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CALCIUM- AND CALMODULIN-DEPENDENT PHOSPHORYLASE KINASE ACTIVITY IN PORCINE UTERINE SMOOTH MUSCLE*

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SUMMARY: Porcine uterine smooth muscle phosphorylase kinase has been partially purified. The enzyme was activated about 1.5-2.0-fold by exogenous calmodulin. Half maximal stimulation was observed at about 100 nM calmodulin. The activation was dependent on calcium and was maximum at pH 7.5 in the range of pH from 6 to 9. This activation was completely abolished by 100 μM trifluoperazine. The result suggested that unlike slow and cardiac muscles, phosphorylase kinase of uterine smooth muscle showed similar response to calmodulin with that of fast muscle. The physiological role of the calcium and calmodulin-dependent activation of myometrium phosphorylase kinase is briefly discussed.

Phosphorylase kinase (EC 2.7.1.38) is known to be a key enzyme in the cascade of reactions that regulate glycogen metabolism. Recently, besides the cAMP-dependent mechanism, calcium-calmodulin-dependent activation of phosphorylase kinase was demonstrated in skeletal (fast) muscle (1) and liver (2). It was, however, reported that the enzyme in slow and cardiac muscles did not appear to be regulated by exogenous calmodulin (3,4). In smooth muscle, the calcium-calmodulin complex has been shown to stimulate the Ca^{2+}-Mg^{2+}-ATPase associated with microsome (5), Ca^{2+} pump in plasma

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Abbreviations: EGTA, ethylene glycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; PMSF, phenyl methylsulfonyl fluoride.

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membrane (6), membrane phosphorylation (7), and the phosphorylation of myosin by myosin light chain kinase (8,9), and also to regulate the adenylate cyclase activity (10). However, the regulation of smooth muscle phosphorylase kinase by calcium-calmodulin remained unexplored. In the present study, we have attempted to isolate phosphorylase kinase from porcine myometrium, which comprises smooth muscle fibers and plays an important role in the maintenance of pregnancy and delivery of the fetus, and further to clarify the interaction of the enzyme involved in calcium and calmodulin system.

MATERIALS AND METHODS

(U-^{14}C)Glucose-1-phosphate was purchased from New England Nuclear. Sepharose CL-4B was purchased from Pharmacia Fine Chemicals. Rabbit skeletal muscle phosphorylase b was purified by the methods of Fischer and Krebs (11). Bovine brain calmodulin was homogeneously prepared by the method of Yazawa et al. (12). Calmodulin-Sepharose 4B was prepared by the method of Roach et al. (13). Other experimental materials were obtained from commercial sources.

Phosphorylase kinase activity was determined by measuring the conversion of phosphorylase b to phosphorylase a and the definition of one unit of this enzyme was the same as described in Taira et al. (14). Protein concentration was determined using Protein Assay (Bio-Rad) with ovalbumin as a standard.

Partial purification of myometrium phosphorylase kinase was performed at 0-4°C. Porcine uteri were obtained from a local slaughter house and treated as described below within 1 h. Three porcine uteri (25g), separated from vaginal regions and serosa, were minced and homogenized in an Ultra-Turrax homogenizer with 2.5 volumes of 50 mM Tris-HCl at pH 7.5 containing 4 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM PMSF and 0.5 mM benzamidine. The composition of this buffer was similar to that used in our previous study (15). A slight modification was made with Cohen's preparation (16). The suspension was centrifuged at 10,000 x g for 33 min and the supernatant was decanted through glass wool. The solution was centrifuged at 130,000 x g for 55 min and the supernatant was carefully decanted through glass wool. Solid ammonium sulfate was added to the enzyme solution to make a final concentration of 40 %. After standing 30 min in ice, the suspension was centrifuged for 10 min at 10,000 x g. The supernatant was discarded and the precipitate was resuspended in a small volume of homogenizing buffer and dialysed for 1 h against this solvent. The dialysate, 3.3 ml, was clarified at 15,000 x g for 2 min and chromatographed on a Sepharose CL-4B column (94 x 2.6 cm) equilibrated in 25 mM Tris-HCl at pH 7.5 containing 10 mM 2-mercaptoethanol, 0.1 mM PMSF, and 20 % (w/v) glycerol (gel buffer). The active fractions were collected and concentrated by Amicon (type 202, membrane filter: PM 10). Then, 1 M $CaCl_2$ was added to this enzyme solution to make a final calcium concentration of 2 mM and the enzyme solution was applied to a calmodulin-Sepharose 4B column (6 x 1.2 cm) equilibrated with gel buffer containing 2 mM $CaCl_2$. Then the column was washed with the same buffer. The flow rate was 0.47 ml/min and fractions of 1.5 ml each were collected. After collection of fraction 25, elu-

tion was continued with gel buffer containing 2 mM EGTA. The most active fraction was used in this experiment. All experiments were carried out with at least 5 preparations and the assays were carried out in duplicate.

RESULTS

The specific activity of the phosphorylase kinase fraction after this procedure was 4.3-10.3 units/mg protein. The activity of myometrium phosphorylase kinase is shown as a function of added calmodulin concentrations (Fig. 1). The stimulation at pH 7.5 was 1.8-fold over the basal activity in average. The concentration of calmodulin required for half-maximal enzymatic activity was 1×10^{-7} M and the maximal activity was obtained at 1×10^{-6} M.

The antipsychotic drug trifluoperazine which is known to abolish the calmodulin-stimulated skeletal muscle phosphorylase kinase activity (1), was tested for its ability to influence the enzyme of smooth muscle (Fig. 2). Trifluoperazine slightly affected the Ca^{2+}-dependent activity in the absence of calmodulin, the inhibi-

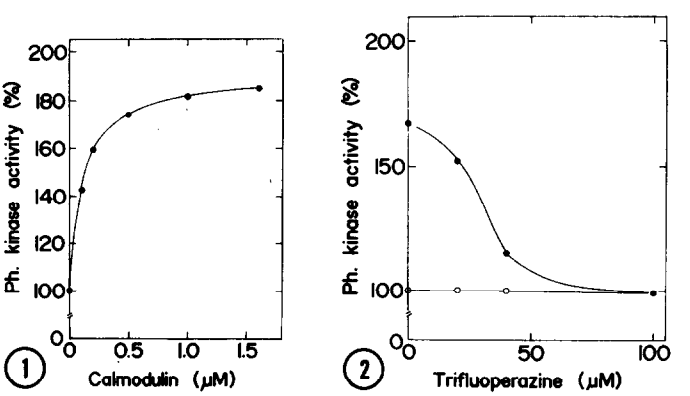

Fig. 1. Activation of myometrium phosphorylase kinase by exo calmodulin. The enzyme activity was determined as described viously (14) at pH 7.5 except that various concentrations of dulin were added to the initial reaction mixture (phosphoryla phosphorylase a reaction). As the enzyme preparations in the tion mixture contained 0.6 mM EGTA, exogenous calcium was ad the final concentration of 0.6 mM. Activity of phosphorylase in the absence of calmodulin is taken as 100 %.

Fig. 2. Effect of trifluoperazine on the activity of myometrium phosphorylase kinase activated by 1.2 μM calmodulin (●). The enzyme activity was assayed as in the legend to Fig. 1 except that various concentrations of trifluoperazine were added to the initial raction mixture. Activity of phosphorylase kinase in the absence of calmodulin (○) is taken as 100 %.

tion from preparation to preparation being about 40 % at 0.1 mM trifluoperazine, which nearly corresponded with the results of Shenolikar et al. (1). The calmodulin-stimulated activity was completely abolished by 0.1 mM trifluoperazine.

The next experiment was conducted to examine whether the activity of myometrium phosphorylase kinase is dependent on the presence of Ca^{2+}. The activity of phosphorylase kinase was measured as a function of EGTA in the presence or absence of exogenous calmodulin (Fig. 3). At almost saturating concentration of Ca^{2+} (10^{-6}-10^{-5} M) (17), phosphorylase kinase was activated about 2-fold in the presence of calmodulin. This activation was abolished by increased concentrations of EGTA.

It has been demonstrated that the activation of skeletal muscle phosphorylase kinase by exogenous calmodulin is dependent on pH (13,18,19). It was therefore of interest to examine whether calmodulin was effective in activating myometrium phosphorylase kinase over a wide range of pH values. Fig. 4 shows the effect of pH on the activity of myometrium phosphorylase kinase with or without

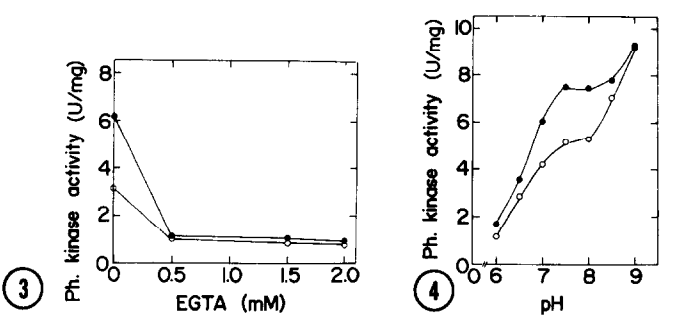

Fig. 3. Calcium dependency of myometrium phosphorylase kinase activity in the presence (●) or absence (○) of 1.2 μM calmodulin. The enzyme activity was assayed as in the legend to Fig. 1. As the enzyme preparations in the reaction mixture contained 0.6 mM EGTA, exogenous calcium was added to the final concentration of 0.6 mM. Then various concentrations of EGTA were added to the initial reaction mixture.

Fig. 4. Effect of pH on myometrium phosphorylase kinase in the presence (●) or absence (○) of 1.2 μM calmodulin. The enzyme activity was assayed as in the legend to to Fig. 1 except that 75 mM Tris-glycerophosphate buffers were employed as the indicated pH.

exogenous calmodulin. Myometrium phosphorylase kinase was obviously stimulated by the addition of exogenous calmodulin especially at pH 7.5.

DISCUSSION

The results obtained in the present studies demonstrate that like the skeletal (fast) muscle enzyme, porcine uterine smooth muscle phosphorylase kinase is activated by the addition of exogenous calmodulin. The activation is also Ca^{2+}-dependent. Although most of the myometrium phosphorylase kinase bound to the calmodulin-Sepharose 4B column with 2 mM Ca^{2+}, a small amount of the enzyme never bound to the column. The reason for this elution profile of the myometrium enzyme must await further studies.

There have been many protein kinases which are controlled by calmodulin (20,21). Although the possibility of the participation of another calmodulin-dependent protein kinase which stimulates the myometrium phosphorylase kinase cannot be ruled out at present, this calcium-calmodulin-dependent activation of myometrium enzyme seems to be a direct stimulation like the liver enzyme reported in our previous study (2). On the other hand, we examined the calmodulin effect on phosphorylase kinase activity using rabbit fast, slow and cardiac muscles, the enzymes of which were obtained after the step of Sepharose CL-4B. The results are essentially consistent with the reports of several investigators (3,4,13,18,19). Namely, no effect of calmodulin on slow and cardiac muscle enzymes was observed. As concerns fast muscle, the enzyme was activated 2-2.5-fold by adding exogenous calmodulin, and this seemed to be true characteristic. After the same purification procedure, the effect of calmodulin on uterine enzyme has been similar to that in the present studies (data not shown).

Uterine contraction is affected by agents such as oxytocin, prostaglandin $F_{2\alpha}$, acetylcholine, epinephrine and estrogens, which

increase intracellular Ca^{2+} in uterine smooth muscle (22). Myosin light chain kinase in smooth muscle, which is one of the calmodulin activatable enzyme (8), has been reported to be involved in the contraction-relaxation cycle by stimulation of the actin-activated Mg^{2+}-ATPase activity of myosin, and may serve for the contractility of the myometrium (22-24). Thus, Ca^{2+} and calmodulin in myometrium may be closely related to the maintenance of pregnancy and delivery of the fetus, and serve for the coordination of glycogen metabolism and contractility in uterine smooth muscle.

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REFERENCES

1. Shenolikar, S., Cohen, P.T.W., Cohen, P., Nairn, A.C. and Perry, S.V. (1979) Eur. J. Biochem. 100, 329-337
2. Nakamura, S., Tsutou, A., Mizuta, K., Negami, A., Nakaza, T., Hashimoto, E. and Yamamura, H. (1983) FEBS Lett. 159, 47-50
3. Sharma, R.K. Tam, S.W., Waisman, D.M. and Wang, J.H. (1980) J. Biol. Chem. 255, 11102-11105
4. Yoshikawa, K., Usui, H., Imazu, M., Takeda, M. and Ebashi, S. (1983) Eur. J. Biochem. 136, 413-419
5. Hogaboom, G.K. and Fedan, J.S. (1981) Biochem. Biophys. Res. Commun. 99, 737-744
6. Morel, N., Wibo, M. and Godfraind, T. (1981) Biochim. Biophys. Acta 644, 82-88
7. Hogaboom, G.K., Emler, C.A., Butcher, F.R. and Fedan, J.S. (1982) FEBS Lett. 139, 309-312
8. Dabrowska, R., Sherry, J.M.F., Aromatorio, D.K. and Hartshorne, D.J. (1978) Biochemistry 17, 253-258
9. Hartshorne, D.J. and Mrwa, U. (1982) Blood Vessels 19, 1-18
10. Piascik, M.T., Babich, M. and Rush, M.E. (1983) J. Biol. Chem. 258, 10913-10918
11. Fischer, E.H. and Krebs, E.G. (1962) Methods Enzymol. 5, 369-376
12. Yazawa, M., Sakuma, M. and Yagi, K. (1980) J. Biochem. 87, 1313-1320
13. DePaoli-Roach, A.A., Gibbs, J.B. and Roach, P.J. (1979) FEBS Lett. 105, 321-324
14. Taira, T., Kii, R., Sakai, K., Tabuchi, H., Takimoto, S., Nakamura, S., Takahashi, J., Hashimoto, E., Yamamura, H. and Nishizuka, Y. (1982) J. Biochem. 91, 883-888
15. Hashimoto, E., Mizuta, K., Tsutou, A., Nakamura, S. and Yamamura, H. (1983) J. Biochem. 93, 939-942
16. Cohen, P. (1973) Eur. J. Biochem. 34, 1-14
17. Bartfai, T. (1979) Adv. Cyclic Nucl. Res. 10, 219-242

18. Walsh, K.X., Millikin, D.M., Schlender, K.K. and Reimann, E.M. (1980) J. Biol. Chem. 255, 5036-5042
19. Cohen, P. (1980) Eur. J. Biochem. 111, 563-574
20. Means, A.R. and Dedman, J.R. (1980) Nature 285, 78-77
21. Cohen, P. (1982) Nature 296, 613-620
22. Huszar, G. and Roberts, J.M. (1982) Am. J. Obstet. Gynecol. 142, 225-237
23. Adelstein, R.S. and Eisenberg, E. (1980) Annu. Rev. Biochem. 49, 921-956
24. Stull, J.T. Blumenthal, D.K. and Cooke, R. (1980) Biochem. Pharmacol. 29, 2537-2543