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The Exogenously Added Small Subunit of Smooth Muscle Myosin Phosphatase Increases the Ca^{2+} Sensitivity of the Contractile Apparatus in the Permeabilized Porcine Renal Artery

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The effects of the small noncatalytic subunit of myosin light chain phosphatase (MLCPsr) on the Ca^{2+}-induced contraction of smooth muscle were investigated in the Triton X-100-permeabilized porcine renal artery. The full-length recombinant chicken MLCPsr obtained by the bacterial expression system induced an additional contraction at a constant $[Ca^{2+}]_i$ and shifted the $[Ca^{2+}]_i$–force relation curve to the left. A deletion mutant containing the N-terminal 78 amino acids of MLCPsr retained the full action, compared with the full-length MLCPsr, while the deletion of this region completely abolished its effect. The process of relaxation was also delayed by the fragment containing the N-terminal 78 amino acids. These results indicated that MLCPsr increases the Ca^{2+} sensitivity of the contractile apparatus while the N-terminal 78 amino acids are responsible for this effect in vascular smooth muscle.

Both in the studies of permeabilized preparations and in the studies of fura-2 fluorometry, it was revealed that the Ca^{2+} sensitivity of contraction changes following receptor activation in the smooth muscle (1–4). Although subsequent studies have indicated that myosin light chain phosphatase (MLCP) may be one of the key molecules mediating the change in Ca^{2+} sensitivity (3), the precise mechanism for the regulation of MLCP activity remains to be elucidated.

MLCP belongs to type 1 protein phosphatase (5, 6) and is composed of three subunits, i.e., one catalytic subunit of 38 kDa (PP1c), and two noncatalytic subunits. Although the involvement of other phosphatases cannot be entirely ruled out (7, 8), the major activity towards MLC in smooth muscle is thought to be due to the heterotrimeric holoenzyme (9). The two noncatalytic subunits have been assumed to be the regulatory subunits of MLCP (10, 11). The large non-catalytic subunit has a molecular weight of 110–130 kDa (M110 in rat aorta or M130 in chicken gizzard). The function of M130 or M110 has been intensively investigated and the activity of PP1c has been shown to be modulated by M110 or M130 (12–15). In contrast, regarding the small regulatory subunit (termed as MLCPsr), only a limited amount of information is available. The cDNA of MLCPsr was originally reported to code for a protein of 186 amino residues with a molecular weight of 21 kDa (16, 17). Recently, a different cDNA for MLCPsr has also been reported by another group (18). The newly reported sequence includes a 53 bp insertion between the 158th and 159th codon of the original one. Because this insertion has a termination codon, this cDNA codes for a molecule of 161 amino residues with 1 to 158 residues being the same as the originally reported one. The C-terminal residues in either sequence show a high homology to the C-terminal residues of M110 and M110′s human equivalent, type 2 regulatory subunit (H/MYPT2) (16–19). In addition, MLCPsr has also been reported to possibly bind to myosin as well as M130 (12, 17). However, up to now, the physiological function of MLCPsr has not been reported.

The present study was thus aimed to determine the physiological function of MLCPsr. For this purpose,

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Abbreviations used: MLC, myosin light chain; MLCP, MLC phosphatase; MLCPsr, small regulatory subunit of MLCP; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; H/MYPT2, human type 2 regulatory subunit of MLCP; RT-PCR, reverse transcription polymerase chain reaction; CSS, cytoplasmic substitution solution; CPK, creatine phosphokinase; EGTA, ethylenglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyanoside.

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chicken gizzard MLCPsr cDNA was obtained by reverse transcription PCR (RT-PCR) and recombinant protein was produced using a bacterial expression system. The effects of recombinant MLCPsr on the Ca^{2+} induced contraction were thus observed in the permeabilized porcine renal artery. As a result, exogenously added MLCPsr was found to increase the Ca^{2+} sensitivity of the contractile apparatus while the N-terminal 78 residues were found to be responsible for this action.

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized by Hokkaido System Science (Sapporo, Japan). dNTPs (dATP, dCTP, dTTP, dGTP), restriction enzymes and LA Taq polymerase were purchased from Takara (Tokyo, Japan). M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase was bought from BRL (Gaithersburg, MD). The expression vectors (pQE vectors) and bacterial host for hexahistidine-tagged proteins were purchased from Qiagen (Hilden, Germany). Creatine phosphokinase (CPK) and calmodulin were obtained from Sigma (St. Louis, MO) and Seikagaku Kogyo (Tokyo, Japan), respectively. Triton X-100 was purchased from Katayama Chemicals (Osaka, Japan). EGTA (ethylenglycol-bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) was purchased from Dojindo (Kumamoto, Japan). All other chemicals were of the highest grade commercially available.

RT-PCR for chicken gizzard MLCPsr and production of recombinant protein. Total RNA was prepared from fresh chicken gizzards according to the method described by Chomczynski and Sacchi (20). The RT-PCR was conducted as previously described (21). The nucleotides sequences for primers are as follows: For the RT reaction, 5′-aagggcagaaagggaacg-3′. For PCR amplification, 5′-agcggtaccatgtcgtcgctgttcaccag-3′ (sense) and 5′-ccccggtacctacagctcggctcccctact-3′ (antisense). The PCR product was digested by *kpn*I and subsequently subcloned into pQE30. The sequence of the subcloned MLCPsr cDNA was determined by the dye-termination method with an automated sequencer ABI Prism 310 (Applied Biosystem, USA). The N- or C-terminal truncation mutants were obtained by PCR using the subcloned MLCPsr cDNA as a template. The expression and purification of wild type or mutant MLCPsr were performed using JM109 strain of *Escherichia coli* as a host, according to the manufacturer's instruction. The final elution was dialyzed with a buffer containing 100 mM K-methane sulfonate, 20 mM Tris-maleate (pH 6.8). The dialysate was clarified by centrifugation at 20,000*g* at 4°C for 10 min and concentrated by using centricon 10 (Amicon, Tokyo, Japan). The concentration of protein was determined by the Bradford method (22) using bovine serum albumin as the standard (Pierce, Rockford, IL).

Permeabilization and force measurements. Fresh porcine kidneys of either sex were obtained from a local slaughterhouse. They were brought back to the laboratory in preaerated physiological salt solution (NaCl 123 mM, KCl 4.7 mM, $NaHCO_3$ 15.5 mM, KH_2PO_4 1.2 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 1.25 mM, and D-glucose 11.5 mM). The kidney was then cut open and the distal portions of interlobular arteries were isolated. Arterial segments with an interior diameter of 200–250 μm were chosen for the experiment. The fat and adventitia were mechanically removed under a binocular microscope. The segments were then cut into 500-μm-wide vascular rings. The preparative procedures were conducted in a physiological salt solution aerated with 95% O_2 and 5% CO_2. The arterial rings thus obtained were permeabilized with 1% Triton X-100 in the Ca^{2+}-free cytoplasmic substitution solution [CSS: 10 mM EGTA, 100 mM K-methane sulfonate, 3.38 mM $MgCl_2$, 2.2 mM Na_2ATP, 10 mM creatine phosphate, 2 μM calmodulin, 50 U/ml CPK and 20 mM Tris(hydroxymethyl)aminomethane-maleate (pH 6.8)] at 24–25°C for 20 min. The measurement of isometric force was performed at 24–25°C as described (2). Briefly, the tissue was mounted onto two tungsten wires bathed in wells filled with Ca^{2+}-free CSS on a plate, by passing the tungsten wires through the lumen of the arterial ring. One of the wires was fixed while the other was connected to a force transducer (U gauge, Minebea, Japan). The tissue was then stretched to two times its resting diameter and thereafter was allowed to be stabilized in Ca^{2+}-free CSS for 30 min. The extent of force development was expressed as a percentage, assigning values in Ca^{2+}-free CSS (resting state) and in 10 μM Ca^{2+}-CSS (maximum contraction) to be 0% and 100%, respectively. Ca^{2+} CSS containing the indicated concentration of the free Ca^{2+} was prepared by adding appropriate amount of $CaCl_2$, using the EGTA-Ca^{2+} binding constant of 10^6/M (23).

Data analysis. The EC_{50} value, namely the concentration required to induce the force to 50% of the maximum response, was determined by fitting the concentration-response curves to a four-parameter logistic model (24). The data were expressed as the means ± standard error (SE). Student's *t* test was used to determine any statistically significant difference. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Cloning, expression, and purification of MLCPsr. The sequence of cDNA of chicken gizzard MLCPsr obtained in the present study was identical to that reported by Zhang *et al.* (18). Compared with the originally cloned 21-kDa MLCPsr (16, 17), this cDNA contains a 53-bp insertion between the 158th and 159th codons. Since the forth codon of the inserted sequence is TAG, this cDNA thus encodes for a protein of 161 amino acid residues with a molecular weight of 18.5 kDa (Fig. 1A). As shown in Fig. 1A, the C-terminal portion of MLCPsr (residues 79–158) was highly homologous to the C-terminal portion of M110 (residues 872–949) and M110's human equivalent, H/MYPT2 (residues 875–954). An SDS–PAGE analysis of the expression and purification of recombinant MLCPsr is shown in Fig. 1B. The expected molecular weight of the recombinant MLCPsr was 20.5 kDa, because of the 6 x His tag and a linker. A thick band that appeared slightly larger than 21 kDa on SDS–PAGE (lane 3) was considered to be a recombinant MLCPsr, because the control *E. coli* without induction did not produce this protein (lane 2). A portion of the final concentrated dialysate of elute was run on lane 4. The majority of the eluted proteins corresponded to the recombinant MLCPsr.

The effect of MLCPsr on the Ca^{2+} contraction in the permeabilized porcine renal artery. Figures 2A and 2B show the effect of MLCPsr on the Ca^{2+} induced contraction in the 1% Triton-X permeabilized porcine renal artery. In the Ca^{2+} free CSS, the application of MLCPsr, even up to 10 μM, was not able to produce any significant tension development (trace not shown). However, when the tissue was precontracted with 0.3 μM Ca^{2+}, the application of 3 μM MLCPsr produced a further contraction by 28.3 ± 5.2% (mean ± SE, $n = 3$), compared with that caused by 10 μM Ca^{2+} (a 100% response) (Fig. 2A and Fig. 3B). In contrast, the application of an equal volume of vehicle did not cause any

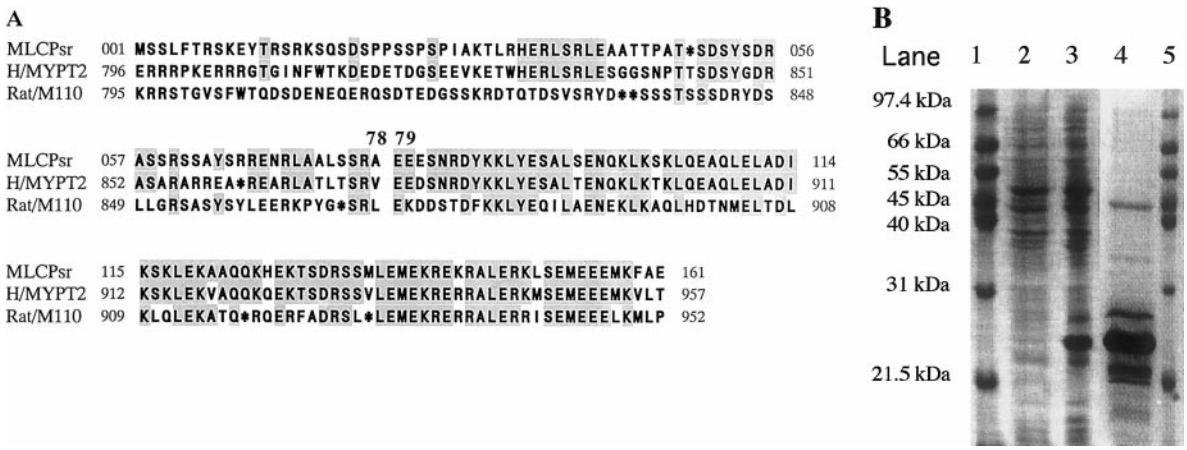

FIG. 1. Expression and purification of chicken MLCPsr. (A) Alignment of deduced amino acid sequences of 18.5 kDa chicken MLCPsr with homologous parts of M110 (16) and H/MYPT2 (19). The shadowed area represents the shared sequences of MLCPsr with rat M110 or H/MYPT2. "*" illustrates the skip in sequences. (B) An SDS–PAGE analysis of the expression and purification of MLCPsr. Lane 1 and lane 5, the size markers. Lane 2, the control, the cells uninduced by IPTG. Lane 3, the cells induced by 0.4 mM IPTG. Lane 4, an analysis of the final concentrated dialysate of elute.

response (data not shown). The effect of MLCPsr on the $[Ca^{2+}]_i$-force relation curve was shown in Fig. 2B. In this protocol, contractions were thus obtained by stepwise increases in Ca^{2+} concentration in the presence of 3 μM MLCPsr or an equal volume of vehicle. It should be noted again that 3 μM MLCPsr was not able to produce any significant tension development when the $[Ca^{2+}]_i$ was less than 0.1 μM. In the control experiment, the force development was observed at Ca^{2+} concentrations higher than 0.18 μM. The EC_{50} value of Ca^{2+} of the control was 0.442 ± 0.040 μM (mean ± SE, $n = 3$). In the presence of 3 μM MLCPsr, the $[Ca^{2+}]_i$-force curve significantly shifted to the left, with a EC_{50} values of Ca^{2+} of 0.267 ± 0.019 μM ($n = 3$, mean ± SE, $P < 0.01$). These results indicated that MLCPsr could enhance the Ca^{2+}-induced contraction in the permeabilized porcine renal artery.

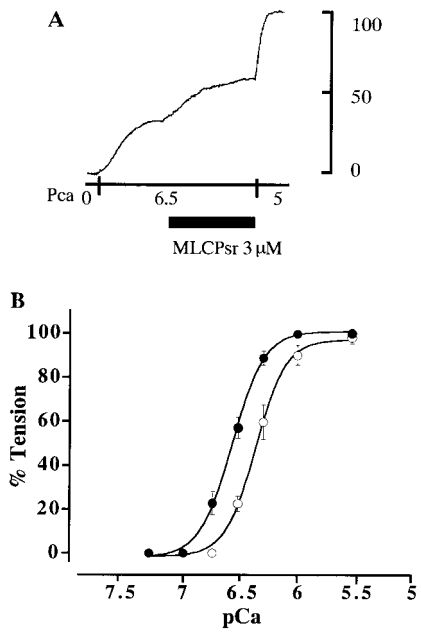

FIG. 2. Effect of MLCPsr on Ca^{2+} induced contraction in 1% Triton X-100-permeabilized porcine renal artery. The force development was expressed as a percentage of that obtained with 10 μM Ca^{2+}. (A) Representative recordings showing the effect of 3 μM MLCPsr on the force development in 0.3 μM Ca^{2+}-CSS. (B) The $[Ca^{2+}]_i$-force relation curves induced by a stepwise increasing in the Ca^{2+} levels in the absence (control; ○) and presence of 3 μM MLCPsr (●). All values are the means ± SE ($n = 3$).

The effect of the N- or C-terminal fragments of MLCPsr on Ca^{2+} contraction. Since MLCPsr demonstrated an ability to enhance Ca^{2+} contraction, we tried to determine the domain that is responsible for this action. We constructed two truncation mutants, one containing residues 1–78 (termed as $MLCPsr^{1-78}$), and the other consisted of residues 79–161 of MLCPsr (termed as $MLCPsr^{79-161}$). Either mutant was also confirmed by plasmid sequence as well as protein expression and showed an high purity on SDS–PAGE (data not shown). As shown in Figs. 3A and 3B, 0.3 μM Ca^{2+} CSS induced a contraction of 27.9 ± 3.6%, (mean ± SE, $n = 3$) compared with 10 μM Ca^{2+} CSS. When this contraction reached a steady state, the application of 3 μM $MLCPsr^{1-78}$ caused a further contraction by 27.2 ± 2.6% (mean ± SE, $n = 3$) (Fig. 3A). In addition, the enhancement of 0.3 μM Ca^{2+} CSS-induced contraction by 3 μM $MLCPsr^{1-78}$ was not significantly different from that of 3 μM full length MLCPsr (Fig. 3B) (from 29.7 ± 3.6% to 55.1 ± 1.2% vs 28.8 ± 3.6% to 57.0 ± 1.8%, mean ± SE, $n = 3$). On the other hand, $MLCPsr^{79-161}$, even up to 10 μM, failed to produce any response in the 0.3 μM Ca^{2+} precontracted tissues (Fig. 3C).

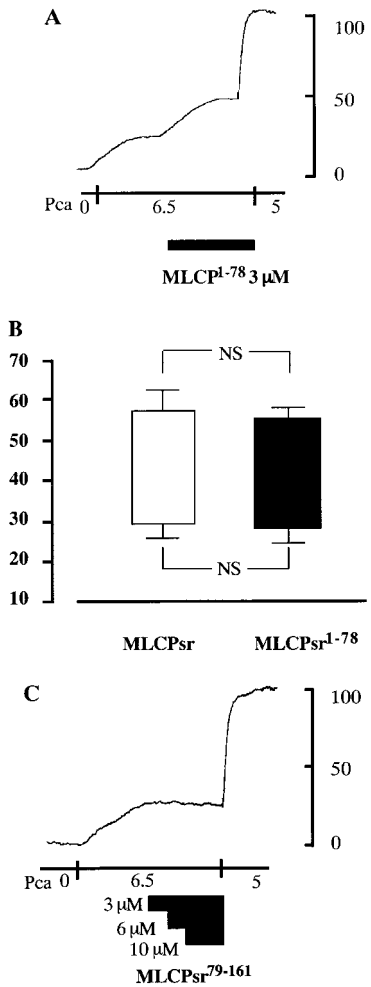

FIG. 3. Effect of truncate mutants of MLCPsr on the Ca^{2+} induced contraction in 1% Triton X-100-permeabilized porcine renal artery. The force development was expressed as a percentage of that obtained with 10 μM Ca^{2+}. (A) Representative recordings showing the force development by 3 μM $MLCPsr^{1-78}$ in 0.3 μM Ca^{2+}-CSS. (B) Comparison of the force developments induced by 3 μM $MLCPsr^{1-78}$ and 3 μM MLCPsr in 0.3 μM Ca^{2+}-CSS. All values are expressed by the mean ± SE ($n = 3$). (C) A representative recording showing that no further force was developed following the application of $MLCPsr^{79-161}$ in 0.3 μM Ca^{2+}-CSS.

The effect of the $MLCPsr^{1-78}$ on the relaxing rate. To clarify whether the above increases in Ca^{2+} contraction was the result of an_inhibition in MLCP activity, we determined the effect of $MLCPsr^{1-78}$ on the process of relaxation. Since MLCK is supposed to be inactive in the absence of Ca^{2+}, the relaxation of Ca^{2+} contraction by exposure to Ca^{2+} free CSS can be considered to be determined by MLCP. The tissues were first contracted by 10 μM Ca^{2+} and allowed to reach a peak contraction, in order to allow the MLC to become maximally phosphorylated. At this point, 3 μM $MLCPsr^{1-78}$ or an equal volume of vehicle was added and incubated for 30 min. As expected, 3 μM $MLCPsr^{1-78}$ did not produce any further contraction. The tissue specimens were then relaxed by Ca^{2+} free CSS containing 3 μM $MLCPsr^{1-78}$ or vehicle. As illustrated in Fig. 4, the relaxation process of maximum contraction by exposure to Ca^{2+} free CSS was significantly delayed by 3 μM $MLCPsr^{1-78}$, compared with that of the control. The value of $t_{1/2}$ (time for the tissue to relax to 50% of peak contraction) thus increased from 278.0 ± 23.4 s of the control to 558.0 ± 40.5 s (mean ± SE, $n = 3$; $P < 0.005$).

DISCUSSION

In the present study, we investigated the effect of the recombinant chicken gizzard MLCPsr, which was produced using the bacterial expression system, on the Ca^{2+} sensitivity of smooth muscle in permeabilized porcine renal artery. We found that (i) MLCPsr induced an additional contraction at a constant Ca^{2+} concentration and shifted the $[Ca^{2+}]_i$-force relationship to the left; (ii) The fragment containing the N-terminal 78 amino acids reserved this action of MLCPsr, and deletion of this region abolished the effect of MLCPsr; (iii) The process of relaxation was delayed by the fragment containing the N-terminal 78 amino acids. These results indicated that MLCPsr is involved in the regulation of smooth muscle Ca^{2+} sensitivity.

To isolate cDNA for MLCPsr, we designed the primers according to the sequence for the originally reported 21-kDa MLCPsr (16, 17). However, we could only obtain clones that had a 53-bp insertion between the 158th and 159th codons. Because of the presence of a termination codon in this insertion, our cDNA clone for MLCPsr encoded for a protein of 161 amino acid residues with a molecular weight of 18.5 kDa. We thus could not examine the effect of 21 kDa MLCPsr on the Ca^{2+}-induced contraction. We believe that 18.5-kDa MLCPsr may be the major isoform of MLCPsr expressed in chicken gizzard smooth muscle, as pointed out previously by Zhang *et al.* (18).

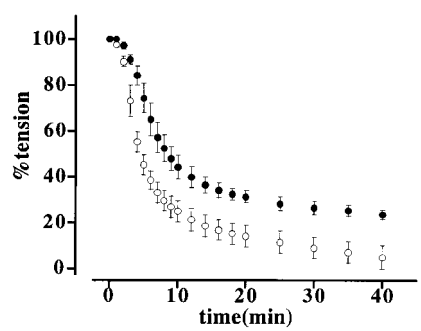

FIG. 4. Effect of $MLCPsr^{1-78}$ on relaxation in 1% Triton X-100-permeabilized porcine renal artery. Permeabilized fibers were contracted with 10 μM Ca^{2+}-CSS, and then relaxed by exposure to Ca^{2+}-free CSS either in the presence (●) or absence (○) of 3 μM $MLCP^{1-78}$. $MLCP^{1-78}$ or vehicle was applied after the contraction became constant, and was incubated for 30 min. All values are expressed as the mean ± SE ($n = 3$).

Because the $[Ca^{2+}]_i$ is clamped in the permeabilized preparations, the increase in contraction can be considered to be an increase in the Ca^{2+} sensitivity of the contractile apparatus. The MLCPsr-induced increase in Ca^{2+} sensitivity is shown in Fig. 2A, in which the application of MLCPsr caused a further force development of the tissue precontracted by 0.3 μM Ca^{2+} CSS. It was further confirmed by the effect of MLCPsr on $[Ca^{2+}]_i$-force curves. As shown in Fig. 2B, MLCPsr significantly shifted the $[Ca^{2+}]_i$-force curve to the left. These results clearly indicated that MLCPsr induces increase in Ca^{2+} sensitivity. The fact that the C-terminal fragment was ineffective up to 10 μM (Fig. 3C) ruled out the possibility that the histidine tag or other possible contaminating bacterial proteins might be responsible for the apparent Ca^{2+}-sensitizing effects (25). In addition, it should be pointed out that this Ca^{2+}-sensitizing effects by MLCPsr involves the pathway which needs a physiological concentration of Ca^{2+}, because MLCPsr did not induce any force development in the nominally Ca^{2+} free medium (0 Ca^{2+} CSS buffered by 10 mM EGTA or $[Ca^{2+}]_i$ less than 0.1 μM; Fig. 1C).

To further determine the domain of MLCPsr that is responsible for this Ca^{2+}-sensitizing action, the effects of the N- ($MLCPsr^{1-78}$) and C-terminal ($MLCPsr^{79-161}$) fragments were also examined. The reason why we chose this truncation is that $MLCPsr^{1-78}$ was thought to be unique for MRCPsr, and $MLCPsr^{79-161}$ was homologous to M110 or M110′s human equivalent, H/MYPT2. As shown in Fig. 3A, $MLCPsr^{1-78}$ also had an enhancing effect on Ca^{2+} sensitivity. In addition, this effect was not significantly different from that of the full length MLCPsr (Fig. 3B). $MLCPsr^{1-78}$ was thus suggested to be the active fragment of MLCPsr and the deletion of the C-terminal amino residues 79–161 does not affect the Ca^{2+} sensitizing effect of MLCPsr. The truncate $MLCPsr^{79-161}$ failed to induce any response even up to 10 μM (Fig. 3C). As mentioned above, we could not examine the effect of 21 kDa MLCPsr on the Ca^{2+}-induced contraction. However, since the N-terminal portion shared by both 21 kDa and 18.5 kDa isoform was responsible for the Ca^{2+} sensitizing effect, both might have a similar effect on the Ca^{2+} sensitivity.

One possible explanation for the above Ca^{2+} sensitizing effect is that the exogenously applied MLCPsr might have an inhibitory action on the endogenous MLCP. This speculation was supported by the observation that the treatment with $MLCPsr^{1-78}$ significantly delayed the relaxation of the tissue that was maximally activated by Ca^{2+} and then exposed to Ca^{2+} free CSS (Fig. 4). This delay in relaxation is probably not due to an increase in MLC phosphorylation before exposure to Ca^{2+} free CSS, because the tissue specimen contracted to a maximum extent due to 10 μM Ca^{2+}, as demonstrated by the observation that the addition of $MLCPsr^{1-78}$ did not produce any further contraction in the steady state of 10 μM Ca^{2+} induced contraction (data not shown). The $MLCPsr^{1-78}$-induced delay in relaxation was also not due to the activation of MLCK in Ca^{2+} free CSS, either, because MLCPsr did not induce any tension development in the nominally Ca^{2+} free medium (0 Ca^{2+} CSS buffered by 10 mM EGTA or $[Ca^{2+}]_i$ less than 0.1 μM; Fig. 2B). It is thus likely that exogenously applied MLCPsr might inhibit the endogenous MLCP activity. However, MLCPsr has been reported to bind to myosin and M130 but not to PP1c (12, 17). MLCPsr therefore does not appear to directly bind to PP1c and thus inhibits its activity. It is also unlikely that this inhibition was caused via binding to M130, since the N-terminal fragment of MLCPsr was unable to bind to M130 (17). One possibility is that MLCPsr binds to the substrate to inhibit the activity of PP1c. This hypothesis is obviously speculative and needs further investigation.

In addition, it is still premature to consider the action of endogenous MLCPsr on PP1c to be inhibitory. This is because there is a possibility that the bacterially synthesized MLCPsr can not function properly as the endogenous MLCPsr, and thus might antagonize the function of endogenous MLCPsr. In other words, the exogenously applied MLCPsr may act as a dominant negative. If so, the action of endogenous MLCPsr on PP1c might be stimulatory. Despite of this possibility, it still appears that MLCPsr is involved to some degree in the modulation of the Ca^{2+} sensitivity of the smooth muscle.

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