Vol. 153, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1988 Pages 156-161

EFFECT OF OKADAIC ACID ON PHOSPHORYLATION-DEPHOSPHORYLATION OF MYOSIN LIGHT CHAIN IN AORTIC SMOOTH MUSCLE HOMOGENATE

Ferenc Erdődi, Anikó Rokolya, Joseph Di Salvo†, Michael Bárány and Kate Bárány*

Departments of Biological Chemistry, and *Physiology and Biophysics, College of Medicine, University of Illinois, Chicago, IL 60612

†Department of Physiology and Biophysics, College of Medicine, University of Cincinnati, Cincinnati, OH 45267

Received April 6, 1988

SUMMARY: Myosin light chain phosphorylation in aortic smooth muscle homogenate reached a maximal level of 0.75 mol phosphate/mol light chain, and then declined. Addition of okadaic acid led to a sustained phosphorylation level of 1.7 mol/mol. In the absence of okadaic acid, phosphorylation was predominantly due to myosin light chain kinase, whereas in the presence of okadaic acid both myosin light chain kinase and protein kinase C were involved in phosphorylation. Okadaic acid inhibited dephosphorylation of the distinct sites in LC phosphorylated by either myosin light chain kinase or protein kinase C, suggesting that it exerts its effect through inhibition of myosin light chain phosphatases present in aortic homogenate.

It is generally accepted that phosphorylation of the 20,000 dalton light chain of myosin (LC) is involved in the initiation of smooth muscle contraction (1). LC phosphorylation may be catalyzed by myosin light chain kinase (MLCK) and protein kinase C on distinct serine and threonine residues (2-4).

We have recently shown that in phosphatase-free actomyosin, LC phosphorylation may exceed 2 mol phosphate/mol LC by the simultaneous action of MLCK and protein kinase C (4). In contrast, during various stimulations of intact artery, LC phosphorylation ranged between 0.7-0.8 mol/mol and was mainly attributable to MLCK (5). Since myosin LC phosphatases are present in muscle, they may modulate the phosphorylation level of LC.

*To whom correspondence should be addressed.

0006-291X/88 $1.50

Okadaic acid (OA), a toxin isolated from black sponges, was shown to inhibit myosin LC phosphatases in smooth muscle homogenate (6). In this paper we show that in aortic homogenate, OA markedly enhances LC phosphorylation by both MLCK and protein kinase C due to the inhibiton of phosphatases.

EXPERIMENTAL PROCEDURES

Aorta segments were obtained from freshly slaughtered hogs. The cleaned and chopped aortic media was homogenized with a Brinkmann Polytron in a solution containing 35 mM KCl, 50 mM 3-(4-morpholino)propane sulfonic acid (MOPS), 1 mM DTT, 0.15 mM phenylmethylsulphonylfluoride (PMSF), 1 mg/liter leupeptin, 2 mg/liter soybean trypsin inhibitor and 70 μM streptomycin, pH 7.0 (Buffer A). The LC in the homogenate was phosphorylated by endogenous kinases in the presence of 5 mM $MgSO_4$, 2 mM [γ-^{32}P]ATP (150-200 cpm/pmol) and 2 mM EGTA, or 0.1 mM $CaCl_2$, or 0.1 mM $CaCl_2$ plus 10 μM okadaic acid at 25°C. The phosphorylation reaction was stopped by the addition of equal volume of buffer containing 0.2% SDS, 12 mM Tris•HCl (pH 6.8), 32 mM DTT and 10% glycerol, and the mixture was boiled for 5 min. The samples were applied on a one-dimensional SDS slab gel, the LC bands were dissected, the radioactivity was determined, and the extent of phosphorylation was calculated (7). Alternatively, LC was eluted from the gel and digested by trypsin. One- and two-dimensional phosphopeptide mapping was performed on Kodak Chromagram 13255 cellulose sheets as described (4,5).

Crude phosphatase was prepared by homogenizing aortic media in 3 vols. of 10 mM Tris•HCl, 250 mM sucrose, 1 mM DTT, 0.15 mM PMSF, 1 mg/liter leupeptin, 2 mg/liter trypsin inhibitor and 70 μM streptomycin, pH 7.2. The homogenate was centrifuged (10,000 g, 20 min) and the supernatant was fractionated with $(NH_4)_2SO_4$ between 30-60% saturation. The pellet was dissolved in 10 mM Tris•HCl, 0.1 mM EGTA and 1 mM DTT, pH 7.2, and dialyzed against this solution.

Phosphatase-free actomyosin (4) in Buffer A was phosphorylated by endogenous kinases in the presence of 5 mM $MgSO_4$, 2 mM [γ-^{32}P]ATP (150-200 cpm/pmol) and 0.1 mM $CaCl_2$ for 120 min at 25°C. The actomyosin (5 mg/ml) in Buffer A was dephosphorylated by addition of 0.5 mg/ml crude phosphatase in the absence or in the presence of 5 μM okadaic acid. Okadaic acid was the generous gift of Dr. Y. Tsukitani, Fujisawa Pharmaceutical Co., Tokyo, Japan.

RESULTS

Fig. 1 shows the time course of LC phosphorylation in aortic homogenate by endogenous kinases. In the presence of Ca^{2+}, phosphorylation increased to 0.75 mol phosphate/mol LC in 2 min, and then declined to less than 0.15 mol/mol in the following 60 min. Substituting Ca^{2+} with EGTA resulted in a marked decrease in the rate and extent of LC phosphorylation indicating that in muscle homogenate LC phosphorylation strongly depends on Ca^{2+}. Addition of OA in the presence of Ca^{2+} enhanced LC phosphorylation to 1.72 mol/mol in 5 min and that level was essentially maintained for 60 min.

To determine which protein kinases are involved in the phosphorylation, the LC in the aortic homogenate was maximally

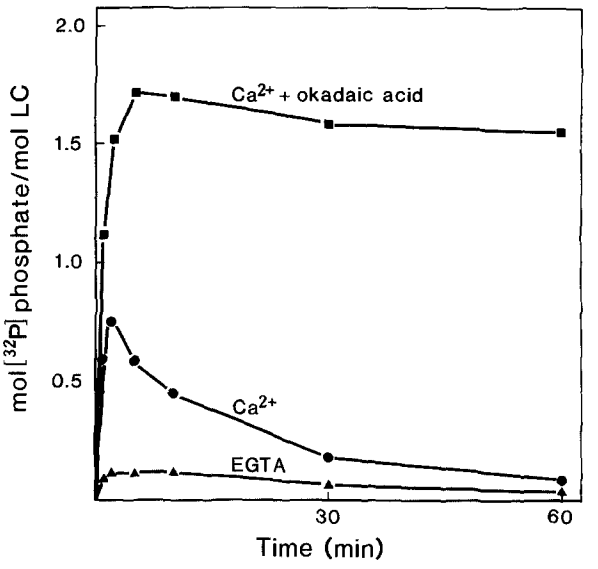

Fig. 1. Time course of LC phosphorylation by endogenous kinases in aortic homogenate. Phosphorylation was determined in the presence of 5 mM $MgSO_4$, 2mM [γ-^{32}P]ATP and 2 mM EGTA (▲), or 0.1 mM $CaCl_2$ (●), or 0.1 mM $CaCl_2$ plus 10 μM okadaic acid (■) at 25°C.

phosphorylated in the absence or in the presence of OA, and then subjected to two-dimensional phosphopeptide mapping. Fig. 2 shows that six phosphopeptides, referred to as A through F, were separated. In the absence of OA (Fig. 2, left part), peptides A and B were preferentially phosphorylated, whereas peptides C, D, E and F, appearing faintly on the autoradiogram, were phosphorylated to a lesser extent. In the presence of OA (Fig. 2, right part), the radioactive labelling in peptides C, D, E and F was substantially increased, suggesting that OA gave rise to increased phosphorylation of these peptides. We have shown that MLCK phosphorylates serine residues in peptides A and B, and predominantly threonine residues in peptides C and D (4). Furthermore, protein kinase C phosphorylates serine residues in peptide E, and threonine residues in peptide F (4). Thus, the data of Fig. 2 suggest that in the absence of OA, LC phosphorylation was catalyzed predominantly by MLCK in peptides A and B, whereas in the presence of OA, MLCK catalyzed also the phosphorylation of peptides C and D, and protein kinase C catalyzed the phosphorylation of peptides E and F.

It has been shown that OA does not affect either MLCK or protein kinase C activities (6,8). On the other hand, dephosphorylation of LC was inhibited by OA (6). We studied the effect of OA on the dephosphorylation of distinct sites in LC. LC was phosphorylated in phosphatase-free actomyosin by endogenous MLCK and protein kinase C under conditions which facilitated phosphorylation in peptides C, D, E and F

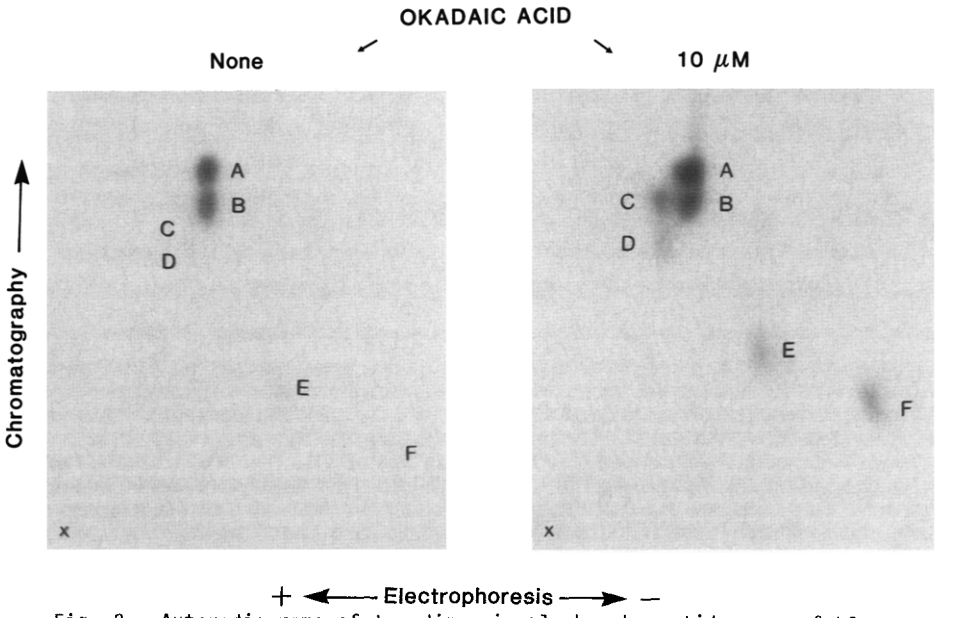

Fig. 2. Autoradiograms of two-dimensional phosphopeptide maps of LC phosphorylated in aortic homogenate. The extent of phosphorylation in the presence of 5 mM $MgSO_4$, 2 mM [γ-^{32}P]ATP and 0.1 mM $CaCl_2$, or 0.1 mM $CaCl_2$ plus 10 μM okadaic acid, was 0.75 mol phosphate/mol LC (left panel) or 1.72 mol/mol (right panel), respectively. The LC was separated by one-dimensional gel electrophoresis, and then subjected to two-dimensional phosphopeptide mapping on cellulose sheets. x, origin; A, B, C, D, E and F, phosphopeptides.

(4). The actomyosin was subjected to dephosphorylation by crude phosphatase isolated from aortic homogenate. Fig. 3 shows that more than 75% of the radioactive phosphate was liberated from LC by crude phosphatase during 60 min of incubation. This dephosphorylation was markedly inhibited by the addition of OA. The phosphopeptides from LC dephosphorylated for 10 and 60 min were separated by one-dimensional phosphopeptide mapping (Fig. 3, right part). In the absence of OA, peptides AB, CD and F were readily dephosphorylated, while peptide E was dephosphorylated at a slower rate. Clearly, OA inhibited the dephosphorylation of all sites.

DISCUSSION

The results of this study strongly suggest that arterial protein phosphatase activity can modulate the extent of LC phosphorylation as well as the sites on the LC which are phosphorylated. Thus, Ca^{2+}-dependent phosphorylation in aortic homogenate was transient; it occurred to the extent of less than 1 mol phosphate/mol LC, and it was limited essentially to sites phosphorylated by MLCK (Figs. 1 and 2). In contrast, when endogenous phosphatase activity was decreased by inhibition of the enzyme(s) with okadaic acid, the extent of phosphorylation was sustained at a markedly increased level of more than

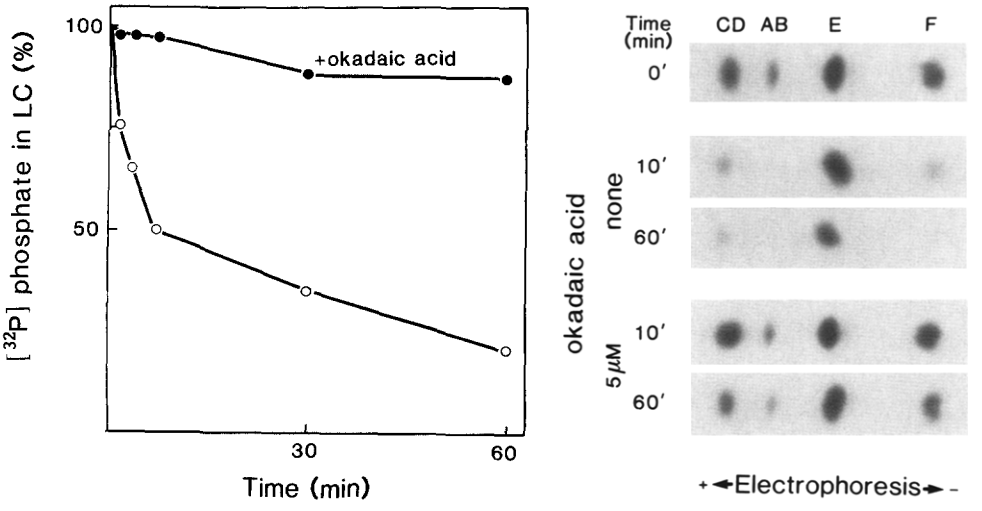

Fig. 3. Dephosphorylation of LC in actomyosin by crude phosphatase. Actomyosin contained 2.1 mol phosphate/mol LC. Left panel shows the time course of LC dephosphorylation in the absence (○) or in the presence of 5 μM okadaic acid (●). Right panel shows the autoradiograms of one-dimensional phosphopeptide maps of LC dephosphorylated for 0, 10 and 60 min in the absence or presence of okadaic acid.

1.5 mol phosphate/mol LC. Moreover, sites ascribable to protein kinase C were also involved in the phosphorylation, in addition to the sites attributable to MLCK (Figs. 1 and 2). Since okadaic acid effectively inhibited dephosphorylation of all phosphorylated sites in the LC (Fig. 3), the contribution of both MLCK and protein kinase C to the overall state of LC phosphorylation was revealed as indicated by increased incorporation of phosphate and by phosphorylation of additional sites.

LC phosphorylation in smooth muscle homogenate is the *in vitro* correlate of the phosphorylation events taking place in intact muscle, concerning the phosphorylation profile, the extent of phosphorylation and the phosphopeptide pattern of LC (5,9). This suggests that the protein phosphatases *in vivo* may participate in limiting LC phosphorylation in a fashion similar to that observed in muscle homogenate. LC phosphorylation by protein kinase C is restricted in intact muscle (5). It is of interest that OA, by inhibiting the phosphatases, can induce LC phosphorylation by protein kinase C. It was shown that the actin activated MgATPase activity of myosin, an *in vitro* correlate of muscle contraction, was affected differently by phosphorylation with MLCK or protein kinase C (10). Since OA also elicits contraction of intact (11) and chemically skinned smooth muscle (6), it is likely to be a useful tool for elucidating the functional role of protein kinase C-catalyzed phosphorylation in smooth muscle contraction.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health, AR 34602 and HL 20196. We thank Kathryn Peregrine for dedicated assistance and Donna M. Lattyak for careful typing of the manuscript.

REFERENCES

1. Sellers, J.R., and Adelstein, R.S. (1987) In the Enzymes (P.D. Boyer and E.G. Krebs, Eds.), Vol. 18, pp. 381-418, Academic Press, New York.
2. Ikebe, M., Hartshorne, D.J., and Elzinga, M. (1986) J. Biol. Chem. 261, 36-39.
3. Bengur, A.R., Robinson, E.A., Appella, E., and Sellers, J.R. (1987) J. Biol. Chem. 262, 7613-7617.
4. Erdődi, F., Rokolya, A., Bárány, M., and Bárány, K. (submitted for publication).
5. Erdődi, F., Bárány, M., and Bárány, K. (1987) Circ. Res. 61, 898-903.
6. Takai, A., Bialojan, C., Troschka, M., and Ruegg, J.C. (1987) FEBS Lett. 217, 81-84.
7. Barron, J.T., Bárány, M., and Bárány, K. (1979) J. Biol. Chem. 254, 4954-4956.
8. Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., Ojika, M., Wakamatsu, K., Yamada, K., and Sugimura, T. (1988) Proc. Natl. Acad. Sci. USA 85, 1768-1771.
9. Csabina, S., Bárány, M., and Bárány, K. (1987) Comp. Biochem. Physiol. 87, 271-277.
10. Nishikawa, M., Hidaka, H., and Adelstein, R.S. (1983) J. Biol. Chem. 258, 14069-14072.
11. Shibata, S., Ishida, Y., Kitano, H., Ohizumi, Y., Habon, J., Tsukitani, Y., and Kikuchi, H. (1982) J. Pharmacol. Exp. Ther. 223, 135-143.