPγS for 10 min. When used, hydroxyfasudil (OH-fasudil, 10 μM) or C3 toxin (1 μg/ml) was pretreated for 15 min before GTPγS. (Photographs) Band reacted with pMBS-T654 (the upper panel) or N-MBS (the lower panel). MBS phosphorylation is expressed as % of the level in the resting state. Data are mean ± S.E.M ($n = 4$). * $P < 0.05$.

Figure 8. PGF2α-induced phosphorylation of myosin binding subunit (MBS) of phosphatase and its inhibition by fasudil.

A, time-course of the PGF2α (10 μM)-induced contraction in the absence (○) or presence (●) of fasudil (10 μM). When present, fasudil was applied 15 min before addition of PGF2α. B, (photographs) example of Western blots with the pMBS-T654 antibody or the N-MBS antibody. Samples were taken before the addition of PGF2α (resting) and 1 min after PGF2α. (Bar graph)

Quantified phosphorylation of MBS (MBS-P) in the resting state, 1 or 15 min after the addition of PGF2α in the absence (open columns) or presence (filled columns) of fasudil. The ratio of the density of a band with the pMBS-T654 antibody to that with the N-MBS antibody in the resting state is referred as 1. Data are mean ± S.E. of 4 experiments. * Significantly different from the resting level ($P < 0.05$). § Significantly different from PGF2α alone ($P < 0.05$).

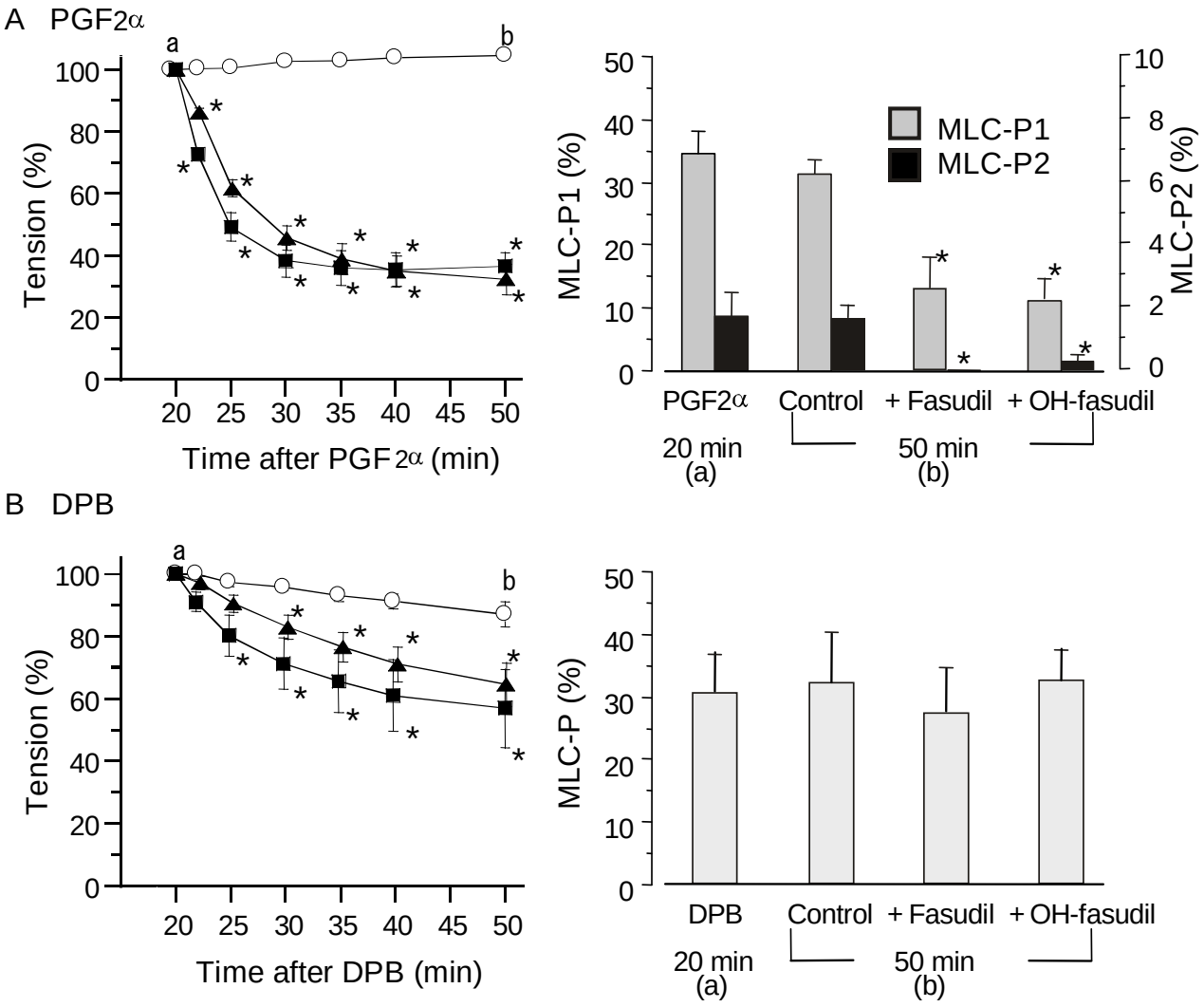
A PGF2α
a
b
Tension (%)
Time after PGF 2α (min)
MLC-P1 (%)
MLC-P2 (%)
MLC-P1
MLC-P2
PGF2α
Control
+ Fasudil
+ OH-fasudil
20 min
(a)
50 min
(b)
B DPB
Tension (%)
Time after DPB (min)
MLC-P (%)
DPB
Control
+ Fasudil
+ OH-fasudil
20 min
(a)
50 min
(b)

Fig. 1

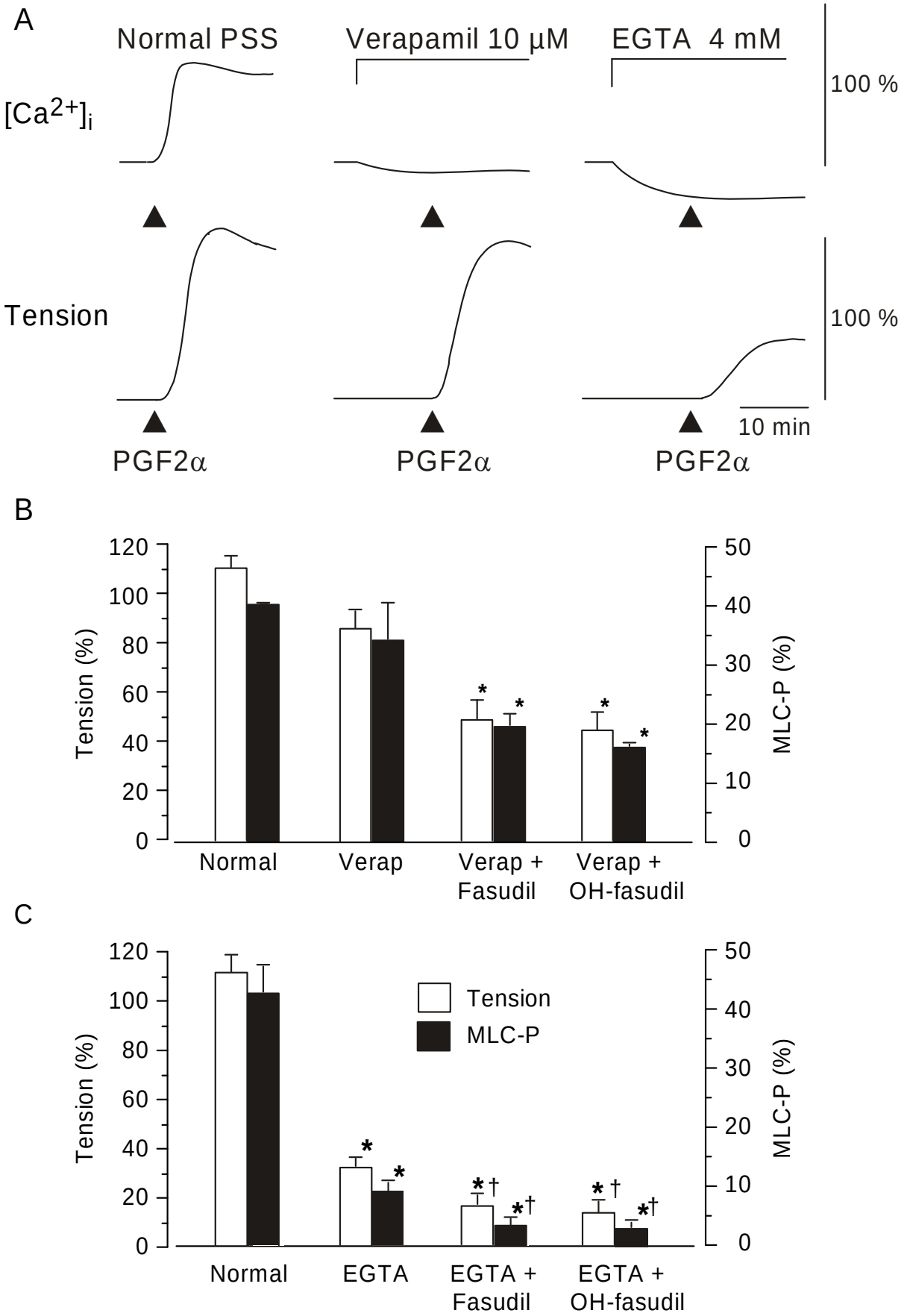
A
Normal PSS
Verapamil 10 µM
EGTA 4 mM
[Ca2+]i
100 %
Tension
100 %
10 min
PGF2α
PGF2α
PGF2α
B
Tension (%)
MLC-P (%)
Normal
Verap
Verap + Fasudil
Verap + OH-fasudil
C
Tension
MLC-P
Tension (%)
MLC-P (%)
Normal
EGTA
EGTA + Fasudil
EGTA + OH-fasudil

Fig. 2

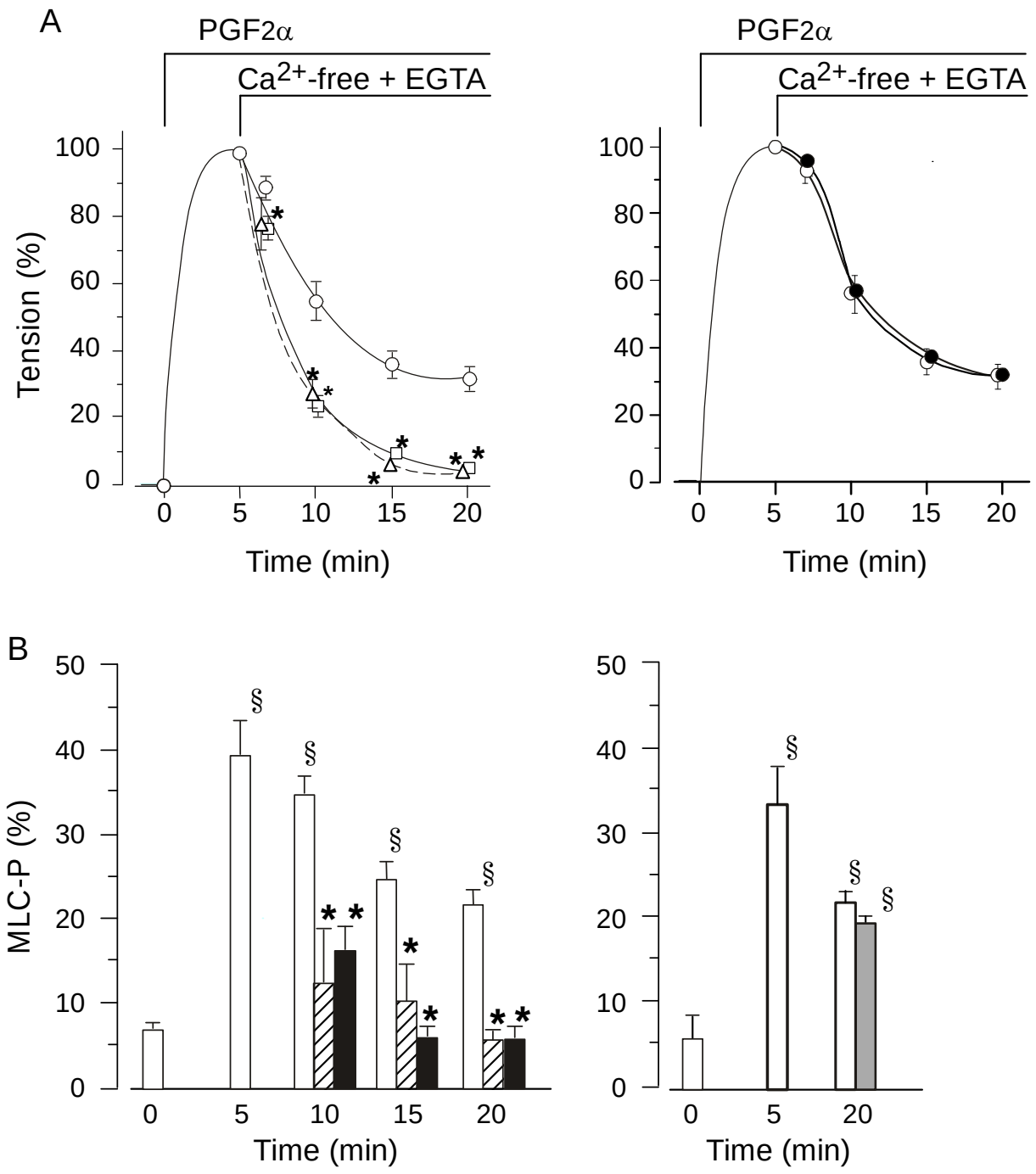
A
PGF2α
Ca2+-free + EGTA
Tension (%)
Time (min)
PGF2α
Ca2+-free + EGTA
Time (min)
B
MLC-P (%)
Time (min)
Time (min)

Fig. 3

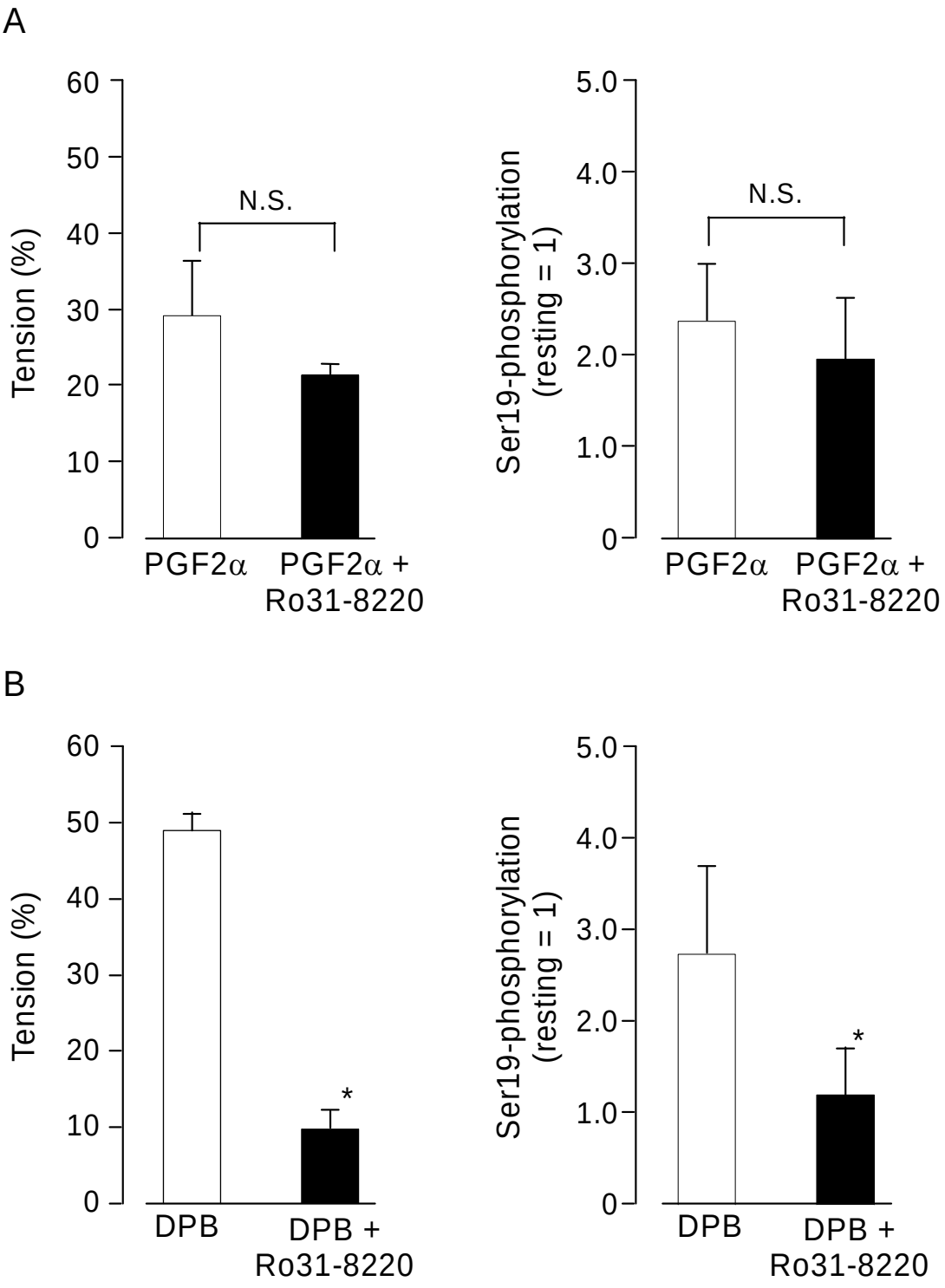
A
Tension (%)
60
50
40
30
20
10
0
N.S.
PGF2α
PGF2α +
Ro31-8220
Ser19-phosphorylation
(resting = 1)
5.0
4.0
3.0
2.0
1.0
0
N.S.
PGF2α
PGF2α +
Ro31-8220
B
Tension (%)
60
50
40
30
20
10
0
*
DPB
DPB +
Ro31-8220
Ser19-phosphorylation
(resting = 1)
5.0
4.0
3.0
2.0
1.0
0
*
DPB
DPB +
Ro31-8220

Fig. 4

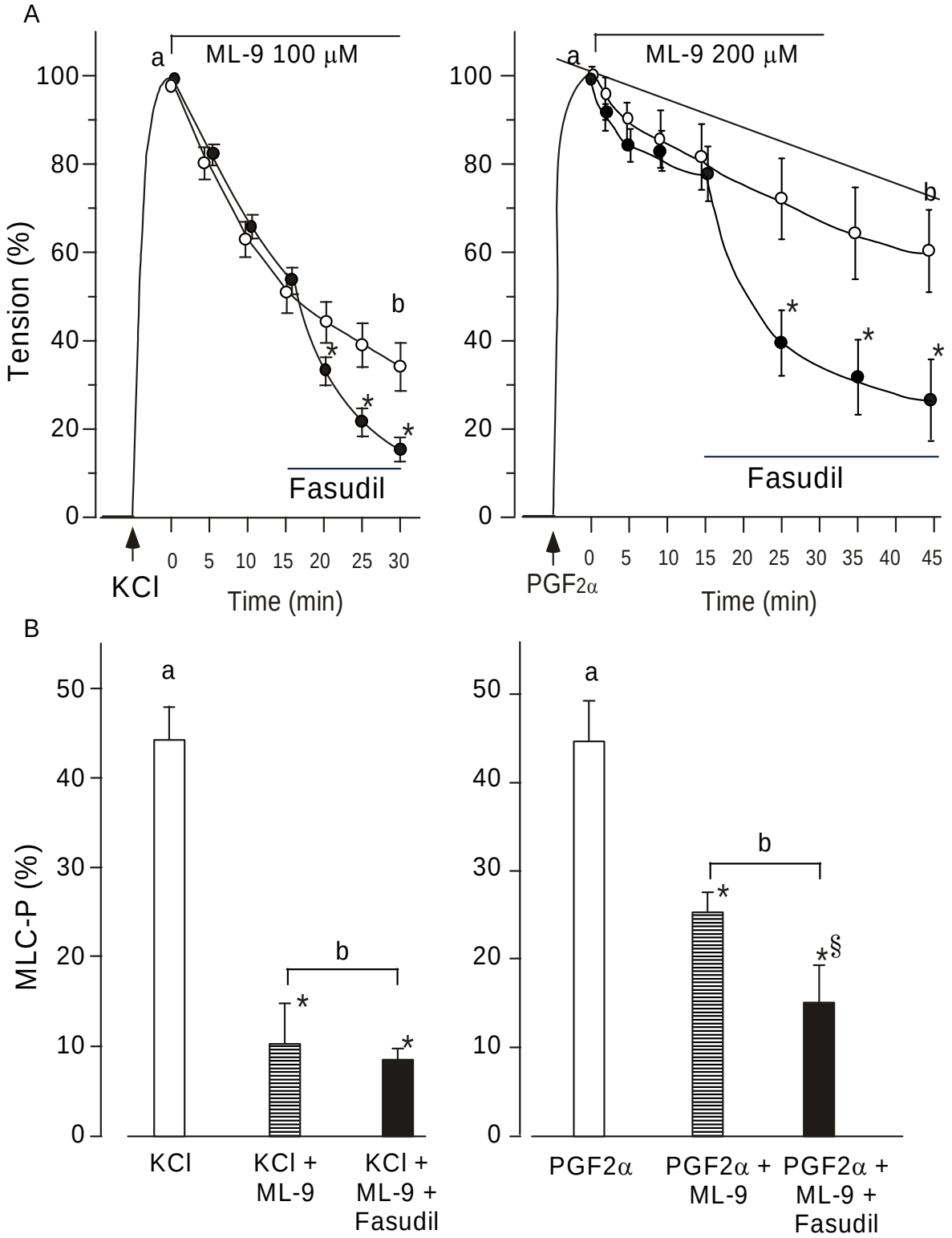
A
ML-9 100 μM
ML-9 200 μM
Tension (%)
Fasudil
KCl
PGF2α
Time (min)
B
MLC-P (%)
KCl
KCl + ML-9
KCl + ML-9 + Fasudil
PGF2α
PGF2α + ML-9
PGF2α + ML-9 + Fasudil

Fig. 5

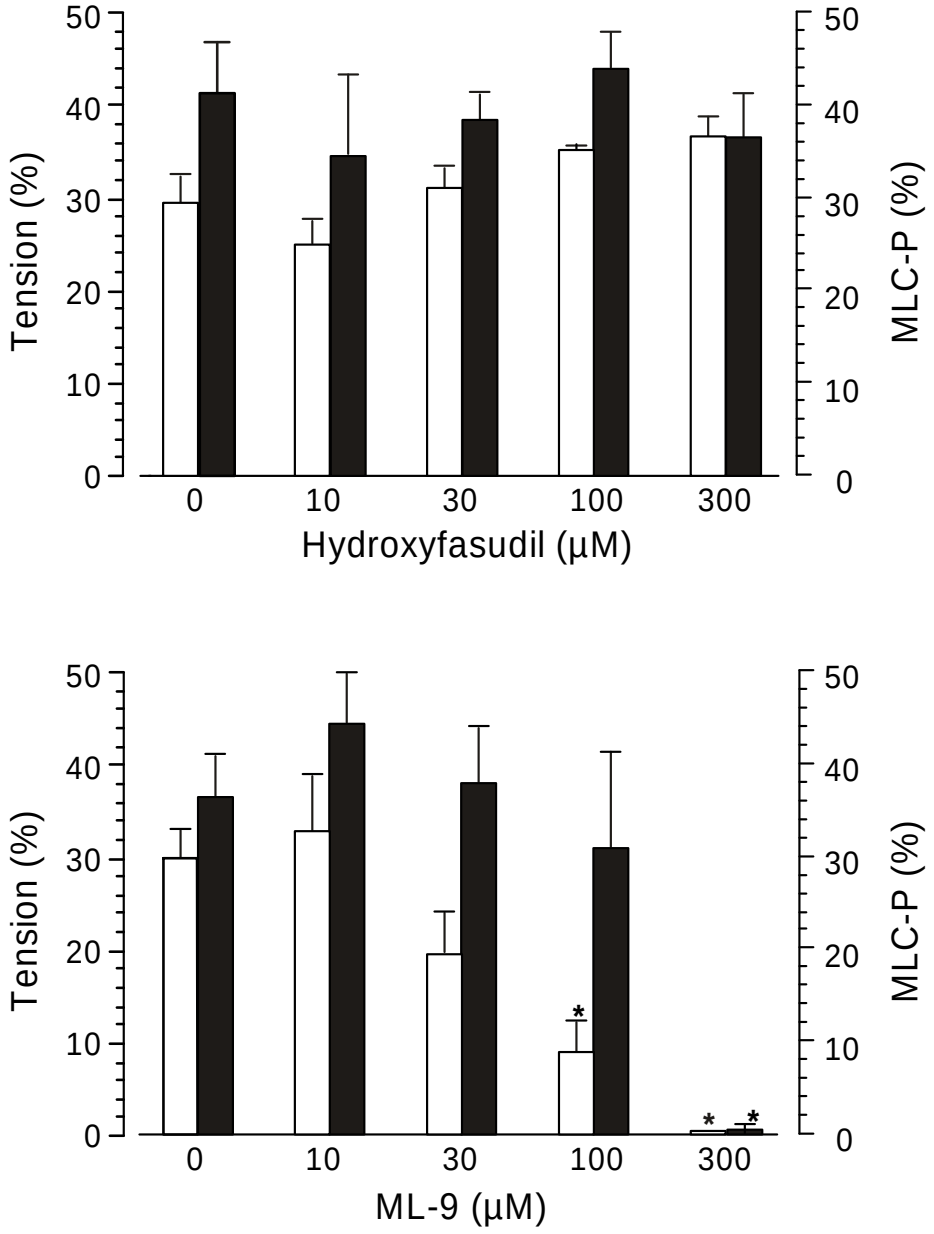
Tension (%)
MLC-P (%)
0
10
20
30
40
50
0
10
30
100
300
Hydroxyfasudil (μM)
*
*
*
ML-9 (μM)

Fig. 6

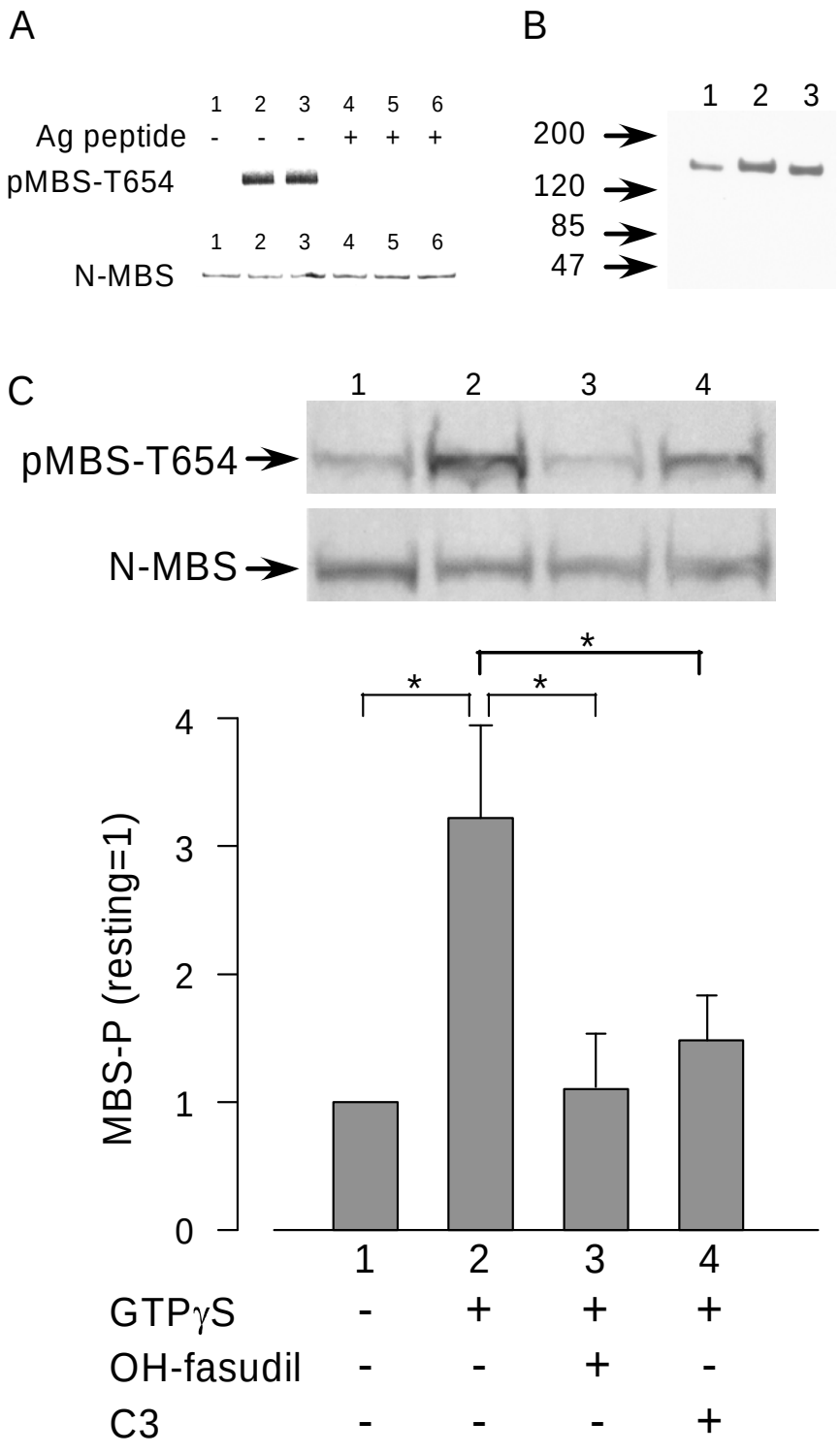
A
1 2 3 4 5 6
Ag peptide - - - + + +
pMBS-T654
1 2 3 4 5 6
N-MBS
B
1 2 3
200
120
85
47
C
1 2 3 4
pMBS-T654
N-MBS
MBS-P (resting=1)
0
1
2
3
4
*
*
*
1 2 3 4
GTPγS - + + +
OH-fasudil - - + -
C3 - - - +

Fig. 7

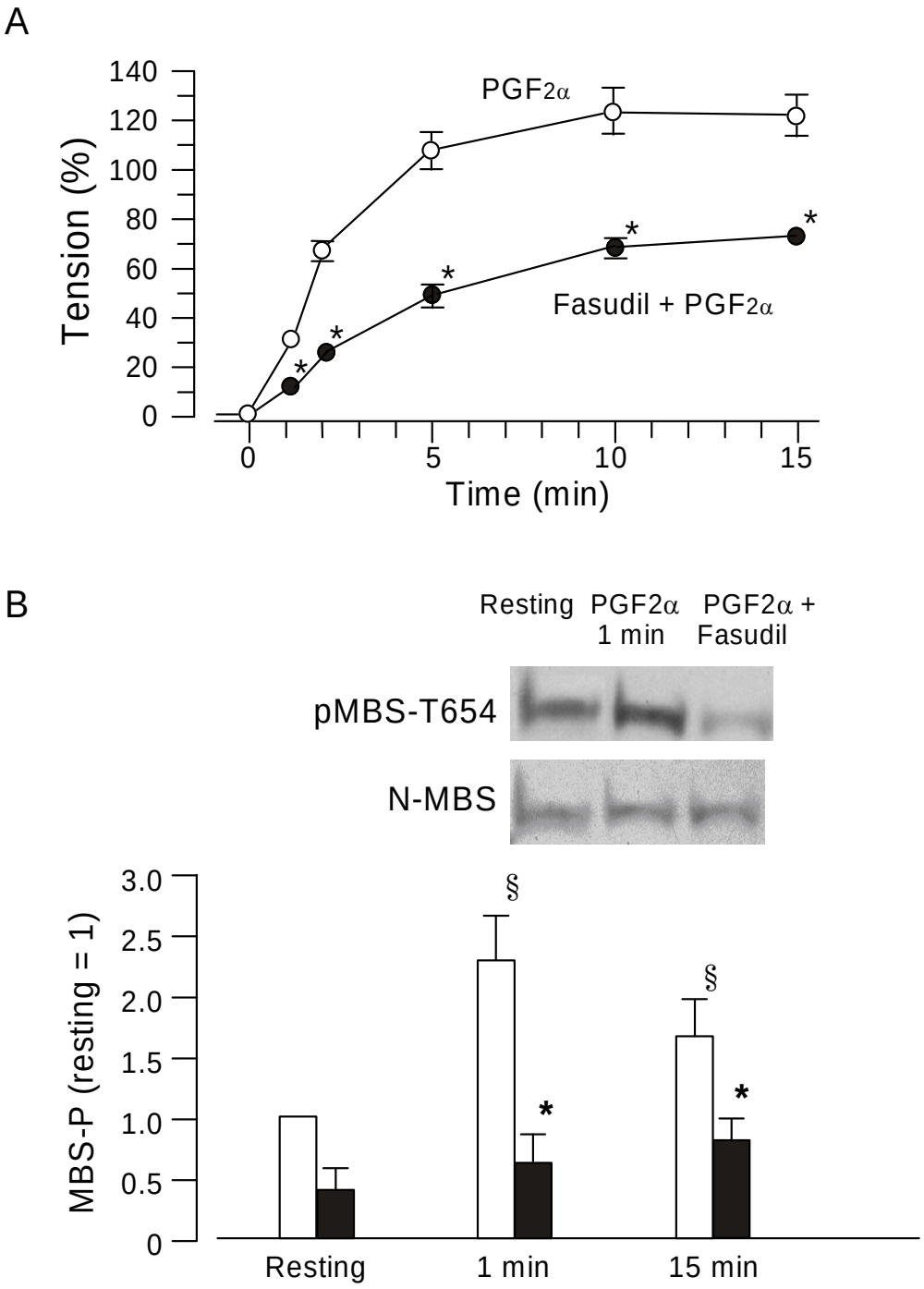
A
140
120
100
80
60
40
20
0
Tension (%)
PGF2α
Fasudil + PGF2α
*
0
5
10
15
Time (min)
B
Resting
PGF2α
1 min
PGF2α +
Fasudil
pMBS-T654
N-MBS
3.0
2.5
2.0
1.5
1.0
0.5
0
MBS-P (resting = 1)
§
§
*
*
Resting
1 min
15 min

Fig. 8