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INCREASED CREATINE KINASE IN THE HORMONE-STIMULATED SMOOTH MUSCLE OF THE BOVINE UTERUS

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SUMMARY

The hormone-stimulated growth of the bovine myometrium is accompanied by a 2-fold increase in creatine kinase (CK)* activity and a decrease in the total cell protein at 90-120 day gestation. The microsome bound fraction of CK increases 4-fold. From identical electrophoretic and immunological behavior of the enzyme with the enzyme from unstimulated muscle, we conclude that the hormone-induced change in activity results from an increase in the quantity of the normal smooth muscle CK isoenzyme.

Creatine Kinase (EC.2.7.3.2) has played a special role in striated muscle metabolism. During differentiation of the myoblast the transition from the embryonic to the muscle form of CK isoenzyme and its rate of synthesis are closely synchronous with the synthesis of myosin (1,2,3) as are the synthesis of other muscle specific proteins (4). In the fully differentiated muscle the enzyme is believed to be involved in harnessing the energy in PCr for contraction by transphorylation (5). During the development and growth of the uterine smooth muscle induced either by administration of estradiol to immature animals or as a consequence of secretion of the hormone by the ovaries in pregnancy, the synthesis of actomyosin is reported to increase (6). This led us to investigate the level, subcellular distribution, isoenzyme pattern and immunochemical behaviour of CK in the bovine myometrium in pregnancy.

MATERIALS AND METHODS

Enzyme Extraction: Fresh bovine uterus was obtained immediately after sacrifice and chilled in ice during transport. The myometrium was carefully dissected from the endometrium and the adherent connective tissues. The muscle was ground

*Abbreviations Used: CK=Creatine Kinase; MM and BB=Homodimers of CK in skeletal muscle and brain respectively; PMSF=Phenylmethyl sulforyl fluoride; PCr= Phosphoryl creatine

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in a mechanical grinder and was homogenized for 30 seconds using a Sorvall Omin-mixer in ice cold solution of 0.3 M KCl, 0.15 M phosphate buffer (pH 6.8), 1 mM β-mercaptoethanol and 0.5 mM PMSF. Extraction was continued for 30 minutes on a mechanical shaker at 4°C. The supernate was obtained by centrifugation at 5000 g for 20 min, the residue was extracted twice more in the cold medium. A fourth extraction solubilized negligible amounts of CK. The pooled supernatant solution was used for determination of CK activity and protein. The endometria of the non-gravid control animals were routinely examined for absence of signs of estrus. Tissues from pregnant animals were used only after establishing the period of pregnancy (90-120 days) by determining the skeletal dimensions of the fetus (7).

Subcellular Fractions: Subcellular fractionation of the myometrium was performed by differential centrifugation in isotonic sucrose (8). 2.0 to 4.0 g of carefully trimmed and minced muscle was homogenized as described above in 12 volumes of 0.3 M sucrose prepared in freshly boiled water. The fractions collected and the centrifugal fields used were: a) nuclei-myofibrils at 700 g for 10 min; b) mitochondria at 11,000 g for 30 min; c) lysosomes by the use of discontinuous sucrose density gradients (0.45 M/0.6 M); d) microsomes at 100,000 g for 60 min. The 100,000 g supernate was used as the cytosol. Enzyme activity and protein content were determined immediately. The pellets were suspended in 0.2 ml of 0.3 M sucrose and dispersed gently with a Dounce homogenizer. The cytosol and the microsomes were diluted with 0.05 M tris-glycine buffer (pH 8.6) containing 1 mM β-mercaptoethanol.

CK Activity: The enzyme activity was measured in the direction of ATP synthesis by the procedure of Oliver (9) either using the Max Pack Kit (Calbiochem, La Jolla, Ca.) or preparing the assay mixture of the composition furnished by the manufacturers. Absence of inhibitors or activators in tissue extracts were ensured by varying the sample size in the assay system. Controls runs (no PCr) showed that adenylate kinase activity did not interfere with the assay. Units of enzyme activity (μmoles of NADPH/min) were computed from absorbance changes using E_M^{340nm} of 6.22×10^3 for NADPH.

Isoenzyme Determination: CK isoenzymes were separated by electrophoresis on cellulose acetate strips in a Beckman Microzone apparatus. Electrophoresis was carried out for 30-40 min at a nominal voltage of 250 V using 0.025 M Tris, 0.190 M glycine and 0.001 M β-mercaptoethanol as the electrode buffer. The active enzyme species was located by a gel overlay technique (10).

Immunochemical Procedures: The brain type homodimer (BB) of CK was purified from bovine brain by the procedure described by Armstrong et al. (11) and antibodies were raised in rabbits by intramuscular injection of the enzyme in complete Freund's adjuvant followed by intradermal injection of booster doses. The specificity of the antibodies was established by double diffusion in agar (12). Titration of the enzyme against the antiserum was done in 0.15 M NaCl, 0.01 M phosphate buffer (pH 7.4) in a total volume of 50 μl using 0-20 μl of an appropriately diluted serum against a constant amount of enzyme activity (equivalent to ΔA_{340}/min fo 0.3 to 0.4). The mixture was incubated for 1 hour at 37°C and for 18 hours at 0°C. After centrifugation at 11×10^3 g for 10 min CK activity was determined on 5 to 20 μl of a carefully siphoned supernatant layer.

Protein was determined by the Lowry method (13) using bovine serum albumin as standard.

RESULTS

In Table I are presented the total CK activity extractable from fresh bovine myometrium. Under the assay conditions the rate was linear for 10 min or more when the enzyme was diluted to yield a A_{340}/min of 0.04 - 0.10. The five samples of the myometrium from non-pregnant animals had a mean activity of 30.4 units/g

Table I: Total creatine kinase activity and soluble protein in the pregnant and non-pregnant bovine myometrium.

	Non-Pregnant (N=5)	Pregnant (N=7)
CK Activity ±S.E.M. (u/g wet weight)	30.4±4.8	65.5±8.8
Protein ± S.E.M. (mg/g wet weight)	80.0±6.2	72.0±8.0

Total enzyme was assayed immediately after extraction as described in Methods at 22°C. The ΔA_{340}/min has been converted to units (1 μ mole NADPH/min) at 30°C by using a temperature coefficient of 1.6 and a molar extinction coefficient of 6.2×10^3 for NADPH.
N=Number of Animals.

wet weight (range 15.1 - 42.7 units) whereas the mean CK activity at 90 to 120 days pregnancy was 65.5 units/g wet weight (36.8 - 86.5 units). The total sacroplasmic proteins from the two groups were 80.0 and 72.0 mg/g wet weight respectively. The slight decrease in the cellular content of the soluble proteins might reflect the effect of estrogen in promoting the influx of electrolytes and water into the cells (14).

In Table II are the results of CK activity found in the various subfractions of the muscle. In the case of the tissue from the gravid, as well as from the control animals, the majority of the CK activity was found in the cytosol fraction A small but reproducible level of activity was found in the microsomal fractions. Neither mitochondria nor lysosomes contained significant CK activity. The "Nuclear-myofibrillar" fraction (700 g sediment) accounted for about 3 to 5 percent of the total, but in view of the heterogeneity of this fraction, including

Table II: Subcellular distribution of creatine kinase in the bovine uterus (units/g wet weight).

	Cytosol	Mitochondria	Lysosomes	Microsomes
Non-Pregnant (N=4)	33.5±2.1	Trace	N.D.	1.1±0.1
Pregnant (N=5)	49.6±7.6	Trace	N.D.	4.5±0.6

2.0 to 4.0 g muscle were homogenized in 0.3 M sucrose and fractionated as described under Materials and Methods. The numbers represent means and standard errors of the mean.
N=Number of Animals N.D.=Not detected

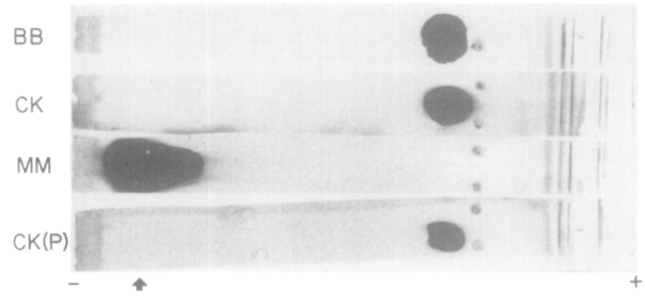

Figure 1. CK isoenzyme patterns obtained by electrophoresis on cellulose acetate in 0.025 M tris-0.190 M glycine (pH 8.6) and 1 mM β-mercaptoethanol. BB=purified bovine brain enzyme, CK=control uterine muscle extract, MM=purified rabbit skeletal muscle CK. CK(P)=extract from hormone-stimulated muscle. Arrow represents origin.

unbroken cells, no results are listed. As shown in Table I and II, the increase in the cytosol fraction in pregnancy reflects the increase in the total cell enzyme activity. In contrast, the microsome-associated enzyme activity shows a 4-fold increase in the hormone-dominated tissue. The microsomal activity was lost after keeping at $4^{o}C$ for 8-10 days or by repeated freeze-thawing. Repeated washing of fresh membranes with cold 0.3 M sucrose did not release the enzyme.

When the total extracts from the uterine smooth muscle were subjected to electrophoresis on cellulose acetate and the active enzyme was visualized the results shown in Figure 1 were obtained. A single spot of activity from both the gravid and the non-gravid tissues with a mobility equal to that of the pure brain enzyme was observed. It is apparent that the more acidic BB form of the enzyme was the predominant species in either tissue. In some experiments when the membranes were excessively loaded, a small amount (about 5% of the total as determined by densitometry) of creatine kinase with the mobility of the MM isoenzyme was found. This was found to be the case from control as well as from the hormone-stimulated muscles.

The specificity of the antiserum raised against the bovine brain CK was established by the fact that a single precipitin band was observed in double diffusion experiments with extracts of bovine brain and myometrium but not against skeletal muscle homogenates (results not shown). Further, prior treatment of the antiserum with pure brain CK abolished reactivity towards the uterine muscle

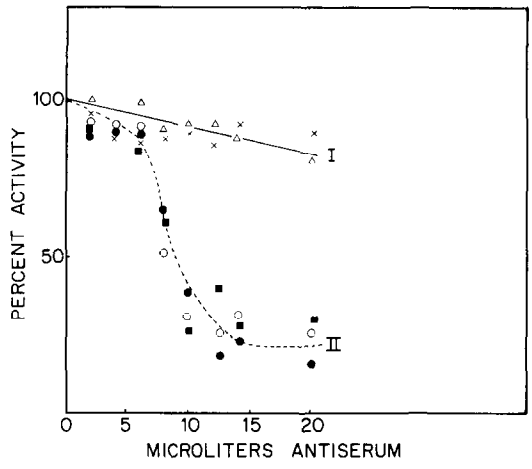

Figure 2. Antibody titration of uterine smooth muscle CK. Total myometrial extract diluted to ΔA_{340}/min of 0.3 - 0.4 was treated with antiserum. I: titration with control (preimmune antiserum); II: titration with antiserum to pure bovine brain enzyme. CK represented by ● and CK(P) represented by ■ are as under Figure 1.

extracts. In Figure 2 is shown the results obtained when a constant amount of CK was treated with increasing amounts of serum. The control serum (preimmune serum) had little effect on the enzyme activity. Increasing amounts of the antiserum, in contrast, resulted in the precipitation of creatine kinase as indicated by the disappearance of activity in the supernatant fraction. The behavior of the enzymes from the control and hormone-stimulated muscles towards the antibody is indistinguishable. The quantitative response in terms of the onset of inhibition, the final degree of inhibition (about 80%) and the steep fall in the activity in between can be seen to be identical.

DISCUSSION

The growth and development of the mammalian uterus during pregnancy and its regression following parturition provides a unique model to study the cyclical processes accompanying cell and tissue hypertrophy. In humans and in cows the weight of the gravid uterus is about 40-fold higher than that of its counterpart in the non-pregnant state (15,16). The growth of the smooth muscle cells of the myometrium and of the associated connective tissue under the stimulus of ovarian hormones parallels the change in tissue weight (15,17). Experiments with immature

and castrate mature animals have established that administration of estrogens alone is sufficient to promote the uterotrophic effects (15,17-20). Estrogen receptors of high affinity have been found in the smooth muscle cells of the myometrium (21,22). Our results show that the hormone-stimulated growth of the smooth muscle results in a doubling of the CK activity in early pregnancy and that this phenomenon is not a reflection of a generalized increase in cell protein In fact, the total soluble protein has slightly decreased. The muscle growth at this stage is mostly due to cell proliferation (14,15) and occurs under the influence of estrogen (14,15). We have found that the cell size is not significantly altered in electron micrographs. Csapo and collaborators have previously reported increase in myometrial actomyosin in pregnancy (6). Thus the muscle specific proteins seem to be selectively enhanced. The only bound form of CK in the uterine smooth muscle was in the microsomes. Absence of the enzyme activity in the other subcellular organelles and the relatively tight binding to the microsomes argues against a non-specific binding especially in view of the high negative charge on both the membrane and the enzyme. It is noteworthy that, in estrogen treated rats, increase in the number of myofilaments and of endoplasmic reticulum were the most prominent changes observed in the myometrium (17,23). The identity of the electrophoretic mobility of the enzyme from pregnant animals on cellulose acetate with that from the control and from brain suggests that there is no change in the isoenzyme by the hormone. This confirms previous reports (24) that the smooth muscle isoenzyme of CK is identical to the brain form (BB). The similarity of interaction of the smooth muscle enzymes with the antibody against the anti-BB antiserum further substantiates the conclusion that the enzyme in the hormone-stimulated tissue is identical to the protein in the unstimulated muscle. Thus, we conclude that the increased CK activity observed in the proliferative phase of the bovine uterus smooth muscle growth represents an increase in the amount of the enzyme. Current studies are directed towards establishing the role of increased protein synthesis and decreased degradation in this process.

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