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DIRECT INHIBITION OF SMOOTH MUSCLE MYOSIN LIGHT CHAIN KINASE BY ARACHIDONIC ACID IN A PURIFIED SYSTEM

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The direct effect of arachidonic acid (AA) on the phosphorylation of smooth muscle myosin light chain (SMLC) by smooth muscle myosin light chain kinase (SMLCK) was assessed in a purified system. AA inhibited the phosphorylation of SMLC by SMLCK in a dose dependent manner. Increasing the amount of calmodulin (59 nM and 590 nM) did not reverse this inhibition. Linoleic acid and oleic acid also inhibited the phosphorylation. The inhibitory potency of these unsaturated fatty acids paralleled the number of cis double bonds. These results show that SMLCK is directly inhibited by unsaturated fatty acids including AA.

Since it was shown that arachidonic acid (AA) directly activates a Ca^{2+}/phospholipid-dependent protein kinase (1),the effect of AA as a second messenger on the regulation of cellular functions has been extensively studied (2-4). The direct effect of AA on Ca^{2+}/calmodulin (CaM)-dependent protein kinases, however, remains to be determined to our knowledge. Smooth muscle myosin light chain kinase (SMLCK), a Ca^{2+}/CaM-dependent protein kinase, is well characterized kinase whose functional role has been established in smooth muscle and nonmuscle cells (5). The present study will show a direct effect of AA on the phosphorylation of smooth muscle myosin light chain (SMLC) by SMLCK.

Methods

SMLC and SMLCK purified from turkey gizzard (6) were the gifts from Dr J.R. Sellers, the Laboratory of Molecular Cardiology,

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NHLBI, NIH. Assays were performed at 30 ℃ for 2 min in a reaction mixture containing 20 mM Tris/ HCl (pH 7.5), 5 mM magnesium acetate, 0.2 mM calcium chloride, 100 μM (γ-^{32}P)ATP (specific activity, 3,000 Ci/mmol, New England Nuclear), 5.9 nM CaM, 1 mg/ml of SMLC and 2 μg/ml of SMLCK in the presence or absence of AA (50 nM - 500 μM) solved in ethanol (final concentration, 5 %). Linoleic acid , oleic acid, diacylglycerol and phosphatidylserine, solved in ethanol, were also used instead of AA. The enzyme reactions were terminated by transferring a 20-μl aliquot to a paper filter (1.5 x 1.5 cm, 3 MM, Whatman) and immersing in ice-cold 10 % (w/v) trichloroacetic acid. Paper filters were washed twice in 10 % (w/v) trichloroacetic acid solution, then once in 95 % (v/v) ethanol solution. Finally, the filters were rinced with acetone, dried, and placed into scintillant for β-spectrometry. In some experiments, the radioactive SMLC was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, autoradiography and densitometry as described previously (7).

Results

As shown in Fig. 1, SMLCK was activated in a Ca^{2+}/CaM-dependent manner even in the presence of solvent (5 % ethanol) (lanes 1, 2 and 3). In this assay condition, the phosphorylation of SMLC

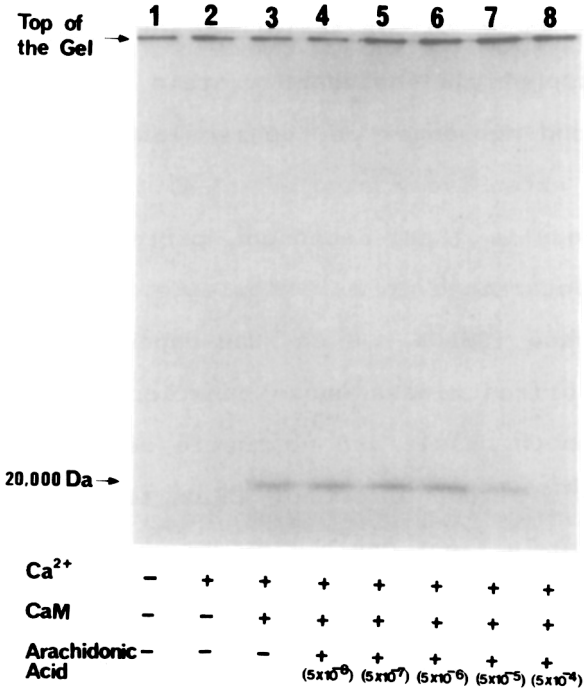

Fig. 1. Autoradiogram showing the effect of AA on the phosphorylation of SMLC by SMLCK. In lanes 1, 2 and 3, the solvent (5 % ethanol) was added instead of AA. In lane 1, 1 mM of EGTA (ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid) was added instead of the addition of calcium chloride.

was inhibited almost completely by 500 μM of AA (n=5) (Fig. 1, lane 8), and partially by 50 μM of AA (48-55 %, n=5) (Fig. 1, lane 7) as compared with that in the presence of solvent alone (Fig. 1, lane 3). Less than 5 μM of AA had no such an inhibitory effect (Fig. 1, lanes 4 and 5).

To assess whether the AA-induced inhibition of the phosphorylation of SMLC is due to the antagonistic action to CaM, the effects of various concentrations of CaM (5.9 nM, 59 nM and 590 nM) on this inhibition were examined (Fig. 2). In these assay conditions, SMLCK activities increased linearly during the incubation time (2 min) (data not shown), and the rate of ^{32}P incorporated in to SMLC reached a plateau at the concentration of 59 nM of CaM as indicated in the center of Fig. 2. Increasing the amount of CaM (59 nM and 590 nM), however, did not reverse the AA induced-inhibition of the phosphorylation of SMLC (Fig. 2).

Next, the effects of other unsaturated fatty acids (linoleic acid and oleic acid) and phospholipids (diacylglycerol and phos-

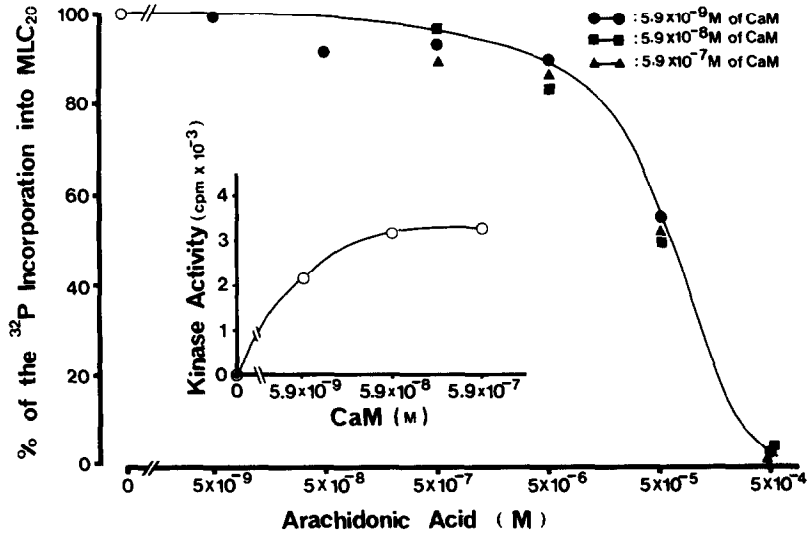

Fig. 2. Effect of AA on the phosphorylation of SMLC by SMLCK in the presence of various concentrations of CaM. The results are expressed as the percentage of the amount of ^{32}P incorporated into SMLC in the presence of AA when compared with that in the presence of the solvent alone as 100 %.

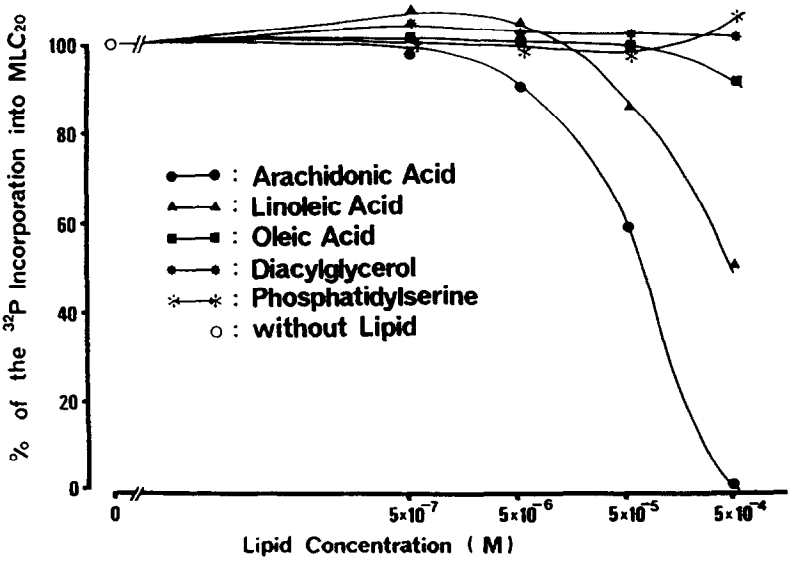

Fig. 3. Effects of unsaturated fatty acids and phospholipids on the phosphorylation of SMLC by SMLCK. Values plotted are the means of three separate experiments.

phatidylserine) on the phosphorylation of SMLC were also examined. As shown in Fig. 3, the phosphorylation of SMLC was inhibited slightly by 500 μM of oleic acid (93-96 %, n=3) and by 50 μM of linoleic acid (88-94 %, n=3), and moderately by 500 μM of linoleic acid (45-51 %, n=3) as compared with that in the presence of solvent alone as 100 % (Fig. 3). The phosphorylation of SMLC was not inhibited by diacylglycerol and phosphatidylserine (Fig. 3). The doses of the half-maximum inhibition of SMLCK were about 50 μM in AA and about 500 μM in linoleic acid, respectively (Fig. 3).

Discussion

The present study clearly demonstrates that SMLCK is directly inhibited by AA in a purified system. SMLCK has been shown to be phosphorylated in vitro by a cAMP-dependent protein kinase (8, 9) and a Ca^{2+}/phospholipid-dependent protein kinase (10). The phosphorylation of SMLCK by these kinases results in a decrease in SMLCK activity through a decrease in its binding affinity to CaM (8-10). These in vitro findings probably imply an intracellular

modulation of SMLCK activity by a cAMP-dependent protein kinase and a Ca^{2+}/phospholipid-dependent protein kinase in smooth muscle and nonmuscle cells. As shown in the present study, however, the way by which AA inhibits SMLCK was different from that of these kinases because increasing the amount of CaM (up to 590 nM) did not reverse the phosphorylation of SMLC by SMLCK. A dose necessary for the half-maximum inhibition of SMLCK by AA (50 μM) was ten times lower than that by linoleic acid (500 μM). Oleic acid of 500 μM slightly inhibited SMLCK. Higher concentrations than 500 μM of these fatty acids were not tested because of insolubility. In addition, phospholipids up to the concentration of 500 μM tested had no inhibitory effect on SMLCK activity. These results suggest that the AA-induced inhibition of SMLCK is specific, and also suggest that the inhibitory potency of unsaturated fatty acids parallels the number of cis double bonds because oleic acid, linoleic acid and AA have one, three and four cis double bonds, respectively, in the structure.

The concentration of AA required for the inhibition of SMLCK was 5 μM or more in the present study. These concentrations of AA were similar to those used in previous *in vitro* studies (1-4, 11-13) in which the effective doses of unsaturated fatty acids ranged from 10 μM to 500 μM. However, whether the concentrations of AA used in the present study are physiological levels remains unclear because intracellular levels of free AA have not been settled. Further studies are needed to assess the physiological significance of the AA-induced inhibition of SMLCK.

In summary, the present study demonstrates that SMLCK is directly inhibited by unsaturated fatty acids including AA in a purified system. Although the precise mechanism by which AA inhibits SMLCK is unclear from the present study, the ability of AA to decrease SMLCK activity *in vitro* may raise a possibility that endogenous AA could serve as an inhibitory signal in the regulation of cellular functions which involve SMLCK.

Acknowledgments

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