Vol. 208, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 8, 1995 Pages 397-404

PHOSPHORYLATION OF CALPONIN MEDIATED BY PROTEIN KINASE C IN ASSOCIATION WITH CONTRACTION IN PORCINE CORONARY ARTERY

Terumasa Mino, Uhito Yuasa, Michiko Naka and Toshio Tanaka*

Department of Molecular and Cellular Pharmacology
Mie University School of Medicine
Edobashi, Tsu, Mie 514, Japan

Received January 30, 1995

SUMMARY: Calponin is an actin-associated regulatory protein in smooth muscle. We report that both endothelin-1 (ET-1) and phorbol 12,13-dibutyrate(PDBu) caused a significant increase in phosphorylation of calponin during contraction of porcine coronary artery, while high levels of KCl were ineffective. This phosphorylation was predominantly catalyzed by activation of protein kinase C(PKC). In addition, the level of phosphorylation of calponin increased closely in association with the size of the contractile force induced by PDBu. Thus, the phosphorylation of calponin *in vivo* by PKC might modulate in part the contraction of smooth muscle that occurs in response to ET-1 or PDBu.

The phosphorylated myosin light chain (MLC) activates actin-activated myosin Mg^{2+}-ATPase and plays a primary role in smooth muscle contraction (1-4). However, this regulatory mechanism does not explain all aspects of the regulation of the contraction of smooth muscle (2,5). Therefore, it has been proposed that additional regulatory mechanisms must exist to modify the smooth muscle contraction. Smooth muscle calponin, which is present at high concentrations in smooth muscle(6), is known to be an excellent substrate for protein kinase C (PKC) *in vitro*(7,8).

* To whom all correspondence should be addressed.

abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

0006-291X/95 $5.00

Purified calponin inhibits the actin-activated myosin Mg^{2+}-ATPase activity of smooth muscle and the phosphorylation of calponin *in vitro* results in a loss of this inhibitory activity(8,9). We also demonstrated that exogenous application of calponin suppressed the Ca^{2+}-sensitive contraction of skinned smooth muscle, but phosphorylated calponin failed(10). Thus, it seems possible that phosphorylation of calponin by PKC might modify the contraction of intact smooth muscle. However, it remains to be determine whether calponin is phosphorylated by PKC in intact smooth muscles. To address this the following studies have been carried out using immunoprecipitation method in porcine coronary arteries.

MATERIALS AND METHODS

Materials

Endothelin-1(ET-1) and phorbol-12,13 dibutyrate(PDBu) were purchased from Sigma(St Louis, U.S.A.). Protein A-Sepharose was obtained from Pharmacia. All other materials, including protein purification methods, were as previously described from this laboratory(7,9).

Preparation of tissue

Strips of fresh porcine coronary artery (about 10 mm long and 3 mm wide) were dissected and the muscles were attached to an isometric transducer (TB-651T Nihon Koden; Tokyo, Japan) for measurements of tension at 37°C. The strips were incubated with [^{32}P]orthophosphate (0.4 mCi/ml) for 5 hr in phosphate-free HBS at 37°C (pH 7.4).

Immunoprecipitation of calponin and determination of phosphorylation stoichiometry

Strips frozen in liquid nitrogen, were solubilized in a solution of 2% SDS in 400 µl of tissue-lysis buffers which contained 50 mM imidazole-HCl (pH 7.3), 190 mM NaCl, 1 mM EDTA, 5 mM EGTA, 3 mM DTT, 1.5% Triton X-100, 1 µM p-APMSF, 50 mM NaF, 50 mM sodium pyrophosphate, 1 µM okadaic acid and 50 µg/ml leupeptin. Each lysate was heated for 10 min at 95°C. Subsequent immunoprecipitation using rabbit anti-calponin antiserum was conducted essentially by the method of Adam,LP *et al*(11). The specific activity of intracellular ATP was determined by the method of England and Walsh(12) with minor modification. The stoichiometry of the phosphorylation of calponin was calculated using the method described by Adam,LP *et al*(11).

Other methods

Two-dimensional phosphopeptide mapping was conducted following tryptic digestion of ^{32}P-labeled calponin by the method described previously(13). Purification of tryptic phosphopeptides by HPLC and analysis of amino acid sequence were performed by the method described previously(9). Purified calponin from porcine stomach was phosphorylated by protein kinase C as described previously(9). Statistical significance was determined by Two-tailed, unpaired t-tests.

RESULTS AND DISCUSSION

The present study was performed to determine, by immunoprecipitation with calponin-specific antiserum and subsequent SDS-PAGE, whether calponin can be phosphorylated by protein kinase C(PKC) in intact porcine coronary artery. When the immunoprecipitated material was analyzed after SDS-PAGE by staining with Coomassie Brilliant Blue and accompanying autoradiography, the extent of phosphorylation of a protein of about 35 kDa was markedly enhanced in response to ET-1 (Fig. 1). This 35 kDa protein was identified as calponin by Western blotting with a calponin-specific antibody (data not shown) and same molecular size of calponin has been found in bovine aorta homogenate(14). Phosphorylation of calponin has also been observed in canine trachea(15), toad stomach(16) and porcine carotid artery(17), but no phosphorylation of calponin has been found in porcine carotid artery(18) or chicken gizzard(19). These

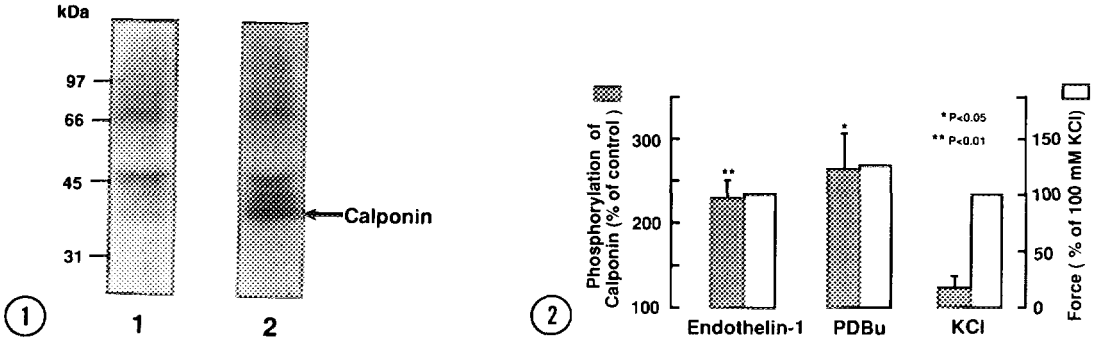

Figure 1. Phosphorylation in vivo of calponin in contracted porcine coronary artery. Arterial strips were prelabeled with [^{32}P]-orthophosphate (0.4 mCi/ml) and then incubated for 20 min in the absence (lane 1) or the presence (lane 2) of endothelin-1 (1 μM). Other conditions were performed as described in "Materials and Methods".

Figure 2. Comparison of the extent of phosphorylation of calponin in porcine coronary arteries after treatment with PDBu (1 μM), endothelin-1 (1 μM) and KCl (100 mM) for the following exposure times: endothelin-1, 5 min; PDBu, 20 min; and KCl, 2 min. The extent of phosphorylation of calponin was determined as described in "Materials and Methods". Results are the means and S.E. (4-5).
* P<0.05, ** P<0.01.

discrepancies relations to phosphorylation of calponin have not been explained. However, in all cases, results were analyzed by two-dimensional gel electrophoresis, whereas we first used on immunoprecipitation procedure to examine the state of phosphorylation of calponin. This method allowed us to purify calponin away from other phosphoproteins and to concentrate it.

When phosphorylation of calponin in ET-1-, PDBu- and KCl-treated arteries was compared, despite establishment of a similar contractile force in each case, ET-1 (1 μM) and PDBu (1μM) caused 2.3-fold and 2.6-fold significant increases in the extent of phosphorylation of calponin, respectively, while 100 mM KCl didn't (Fig. 2). Calponin is known as an excellent substrate for PKC (7,20) and for Ca^{2+}/calmodulin kinase II(20). The former enzyme is activated by PDBu, and the latter by high level of KCl. Therefore, ET-1 and PDBu-induced phosphorylation of calponin appear to be mediated predominantly by PKC in intact porcine coronary artery. To obtain more directed evidence, we performed two-dimensional mapping of tryptic phosphopeptide of calponin that had been immunoprecipitated from extracts of porcine coronary artery or phosphorylated in vitro by PKC. Treatment with PDBu produced one major spot and one minor spot on a two-dimensional tryptic phosphopeptide map of calponin immunoprecipitated from ^{32}P-labeled artery (Fig. 3A). When the artery has been treated with ET-1, only one spot was observed (Fig. 3B). This spot was identified as the major spot observed in the case of PDBu-treated artery by co-mapping of phosphopeptides(Fig. 3C). Furthermore, both the major spot and the minor spot from PDBu-treated artery were also observed in the phosphopeptide map of porcine calponin that has been phosphorylated in vitro by PKC (Fig. 3D).

When the major phosphopeptide, which corresponded to the major spot in two-dimensional peptide map, from calponin phosphorylated

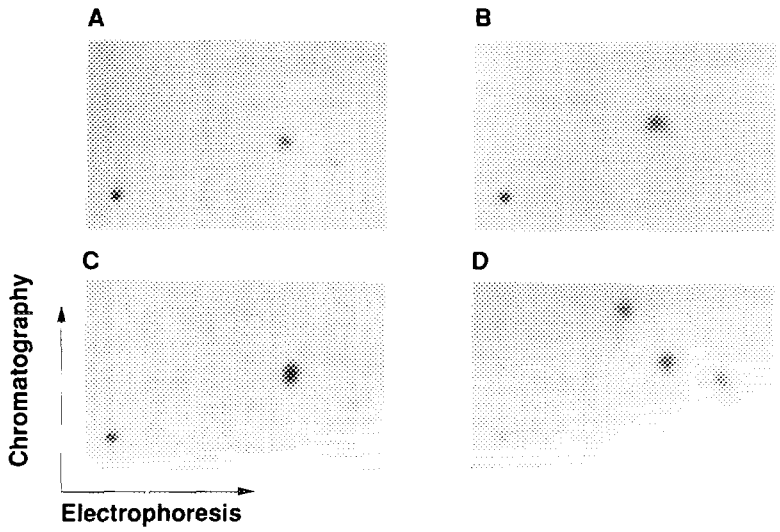

Figure 3. Two-dimensional tryptic phosphopeptide mapping of porcine calponin that had been phosphorylated *in vivo* and *in vitro* was performed as described in "Materials and Methods". Panel A: after treatment with PDBu (1 μM); panel B: after treatment with endothelin-1 (1 μM); panel C: co-mapping of samples after separate treatments with PDBu and endothelin-1; panel D: after treatment with PKC *in vitro*.

by PKC was analyzed with a protein sequencer, its thirteen N-terminal residues were found to correspond to amino acid residues 173-185 of calponin. In addition, Thr-184 was identified as the preferred phosphorylation site by protein kinase C resulting in a marked drop in yield at cycle 12 of the tryptic phosphopeptide (data not shown). These findings identified with our previous results as to phosphorylation *in vitro* of gizzard calponin by PKC(9) and provided evidence that PKC was responsible for the phosphorylation of calponin in ET-1- or PDBu-treated arteries.

Figure 4(A) and 4(B) show a representative concentration-response curve and time course, respectively, for phosphorylation of calponin and the isometric force produced by stimulation with PDBu in porcine coronary arteries. Treatment with PDBu (0.001-1 μM) increased the phosphorylation of calponin in a concentration-dependent manner, to 0.23 mol Pi/mol calponin at a final concentration of 1 μM. Also, PDBu resulted in the concentration-

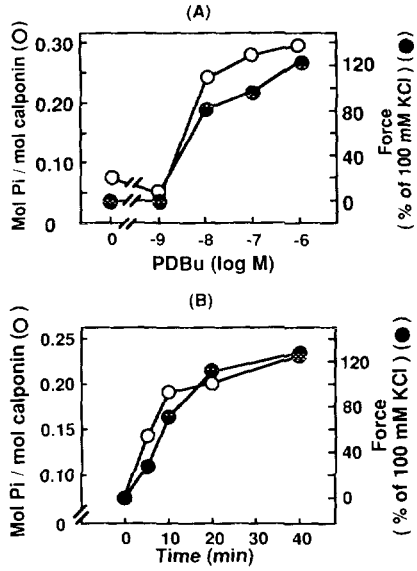

Figure 4. Concentration-response curves(A) and time courses(B) for the generation of contractile force and the corresponding extent of phosphorylation of calponin in coronary arterial smooth muscles. (A) Strips were incubated with increasing concentrations of PDBu for 40 min. (B) Strips were stimulated by 1μM PDBu. Other conditions were performed as described in "Materials and Methods". Each result is representative of the results of two separate experiments.

dependent generation of contractile force at concentrations above 0.01 μM as well as the phosphorylation of calponin(Fig.4A). In addition, the time course of phosphorylation of calponin closely resembled it of the contractile response to PDBu(Fig.4B). These findings suggest a close relationship between phosphorylation of calponin and contraction in porcine coronary artery. Caldesmon, an actin-binding protein, inhibits the ATPase activity of actomyosin *in vitro*(21) and its inhibition is diminished following phosphorylation of caldesmon(22). Adam et al. (11,23) reported that, in porcine artery stimulated by PDBu, the extent of phosphorylation of caldesmon increased slowly in association with the force development, but the phosphorylation was not catalyzed by activation of PKC. Therefore, calponin is a novel actin-associated protein that can be phosphorylated by PKC during

contraction of arterial smooth muscle and the phosphorylation of calponin may modulate partly the contraction of vascular smooth muscle.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aide for Scientific Research from the Ministry of Education, Science and Culture and from the Ministry of Health and Welfare, Japan. It was also supported in part by grants for the Cell Signaling Project of Mie University School of Medicine and from Bayer Yakuhin,Ltd., the Mochida Memorial Foundation for Medical and Pharmaceutical Research, Uehara Memorial Foundation, Kuma Hospital and Minami Hospital. We thank Dr. A. M. Korner for critical reading of the manuscript.

REFERENCES

1. Kamm,K.E.and Stull, J.T.(1985) *Ann. Rev. Pharmacol. Toxicol.*25, 593-620.
2. Hai,C-H.and Murphy, R.A.(1989) *Ann. Rev. Physiol.* 51, 285-298.
3. Hartshorne,D.J.(1987) *In Physiology of the Gastrointestinal Tract* (Johnson, L. R., ed,) 2nd, pp.423-482, Raven Press, New York.
4. Adelstein,R.S.and Sellers,J.R.(1987) *Am. J. Cardiol.* 59, 4B-10B.
5. Stull,J.T.,Gallagher,P.J.,Herring,B.P.and Kamm,K.E.(1991) *Hypertension* 17, 723-732.
6. Takahashi,K.,Hiwada,K.and Kokubu,T.(1988) *Hypertension* 11, 620-626.
7. Naka,M.,Kureishi,Y.,Muroga,Y.,Takahashi,K.,Ito,M.and Tanaka,T.(1990) *Biochem. Biophys. Res. Commun.*171, 933-937.
8. Winder,S.J.and Walsh,M.P.(1990) *J. Biol. Chem.* 265, 10148-10155.
9. Nakamura,F.,Mino,T.,Yamamoto,J.,Naka,M.and Tanaka,T.(1993) *J. Biol. Chem.* 268, 6194-6201.
10. Itoh,T.,Suzuki,S.,Suzuki,A.,Nakamura,F.,Naka,M.and Tanaka, T.(1994) *Eur. J. Physiol.* 427, 301-308.
11. Adam,L.P.,Haeberle,J.R.and Hathaway,D.R.(1989) *J. Biol. Chem.* 264, 7698-7703.
12. England,P.J.and Walsh,D.A.(1976) *Anal. Biochem.* 75, 429-435.

13. Naka,M.,Nishikawa,M.,Adelstein,R.S.and Hidaka,H.(1983) *Nature* 306,490-492.
14. Takahashi,K.,Hiwada,K.and Kokubu,T.(1987) *Life Sciences* 41, 291-296.
15. Pohl,J.,Walsh,M.P.and Gerthoffer,W.T.(1991) *Biophys.J.* 59, 58a.
16. Winder,S.J.,Allen,B.G.,Fraser,E.D.,Kang,H-M.,Kargacin,G.J.and Walsh,M.P.(1993) *Biochem. J.* 296, 827-836.
17. Rokolya,A.,and Moreland,R.S.(1993) *Biophys. J.* 64, A31.
18. Barany,M.and Barany,K.(1993) *Biochim. Biophys. Acta* 1179, 229-233.
19. Gimona,M.,Sparrow,M.P.,Strasser,P.,Herzog,M.and Small,J.V. *Eur. J. Biochem.* 205, 1067-1075.
20. Winder,S.J.and Walsh,M.P.(1990) *J. Biol. Chem.* 265, 10148-10155.
21. Nagai,P.K.,and Walsh, M.P.(1984) J.Biol.Chem. 259, 13656-13659.
22. Nagai,P.K.,and Walsh, M.P.(1985) Biochem.J. 230, 695-707.
23. Adam,L.P.and Hathaway,D.R.(1993) *FEBS Lett.* 322, 56-60.