Interactive Role of Protein Phosphatase 2A and Protein Kinase C α in the Stretch-Induced Triphosphorylation of Myosin Light Chain in Canine Cerebral Artery

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Key Words

Cerebral artery • CPI-17 • Mechanical stretch • Myosin phosphorylation • Okadaic acid • Protein kinase C • Protein phosphatase 2A • Rho kinase

Abstract

The interactive role of protein kinase C (PKC) isoforms and protein phosphatase 2A (PP2A) in the mechanisms underlying the gradual reduction in stretch-induced contraction through triphosphorylation of 20-kDa myosin light chain (MLC₂₀) was investigated in the canine basilar artery. In the presence of 5 mM tetraethylammonium, stretching at a rate of 1 mm/s from the initial length (L_i) to 1.5 L_i produced a contraction. Maintaining the stretched state for 15 min (15-min stretch) produced triphosphorylation of MLC₂₀ at Ser-19, Thr-18 and Thr-9, and a gradual reduction in the contraction, both of which were reversed by Gö6976 (1 μ M), an inhibitor of conventional PKC. The 15-min stretch increased PKC α activity whereas it decreased PP2A activity, both of which were blocked by Y-27632, an inhibitor of rho kinase. Okadaic acid (OA; 1 μ M), a PP2A inhibitor, also produced triphosphorylation of MLC₂₀ at the same amino acid residues and activated PKC α , which was inhibited by Gö6976. Stretching and OA in-

creased phosphorylation of 17-kDa PKC-potentiated inhibitory phosphoprotein (CPI-17), and this phosphorylation was inhibited by Gö6976. The present results suggest that activation of PKC α mediated by an inhibitor of PP2A is involved in the stretch-induced triphosphorylation of MLC₂₀, and that this triphosphorylation counteracts the stretch-induced contraction.

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Introduction

Mechanical stretching of the vascular wall acts as a facilitating stimulus on blood vessels, and elicits a variety of cellular responses, including myogenic contraction and relaxation. This myogenic response contributes to the autoregulation of blood flow, and is also considered to be one of the primary mechanisms responsible for basal vascular tone [1–5]. We previously reported that the cerebral artery of various animal species including dogs [6–8], rabbits [2, 9] and rats [10, 11] was particularly sensitive to mechanical stretching and pressure, leading to a contraction. We found that slow stretching produces contraction only when the large conductance Ca²⁺-activated

K⁺ channels (BK channels) of the artery are inhibited by iberitoxin and tetraethylammonium (TEA) [7] or by 20-hydroxyeicosatetraenoic acid (20-HETE) [12], an endogenous cytochrome P450 metabolite of arachidonic acid. More recently, it has been reported that the inhibition of BK channels under pathophysiological conditions, such as intracellular acidosis [13] and type 1 diabetes [14], results in increased vasoconstriction of cerebral artery. Therefore, a change in BK channel activity may occur under physiologic and pathologic conditions.

We recently reported that slow stretching gradually increased the levels of triphosphorylated 20-kDa myosin light chain (MLC₂₀), and that the triphosphorylated MLC₂₀ in contrast counteracted stretch-induced contraction in the canine cerebral artery treated with TEA. As to the mechanism of MLC₂₀ triphosphorylation, we suggested that myosin-light-chain kinase (MLCK), protein kinase C (PKC), and rho kinase play important roles in this triphosphorylation [8]. However, the interactive roles of these kinases in the sequence of events involved in the mechanotransduction of vascular tissue still remain to be elucidated.

We previously reported that okadaic acid (OA), a black sponge toxin and a cell-permeable inhibitor of protein phosphatase 2A (PP2A) [15], at a relatively low concentration (less than 3 μ M), produced a relaxing effect on canine cerebral and porcine coronary arteries precontracted with a high concentration of KCl [16]. Furthermore, our recent study indicated that this relaxing action on the canine cerebral artery produced by OA was concomitant with triphosphorylation of MLC₂₀ [17]. It has been reported that PKC α activity is regulated by phosphorylation of 3 conserved residues in its kinase domain [18]. Furthermore, our previous findings clearly indicate that of the 4 PKC isoforms (PKC α , δ , ζ and η) present in the canine basilar artery [19], PKC α and PKC δ are translocated from the cytosol to the membrane fraction by slow stretching [20]. It has been reported that both a physical and a functional interaction between PP2A and PKC α occurs in the mast cells [21]. Based on these observations and circumstantial evidence, we hypothesize that an intimate interaction between PP2A and PKC α may be involved in the mechanism for stretch-induced triphosphorylation of MLC₂₀.

The present study was thus aimed at further clarifying the mechanisms underlying the reduction in the stretch-induced contraction in the TEA-treated canine cerebral artery. For this purpose, we used OA as a pharmacological probe to specifically inhibit PP2A. Our results suggest that PKC α mediated by PP2A inhibition is involved in

stretch-induced triphosphorylation of MLC₂₀. Inhibition of basal activity of PP2A by stretching seems to be implicated in this stretch-induced triphosphorylation of MLC₂₀ and the concomitant reduction in stretch-induced contraction.

Materials and Methods

All experiments were carried out in accordance with the Institutional Guidelines for Animal Experiments of the University of Shizuoka, and the Guiding Principle of the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

Isolation of Basilar Artery

Healthy mongrel dogs of either sex weighing 7–15 kg were anesthetized with pentobarbital sodium (30 mg/kg, i.v.) and exsanguinated by bleeding from the carotid arteries. A cylindrical segment of the basilar artery, 2 cm long, was isolated and cut into ring segments about 0.5 mm in width. The preparation was horizontally mounted in an organ bath containing 5 ml of Tyrode's solution of the following composition: 158.3 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1.05 mM MgCl₂, 10 mM NaHCO₃, 0.42 mM NaH₂PO₄, and 5.6 mM glucose, and bubbled with 97% O₂ and 3% CO₂ (pH 7.35 at 35°C).

Mechanical Stimulation and Recording of Isometric Contraction

The experimental method for stretching ring segments of the canine cerebral artery has previously been described in detail [7]. Briefly, the endothelium was eliminated by gentle rubbing of the intimal layer of the artery with a moist cotton pledget. For the mechanical stimulation, a ring segment of the artery was mounted between two tungsten wires connected with a force transducer and an arm whose position was controlled by a mechanical stimulator (DPS-265; Dia Medical System Co., Tokyo, Japan), and the distance between the tungsten wires with the ring segments was controlled. The distance was adjusted to half of the initial length (L_i) of the ring segment, at which no measurable increase in passive tension was observed. L_i is the circumference of the ring segment assuming the segment to be cylindrical in shape [22]. In order to verify the responsiveness of the artery segments, an 80 mM KCl-induced contraction was produced at the beginning of each experiment. Isotonic 80 mM KCl-Tyrode's solution was prepared by replacement of NaCl with an equimolar amount of KCl. We previously reported that a slow stretch at a rate of 1 mm/s from L_i to 1.5 L_i elicited a reproducible contraction when the artery was treated with TEA (5 mM) or iberitoxin (100 nM) [7]. The slow stretch-induced contraction reached a maximum at almost 1 min after stretching and then gradually decreased to near basal level within 15 min. Therefore, in this study, the ring segments were stretched from L_i to 1.5 L_i at a rate of 1 mm/s and then kept in the stretched state for 15 min in the presence of TEA (5 mM). The active tension produced by the slow stretching was defined as the difference in amplitude between the tension in the absence of papaverine and that in the presence of 100 μ M papaverine (passive tension) (fig. 1a) as previously described [7]. To investigate the in-

Fig. 1. Slow stretch-induced contraction and MLC_{20} phosphorylation in the canine basilar artery. **a** Typical tracings of slow-stretch-induced contraction. Artery segments were treated with or without Gö6976 ($1\ \mu\text{M}$) or papaverine (PPV, $100\ \mu\text{M}$) for 15 min. Then they were stretched at a rate of $1\ \text{mm/s}$ from L_i to $1.5\ L_i$ and maintained in the stretched state for 15 min (ST) in the presence of TEA ($5\ \text{mM}$). **b** Typical phosphorylation patterns of MLC_{20} detected by the immunoblot technique. The segments were incubated in a solution containing vehicle (ST; 0.1% DMSO) or Gö6976 (ST + Gö; $1\ \mu\text{M}$) for 15 min, and then were stretched for 15 min. In a separate series of experiments, the segments were pretreated with vehicle (OA; 0.1% DMSO) or Gö6976 (OA + Gö; $1\ \mu\text{M}$) for 15 min before application of OA ($1\ \mu\text{M}$). Nontreated segments (control), and ST- or OA-treated segments were frozen in an acetone-dry ice. Note that four immunoreactive bands to anti- MLC_{20} (MLC, MLC-p, MLC-pp and MLC-ppp corresponding to non-, mono-, di-, and tri-phosphorylated MLC_{20} , respectively) were detected. **c** Summarized results are depicted. The ordinate indicates the percent area for a given band calculated as described previously [8]. Data are means $\pm$ SEM of 5 experiments using 5 artery rings obtained from 5 different dogs. ^a $p < 0.01$ compared with before stretch and ^b $p < 0.01$ compared with vehicle after stretch.

terplay between PKC isoforms and PP2A in MLC_{20} phosphorylation and contraction after the 15-min stretch, ring segments were treated with an inhibitor against one of the indicated molecules for 15 min before the stretch.

Measurement of MLC_{20} Phosphorylation

The level of MLC_{20} phosphorylation was quantified by the method described previously [8]. Ring segments were frozen in 10% trichloroacetic acid (TCA)- 90% acetone cooled on dry ice

before and after the 15-min stretch. The frozen segments were stored in the same solution for 20–30 min, and then immersed in acetone for 24 h to remove the TCA. The segments were then smashed in liquid nitrogen and sonicated in $100\ \mu\text{l}$ of an extracting solution containing $10\ \text{M}$ urea, $2\ \text{mM}$ ethylenediaminetetraacetic acid (EDTA), $5\ \text{mM}$ dithiothreitol, 0.01% bromophenol blue, and $20\ \text{mM}$ Tris-HCl (pH 7.5). Samples were subjected to isoelectric focusing (IEF)-polyacrylamide gel electrophoresis (PAGE) using pH 4–6 ampholyte, transferred to nitrocellulose

Fig. 2. Two-dimensional phosphopeptide maps of MLC₂₀ from the canine basilar artery. Representative autoradiographic maps obtained from at least 3 mappings for each procedure. **a** Purified MLC₂₀ from a canine basilar artery phosphorylated by MLCK. **b** Purified MLC₂₀ from a canine basilar artery phosphorylated by PKC α . **c** Nonstimulated segment (control). **d** The segment was stretched at a rate of 1 mm/s from L_i to 1.5 L_i and maintained in the stretched state for 15 min in the presence of TEA (5 mM). **e** The segment was pretreated with Gö6976 (1 μ M) for 15 min and then stretched. **f** The segment was pretreated with OA (1 μ M) for 15 min. **g** The segment was pretreated with Gö6976 (1 μ M) for 15 min before application with OA (1 μ M). Spots a and b correspond to peptides containing Ser-19 and Thr-18, respectively, and were phosphorylated by MLCK. Spots c and d correspond to peptides containing Thr-9 and Ser-1/2, respectively, and were phosphorylated by PKC.

membranes, and blotted with MLC₂₀-specific mouse antibodies. The extent of MLC₂₀ phosphorylation was quantified from densitometric scans of immunostained nitrocellulose blots.

Two-Dimensional Phosphopeptide Mapping

Two-dimensional tryptic phosphopeptide mapping was carried out based on the method described by Erdodi et al. [23]. MLC₂₀ was purified from the canine basilar artery and phosphorylated in vitro using the method described by Hathaway and Haeberle [24]. In order to perform the mappings, a total of 20 artery

rings were isolated from 5 dogs, and subjected to each procedure as depicted in figure 2a–g. At least 3 mappings were carried out for each procedure. The ring segments were preincubated with [³²P]orthophosphate (1.2 mCi/ml) for 60 min in Tyrode's solution that did not contain Na₂HPO₄ and were stretched for 15 min. IEF gel electrophoresis was performed with extracts from stretched arteries. Following staining with Coomassie blue, MLC₂₀ was eluted from gels and digested at 37°C by 5 repeated additions of 5 μ l of 1 μ g/ml N-p-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin at 45-min intervals; the digestion was continued

for a total of 6 h. Gel pieces up to 20, depending on the radioactivity incorporated into the proteins of the individual bands, were pooled before elution. The samples were subjected to electrophoresis performed in acetic acid/formic acid/water (150:50:80 vol/vol) at pH 1.9 at 300 V for 90 min on a cellulose sheet (first dimension), followed by ascending chromatography (second dimension) in butanol/pyridine/acetic acid/water (15:10:3:12 vol/vol) for 3 h. After drying of the cellulose sheet, phosphopeptides on the sheet were subjected to autoradiography.

Measurement of Enzyme Activity of PKC α

The activity of PKC α was assessed as follows: translocation of PKC α from the cytosol to the membrane fraction was measured by immunoblot analysis as described previously [25]. Briefly, canine basilar artery rings were homogenized with a Polytron in ice-cold homogenization buffer composed of 50 mM Tris-HCl, 5 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 10 mM benzamide, 25 μ g/ml leupeptin, and 250 mM sucrose, and then centrifuged at 100,000 g for 30 min at 4°C. 'Cytosol' and 'crude membrane' fractions were derived from the supernatant and the pellet, respectively. PKC isoforms in each fraction were separated by sodium dodecylsulfate-PAGE (SDS-PAGE) on 10% acrylamide gels, transferred to nitrocellulose membranes, and blotted with anti-PKC α antibody.

Phosphorylation of PKC α at Ser-657, another measure of the enzyme activity [18], was also checked. The cytosol fractions were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-PKC α or anti-phospho-PKC α antibodies. Immunoblot analysis was performed to detect each protein molecule by the standard procedures as described previously [25] unless otherwise stated in the text.

The activity of PKC α was measured with a nonradioactive protein kinase assay kit (TruLight™ PKC α Assay Kit; Calbiochem) according to the manufacturer's protocol. The cytosol fraction (50 μ l) was mixed with 50 μ l of the reaction solution containing 1 μ M PKC α substrate (RFARKGSLRQKNV) and 2 μ M ATP, and incubated for 1 h at room temperature. After addition of the microsphere, a kit component (No. KP31689), which is coated with highly fluorescent polymers and binds to the phosphorylated PKC α substrate, resulting in the change of fluorescence, the fluorescence intensity was measured at an excitation wavelength of 450 nm and an emission wavelength of 490 nm.

Measurement of PP2A Activity

PP2A activity was measured with a Ser/Thr phosphatase assay kit (Upstate Biotechnology) using the method described by Yu et al. [26]. Canine basilar artery rings before and after the 15-min stretch were quick-frozen in liquid nitrogen and lysed for 30 min on ice in lysis buffer (137 mM NaCl, 1 mM CaCl₂, 1% Nonidet P-40, 10% glycerol, 20 mM Tris-HCl, pH 8.0, 1 mM sodium vanadate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 20 μ g/ml leupeptin, and 20 μ g/ml aprotinin). Lysates were centrifuged at 20,000 g for 15 min at 4°C, and the supernatants were incubated with 2.5 μ g of anti-PP2A antibody for 2 h and with protein A/G agarose beads for a further hour at 4°C. The immunoprecipitates were washed twice with lysis buffer, washed once with 50 mM Tris buffer (50 mM Tris-HCl, pH 7.0; 0.1 mM CaCl₂), resuspended in assay buffer (50 mM Tris-HCl, pH 7.0, 0.1 mM CaCl₂, 2.5 mM NiCl₂, and 1 mg/ml *p*-nitrophenyl phosphate), and incubated at 37°C for 30 min. The reaction was stopped by the addition of 13%

K₂HPO₄, and the absorbance was read at 405 nm. Protein complexes bound to beads were solubilized in SDS-PAGE sample buffer, and the amount of PP2A was quantified by immunoblot analysis with anti-PP2A antibody.

Determination of Association between PKC α and PP2A

Canine basilar artery rings before and after the 15-min stretch were lysed in 1 ml of ice-cold RIPA (Radio Immunoprecipitation Assay) buffer composed of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM Na₂HPO₄, 0.25% sodium deoxycholate (w/v), 0.1% Nonidet P-40, 1 mM Na₃VO₄, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 5 mM EDTA and 5 mM EGTA. At least 1 μ g of primary antibody [anti-PKC α or anti-PP2A catalytic subunit (PP2Ac) antibodies] was added to 1 ml of clarified lysate, and the sample was incubated for 1 h at 4°C with mixing. Immunoreactive proteins and protein complexes were then precipitated with the addition of 20 μ l of protein A/G agarose beads and incubated at 4°C overnight with mixing. The beads were pelleted by centrifugation at 1,200 g for 5 min at 4°C, and the supernatant was discarded. The pellet was washed 4 times with ice-cold PBS (NaCl concentration adjusted to 1.0 M) before the addition of 40 μ l of 3 $\times$ SDS-PAGE sample buffer and storage at -20°C until further SDS-PAGE and Western blot analysis with anti-PKC α or anti-PP2Ac antibodies.

Measurement of CPI-17 Phosphorylation

Phosphorylation of 17-kDa PKC-potentiated inhibitory phosphoprotein (CPI-17) was measured by immunoblot analysis using anti-phospho-CPI-17 (Thr-38) antibody. Canine basilar artery rings before and after the 15-min stretch were quick-frozen in liquid nitrogen and homogenized with a Polytron in ice-cold homogenization buffer composed of 50 mM Tris-HCl, 5 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 10 mM benzamide, 25 μ g/ml leupeptin, and 250 mM sucrose. Homogenates were subjected to immunoblotting. The band intensity of phosphorylated CPI-17 on the X-ray film was quantified by use of a densitometer and analyzing software (NIH Image version 1.62).

Drugs

Anti-MLC₂₀ and anti-PKC α isoform antibodies were obtained from Sigma (St. Louis, Mo., USA). OA was purchased from Wako (Osaka, Japan). Gö6976 [12-(2-cyanoethyl)-6,7,12,12-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a) pyrrolo(3,4-c)-carbazole], Y-27632 [(R)-(+)-trans-N-(2-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide], and TruLight™ PKC α Assay Kit were obtained from Calbiochem (Darmstadt, Germany). Anti-CPI-17, anti-phospho-CPI-17 (Thr-38), anti-PP2A (clone 1D6), anti-PP2Ac, and anti-phospho-PKC α (Ser-657) antibodies, Ser/Thr phosphatase Assay Kit, myosin light chain kinase (active), and PKC α (active) were obtained from Upstate Biotechnology (Lake Placid, N.Y., USA). Protein A/G agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Gö6976 and OA were stocked in 10 mM solution in 100% dimethylsulfoxide (DMSO) and were diluted over 100 times into the bath solution, the final concentration of DMSO being kept under 1%. We confirmed that 1% DMSO had no apparent effect on any of the stretch-induced contractions. All other drugs used in the present study were reagent grade.

Statistical Analysis

Data were expressed as means $\pm$ SEM. Statistical analyses were made by paired or unpaired Student's *t* tests, or by Fisher's protected least significant difference test after analysis of variance (ANOVA). A *p* value less than 0.05 was considered statistically significant.

Results

Slow-Stretch-Induced MLC₂₀ Phosphorylation and Contraction

Figure 1a shows typical tracings of developed tension in response to slow stretching before and after treatment with Gö6976, an inhibitor of conventional PKC, which was superimposed on the passive tension after treatment with papaverine. The slow stretching at a rate of 1 mm/s to 1.5 times *L*_i produced a delayed contraction of the canine basilar artery in ring form pretreated with TEA (5 mM), which reached a maximum (active tension: 6.3 ± 1.1 mN, *n* = 5) at 1–2 min after the application of stretching (fig. 1a), as has been reported previously [7, 8]. The developed tension then gradually decreased, but it remained slightly higher than its initial level. Although Gö6976 (1 μ M) had no apparent effect on the maximum tension induced by slow stretching, it slowed the gradual reduction in tension at 15 min after stretching (active tension, control: 0.6 ± 0.1 mN; after Gö6976: 2.9 ± 0.3 mN, *n* = 5, *p* < 0.01).

Furthermore, we confirmed our previous findings that the phosphorylation patterns of MLC₂₀ seen during the 15-min interval were very different from those of the 1-min interval and those of 80 mM KCl [8]. In the artery segments stretched for 15 min, the typical IEF-PAGE detected multiple bands indicating non-, mono-, di-, and triphosphorylated MLC₂₀ (fig. 1b). Summarized data shown in figure 1c indicate that Gö6976 (1 μ M) decreased di- and triphosphorylated MLC₂₀. Low concentrations of OA (1 μ M), an inhibitor of PP2A, also increased mono-, di-, and triphosphorylated MLC₂₀, and these increases were inhibited by Gö6976 (1 μ M) (fig. 1b, c). Rottlerin (5 μ M), an inhibitor of PKC δ , had no apparent effect on MLC₂₀ phosphorylation and contraction induced by stretching (data not shown), indicating no primary role of PKC δ in the MLC₂₀ triphosphorylation.

The sites of phosphorylation of MLC₂₀ were identified by two-dimensional phosphopeptide maps. Figure 2a–g shows each representative autoradiographic map obtained from at least 3 mappings for each procedure. When the purified MLC₂₀ from the canine basilar artery was phosphorylated by MLCK, 2 radioactive spots (a and

b) were observed (fig. 2a). When the same purified MLC₂₀ was phosphorylated by PKC α , another 2 radioactive spots (c and d) were observed (fig. 2b). Maps of the canine basilar artery MLC₂₀ phosphorylated by MLCK and PKC α revealed patterns similar to those for gizzard MLC₂₀ reported by Ikebe et al. [27] and by Singer et al. [28], respectively. Our observations indicate that spots a and b correspond to peptides containing Ser-19 and Thr-18, respectively, which were phosphorylated by MLCK, and that spots c and d correspond to peptides containing Thr-9 and Ser-1/2, respectively, which were phosphorylated by PKC. The digests from the nonstimulated ring segments contained 1 phosphopeptide (spot a) (fig. 2c, control). When the ring segments were stretched for 15 min, 3 phosphopeptides (spots a–c) were observed (fig. 2d). Treatment with Gö6976 (1 μ M) for 15 min before stretching resulted in the disappearance of spot c (fig. 2e). Our preliminary studies indicated 1 peptide (spot a) and 2 peptides (spots a and b) were present in the maps of the ring segments stretched for 1 and 5 min, respectively (data not shown). The digests from the ring segments treated with OA (1 μ M) for 15 min also contained 3 phosphopeptides (spots a–c) (fig. 2f). Of these, 2 spots (b and c) disappeared by treatment with Gö6976 (1 μ M) for 15 min before application of OA (fig. 2g). These results indicate that PKC α may play a dominant role in OA-induced MLC₂₀ triphosphorylation, while it may partially participate in the stretch-induced MLC₂₀ triphosphorylation.

Effects of Stretching and OA on Activation of PKC α

Figure 3a shows the effects of stretching and OA on the translocation of PKC α from the cytosol to the membrane fraction, which is a measure of PKC α activation. In the resting state (control), PKC α was abundant in the cytosol ($66.3 \pm 3.8\%$, *n* = 5). We have previously reported that PKC α transiently translocates about 1 min after stretching [20], but its translocation ceases after a few minutes. Consistently, we noticed here no apparent translocation of PKC α at 15 min after the stretch (fig. 3a). OA (1 μ M) had no apparent effect on the translocation of PKC α at either 1 (data not shown) or 15 min (fig. 3a) after treatment.

The phosphorylation of PKC α at Ser-657 causes accumulation of active enzyme [18]. To clarify why PKC α plays a dominant role in the triphosphorylation of MLC₂₀ without any sustained translocation from the cytosol to the membrane fraction at 15-min stretch, we examined the effects of stretching and OA on the phosphorylation of PKC α at Ser-657 in the cytosol (fig. 3b). The phosphor-

Fig. 3. Activation of PKC α induced by stretching and OA. **a** Distribution of PKC α in the membrane fraction. Artery segments were stretched at a rate of 1 mm/s from L_i to 1.5 L_i and maintained in the stretched state for 15 min in the presence of TEA (5 mM), or they were treated with OA (1 μ M) for 15 min. The subcellular distributions of PKC α in the cytosol (C) and the membrane (M) fractions were detected by Western blot analysis with anti-PKC α antibody (**inset**). The results were expressed as a percentage of the total amount of PKC α . **b** Phosphorylation of PKC α at Ser-657. PKC α and phospho-PKC α (p-PKC α) were identified by Western blot analysis with each specific antibody (**inset**). The segments were pretreated with Gö6976 (Gö; 1 μ M) or Y27632 (Y; 1 μ M) for 15 min before application of stretching (ST) or OA (1 μ M). The cytosol fraction was subjected to SDS-PAGE and immunoblotted with anti-PKC α or anti-phospho-PKC α antibodies. Activity of PKC α was expressed as the ratio of phospho-PKC α to total PKC α . **c** PKC α activity in the cytosol was measured using a non-radioactive protein kinase assay kit. Artery segments were stretched at a rate of 1 mm/s from L_i to 1.5 L_i and maintained in the stretched state for 15 min, or they were treated with OA (1 μ M) for 15 min. The segments were pretreated with Gö6976 (Gö; 1 μ M) or Y27632 (Y; 1 μ M) for 15 min before application of ST or OA (1 μ M). Data are means $\pm$ SEM of 5 experiments using 5 artery rings obtained from 5 different dogs. ^a $p < 0.01$ compared with control group and ^b $p < 0.01$ compared with nontreated group.

ylation of PKC α was increased by the 15-min stretch, and this increase was inhibited by Gö6976 (1 μ M) or Y-27632 (1 μ M), an inhibitor of rho kinase. OA (1 μ M) also increased the phosphorylation of PKC α . This phosphorylation was inhibited by Gö6976 (1 μ M), but not by Y-27632 (1 μ M). The increase in phosphorylated PKC α was not the result of changes in the total amount of PKC α because immunoblot analysis with anti-nonphosphorylated

PKC α antibody showed that lysate contained a similar amount of PKC α after stretching or OA treatment.

The 15-min stretch stimulated PKC α activity in the cytosol measured using a nonradioactive protein kinase assay kit, and the PKC α activity was inhibited by Gö6976 or Y-27632 (fig. 3c). OA also stimulated PKC α activity in the cytosol. The PKC α activity was inhibited by Gö6976, but not by Y-27632. Taken together, the results suggest

Fig. 4. Effects of stretching on PP2A. PP2A activity was measured with a Ser/Thr phosphatase assay kit. Artery segments were treated with Gö6976 (1 μ M) or Y-27632 (1 μ M) for 15 min. Then they were stretched at a rate of 1 mm/s from L_i to 1.5 L_i and maintained in the stretched state for 15 min in the presence of TEA (5 mM). Artery segments were quick-frozen in liquid nitrogen and were lysed for 30 min on ice in lysis buffer. The amount of PP2A in each reaction mixture was measured after immunoprecipitation with anti-PP2A antibody (inset). PP2A activity was expressed in arbitrary units (AU). Data are means $\pm$ SEM of 5 experiments using 5 artery rings obtained from 5 different dogs. ^a $p < 0.01$ compared with basal and ^b $p < 0.01$ compared with the nontreated group.

that phosphorylation of PKC α at Ser-657 causes its activation in the cytosol without any sustained translocation of PKC α from the cytosol to the membrane.

Effects of Stretching on PP2A Activity

We recently reported that OA at a low concentration (1 μ M) relaxed 80 mM KCl-induced contraction of the canine basilar artery with a concomitant decrease in the activity of PP2A, and an increase in triphosphorylated MLC₂₀ [17]. Thus, we investigated whether or not stretching also inhibited PP2A activity, leading to subsequent events such as triphosphorylation of MLC₂₀ and inhibition of contraction. The activity of PP2A, expressed in arbitrary units, which was significantly inhibited by the 15-min stretch, was reversed by Y-27632 (1 μ M) but not by Gö6976 (1 μ M) (fig. 4), indicating the involvement of

the rhoA/rho kinase system in the PP2A inhibition induced by stretching. The amount of PP2A measured using immunoblot analysis was not significantly different in any of the reaction mixtures (fig. 4, inset).

Physical Association of PKC α with PP2A

It has been proposed that activation of PP2A by stimuli leads to dephosphorylation and inactivation of PKC α and subsequent responses in smooth muscle cells [29]. A physical association between PKC α and PP2A was determined (fig. 5). Immunoprecipitation and Western blot analysis showed the constitutive expression of both PKC α and PP2A in both unstimulated and stretched basilar arteries (fig. 5a, b). Immunoprecipitates of PKC α , when probed with anti-PP2A catalytic subunit (PP2Ac) antibody, demonstrated the presence of PP2A in the unstimulated artery but not in the stretched artery (fig. 5c). To confirm further the association of PKC α and PP2A, lysates were immunoprecipitated with anti-PP2Ac antibody and then blotted with anti-PKC α antibody. The presence of PKC α was observed in the immunoprecipitations of PP2A from unstimulated basilar artery but not from the stretched artery (fig. 5d). Thus, PKC α and PP2A were physically associated in the basilar artery, and this association was inhibited by stretching.

Effect of Stretching and OA on Phosphorylation of CPI-17

Finally, we examined whether or not CPI-17 activity was involved in MLC₂₀ triphosphorylation by stretching or OA (fig. 6) because it has been reported that the inhibition of MLC phosphatase (MLCP) (PP1) through CPI-17 phosphorylated by PKC α and PKC δ also augments MLC₂₀ phosphorylation [30]. In the resting state (basal), a small amount of CPI-17 was phosphorylated. The phosphorylation of CPI-17 was increased by stretching, and this increase was inhibited by either Gö6976 (1 μ M) or Y-27632 (1 μ M). The phosphorylation of CPI-17 was also increased by OA (1 μ M). However, this increase was inhibited by Gö6976 (1 μ M) but not by Y-27632 (1 μ M).

Discussion

In the present study, we have demonstrated that activation of PKC α mediated by inhibition of PP2A is primarily involved in the stretch-induced triphosphorylation of MLC₂₀ in the canine basilar artery. Moreover, this mechanism has a self-inhibitory action on the contraction induced by stretching.

Fig. 5. Physical association between PKC α and PP2A. Representative Western blot obtained from 5 blotting analyses for each procedure. **a** Lysates of unstimulated (C) and stretched (ST) basilar arteries were immunoprecipitated (IP) with anti-PKC α antibody and probed (WB) with anti-PKC α antibody. **b** Lysates were immunoprecipitated with anti-PP2Ac antibody and probed with anti-PP2Ac antibody. **c** Lysates were immunoprecipitated with anti-PKC α antibody and probed with anti-PP2Ac antibody. **d** Lysates were immunoprecipitated with anti-PP2Ac antibody and probed with anti-PKC α antibody.

It has been reported that PKC α may be involved in stretch-induced contraction of the rat basilar artery [31, 32] and in pressure-induced contraction of the ferret coronary artery [33], and that stretching produces a sustained translocation of PKC α from the cytosol to the cell membrane. In the present study, however, the 15-min stretching caused triphosphorylation of MLC₂₀ mediated by PKC α without any sustained translocation of PKC α (fig. 1, 3). This response was accompanied by a stimulation of PKC α phosphorylation at Ser-657, and PKC α activity in the cytosol and was significantly blocked by a cPKC inhibitor Gö6976 (fig. 3). Phosphorylation of PKC α at Ser-657 accumulates the active enzyme [34]. To our knowledge, this is the first evidence demonstrating the activation of PKC α without any sustained translocation.

As is well documented, PP2A and PKC α are physically and functionally associated in various cells, including human and mouse mast cells [21], smooth muscle cells [29], and COS cells [35]. In vitro, PKC α activity can thus be inhibited through dephosphorylation by PP2A [36]. Our findings also showed that PKC α and PP2A were physically associated in the nonstretched canine basilar

artery, and these were dissociated by stretching through inhibition of PP2A activity (fig. 4, 5). OA, similar to stretch, increased PKC α phosphorylation at Ser-657 and PKC α activity (fig. 3) as well as MLC₂₀ triphosphorylation (fig. 1). PKC α phosphorylation at Ser-657 and PKC α activity stimulated by OA has been also reported in mast cells [21]. Thus, it is likely that the activity of PKC α is increased not only by phosphorylation of Ser-657 but also by the inhibition of PP2A in the cytosol, which may play an essential role in the stretch-induced MLC₂₀ triphosphorylation without any sustained translocation of PKC α .

Five possible sites of phosphorylation have been reported for MLC₂₀ of chicken gizzard: Ser-19 and Thr-18 for MLCK [37], and Ser-1, Ser-2 and Thr-9 for PKC [38–40]. In this study, stretching and OA increased MLC₂₀ triphosphorylation at both MLCK sites (Ser-19 and Thr-18) and the PKC site (Thr-9) in the canine basilar artery (fig. 1, 2). Of these sites, phosphorylation of the PKC site (Thr-9) induced by either stretching or OA was completely inhibited by Gö6976, indicating the primary role of PKC α in the phosphorylation of Thr-9. On the other

Fig. 6. Phosphorylation of CPI-17 induced by stretching and OA. **a** CPI-17 and phospho-CPI-17 (p-CPI-17) were identified by Western blot analysis with each specific antibody. The segments were pretreated with Gö6976 (1 μ M) or Y-27632 (1 μ M) for 15 min before application with stretch (ST) or OA (1 μ M). The segments were frozen in an acetone-dry ice and then homogenated. Homogenates were subjected to SDS-PAGE and immunoblotted with anti-CPI-17 or anti-phospho-CPI-17 (Thr-38) antibodies. **b** Quantitative data of phosphorylation of CPI-17. The results are expressed as the ratio of phospho-CPI-17 to the total CPI-17. Data are means $\pm$ SEM of 5 experiments using 5 artery rings obtained from 5 different dogs. ^a $p < 0.01$ compared with basal and ^b $p < 0.01$ compared with the Gö6976-untreated group.

hand, Gö6976 inhibited phosphorylation of Ser-19 and Thr-18 (MLCK sites) induced by OA to the resting level, but partially reduced these phosphorylations induced by stretching. These results suggest that in addition to phosphorylation of Thr-9 by PKC α , PKC α -dependent and -independent phosphorylation of Ser-19 and Thr-18 are involved in stretch-induced triphosphorylation of MLC₂₀ while only their PKC α -dependent phosphorylation is involved in OA-induced triphosphorylation of MLC₂₀.

We have reported that rhoA/rho kinase plays a key role in the mechanism underlying stretch-induced triphosphorylation in canine basilar artery [8]. Y-27632, an inhibitor of rho kinase, eliminated only the responses to the 15-min stretch, such as augmentation of PKC α activity (fig. 3) and inhibition of PP2A activity (fig. 4), whereas the inhibitor had no apparent effect on the similar responses to OA. These results suggest that activation of rho/rho kinase by stretching has an inhibitory action on PP2A; however, further studies will be necessary to determine whether rho/rho kinase phosphorylates PP2A directly.

Rho kinase phosphorylates MLCP at its myosin binding subunit (MYPT1), which leads to an elevation of MLC₂₀ phosphorylation at MLCK sites [41, 42]. We have suggested that MLCP inactivation by rho kinase seems to be involved in the stretch-induced phosphorylation of MLC₂₀ at Ser-19 and Thr-18 [8]. On the other hand, the crosstalk between PKC α and CPI-17 has also been reported as an alternative pathway, which leads to augmentation of phosphorylation of MLC₂₀ at MLCK sites [30, 43, 44]. Accordingly, CPI-17 phosphorylated by PKC α or PKC δ accelerates the inactivation of MLCP [30]. Phosphorylation of CPI-17 induced by stretching and OA was inhibited by Gö6976 (fig. 6). Thus, it seems possible that stretching and OA increase MLC₂₀ phosphorylation at Ser-19 and Thr-18 through the inhibition of MLCP by PKC α -mediated CPI-17 phosphorylation. MLC₂₀ phosphorylation at Thr-9 induced by stretching and OA was inhibited by Gö6976 (fig. 2). Taken together, the results suggest that the triphosphorylation of MLC₂₀ in response to stretching involves not only diphosphorylation of MLC₂₀ at Ser-19 and Thr-18 by the MLCP inhibition, which is mediated through phosphorylation by rho kinase and PKC α -mediated phosphorylation of CPI-17, but also additional phosphorylation of MLC₂₀ at Thr-9 by PKC α .

In vitro, OA blocks PP2A and 1 (PP1) activities at 0.1–10-nM concentrations, although it is 100-fold more effective against PP2A [45, 46]. In intact cells, higher concentrations (up to 1 μ M) are required to achieve an effect similar to that seen in vitro [47, 48]. MLC₂₀ phosphorylation induced by OA (1 μ M) was practically inhibited by Gö6976 (fig. 1, 2). OA-stimulated phosphorylation of CPI-17 was also inhibited by Gö6976 (fig. 6). Therefore, the present results suggest that OA (1 μ M) does not directly inhibit MLCP (PP1) in canine basilar artery.

Diphosphorylation of MLC₂₀ at Ser-1/2 and Thr-9 [38, 49] or monophosphorylation at Thr-9 [50], induced by PKC, inhibits the MLCK-induced increase in actin-activated myosin ATPase activity in vitro, and hence, would

be expected to counteract smooth muscle contraction. However, the studies with intact and permeabilized smooth muscle fibers, including rabbit aorta [28], porcine carotid artery [51] and bovine trachea [52], do not support the idea that PKC-catalyzed MLC₂₀ phosphorylation at Ser-1/2 plays a role in such physiological functions as smooth muscle contraction. In the present study, stretching produced the triphosphorylation of Ser-19, Thr-18 and Thr-9 without contraction (fig. 1, 2). This triphosphorylation has produced decreased shortening velocity and stiffness [8]. Gö6976 slowed the gradual reduction in stretch-induced contraction (fig. 1a). It has been considered that the phosphorylation of MLC₂₀ at Ser-19 and Thr-18 mediated by rho kinase and CPI-17 is involved in Ca²⁺ sensitization of smooth muscles to agonists [44]. We also recently reported that OA phosphorylated Thr-9 of MLC₂₀ in the canine basilar artery via PKC α , which led to the relaxation of 80 mM KCl-induced contraction [17]. Therefore, phosphorylation of MLC₂₀ at Thr-9 may act in a self-inhibitory manner on stretch-induced tension development.

We previously showed that BK channel blockers increased the contractile responsiveness to stretch [7]. Considering that the inhibition of BK channels under pathophysiological conditions such as intracellular acidosis [13] and type 1 diabetes [14] results in increasing vasoconstriction of cerebral artery, the inhibitory action mediated by MLC₂₀ triphosphorylation may play a role in the regulation of vascular smooth muscle contractility under physiological and pathophysiological conditions.

Taken together, we propose here the sequence of events involved in the triphosphorylation of MLC₂₀ and the concomitant reduction in the contraction in response to stretching (fig. 7). Stretching activates rho/rho kinase, and the activated rho kinase phosphorylates MLCP at its myosin-binding subunit, inactivating MLCP. Alternatively, rho/rho kinase also leads to the inactivation of PP2A, resulting in activation of PKC α . The activated PKC α also inactivates MLCP through CPI-17 phosphorylation. The inactivation of MLCP leads to an increase in the level of MLC₂₀ phosphorylation at Ser-19 and Thr-18 by MLCK. Furthermore, the activated PKC α phosphorylated MLC₂₀ at an additional site (Thr-9), resulting in the triphosphorylation of MLC₂₀ and the concomitant reduction in stretch-induced contraction. Inhibition of PP2A by a low concentration of OA also activates PKC α . Thus, a similar mechanism with the exception of rho-kinase-mediated phosphorylation of MLCP appears to underlie OA-induced triphosphorylation of MLC₂₀.

We have previously mentioned the physiological/pathophysiological significance of the multiple phos-

Fig. 7. Possible mechanisms involved in the stretch-induced MLC₂₀ triphosphorylation. Stretching activates the rho/rho kinase pathway in the canine basilar artery. Activated rho/rho kinase phosphorylates and inactivates MLCP. Inactivation of MLCP further produced MLC₂₀ diphosphorylation at MLCK sites (Ser-19 and Thr-18) (MLC₂₀-pp). MLC₂₀ diphosphorylation facilitates myogenic contraction. On the other hand, the activated rho/rho kinase also inactivated PP2A activity, resulting in activation of PKC α . The activated PKC α phosphorylates CPI-17 and inactivates MLCP, producing MLC₂₀ diphosphorylation at MLCK sites. Furthermore, the activated PKC α directly phosphorylates MLC₂₀ at an additional site (Thr-9), resulting in triphosphorylation of MLC₂₀ (MLC₂₀-ppp). The triphosphorylated MLC₂₀ counteracted the myogenic contraction. OA also activated PKC α through inactivation of PP2A, and produced triphosphorylation of MLC₂₀ (see Discussion).

phorylation of MLC₂₀ with special reference to the 'Bayliss effect' [8]. Briefly, we emphasize the notion that stretch-induced triphosphorylation of MLC₂₀, especially Thr-9 phosphorylation, may act in a self-inhibitory manner on tension development. We consider the Bayliss effect in a modern sense in that the myogenic response to hemodynamic factors such as pressure and stretch play an important role not only in the genesis of basal vascular

tone, but also in a brake mechanism via stretch-induced triphosphorylation of MLC₂₀ for excessive vasoconstriction (fig. 7).

In summary, we have provided evidence demonstrating that slow stretching induces inhibition of PP2A activity leading to activation of PKC α , which augments triphosphorylation of MLC₂₀. Of the triphosphorylated amino residues of MLC₂₀, the phosphorylated Thr-9 via PKC α has a self-inhibitory action on the stretch-induced contraction.

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