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BBA Report

Modification of myosin light chain phosphorylation in sustained arterial muscle contraction by phorbol dibutyrate

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The decrease in phosphorylation of the 20 kDa myosin light chain during prolonged K^+-stimulation of arterial smooth muscle was counteracted by treating this muscle with phorbol dibutyrate. Quantitative phosphopeptide analysis revealed that phorbol dibutyrate induced phosphorylation of serine and threonine residues in the light chain by protein kinase C and phosphorylation of a threonine residue by myosin light chain kinase. The same residues of light chain were also phosphorylated when phorbol dibutyrate was added to muscles pretreated either with the Ca^{2+}-channel-blocking agents nifedipine and verapamil, or with the Ca^{2+}-chelating agent ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid. The results indicate an interrelationship between protein kinase C and myosin light chain kinase phosphorylated sites of light chain in intact arterial smooth muscle.

Recently, we reported in this journal the analysis of phosphopeptides of the 20 kDa myosin light chain (LC) in phorbol dibutyrate (PDBu) contracted artery [1]. At the same time, we observed that addition of PDBu to 1-min K^+-contracted arteries, containing maximally phosphorylated LC, prevented LC dephosphorylation known to occur during sustained K^+ -contraction [2,3]. This phenomenon was investigated by quantitative phosphopeptide analysis and is the subject of this report. We also describe the lack of effect of Ca^{2+}-channel blocking agents and that of EGTA on PDBu-induced tonic contraction of arterial muscle and on the associated LC phosphorylation.

The preparation of porcine carotid arterial muscles, their ^{32}P-labeling in physiological salt solution (PSS), the isolation of LC by two-dimensional gel electrophoresis, the quantitation of [^{32}P]phosphate incorporation into LC and two-dimensional tryptic phosphopeptide mapping of LC are described in Ref. 1.

Fig. 1 shows typical experiments for the effect of PDBu on the force development of arterial muscle. In the first experiment (top), the muscle was stimulated with KCl raising the force from the resting tension of about 25 g to 75 g within 2 min; the subsequent PDBu treatment (in the presence of KCl) raised the force gradually to about 100 g in 60 min. In the second

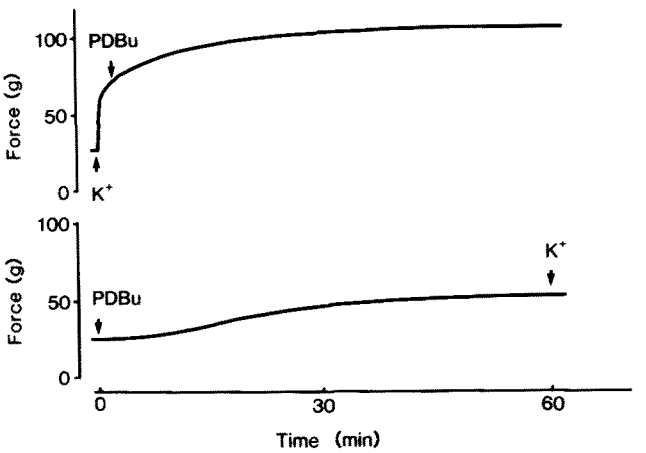

Fig. 1. Time-course of force development of PDBu-treated porcine carotid arterial muscles. Top: After applying a resting tension simulating 100 mmHg mean arterial pressure, the PSS solution in the bath was exchanged for the 100 mM K^+-stimulating solution [3]; 2 min later a stock PDBu solution was added to the bath to give 0.8 μM PDBu concentration. Bottom: The muscle was incubated in Ca^{2+}-free PSS containing 1.0 mM EGTA for 5 min, then the resting tension was adjusted and PDBu was added to 0.8 μM; after 60 min, saturated KCl solution was added to 100 mM K^+ final concentration.

Abbreviations: LC, light chain; PDBu, phorboldibutyrate; MLCK, myosin light chain kinase; PKC, protein kinase C.

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experiment (bottom), the muscle was pretreated with EGTA for 5 min which had no effect on the subsequent PDBu-induced contraction of the muscle (in the presence of EGTA), i.e., about 25 g force was produced in 60 min. Furthermore, upon addition of K^+ to the EGTA and PDBu treated muscle, no change in force occurred.

The results of the experiment shown in Fig. 1 may be compared with those of control experiments in which K^+-stimulation alone elicited maximal force within few minutes that was maintained up to 62 min [1]. Thus, the combined K^+ and PDBu treatment results in a force larger than that of K^+ alone. We also described [1] that addition of K^+ to 60-min PDBu-treated artery produces about 50 g force over that produced by PDBu. This effect of K^+ is apparently abolished in muscles which have been treated with EGTA and PDBu.

Force development curves similar to that shown in the bottom part of Fig. 1 were obtained with muscles pretreated for 30 min with 10 μM nifedipine or 0.2 mM verapamil. That is, these Ca^{2+}-channel blockers had no effect on the subsequent PDBu-induced contraction. Furthermore, addition of K^+ to the PDBu-contracted arteries in the presence of nifedipine or verapamil did not cause a change in force development.

Fig. 2 compares the two-dimensional gel electrophoretic pattern of LC isolated from a 62-min K^+-treated muscle with that from a muscle treated with K^+ for 2 min followed by K^+ and PDBu for 60 min. Four LC spots are seen in the stained gels (top), called Spots 1, 2, 3 and 4 from the acidic toward the alkaline pH. The percentage distribution of stain among these spots showed distinct differences between the muscles. For the K^+ 62-min-treated muscle, the distribution was 4, 9, 23 and 64% for Spots 1, 2, 3 and 4, respectively, whereas for the K^+ 2-min, K^+ and PDBu 60-min-treated muscle, the distribution was 5, 13, 32, and 50%. In the corresponding autoradiograms of these gels (bottom), the K^+ 62-min-treated muscle shows three LC spots which correspond to staining Spots 1, 2 and 3. The autoradiogram of LC from the K^+ 2-min, K^+ and PDBu 60-min-treated muscle shows four spots which correspond to staining Spots 1, 2, 3, and to a spot not observable by staining but only by radioactivity, called Spot 0. This radioactive Spot 0 could also be detected in the gel of the K^+ 62-min-treated muscle when the corresponding area was excised, digested and counted. The percentage distribution of radioactivity in the spots was the following: for the K^+ 62-min-treated muscle, 4, 14, 16, and 66% for Spots 0, 1,2 and 3, respectively, and for K^+ 2-min, K^+ and PDBu 60-min-treated muscle 7, 16, 26, and 51%. Thus, the effect of PDBu is manifested in the staining pattern by decreasing the relative intensity of Spot 4, and in the radioactivity distribution pattern by increasing the relative contribution of Spot 2.

The effect of PDBu on LC phosphorylation under various conditions is summarized in Table I. LC phosphorylation in sustained (62-min) K^+-contraction, 0.4 mol [^{32}P]phosphate/mol LC, is potentiated by PDBu to 0.69 mol/mol. This latter value approximates the maxi-

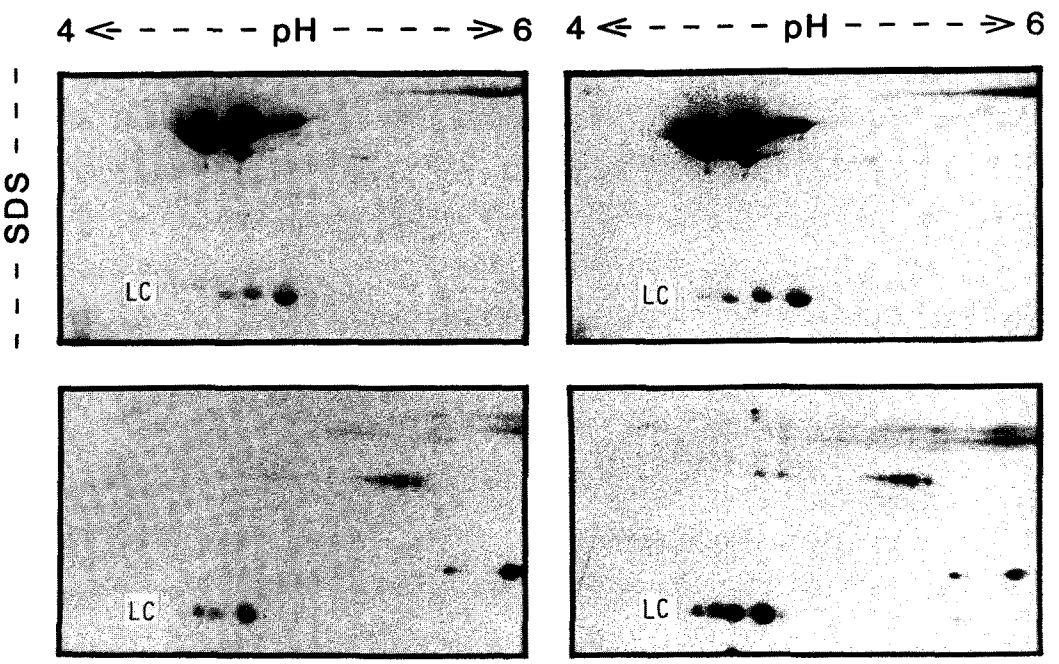

Fig. 2. Two-dimensional gel electrophoretograms of arterial proteins. Top: portion of staining patterns, showing tropomyosin and myosin light chains. Bottom: corresponding autoradiograms. Left: muscle treated with 100 mM K^+-stimulating solution for 62 min. Right: muscle treated with 100 mM K^+-stimulating solution; 2 min later a stock PDBu solution was added to the bath to give 0.8 μM PDBu concentration; total treatment time 62 min. LC, 20 kDa myosin light chain.

TABLE I

Myosin light chain phosphorylation in PDBu-treated arteries

Concentrations: K^+ 100 mM, PDBu 0.8 μM, Ca^{2+} 10 mM, EGTA 1 mM, nifedipine 10 μM, verapamil 200 μM.

Treatment	mol [^{32}P] phosphate / mol LC	*n*
K^+ 62 min	0.40 ± 0.04	8
K^+ 2 min, K^+ and PDBu 60 min	0.69 ± 0.10	8
K^+ 2 min, K^+ and Ca^{2+} 60 min	0.41	2
EGTA 5 min, EGTA and PDBu 62 min	0.30	2
EGTA 5 min, EGTA and PDBu 60 min, EGTA, PDBu and K^+ 2 min	0.28	2
Nifedipine 30 min, nifedipine and PDBu 62 min	0.34	2
Nifedipine 30 min, nifedipine and PDBu 60 min, nifedipine, PDBu and K^+ 2 min	0.35	2
Verapamil 30 min, verapamil and PDBu 62 min	0.29	2
Verapamil 30 min, verapamil and PDBu 60 min, verapamil, PDBu and K^+ 2 min	0.28	2

mal LC phosphorylation, 0.7 mol/mol, found in muscles stimulated with K^+ for 1 to 2 min [1]. On the other hand, raising the Ca^{2+} concentration to 10 mM in the presence of K^+ leaves LC phosphorylation at the same level as that in the 62-min K^+-treated muscles. Treatment of the muscle with EGTA and PDBu for 62 min results in LC phosphorylation of only 0.3 mol/mol, and this value is not changed upon K^+ treatment for 2 min. In the presence of the Ca^{2+}-channel-blocking agent, verapamil, PDBu produces phosphorylation similar to that obtained with EGTA. Slightly higher phosphorylation was observed with the Ca^{2+}-channel-blocking agent, nifedipine.

Fig. 3 compares the two-dimensional tryptic phosphopeptide maps of LC isoforms eluted from Spots 0, 1, 2 and 3 from muscles treated either with K^+ for 62 min or treated with K^+ for 2 min followed by K^+ and PDBu for 60 min. In the maps from the muscle treated with K^+ alone, A and B are the predominant peptides, called MLCK/Ser, because these peptides contain phosphoserine generated by myosin light chain kinase (MLCK), described in Ref. 4. These maps also contain small quantities of peptides C and D (MLCK/Thr) and the protein kinase C (PKC)-generated peptides: E (PKC/Ser) and F (PKC/Thr). On the other hand, in the maps from the K^+ and PDBu-treated muscles, peptides A and B are only predominant in Spots 1 and 3, whereas in Spots 0 and 2 peptides C and D appear in

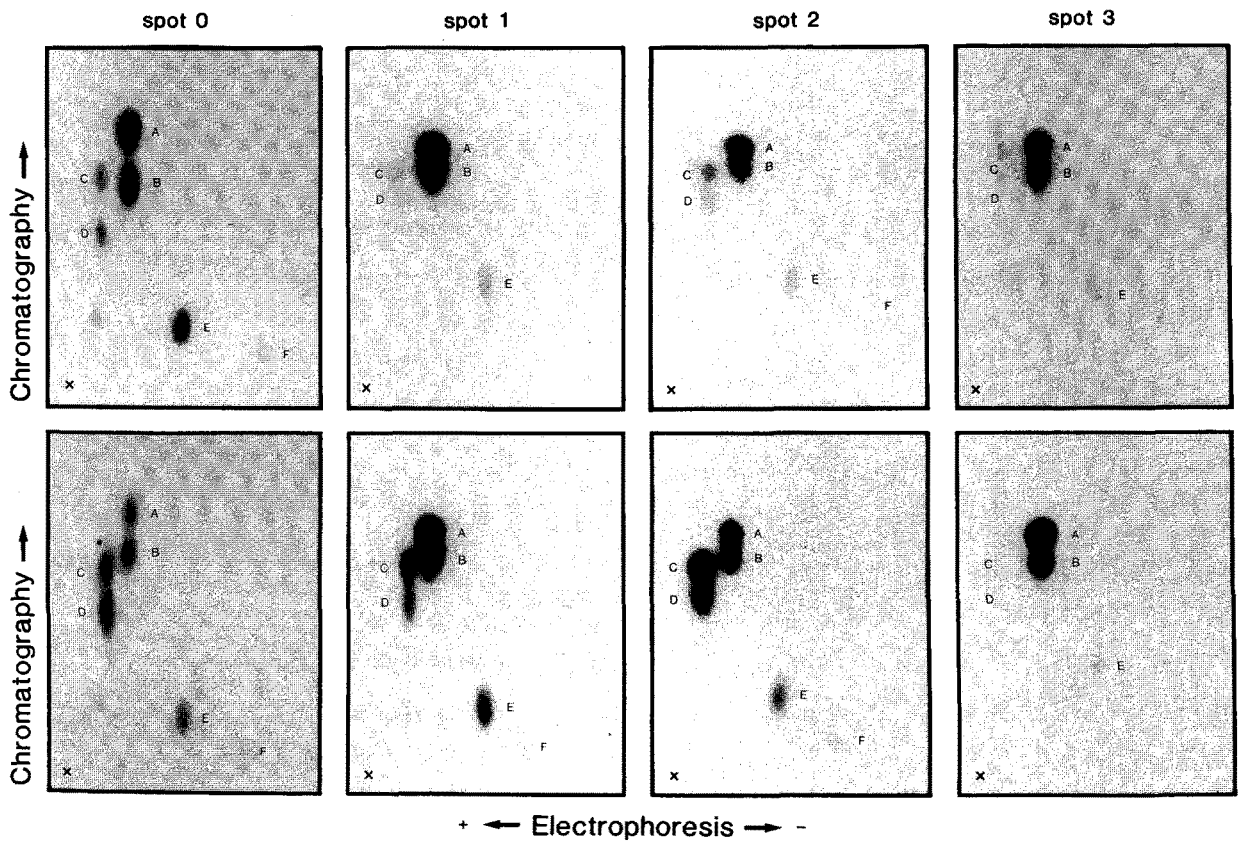

Fig. 3. Autoradiograms of two-dimensional tryptic phosphopeptide maps of individual LC spots isolated by two-dimensional gel electrophoresis, shown in Fig. 2. The upper row shows the peptides of LC isolated from K^+ 62-min-treated muscles and the bottom row shows the peptides of LC isolated from muscles treated with K^+ for 2 min followed by K^+ and PDBu for 60 min. A and B, peptides containing serine residue phosphorylated by MLCK; C and D, peptides containing threonine residue phosphorylated by MLCK; E, peptide containing serine phosphorylated by PKC; F, peptide containing threonine phosphorylated by PKC.

larger quantities than A and B. Furthermore, Spots 0, 1 and 2 contain a significant amount of peptide E, and traces of peptide F.

We calculated the [^{32}P]phosphate content of LC in the major peptide groups, MLCK/Ser, MLCK/Thr, PKC/Ser,Thr, from the total incorporation and the distribution of radioactivity among the spots. Table II shows that in K^+ 62-min-treated muscles about 0.4 mol [^{32}P]phosphate is incorporated per mol LC. Most of the [^{32}P]phosphate (89%) is incorporated into the MLCK/Ser peptides. In the K^+ 2 min, K^+ and PDBu 60-min-treated muscles, the total incorporation is 0.68 mol [^{32}P]phosphate/mol LC. This is distributed among the peptides: MLCK/Ser 71%, MLCK/Thr 19% and PKC/Ser,Thr 10%. The actual increment in the PDBu-induced incorporation (K^+ 2 min, K^+ and PDBu 60 min versus K^+ 62 min) is the following: MLCK/Ser 127 mmol, MLCK/Thr 111 mmol and PKC/Ser,Thr 44 mmol; that is, the increment in the MLCK-catalyzed LC phosphorylation is greater than the PKC-catalyzed LC phosphorylation.

The data of Table II and Fig. 3 identify the LC isoforms separated by two-dimensional gel electrophoresis: Spots 3 and 1 correspond to the monophosphorylated major and minor isoforms, respectively, whereas Spots 2 and 0 correspond to the diphosphorylated major and minor isoforms, respectively [4–6]. It may be calculated from the data of Table II that 18% of the total [^{32}P]phosphate is located in diphosphorylated LC in the muscle treated with K^+ for 62 min and 33% in the muscle treated with K^+ for 2 min and, subsequently, with K^+ and PDBu for 60 min. Elevated diphosphorylation can also be recognized by the ratio of total counts in Spot 2 over Spot 3; this ratio is 0.21 in the K^+ 62-min-treated muscle but 0.51 in the K^+ 2 min, K^+ and PDBu 60-min-treated muscle (Table II). Qualitatively, diphosphorylation is detected by visual comparison of autoradiographic intensities in Spot 2 over Spot 3 of two-dimensional gels of these muscles (bottom part of Fig. 2).

TABLE II

Incorporation of [^{32}P]phosphate into peptides of LC isolated from muscles treated with K^+ alone or K^+ and PDBu

The powder containing the radioactive peptides, as visualized by the autoradiogram, was scraped from the cellulose sheets and the radioactivity was determined by liquid scintillation counting. MLCK/Ser, peptide containing Ser phosphorylated by MLCK; MLCK/Thr, peptide containing Thr phosphorylated by MLCK; PKC/Ser,Thr, peptides containing Ser or Thr phosphorylated by PKC.

Treatment	Spot	mmol [^{32}P]phosphate/mol LC in tryptic peptides			
		MLCK/Ser	MLCK/Thr	PKC/Ser,Thr	total
K^+ 62 min	0	9	3	4	16
K^+ 62 min	1	54	4	5	63
K^+ 62 min	2	36	11	8	55
K^+ 62 min	3	252	2	5	259
		351	20	22	393
K^+ 2 min, K^+ and PDBu 60 min	0	16	23	9	48
K^+ 2 min, K^+ and PDBu 60 min	1	78	16	14	108
K^+ 2 min, K^+ and PDBu 60 min	2	61	88	26	175
K^+ 2 min, K^+ and PDBu 60 min	3	323	4	17	344
		478	131	66	675

Analyses similar to those described in connection with Table II gave the following results. Raising the Ca^{2+} concentration from 2.5 to 10 mM in PSS did not result in elevated LC diphosphorylation. This supports the idea that PDBu does not act through increasing the myoplasmic Ca^{2+} concentration from the extracellular space. In contrast, significant LC diphosphorylation was found in muscles treated with PDBu in the presence of nifedipine, verapamil or EGTA involving both PKC/Ser,Thr and MLCK/Thr peptides. These results indicate that the increase in myoplasmic [Ca^{2+}] in PDBu-treated porcine carotid arterial muscles [7] takes place from intracellular Ca^{2+} stores. Substantial LC diphosphorylation through PKC/Ser,Thr peptides was described in PDBu-treated arterial muscles [1,8] or bovine tracheal muscles [9]. Additional K^+-stimulation of the PDBu-treated arterial muscle altered the phosphorylation profile. We found that in PDBu 60-min, PDBu and K^+ 2-min-stimulated muscles, the percentage distribution of [^{32}P]phosphate in the peptides was the following: MLCK/Ser 65, MLCK/Thr 22 and PKC/Ser,Thr 13. This indicates a change in LC diphosphorylation pattern shifting from the PKC/Ser,Thr toward the MLCK/Thr, similarly as shown in Table II for the K^+ 2-min, K^+ and PDBu 60-min-treated muscles.

From all these data, the following mechanism may be proposed for the PDBu-induced maintenance of LC phosphorylation during sustained K^+-contraction of arterial muscle. During the prolonged K^+-contraction of the muscle, the free Ca^{2+} concentration decreases [10] and this reduces MLCK activity and, consequently, LC phosphorylation. Addition of PDBu to the K^+-contracted muscle releases new Ca^{2+} from an internal source which will activate PKC to phosphorylate Ser and Thr residues in LC and MLCK to phosphorylate another Thr residue. This proposal is the extension of in vitro findings to in vivo system. Namely, studies with pure arterial LC demonstrated that PKC/Ser,Thr phosphorylation promotes MLCK/Thr phosphorylation, indicating that the phosphorylation of distinct sites in LC by PKC and MLCK is interrelated [4]. The interrela-

tionship between PKC and MLCK phosphorylated sites of LC in intact arterial smooth muscle is clearly shown in this paper.

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References

1 Bárány, K., Rokolya, A. and Bárány, M. (1990) Biochim. Biophys. Acta 1035, 105–108.
2 Dillon, P.F., Aksoy, M.O., Driska, S.P. and Murphy, R.A. (1981) Science 211, 495–497.
3 Bárány, K., Ledvora, R.F. and Bárány, M. (1985) in Calmodulin Antagonist and Cellular Physiology (Hidaka, H. and Hartshorne, D.J., eds.), pp. 199–223, Academic Press.
4 Erdödi, F., Rokolya, A., Bárány, M. and Bárány, K. (1988) Arch. Biochem. Biophys. 266, 583–591.
5 Mougios, V. and Bárány, M. (1986) Biochim. Biophys. Acta 872, 305–308.
6 Erdödi, F., Bárány, M. and Bárány, K. (1987) Circul. Res. 61, 898–903.
7 Rembold, C.M. and Murphy, R.A. (1988) Am. J. Physiol. 255, C719–C723.
8 Singer, H.A., Oren, J.W. and Benscoter, H.A. (1989) J. Biol. Chem. 264, 21215–21222.
9 Kamm, K.E., Hsu, L.C., Kubota, Y. and Stull, J.T. (1989) J. Biol. Chem. 264, 21223–21229.
10 Murphy, R.A., Rembold, C.M. and Hai, C.M. (1990) in Frontiers in Smooth Muscle Research (Sperelakis, N. and Wood, J.D., eds.), pp. 39–50, Alan R. Liss, New York.