Arachidonic Acid Inhibits Myosin Light Chain Phosphatase and Sensitizes Smooth Muscle to Calcium*

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Arachidonic acid (AA) increases, at constant Ca\(^{2+}\), the levels of force and 20-kDa myosin light chain (MLC\(_{20}\)) phosphorylation in permeabilized smooth muscle, and slowed relaxation and MLC\(_{20}\) dephosphorylation. The Ca\(^{2+}\)-sensitizing effect of AA was not inhibited by inhibitors of AA metabolism (indomethacin, nordihydroguaiaretic acid, or propyl gallate), of protein kinase C (pseudopeptide) or by guanosine-5'-O-(\(\beta\)-thiodiphosphate) and was abolished by oxidation of AA in air. A non-metabolizable AA analog, 5,8,11,14-eicosatetraynoic acid also had Ca\(^{2+}\)-sensitizing effects. Extensive treatment with saponin abolished the Ca\(^{2+}\)-sensitizing effects of phorbol 12,13-dibutyrate and guanosine-5'-O-(\(\gamma\)-thiotriphosphate), but not that of AA. A purified, oligomeric MLC\(_{20}\) phosphatase isolated from gizzard smooth muscle was dissociated into subunits by AA, and its activity was inhibited toward heavy meromyosin but not phosphorylase. We conclude that AA may act as a messenger-promoting protein phosphorylation through direct inhibition of the form of protein phosphatase(s) that dephosphorylate MLC\(_{20}\) in vivo.

Calcium is the primary messenger of excitatory signal transduction in smooth muscle, and a major component of the rise in cytoplasmic Ca\(^{2+}\) evoked by excitatory agonists is the Ca\(^{2+}\)-released by inositol 1,4,5-trisphosphate from the sarcoplasmic reticulum as a result of activation of the phosphatidylinositol cascade (Somlyo et al., 1988). Pharmacomechanical coupling, the mechanism of contractile regulation in smooth muscle that can operate independently of the membrane potential (Somlyo and Somlyo, 1988), utilizes, in addition to the 1,4,5-trisphosphate message, a second physiological pathway that can modulate (increase or decrease) the Ca\(^{2+}\)-sensitizing force (Himpens et al., 1988; Kitazawa and Somlyo, 1990). Both pharmacomechanical Ca\(^{2+}\) release and Ca\(^{2+}\) sensitization are coupled to excitatory receptors by G-proteins. However, the two processes can be dissociated (Kobayashi et al., 1991), and certain agonists, such as the thromboxane analog, U46619 (Himpens et al., 1990; Bradley and Morgan, 1987), can strongly modulate (enhance) Ca\(^{2+}\) sensitivity, while causing only minimal or (in the absence of extracellular Ca\(^{2+}\)) no Ca\(^{2+}\) release.

Contraction of smooth muscle is triggered by the activation, by Ca\(^{2+}\)-calmodulin, of myosin light chain kinase that phosphorylates Ser-19 on the regulatory (20 kDa) myosin light chain (MLC\(_{20}\)), permitting the activation of myosin ATPase by actin (reviewed by Hartshorne (1987)). The major mechanism of relaxation, in turn, is dephosphorylation of MLC\(_{20}\) by myosin light chain phosphatase(s). Therefore, we had suggested that inhibition of MLC\(_{20}\) phosphatase is the mechanism of G-protein-coupled Ca\(^{2+}\) sensitization (Somlyo et al., 1989), and subsequently demonstrated that Ca\(^{2+}\)-sensitizing agonists and GTP\(\gamma\)S inhibit MLC\(_{20}\) phosphatase in permeabilized smooth muscle (Kitazawa et al., 1991b). However, MLC\(_{20}\) phosphatase is thought to be strongly associated with myosin filaments (Alessi et al., 1992; Dent et al., 1992; Sellers and Pato, 1984), whereas the surface receptors of Ca\(^{2+}\)-sensitizing agonists and, presumably, the G-proteins that couple the effector system that inhibits MLC\(_{20}\) phosphatase, are associated with the plasma membrane. Therefore, the messenger or cascade that relays the inhibitory message from a surface membrane-bound G-protein to the filament-bound protein phosphatase remains to be identified. In the present study, we explored the possibility that AA, or related lipid metabolites that can affect their respective surface receptors and act as potential autocrine regulators (Needleman et al., 1986). In addition, AA itself can activate protein kinase C (Kikkiawa et al., 1988; Nishizuka, 1989; McPhail et al., 1984), and phorbol esters, well-known activators of protein kinase C, have Ca\(^{2+}\)-sensitizing effects on smooth muscle (Chatterjee and Tejada, 1986; Park and Rasmussen, 1985; Itoh et al., 1988).

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¶¶ The abbreviations used are: MLC\(_{20}\), 20-kDa myosin light chain; AA, arachidonic acid; ML-9, 1-(5-chloronaphthalenesulfonyl)-1H-hexahydropyrimidin-1-4-diazepine; ETYa, 5,8,11,14-eicosatetraynoic acid; Me3SO, dimethyl sulfoxide; HMM, heavy meromyosin; PDBu, phorbol-12,13-dibutyrate; PP1, protein phosphatase-1; SM-PP1M, smooth muscle protein phosphatase-1M; GTP\(\gamma\)S, guanosine-5'-O-(\(\gamma\)-thiotriphosphate; GDT\(\gamma\)S, guanosine-5'-O-(\(\beta\)-thiodiphosphate).

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Nishimura et al., 1990; Ruzycky and Morgan, 1989; Gupta et al., 1990). In several cell systems, agonists can activate phospho-
kinase C; and increase of AA through a G-
protein-coupled pathway that can also be activated by GTPγS or aluminum fluoride (Buckley et al., 1991; Burch et al., 1986; Jelsma, 1987; Narasimhan et al., 1990). Consequently, it has been proposed that AA can act as a cellular messenger (Axelrod et al., 1988; Burch, 1989).

In the present study, we examined the effects of AA on force development and relaxation, and on myosin light chain phosphorylation and dephosphorylation, in permeabilized smooth muscle. We also determined the effects of AA on the activity and oligomeric state of an isolated smooth muscle myosin light chain phosphatase. Our results show that (un-
metabolized) AA can inhibit both purified myosin light chain phosphatase and myosin light chain dephosphorylation in situ, while sensitizing contraction to Ca++/through a mecha-
nism that requires neither activation of protein kinase C nor the "downstream" intervention of a G-protein.

MATERIALS AND METHODS

Small strips (2-3 mm long and 100-200 μm wide for tension measurements or 300-400 μm wide for MLCP₀ phosphorylation mea-
surements) of rabbit femoral artery were dissected and stretched to 1.5 times resting length. Isometric tension was measured with a force
transducer (AE 801; AME, Horten, Norway) in a well on a "bubble"
plate (Horiuti, 1988). In preliminary experiments, we found that
reducing the temperature from 37 °C to room temperature (22-24 °C)
lengthened the lag time (between addition of AA to the bath and the
onset of contraction) and the contraction half-time from, respectively,
1 ± 0.3 min (n = 5) to 12 ± 3.5 min (n = 5) (p < 0.05) and from 14
± 0.4 min (n = 4) to 46 ± 11.6 min (n = 4) (p < 0.01). Therefore,
unless noted otherwise, experiments were conducted at 37 °C. After
steady responses to high K⁺ were observed, the strips were incubated
in normal relaxing solution (Ca++/free, 1 mM EGTA) for about 5 min,
and permeabilized by 90-75 min of incubation, at room temperature,
with 5,000-10,000 units/ml (based on rabbit red blood cell hemolysis)
of Staphylococcus aureus α-toxin (GIBCO). The higher concentration
of α-toxin (10,000 units/ml) and longer incubation time (75 min)
were used when multiple strips were permeabilized simultaneously.
For permeabilization with saponin, the strips were incubated with
200 μg/ml saponin for 22 min. Saponin was freshly dissolved in
Me₂SO just before use: the final Me₂SO concentration in the bath
was 1%.

For the introduction of protein kinase C pseudosubstrate peptide into the striated muscle, smooth muscle strips were permeabilized by 20 min of incubation with 50 μM β-escin (Kobayashi et al., 1989).

To deplete the sarcoplasmic reticulum of calcium, all permeabilized strips were treated with A23187 (10 μM) for 10 min in relaxing
solution (Kitazawa et al., 1989; Kobayashi et al., 1991a).

Phosphorylation of MLCP₀ was measured with two-dimensional isoelectric focusing and sodium dodecyl sulfate-gel electrophoresis, as previously published (Kitazawa et al., 1991a). The dephosphorylation and relaxation rate measurements were carried out at 20 °C (Kitazawa et al., 1991b) on strips rapidly frozen in freon-22 cooled by liquid nitrogen at the indicated intervals.

Details of the solutions used for studies on permeabilized strips were described previously (Kitazawa et al., 1988; Kobayashi et al., 1989, 1991). Calmodulin (1 μM) was added to the Ca++/containing activating solution for experiments employing saponin and β-escin permeabilization.

AA α-toxin was purchased from GIBCO/BRL, GDPS, and GTPγS from Boehringer Mannheim, and saponin from ICN Nutri-
tional Biochemicals, Cleveland, OH. AA, myristic acid, oleic acid, phosphatidic acid (dimyristoyl- and dioleoyl-phosphatidic acid), lysophosphatidic acid (myristoyl- and oleoyl-lysophosphatidic acid), β-escin, PDBu (phorbol-12,13-dibutyrate), and ML-9 (1-(5-chloronaphthalenyl)-1H-hexahydro-1,4-diazepine) were pur-
chased from Sigma and EHTA (5,8,11,14-eicosatetraynoic acid) from Cayman Chemical Co., Ann Arbor, MI. Protein kinase C pseudosub-
strate peptide (19-36 sequence: Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-
Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn) was purchased from Bachem, Torrence, CA. AA was dissolved in ethanol or in Me₂SO and stored at -20 °C in the dark under nitrogen. The

stock solutions were diluted immediately before use, and the final solvent concentrations in bath were 1%. In control experiments in which the strips were exposed to Me₂SO or ethanol (1% each) for the same length of time as the exposures to AA, 1% of either solvent was added to the strips before addition of the fatty acids with the given concentration, to compensate for any solvent-dependent, "onset of
Ca++/" sensitizing effect, whereas 1% Me₂SO had a slight depressant effect on contraction. The solutions containing Me₂SO 1% or ethanol 1% were exchanged for a solution containing Me₂SO 1% or ethanol 1% in addition to AA or the other fatty acids used. The effect of AA was not significantly different regardless of the solvent used (p > 0.05 at 1, 30, 100, and 300 μM AA), and the results are combined. Similarly, there was no significant difference between the Ca++/sensitizing effect of AA purchased from either Sigma or from Cayman Chemical Co. Smooth muscle protein phosphatase-1M (SM-PP1M) and 32P-

labeled smooth muscle heavy meromyosin (Deni et al., 1992) and 32P-
labeled phosphorylase (Cohen et al., 1988) were prepared as described. 32P-Labeled substrates were prepared using [γ³²P]ATP with a specific radioactivity of 1-2 × 10⁶ cpmp/mmol. 32P-Labeled phosphorylase contained 1.0 mol of phosphate/mol of subunit and 32P-labeled heavy meromyosin (0.9-1.0 mol of phosphate/mol of P-light chain).

Protein phosphatase assays were carried out as described (Cohen et al., 1988; Cohen, 1991) in 50 mM Tris-HCl, 0.1 mM EGTA (pH 7.0 at 25 °C), 0.1% (v/v) 2-mercaptoethanol, 0.03% Brij 35 (solution A). The substrate concentrations were 10 μM (phosphorylase) and 0.3 μM (heavy meromyosin (HMM)). Assays (30 μl) were carried out at 30 °C in the presence of varying concentrations of AA or oleic acid, and caffeine (5 mM) was added when phosphorylase was the substrate. The AA was obtained from a 100 mM stock solution dissolved in Me₂SO and stored at -70 °C. The small amount of Me₂SO carried over into the assays (<0.5% by volume) had no effect on either phospholipase phosphatase or myosin phosphatase activity. Oleic acid (sodium salt) was stored at -20 °C, as a 1 mM aqueous solution in solution A. The dephosphorylation of both heavy meromyosin and phosphorylase was limited to <20% to ensure that rates of dephos-
phorylation were linear with respect to time. 1 milliunit of activity
was defined as the rate of dephosphorylation of 1 μmol of substrate
per mg of protein per hour. 

The Effect of Free Fatty Acids on the Ca++/sensitivity of α-
Toxin-permeabilized Femoral Artery Smooth Muscle—Free AA, dissolved in ethanol or Me₂SO, caused contraction of α-
toxin-permeabilized rabbit femoral artery strips in the pres-
ence of submaximal Ca++/ (pCa 6.7) buffered with 10 mM EGTA (Figs. 1 and 2). AA (300 μM) caused 79 ± 2.4% (n = 9) of the maximal Ca++/induced contraction (Figs. 1 and 2) and increased MLCP₀ phosphorylation from 11 ± 2.3% (n =

\[ \text{pC}\text{a}_{\text{Ca}} \text{= 6.7, Me}_2\text{SO or ethanol} \]
depolarizing the membrane with 30 mM K\(^+\), 300 deflection in each force record, from 26 to 37

\[\pm 5.0\%\], \(n = 3, p < 0.001\) of the maximal, 154 mM K\(^+\)-induced contraction; these contractions were transient (reached peak at about 5 min) and were followed by relaxation near basal tension. ETYA (100 \(\mu\)M), a non-metabolizable analog of AA, also had a biphasic (small contraction followed by relaxation) effect, similar to that of AA, on submaximally contracted, non-permeabilized smooth muscle (\(n = 3\)).

Ca\(^{2+}\) Sensitization Is Not Mediated by the Metabolic Products of Fatty Acids—AA can be metabolized to biologically active products in cells (Needleman et al., 1986). To determine whether Ca\(^{2+}\) sensitization is the direct effect of AA or of its metabolites, we determined the effects of inhibition of AA metabolism on the Ca\(^{2+}\)-sensitizing effect of AA.

Propyl gallate (100 \(\mu\)M), an antioxidant that inhibits both cyclooxygenase and lipoxygenase (Smith et al., 1985; Rainesford, 1988), did not inhibit, but rather increased the effect of AA by shifting the dose-response curve to the left (Fig. 3). The combination of both a cyclooxygenase inhibitor, indomethacin (100 \(\mu\)M), and a lipoxygenase inhibitor, nordihydroguaiaretic acid (100 \(\mu\)M), also did not inhibit AA-induced Ca\(^{2+}\) sensitization (data not shown).

ETYA is an isomorphomic competitive inhibitor of AA in which four alkyne bonds replace four alkene bonds. ETYA competitively inhibits the uptake of AA into cellular membranes and inhibits lipoxygenase and, at higher concentrations, cyclooxygenase enzymes (Anderson et al., 1989). ETYA also had a Ca\(^{2+}\)-sensitizing effect on \(\alpha\)-toxin-permeabilized femoral artery. 100 \(\mu\)M ETYA caused 28 ± 7.6\% (\(n = 4\)) of the maximal Ca\(^{2+}\)-induced contraction in the presence of a submaximally activating concentration of Ca\(^{2+}\) (pCa 6.7).

The Ca\(^{2+}\)-sensitizing effect of AA was significantly decreased by exposing it for 48 h to air and light at room temperature. 100 \(\mu\)M of this oxidized AA did not cause any Ca\(^{2+}\) sensitization (pCa 6.7) but freshly prepared AA subsequently added to the same strips caused 47 ± 28\% (\(n = 4\)) of the maximal Ca\(^{2+}\)-induced contraction. This response is lower than in the absence of oxidized AA and suggests an inhibitory effect of the latter.

\textbf{Arachidonic Acid-induced Ca\(^{2+}\) Sensitization Is Not Mediated by a G-protein or by Protein Kinase C—The Ca\(^{2+}\) sensitizing effect of AA was not inhibited by 1 mM GDP-\(\beta\)S (Fig. 4, \(n = 5\)), whereas similar exposure to 1 mM GDP-\(\beta\)S inhibited by 87 ± 6.7\% (\(n = 7\)) the Ca\(^{2+}\) sensitization induced by AA (control = 5.0\%, \(n = 15, p < 0.001\)).

![Fig. 2. Dose-response curve of free fatty acid-induced Ca\(^{2+}\) sensitization of force in pCa 6.7 solution in \(\alpha\)-toxin-permeabilized rabbit femoral artery. Same protocol was used as in Fig. 1. Single doses of each concentration of fatty acids were added to individual strips (\(n = 3-6\) for each point). Force is normalized to the second (maximal) concentration elicited with pCa 5 solution.](image)

![Fig. 3. Propyl gallate- (100 \(\mu\)M) induced shift of the force dose-response curve to AA. Strips were preincubated for 15 min with 100 \(\mu\)M propyl gallate prior to non-cumulative addition of AA (\(n = 3-9\) for each point). \(*p < 0.05; \**p < 0.01.](image)
in the same strips by 100 μM phenylephrine with 10 μM GTP (Fig. 4).

Extensive permeabilization with saponin removes the G-protein or uncouples it from the Ca2+-sensitizing effector (myosin light chain phosphatase inhibitory) system (Kitazawa et al., 1991b). As shown in Fig. 5, the G-protein-mediated step(s) was (were) abolished in strips extensively permeabilized with saponin (200 μg/ml, 22 min of incubation), as indicated by the lack of response to GTPγS (100 μM). After such treatment, there was also no detectable response to a saturating concentration (10 μM) of PDBu, an activator of protein kinase C, whereas in α-toxin-permeabilized femoral artery strips PDBu induced 74 ± 2.0% (n = 4) of the maximal Ca2+-induced contraction. AA (300 μM) caused 61 ± 4.8% (n = 5) of the maximal Ca2+-induced contraction in the same strips (Fig. 5). A peptide pseudosubstrate inhibitor of kinase C (House and Kemp, 1988) (10 μM), added 15–20 min before AA (100 μM) also did not affect the AA-induced Ca2+ sensitization in β-escin-permeabilized femoral artery.

**MLC20 Dephosphorylation and Relaxation Are Inhibited by Arachidonic Acid—Agonist- and GTPγS-induced Ca2+ sensitization is mediated through inhibition of MLC20 phosphatase (Kitazawa et al., 1991b) Therefore, to determine whether AA operates through the same mechanism, we examined its effect on MLC20 dephosphorylation.**

The effects of AA on the rates of contraction and relaxation and on the rate of MLC20 dephosphorylation were determined at 20 °C. In the absence of Ca2+, at this temperature, the preincubation with AA itself did not cause contraction. Preincubation with 300 μM AA for 30 min did not change the rate of force development in response to pCa 5, but it significantly slowed relaxation. The half-time of pCa 5-induced contraction was 1.9 ± 0.06 min (n = 11) in the absence and 2.0 ± 0.05 min (n = 13, p > 0.05) in the presence of AA (300 μM, 30 min of preincubation). To measure the rate of relaxation, strips were activated with pCa 5 and, after force reached a plateau, incubated in relaxing solution containing 10 mM EGTA (pCa > 8) and 100 μM ML-9 to inactivate MLC20 kinase (Kitazawa et al., 1991b). AA (300 μM) prolonged the half-time of relaxation from 3.5 ± 0.45 min (n = 6) to 6.7 ± 0.82 min (n = 6, p < 0.01).

To assess more directly the effect of AA on MLC20 phosphatase activity in situ, we determined the rate of MLC20 dephosphorylation of muscles in Ca2+- and ATP-free, 10 mM EGTA and 100 μM ML-9-containing relaxing solution. After activation with maximal Ca2+ (pCa 5) for 15 min, strips were incubated in the above Ca2+-free solution and rapidly frozen after 1, 2, 3, and 6 min. As shown in Fig. 6, AA slowed down the rate of MLC20 dephosphorylation (p < 0.001 at 2 and 3 min).

**Arachidonic Acid and Oleic Acid Inhibit Purified Smooth Muscle Protein Phosphatase 1M by Dissociating It into Subunits—**The major protein phosphatase that dephosphorylates MLC20 of smooth muscle myosin was recently purified from avian gizzard and shown to be composed of three subunits, with apparent molecular masses of 130, 37, and 20 kDa. The 37-kDa component was identified as the β-isoform of protein phosphatase-1 (PP1), while the 130- and 20-kDa components formed a regulatory complex that enhanced, by about 3-fold, the myosin phosphatase activity of PP1 and suppressed phosphorylase phosphatase activity by 80% (Alessi et al., 1992; Cohen et al., 1992).

In the present study, heavy meromyosin was used in the assays because of its much greater solubility in the low ionic strength buffers used to assay the phosphatase. As shown in Fig. 7A, AA (20 μM) produced a small (30%) activation of the myosin phosphatase activity of SM-PP1M but was strongly inhibitory at higher concentrations, with 50% inhibition occurring at about 60 μM AA. In contrast, phosphorylase phosphatase activity was inhibited by about 50% up to 20 μM AA and activated at the higher concentrations which suppressed myosin phosphatase activity. Oleic acid had effects similar to those of AA, except that higher concentrations were required.
to observe equivalent inhibition and activation of myosin phosphatase and phosphorylase phosphatase activity (Fig. 8B). For example, 250 μM oleic acid was required for 50% inhibition of the myosin phosphatase activity as compared to 60 μM for AA.

The inhibition of myosin phosphatase which occurs above 30 μM AA, or above 150 μM oleic acid, is similar to that observed previously when the regulatory 130-20 kDa complex is dissociated from the 37-kDa catalytic subunit in the presence of high concentrations of the chaotrope LiBr (Alessi et al., 1992). In order to investigate whether the effects of AA and oleic acid also resulted from dissociation of the subunits of SM-PP1M, the purified phosphatase was incubated for 15 min at 30 °C with 300 μM AA and then subjected to gel filtration on Superose 12 in buffer containing 300 μM AA. As shown in Fig. 8, the catalytic activity eluted from the column with an apparent molecular mass of about 35 kDa, whereas the native enzyme elutes from Superose 12 with an apparent molecular mass of about 500 kDa (Fig. 8, open circles). Similar results were obtained when SM-PP1M was incubated with 400 μM oleic acid and subjected to gel filtration in buffer containing 400 μM oleic acid (data not shown). The dissociated catalytic subunit of SM-PP1M was not inhibited by AA up to 200 μM.

The major findings of this study are that AA can sensitize smooth muscle to Ca²⁺ by inhibiting dephosphorylation of MLC₂₀ in situ, and that it also dissociates and inhibits an oligomeric myosin light chain (MLC₂₀) phosphatase isolated from smooth muscle.

The increase in force elicited by AA at constant Ca²⁺ concentration was accompanied by an increase in MLC₂₀ phosphorylation. This response is identical to that seen during Ca²⁺ sensitization induced by excitatory agonists (Kitazawa et al., 1991a) and GTPγS (Kitazawa et al., 1991a; Fujiwara et al., 1989; Nishimura et al., 1990; Kubota et al., 1992). The slowing of relaxation and MLC₂₀ dephosphorylation by AA (this study) also parallels the inhibition of relaxation and dephosphorylation by Ca²⁺-sensitizing agonists and GTPγS (Kitazawa et al., 1990); GTPγS also inhibits the HMM-phosphatase activity of smooth muscle homogenates (Kubota et al., 1992).

The Ca²⁺-sensitizing effects of AA do not appear to be mediated by any of the products of the known metabolic pathways of AA, as they were not inhibited by either cyclooxygenase (indomethacin) or lipoxygenase (nordihydroguaiaretic acid) inhibitors, but to the contrary, were enhanced by propyl gallate (Fig. 3), a general inhibitor of AA metabolism. Furthermore, both oleic acid, that is not a direct source of AA metabolites, and a non-metabolizable AA analog, ETYA, had similar, although less pronounced, Ca²⁺-sensitizing effects on force development, while oxidation of AA in air abolished its Ca²⁺-sensitizing effect. Finally, the inhibitory effect of AA on a purified smooth muscle myosin light chain phosphatase provides clear evidence of the effectiveness of the non-metabolized fatty acid.

The effects of AA do not appear to be mediated by a G-protein because, unlike the effects of Ca²⁺-sensitizing agonists and GTPγS (Fig. 4) (Himpens et al., 1990; Kitazawa et al.,

![Fig. 7. Effect of AA (panel A) and oleic acid (panel B) on heavy meromyosin phosphatase and phosphorylase-phosphatase activities of purified SM-PP1M. Heavy meromyosin dephosphorylation was assayed at the indicated concentrations of unsaturated fatty acids for 5 min at 0.02 millunits/ml myosin phosphatase activity (0), and phosphorylase-dephosphorylation for 10 min at 0.3 millunits/ml phosphorylase-phosphatase activity (●).](image)

![Fig. 8. Dissociation of the 37-kDa catalytic subunit from the regulatory subunits of SM-PP1M in the presence of 300 μM AA. A 0.02-ml aliquot of SM-PP1M at 290 millunits/ml phosphorylase-phosphatase activity was made 300 μM in AA by the addition of an equal volume of 600 μM AA (in solution A) and incubated for 15 min at 30 °C. The phosphatase was then subjected to gel filtration on a 30 x 1.0-cm column of Superose 12 equilibrated in freshly prepared solution A containing 5% (v/v) glycerol, 0.2 M NaCl, and 300 μM AA. The flow rate was 0.5 ml/min. Fractions of 0.25 ml were collected and assayed for phosphorylase-phosphatase (●). The open circles show a control experiment in which AA was omitted. The arrows denote the positions of the marker proteins, ferritin (450 kDa), bovine serum albumin (66 kDa), and ovalbumin (43 kDa).](image)
Phosphatase activity (Fig. 4) and dissociation of the oligomeric enzyme can account for the Ca\(^{2+}\)-sensitizing effects of AA were observed even after the effects of GTPgammaS were abolished by extensive permeabilization with saponin (Fig. 5).

Protein kinase C is also unlikely to be a necessary mediator of the Ca\(^{2+}\)-sensitizing effect of AA, although AA and other unsaturated fatty acids (Nishizuka, 1989; Kikkawa et al., 1988; Khan et al., 1992; McPhee et al., 1984; Sutton and Haebeler, 1990) can activate kinase C in several systems. Phorbol esters, activators of kinase C, are also reported to stimulate phospholipase A2 (Carter et al., 1989) and have Ca\(^{2+}\)-sensitizing effects on smooth muscle (Chattejee and Tejada, 1986; Ruzyczky and Morgan, 1989). However, a pseudosubstrate peptide inhibitor of kinase C (House and Kemp, 1988) did not inhibit AA-induced Ca\(^{2+}\)-sensitization that, furthermore, was also present after extensive permeabilization with saponin that abolished the effect of PDBu (Fig. 5).

The effects of AA on permeabilized smooth muscle could also not be ascribed to stimulation of MLC\(_{\text{cp}}\) kinase; AA did not affect the rate of force development and, furthermore, it is reported to inhibit purified MLC\(_{\text{cp}}\) kinase (Kigoshi et al., 1990). The relaxing effect of AA (and of its non-metabolizable analog, ETYA) on depolarized, submaximally contracted, intact (non-permeabilized) smooth muscle may have been due to inhibition of Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels (Shimada and Somlyo, 1992) and/or to stimulation of guanylate cyclase (Laychock, 1989). However, since we neither monitored nor buffered cytoplasmic Ca\(^{2+}\) in these experiments (on intact smooth muscle), the mechanism(s) of AA action on intact smooth muscle remains to be identified.

The catalytic subunit of MLC\(_{\text{cp}}\) phosphatase in avian smooth muscle is identical to PP1, and it is now well established that PP1 is complexed to a 130-kDa subunit which, in turn, interacts with a 20-kDa protein (Cohen et al., 1992; Alessi et al., 1992). The inhibition of this trimeric MLC\(_{\text{cp}}\) phosphatase by AA is of considerable interest, not only because it provides evidence of the direct effect of AA on the enzyme, but also because the inhibitory effect appears to result from dissociation of the catalytic subunit. It has been established previously that dissociation of the catalytic subunit induced by high concentrations of LiBr decreases the rate of dephosphorylation of myosin, while at the same time increasing the rate of dephosphorylation of glycogen phosphorylase, and that these effects are reversed when the catalytic subunit and the regulatory subunits are recombined (Alessi et al., 1992). Similarly, AA (above 20 \(\mu M\)) increases phosphorylase phosphate and decreases myosin phosphate activity (Fig. 7) and promotes dissociation of the MLC\(_{\text{cp}}\) phosphatase holoenzyme (Fig. 8). It will be of great interest to determine whether in muscle, as in solution, such dissociation of the oligomeric enzyme can account for the inhibitory effect of AA and possibly other endogenous (e.g. fatty acid) phosphatase inhibitors. The reason for the increase in myosin phosphatase activity and decrease in phosphorylase phosphatase activity in the presence of up to 20 \(\mu M\) AA and up to 100-150 \(\mu M\) oleic acid is unknown, although preliminary experiments suggest that it may be explained by dissociation of the 20-kDa subunit from the 130-kDa component. However, the low concentrations of AA that activated HMM phosphatase in solution did not relax pre-contracted smooth muscle (unpublished observation), and 30 \(\mu M\) AA had a contractile effect, indicative of inhibition of MLC\(_{\text{cp}}\) phosphatase (Fig. 2). The reason for this discrepancy, whether due to different experimental conditions or dissociation between force and MLC\(_{\text{cp}}\) phosphorylation due to cooperativity (Somlyo et al., 1988), remains to be determined.

The question arises whether, as suggested in the scheme below, AA is also a Ca\(^{2+}\)-sensitizing messenger released by agonists under physiological conditions.

\[
\text{Agonist} \rightarrow \text{receptor} \rightarrow \text{G-protein} \rightarrow^{+} \text{PLA}_2 \rightarrow^{+} \text{AA} \rightarrow^{+} \text{MLC}_{\text{cp}} \text{phosphatase}
\]

The concentrations of AA required for Ca\(^{2+}\)-sensitization and for inhibition of MLC\(_{\text{cp}}\) phosphorylation (present study) are similar to the increase (38-75 \(\mu M\)) in AA content of pancreatic islets stimulated with glucose (Wolf et al., 1991). Prolonged elevation (6-fold over 8 min) of AA following stimulation with vasopressin has been observed in a cultured smooth muscle cell line (Grillone et al., 1988). Ca\(^{2+}\) sensitization by agonists and GTPgammaS (Somlyo et al., 1989) is also similar to that by AA (present study), in being associated with a relatively long lag period preceding force development. However, at room temperature (present study), the time course of the response to AA is much slower than the Ca\(^{2+}\)-sensitizing effect of agonists. It will be necessary, therefore, to pursue the kinetics of AA transients (in progress) to determine whether the requisite increase in AA concentration occurs during agonist-induced Ca\(^{2+}\) sensitization in smooth muscle, and whether the slower time course of the effect of added AA than that of agonists reflects the slower diffusion and access of exogenous, rather than endogenous AA to its myosin light chain phosphatase target. It is also possible that transport of endogenous AA (or other lipid) is accelerated by a fatty acid-binding protein. Finally, as in the case of G-protein-mediated MLC\(_{\text{cp}}\) phosphatase inhibition (Kitazawa et al., 1991), we suggest that inhibition of protein phosphatase(s) by AA and/or other fatty acid messengers may also have regulatory functions in other cell systems.

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**Note Added in Proof**—After this paper had been completed, we became aware of studies (De Mey and Vanhoutte, 1982; Singer and Peach, 1983) implicating endothelial cell derived factors in the relaxation of intact (non-permeabilized) vascular smooth muscle by arachidonic acid. We note that the relaxant effect of arachidonic acid on non-permeabilized femoral artery smooth muscle observed in the present study was still present after surgical stripping of the endothelium. The endothelium was removed by scraping from all the permeabilized smooth muscles used for the studies of the Ca\(^{2+}\)-sensitizing effects of arachidonic acid.

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**2** D. Alessi, unpublished results.
