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PHOSPHORYLATION OF CALDESMON BY PROTEIN KINASE C

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Protein kinase C catalyzes phosphorylation of caldesmon, an F-actin binding protein of smooth muscle, in the presence of Ca^{2+} and phospholipid. Protein kinase C incorporates about 8 mol of phosphate/mol of chicken gizzard caldesmon. When calmodulin was added in the medium, there was an inhibition of phosphorylation. The fully phosphorylated, but not unphosphorylated, caldesmon inhibited myosin light chain kinase activity. The possibility that protein kinase C plays some role in smooth muscle contractile system through caldesmon, warrants further attention.

Recent evidence indicates that muscle contraction is regulated by intracellular Ca^{2+} concentrations ranging from 10^{-7} - 10^{-8} M to 5 x 10^{-6} M. It is now generally accepted that in smooth muscle and non-muscle cells, the regulation of contraction is due to the phosphorylation and dephosphorylation of the 20,000-dalton light chain of myosin (1,2). This key reaction is mediated by myosin light chain kinase (MLC kinase), which requires Ca^{2+} and calmodulin. As a result of the covalent modification, the activity of the actin-activated Mg^{2+}-ATPase is markedly enhanced (3-5), and myosin filaments are stabilized (6). However, there seems to be another type of Ca^{2+}-dependent mechanism linked to smooth muscle, namely the F-actin severing and capping proteins (7-9). Caldesmon, first described Sobue et al. (10), is a F-actin binding protein and also interacts with calmodulin in the presence of Ca^{2+}. They proposed that caldesmon may be involved in regulation of the actin- myosin interaction in a Ca^{2+} dependent manner and

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The abbreviations used are: protein kinase C, Ca^{2+}-activated phospholipid-dependent protein kinase; MLC kinase, myosin light chain kinase, EGTA, ethylene glycol bis (ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid; CaD, caldesmon.

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"flip-flop mechanism" was the term used (11). Recent studies indicated that caldesmon causes a significant inhibition of both superprecipitation and actin-activated Mg^{2+}-ATPase activity (12), and that caldesmon induces polymerization of G-actin into filaments in a very low ionic strength solution (13).

Protein kinase C is a Ca^{2+}-dependent protein kinase that requires phosphatidylserine but not calmodulin (14) for its activity. This kinase is distributed in a wide variety of mammalian tissues, including smooth muscle (15,16). There are several proteins known to be phosphorylated by protein kinase C (15-25). However, the physiological significance of protein kinase C-induced phosphorylation remains to be established.

We have reported that two different actin binding proteins, vinculin and filamin were phosphorylated by protein kinase C (20). The present manuscript describes the phosphorylation of caldesmon catalyzed by protein kinase C.

MATERIALS AND METHODS

Caldesmon was freshly prepared from chicken gizzard smooth muscle according to Bretscher (26) and an extinction coefficient $E^{1\%}_{276}$=3.0 was used to determine the protein concentration. Protein kinase C was partially purified from rabbit brain (27). Calmodulin was isolated from bovine brain, as described (28). MLC kinase was purified from chicken gizzard, according to Adelstein and Klee (29). Protein kinase C was assayed in a volume of 0.2 ml of 25 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 50 µg/ml phosphatidylserine, 0.5 mM [γ-^{32}P]ATP, 5 µM caldesmon and 1.0 µg of Protein Kinase C at 28°C for 20 min. Phosphorylated caldesmon was isolated by gel filtration on Sephacryl S-300 column (2.5 x 110 cm) equilibrated with 10 mM imidazole (pH 7.0) 0.1 M NaCl, 0.1 mM EGTA, 0.1 mM DTT, after the phosphorylation of caldesmon by protein kinase C as described in the text. MLC kinase was assayed in a volume of 0.2 ml of 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.4 µM calmodulin, 0.2 mg/ml isolated 20,000-dalton myosin light chain, 10 µM [γ-^{32}P]ATP, and 52 ng of MLC kinase at 30°C for 5 min. SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (30), using a 3% acrylamide stacking gel and a 7.5 % acrylamide resolving gel. The phosphorylated protein was visualized by autoradiography using Kodak-X-O mat film. [γ-^{32}P]ATP was obtained from Amersham, phosphatidylserine (beef brain) was from Serdary Research laboratory, Inc. All other regents were of the highest grade available.

RESULTS AND DISCUSSION

Chicken gizzard muscle caldesmon was phosphorylated by protein kinase C in the presence of Ca^{2+} and phospholipid (Table 1). Ca^{2+} did not stimulate

Table 1
Ca^{2+} and phospholipid dependence
of caldesmon phosphorylation by protein kinase C

Activator	CaD phosphorylation
	pmol/min
EGTA (1 mM)	0.59
Ca^{2+} (1 mM)	0.87
Ca^{2+}+phosphatidylserine (50 µg/ml)	81

Phosphorylation assay was measured as described under "Materials and Methods". Protein kinase C activity was assayed using 1.5 mg/ml of caldesmon in the presence of 1 mM $CaCl_2$ and 50 µg/ml phosphatidylserine or 1 mM EGTA.

the phosphorylation of caldesmon by protein kinase C in the absence of phosphatidylserine. Phosphorylation of caldesmon by protein kinase C was further supported by autoradiographic analysis of the SDS-polyacrylamide gel electrophoresis (Fig. 1). Incorporation of ^{32}P in the presence Ca^{2+} alone was scanty. Similar observations have been reported for the phosphorylation of smooth muscle heavy meromyosin (31). In contrast to caldesmon, other substrate proteins such as tyrosine hydroxylase or guanylate cyclase are

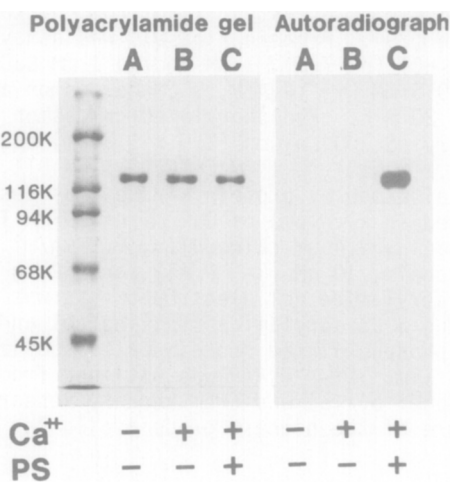
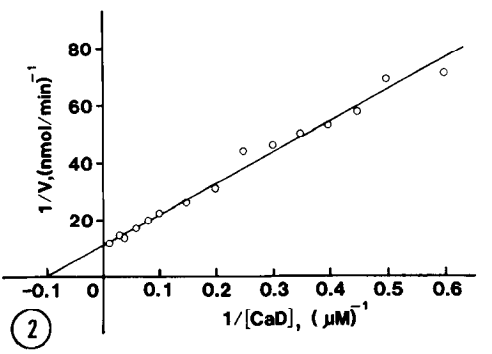


Figure 1. Phosphorylation of caldesmon by protein kinase C
The left panel shows Coomassie blue stain of SDS-polyacrylamide gel electrophoresis of caldesmon and the right panel shows an autoradiograph of the gel. Lane A, 1 mM EGTA; Lane B, 1 mM $CaCl_2$; Lane C, 1 mM $CaCl_2$ + 50 µg/ml phosphatidylserine. Other assay conditions were described under "Materials and Methods".

appreciably phosphorylated by protein kinase C in the presence of Ca^{2+} alone (18,23). A recent report indicated that caldesmon was phosphorylated by a Ca^{2+}/calmodulin-dependent protein kinase contaminating the crude caldesmon preparation (12). In our preparation, endogenous phosphorylation of caldesmon was not evident when caldesmon was incubated without protein kinase C and in the presence of Ca^{2+} and calmodulin. MLC kinase also did not induce the phosphorylation of caldesmon in the presence of Ca^{2+} and calmodulin (data not shown).

The initial rate of phosphorylation of caldesmon by protein kinase C was studied over a wide range of substrate concentrations (Fig. 2). The apparent Km value for caldesmon was 9.7 μM and the Vmax value was 91 nmol/min/mg protein. The Km value was higher than the values of 3.6 and 2.4 μM for histone H1 and heavy meromyosin, respectively, and was lower than the value of 28.6 μM for the isolated 20,000-dalton myosin light chain (31).

The time course of phosphorylation of caldesmon by protein kinase C is shown in Fig. 3. Incorporation reached 7.7 mol of phosphate per mol of caldesmon at the end of 2 hr. Caldesmon served as an excellent substrate for protein kinase C, compared with the other acidic substrate proteins.



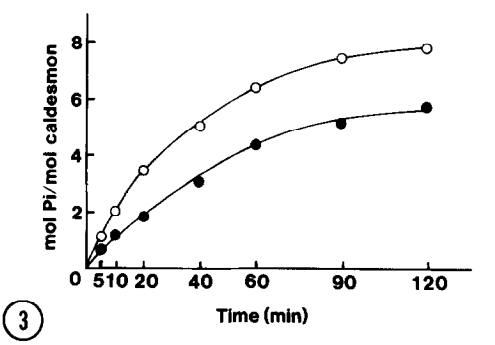


Figure 2. Double reciprocal plots of phosphorylation of caldesmon by protein kinase C
The following concentrations (μM) of caldesmon were used: 1.67, 2.0, 2.22, 2.5, 2.86, 3.33, 4, 5, 6.67, 10, 12.5, 16.7, 25, 33.3, 40. Other assay conditions were described under "Materials and Methods". The molecular weight of caldesmon was 300,000 daltons.

Figure 3. Phosphorylation of caldesmon by protein kinase C in the presence (●) or absence (○) of calmodulin.
Caldesmon (1.5 mg/ml) was phosphorylated, with or without calmodulin (1 mg/ml), as described under "Materials and Methods".

Stoichiometric studies, with troponin I, troponin T, and heavy meromyosin as substrates, showed that protein kinase C incorporated about 1.7, 2.0, and 2.0 mol of phosphate per mol of the proteins, respectively (21,31). In the presence of calmodulin, phosphorylation of caldesmon by protein kinase C was less (5.7 mol Pi/mol caldesmon). Since calmodulin binds to caldesmon in the presence of Ca^{2+}, some phosphorylation sites may be located in the calmodulin-binding region of caldesmon.

Fig. 4 shows the effects ofnative unphosphorylated caldesmon and of caldesmon phosphorylated by protein kinase C on MLC kinase activity, using the isolated 20,000-dalton myosin light chain as a substrate for MLC kianse. Unphosphorylated caldesmon had essentially no effect on MLC kinase activity, up to 10 μg/ml. On the other hand, in the presence of phosphorylated caldesmon by protein kinase C, there was approximately a 40% decrease in MLC kinase activity.

Nagai and Walsh reported that caldesmon caused a inhibition of actin activated myosin Mg^{2+} ATPase activity and did not affect the phosphorylation of myosin. Caldesmon phosphorylated by Ca^{2+}/calmodulin-dependent kinase, did not inhibit Mg^{2+} ATPase activity (12). They also stated that cAMP-dependent protein kinase and MLC kinase are unable to phosphorylate caldesmon (32). These findings suggest that two different kinases, Ca^{2+}/

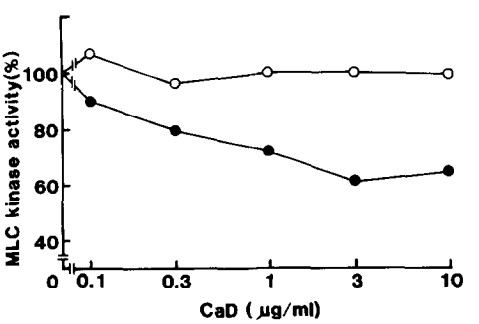


Figure 4. Effect of caldesmon phosphorylated by protein kinase C on MLC kinase
Islated 20,000-dalton myosin light chain (0.2 mg/ml) was phosphorylated by MLC kinase in the presence of unphosphorylated caldesmon (○) or phosphorylated caldesmon by protein kinase C (●). MLC kinase assay condition was described under "Materials and Methods".

calmodulin dependent kinase and protein kinase C, might be involved in the contraction of smooth muscle, via phosphorylation of caldesmon by these kinases.

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REFERENCES

1. Adelstein, R.S. and Eisenberg, E. (1980) Annu. Rev. Biochem. 49, 921-956
2. Walsh, M.P. and Hartshorne, D.J. (1982) In Calcium and Cell Function (Cheung, W.Y., Ed) Vol. 3, pp 223-269, Academic Press, New York
3. Sobieszek, A. and Small, J.V. (1977) J. Mol. Biol. 112, 559-576
4. Ikebe, M., Onishi, H. and Watanabe, S. (1977) J. Biochem. 82, 299-302
5. Sellers, J.R., Pato, M.D. and Adelstein, R.S. (1981) J. Biol. Chem. 256, 13137-13142
6. Suzuki, H., Onishi, H., Takahashi, K. and Watanabe, S. (1978) J. Biochem. 84, 1529-1542
7. Bretscher, A. and Weber, K. (1980) Cell, 20, 839-847
8. Mooseker, M.S., Graves, T.A., Wharton, K.A., Falcon, N. and Howe, C.L. (1980) J. Cell Biol. 87, 809-822
9. Yin, H.L. and Stossel, T.P. (1980) J. Biol. Chem. 255, 9490-9493
10. Sobue, K., Muramoto, Y., Fujita, M. and Kakiuchi, S. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 5652-5655
11. Sobue, K., Morimoto, K., Inui, M., Kanada, K. and Kakiuchi, S. (1982) Biomed. Res. 3, 188-196
12. Nagai, P.K. and Walsh, M.P. (1984) J. Biol. Chem. 259, 13656-13659
13. Galazkiewicz, B., Mossakowska, M., Osińska, H. and Dabrowska, R. (1985) FEBS Lett. 184, 144-149
14. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692-3695
15. Kuo, J.F., Andersson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, I., Brackett, N.L., Katoh, N., Shoji, M. and Wrenn, R.W. (1980) Proc. Natl. Acad. Sci. U.S.A. 7039-7043
16. Minakuchi, R., Takai, Y., Yu, B. and Nishizuka, Y. (1981) J. Biochem. 89, 1651-1654
17. Endo, T., Naka, M. and Hidaka, H. (1982) Biochem. Biophys. Res. Commun. 105, 942-948
18. Wu, W.C-S., Walaas, S.I.I., Nairn, A.C. and Greengard, P. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 5249-5253
19. Limas, C.J. (1980) Biochem. Biophys. Res. Commun. 96, 1378-1383
20. Kawamoto, S. and Hidaka, H. (1984) Biochem. Biophys. Res. Commun. 118, 736-742
21. Katoh, N., Wise, B.C. and Kuo, J.F. (1983) Biochem. J. 209, 189-195
22. Beg, Z.H., Stonik, J.A. and Brewer, H.B. (1985) J. Biol. Chem. 260, 1682-1687
23. Zwiller, J., Revel, M.O. and Malviya, A.N. (1985) J. Biol. Chem. 260, 1350-1353
24. Brocklehurst, K.W. and Hutton, J.C. (1984) Biochem. J. 220, 283-290
25. Umekawa, H., Naka, M., Inagaki, M., Onishi, H. Wakabayashi, T. and Hidaka, H. (1985) J. Biol. Chem. in press
26. Bretscher, A. (1984) J. Biol. Chem. 259, 12873-12880

27. Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692-3695
28. Endo, T., Tanaka, T., Isobe, T., Kasai, H., Okuyama, T. and Hidaka, H. (1981) J. Biol. Chem. 256, 12485-12489
29. Adelstein, R.S. and Klee, C.B. (1981) J. Biol. Chem. 256, 7501-7509
30. Laemmli, U.K. (1970) Nature 227, 680-685
31. Nishikawa, M., Sellers, J.R., Adelstein, R.S. and Hidaka, H. (1984) J. Biol. Chem. 259, 8808-8814
32. Nagai, P.K., Carruthers, C.A. and Walsh, M.P. (1984) Biochem. J. 218, 863-870