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COMPARTMENTATION OF CREATINE KINASE ISOENZYMES IN MYOMETRIUM OF GRAVID GUINEA-PIG

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SUMMARY

1. This study was performed to investigate the possible presence and role of the creatine kinase (CK) system in the contraction and relaxation of skinned guinea-pig uterus as well as the changes of the CK system during gestation. Experiments were performed on isolated longitudinal fibres of gravid and non-gravid myometrium.

2. Total CK activity increased from 74 ± 11 to 196 ± 39 IU (g wet wt)$^{-1}$ during gestation.

3. The four isoenzymes of CK: muscle (MM), muscle–brain (MB), brain (BB) and mitochondrial (mt-CK) were found in myometrium. MM, MB and BB isoenzymes represented respectively $20{\cdot}3 \pm 2{\cdot}6$, $10{\cdot}3 \pm 4{\cdot}4$ and $72{\cdot}7 \pm 2{\cdot}2$ % of total activity. The distribution of isoenzymes did not significantly change with gestation, the contribution of mt-CK increasing from trace to 5 % of total activity.

4. BB-CK was specifically bound to Triton X-100-skinned fibres with the non-gravid uterus containing $6{\cdot}7 \pm 1{\cdot}9$ IU (g wet wt)$^{-1}$ and the gravid uterus containing 44 ± 13 IU (g wet wt)$^{-1}$.

5. Active tension of Triton X-100-treated fibres increased from $6{\cdot}06 \pm 0{\cdot}68$ to $19{\cdot}3 \pm 1{\cdot}9$ mN mm^{-2} during gestation.

6. Submaximal tension ($43{\cdot}3 \pm 4{\cdot}4$ % of maximal tension) can be developed in the absence of ATP and in the presence of 12 mM phosphocreatine (PCr) and 250 μM MgADP from endogenous CK in non-gravid uterine fibres while the gravid uterus was able to generate $65{\cdot}4 \pm 3{\cdot}9$ % of maximal tension via the CK system.

7. The endogenous CK system was able to relax the skinned fibres from high-tension rigor conditions by $47{\cdot}3 \pm 4{\cdot}2$ % of total relaxation in non-gravid fibres and $60{\cdot}6 \pm 3{\cdot}2$ % of total relaxation in gravid fibres.

8. Non-gravid and gravid uteri both contained mt-CK of $17{\cdot}5 \pm 8{\cdot}4$ and 140 ± 22 μg (g wet wt)$^{-1}$ respectively as determined with antibodies against mt-CK.

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9. Oxygen consumption was studied in fibres where the plasmalemma was solubilized with 50 μg ml^{-1} saponin. Maximal respiration was increased from $0{\cdot}91 \pm 0{\cdot}05$ to $2{\cdot}61 \pm 0{\cdot}16$ μmol oxygen min^{-1} (g dry wt)$^{-1}$ in the gravid uterine fibres. However, creatine did not stimulate respiration in the uterine fibres treated with saponin.

10. It is concluded that the CK system undergoes qualitative as well as quantitative changes during gestation. BB-CK is specifically localized in the myofilaments and mt-CK is present in the uterine mitochondria. During gestation both these isoenzymes respond with significant increase in their specific activities in myofibrils and mitochondria. The possible role of the CK system in smooth muscle is discussed.

INTRODUCTION

Creatine kinase (CK) is an important enzyme of muscle cells catalysing the reversible transfer of a phosphate moiety between ATP and creatine. Cytosolic CK exists as a dimer composed of two subunit types, muscle (M) and brain (B), giving three isoenzymes, MM, MB and BB. In addition, there is a fourth CK isoenzyme in the mitochondria (mt-CK), which differs from the cytosolic forms (Jacobs, Helot & Klinberger, 1964). This isoenzyme forms interconvertible dimeric or octameric structures (Schlegel, Zurbriggen, Wegmann, Wyss, Eppenberger & Wallimann, 1988). CK isoenzymes are present in the cytosol or bound to intracellular structures at the sites of ATP production and utilization such as plasma membranes, sarcoplasmic reticulum, nuclei, myofibrils, and mitochondria (Saks, Rosenshtraukh, Smirnov & Chazov, 1978; Wallimann & Eppenberger, 1985). However, the distribution and role of CK seem to be different in different muscle types. In fast glycolytic skeletal muscle, MM-CK is the main isoenzyme and is largely cytosolic, playing a role of spatio-temporal buffering of ATP (Meyer, Sweeney & Kushmerick, 1984). In heart muscle, up to 60 % of total CK is bound to intracellular structures, and the various locations of the CK isoenzymes permit microcompartmentation and functional coupling near the sites of energy production and consumption as well as integration of cellular metabolism and function (Saks *et al.* 1978; Bessman & Carpenter, 1985; Wallimann & Eppenberger, 1985). CK isoenzymes microcompartmentation takes place during perinatal maturation in cardiac cells (Hoerter, Kuznetsov & Ventura-Clapier, 1991).

The uterus is a unique tissue to study biochemical events associated with an increase in muscle activity and differentiation in preparation for parturition. The uterine smooth muscle undergoes many metabolic, functional and structural modifications during gestation (for reviews see: Garfield, 1984; Broderick & Broderick, 1990; Dawson & Raman, 1990). These changes include uterine innervation, vascularization, extracellular matrix, and also the muscle cell mass which undergoes hyperplasia and hypertrophy. Agents such as growth factors, cytokines, hormones, eicosanoids and neurotransmitters induce these changes. The concentrations of high-energy phosphorus metabolites and more specifically of phosphocreatine and ATP also increase (Dawson & Raman, 1990).

One of the well-characterized uterine responses to oestrogen is the induced protein which was so named because of the rapidity of its inducibility in the uterus

(Walker & Kaye, 1981). Reiss & Kaye (1981) determined that BB-CK was in fact the induced protein of the gravid uterus. The level of creatine kinase in the myometrium is responsive to oestrogen treatment, both *in vitro* and *in vivo*; its synthesis is induced by a wide variety of oestrogens, is dependent on RNA synthesis, and is linearly related to the concentration of nucleus-bound oestrogens (Reiss & Kaye, 1981; Walker & Kaye, 1981). Creatine kinase activity doubles during gestation and NMR studies using magnetization transfer have found that the forward rate constant (PCr to ATP) increases in uteri treated with oestrogens (Degani, Victor & Kaye, 1988). Even though this enzyme undergoes a large increase in synthesis during gestation, its role and importance in the metabolism of the uterus has not been fully explored. Iyengar, Fluellen & Iyengar (1980) have shown that the main isoform present in uterine muscle is the BB form; during pregnancy total CK activity increases and there is a 4-fold increase in the microsome bound fraction. These authors also found traces of mitochondrial CK in the uterine mitochondria of non-gestating myometrium and after 90–120 days of gestation in bovine myometrium. This indicates that there is some form of CK localization in different metabolic compartments. CK from the bovine myometrium has a high affinity for MgADP and exhibits a MgADP facilitation of PCr binding (Iyengar, Fluellen & Iyengar, 1982). Although the main isoenzyme of creatine kinase in smooth muscle is the BB isoenzyme, MM and MB isoenzymes were also found (Ishida, Wyss, Hemmer & Wallimann, 1991; Clark, Khuchua & Ventura-Clapier, 1992). Mitochondrial creatine kinase activity has been found in different smooth muscles (Jacobus & Lehninger, 1973; Ishida & Paul, 1989, 1990; Haas & Strauss, 1990) predominantly in octameric form in guinea-pig (Ishida *et al.* 1991) and encoded by a ubiquitous mt-CK gene different from the specific mt-CK gene found in sarcomeric tissues (Hossle *et al.* 1988; Haas & Strauss, 1990). We have shown recently that CK is also structured into microcompartments in smooth muscle; in taenia coli there are at least three isoforms of creatine kinase, and the main form (BB-CK) is associated with the contractile structures of smooth muscle (Clark *et al.* 1992). This is consistent with the observation of a compartmentation of metabolism in vascular smooth muscle (Ishida & Paul, 1989, 1990). Little is known concerning the role and localization of the other CK isoenzymes. It also remains to be determined if there are differential functions for the different isoenzymes within the smooth muscle. Moreover, it is not known if CK is present in the uterine myofibrils, which isoenzyme is present and what role if any CK has in the contraction and relaxation processes. In view of the remarkable changes in contractile capacity necessitated by parturition, it seems logical to assume that metabolic adaptation would closely follow and consequently the CK system's capacity to be organized as an intracellular energy transducing system may be an important part of these adaptations.

This study was performed to investigate the possible presence and role of CK in the contraction and relaxation of skinned guinea-pig uterus as well as the possible changes of the CK system during gestation. The results show the presence of four isoenzymes of CK in uterine muscle. The results indicate association of BB-CK with the uterine myofilaments, the presence of intra-mitochondrial CK, and quantitative as well as qualitative changes of the CK system during gestation.

METHODS

Materials

Control ($n = 8$) or gravid ($n = 6$) guinea-pigs weighing respectively 400–600 g or 900–1100 g were anaesthetized with an intraperitoneal injection of pentobarbitone according to the recommendations of the Institutional Animal Care Committee (INSERM, Paris, France). While under the anaesthetic the animal was exsanguinated. Young adult virgin female guinea-pigs were used as controls. Gravid guinea-pigs about 1 year old were used between 60 and 65 days of gestation (68 days average term). Longitudinal uterine fibres were isolated from circular fibres and adventitia by blunt dissection, collected and rinsed in a Krebs solution containing (mM): 118 NaCl, 25 $NaHCO_3$, 4·7 KCl, 1·2 KH_2PO_4, 1·2 $MgSO_4$, 0·6 $CaCl_2$, and 5 glucose at pH 7·4. Dissection was performed at 21 °C in a solution containing (mM): 30 imidazole, 50 potassium acetate, 5 EGTA, 0·3 dithiothreitol (DTT), and 180 sucrose at pH 6·8. Preparations of approximately equal length and diameter were dissected from longitudinal myometrium of control or gravid guinea-pigs using microtools and a dissecting microscope.

Mechanical experiments

Fibre preparation. Skinning was accomplished by the addition of 1 % Triton X-100 to this solution for 1 h at 21 °C with continuous stirring (Chatterjee & Murphy, 1983). In heart muscle, Triton X-100 treatment induces complete disruption and vesicularization of all cellular membranes resulting in removal of the cytosolic and membrane associated fractions of proteins (Ventura-Clapier, Saks, Vassort, Lauer & Elizarova, 1987).

After the skinning procedure, the fibre was mounted in the experimental set-up, adjusted to slack length and stretched by 20 % of slack length in control relaxing solution (see below). The length and diameter of the muscles were measured using a graticule in the dissecting microscope.

Experimental apparatus. The fibres were mounted between two stainless-steel hooks. One hook was connected to a transducer (model AE 801, SensoNor Microelectroniks, Horten, Norway). Muscles were immersed in 2·5 ml chambers arranged around a disk and immersed in a temperature-controlled bath positioned on a magnetic stirrer. Each solution was well stirred at high speed (> 1000 r.p.m.) during the experiments. All experiments were performed at 22 °C.

Solutions. All solutions were calculated using the program of Fabiato (1988). The specific pH, ionic strength and composition of solutions were designed according to Nayler & Sparrow (1986) for smooth muscle experiments. All solutions contained 10 mM EGTA, 30 mM imidazole, 0·3 mM DTT at a pH of 6·8 with an ionic strength of 0·085 M using potassium acetate. Control relaxing (pCa 9) and stimulating (pCa 4·5) solutions also contained 3·16 mM MgATP and 12 mM PCr. CK relaxing (pCa 9) and CK stimulating (pCa 4·5) solutions (designed to check the CK efficacy) contained 250 μM MgADP, 12 mM PCr with no ATP. Rigor solutions contained neither ATP nor PCr. EGTA was obtained from Sigma Chemicals (St Louis, MO, USA). PCr (Neoton, Schiapparelli Farmaceutica, Turin, Italy) was a generous gift of Professor E. Strumia.

Mitochondrial respiration experiments

For determination of mitochondrial respiratory parameters in saponin-skinned fibres we used the method described previously for heart muscle (Veksler, Kuznetsov, Sharov, Kapelko & Saks, 1987), with the minor changes given below. The selective effect of saponin is easily explained by its affinity for cholesterol whose content is higher in sarcolemma than in mitochondria and sarcoplasmic reticulum; thus, mitochondria and sarcoplasmic reticulum remain functional (Endo, Yagi & Iino, 1982).

Fibre preparation. Fibre bundles with a diameter of 50–200 μm were isolated from the external longitudinal muscle layer of the myometrium and transferred into skinning solution (S, see below). Bundles were incubated with intense shaking for 30 min in solution S containing 50 μg ml^{-1} of saponin. Bundles were then washed for 10 min in respiration solution (R, see below) without high energy phosphates. All these procedures were carried out at 4 °C.

Solutions. Solutions S and R contained (mM): 10 EGTA (pCa 7), 3 free Mg^{2+}, 20 taurine, 0·5 dithiothreitol, and 20 imidazole (pH 7·0). Ionic strength was adjusted to 0·16 M by addition of potassium 2-(*N*-morpholino)ethanesulphonate. Solution S also contained 5 mM MgATP and 15 mM PCr. In place of high energy phosphates, solution R contained 5 mM glutamate, 2 mM malate, 3 mM phosphate and 2 mg ml^{-1} fatty acid-free bovine serum albumin.

Measurement of respiratory parameters. The respiratory rates were determined by a Clark oxygen electrode (Yellow Spring Instruments CO, Yellow Springs, OH, USA) in an oxygraph cell containing 2–5 mg dry weight of fibres in 3 ml solution R at 22 °C with continuous stirring. The solubility of oxygen at 22 °C was taken to be 0·230 μmol oxygen ml^{-1} for solution saturated in air. Bundles were introduced into the oxygraph cell and various respiratory parameters were measured by sequential substrate addition. The parameters measured were as follows: $\dot{V}_0$ basal respiration rate after addition of 5 mM glutamate and 2 mM malate; $\dot{V}_{ADP}$, respiration rate after addition of 100 μM ADP; $\dot{V}_{Cr}$, respiration after addition of 20 mM Cr; $\dot{V}_{max}$, maximal respiration rate after further addition of 1 mM ADP and $\dot{V}_{CAT}$, respiration rate after addition of 35 μM carboxyatractyloside (CAT, an inhibitor of ATP–ADP translocase). Fibres were then dried and weighed. Respiration rates were expressed as micromoles of oxygen per minute per gram of dry weight.

Isolation of mitochondria. Mitochondria were isolated using a trypsin digestion procedure according to Saks, Kuznetsov, Kupriyanov, Miceli & Jacobus, (1985). Longitudinal fibres were minced in a solution of 0·3 M sucrose, 10 mM Na-Hepes, 0·2 mM EDTA and pH 7·2, and digested with 125 mg ml^{-1} trypsin followed by gentle homogenization at 0 °C. The homogenate was centrifuged for 10 min at 600 *g*. The supernatant was saved and centrifuged at 8000 *g* for 15 min. The mitochondria in the resulting pellet were resuspended with isolation medium containing 1 mg ml^{-1} BSA.

Biochemical determinations

Intact muscle or skinned fibre bundles prepared from the external longitudinal muscle layer of the uterus were used to measure CK activities and to determine isoenzyme distribution.

Creatine kinase activity. Fibres were dissected from myometrium and divided into two groups. One group remained intact while the other group was skinned for 1 h in skinning solution containing 1 % Triton X-100. Intact or skinned fibres were minced with scissors, placed into cold solution (1 g wet wt per 10 ml) containing (mM): 100 K_2HPO_4 (pH 8·7), 1 EGTA, 15 *N*-acetyl cysteine, and homogenized in a Polytron homogenizer. Tissue homogenates were incubated for 30 min at 0 ° C for complete extraction of CK, centrifuged at 20000 *g* for 30 min and the supernatant was used in the experiments. CK activity was determined using the coupled enzyme assay of glucose-6-phosphate dehydrogenase (G-6-PDH) and hexokinase (HK) producing NADPH. NADPH production was measured spectrophotometrically at 340 nm (Gilford Spectrophotometer, Corning, NY). The CK activity was assayed in a solution containing (mM): 30 Hepes, 6 magnesium acetate, 0·6 DTT, 1 ADP, 10 PCr, 12 glucose, 0·6 NADP, 13 AMP (to inhibit adenylate kinase), and 2 IU ml^{-1} of HK and G-6-PDH at a pH of 7·4 and 30 °C.

Isoenzyme fractionation. Isoenzymes were determined using agarose electrophoresis (1 %) performed at 200 V and 4 °C for 1 h. Individual isoenzymes were observed by incubating the gel with a staining solution-soaked paper for 30 min. Staining solution contained (mM): 22 Mes, 50 magnesium acetate, 70 glucose, 15 AMP, 120 *N*-acetyl cysteine, 9 ADP, 9 NADP, 120 PCr, 18 IU ml^{-1} HK and 6 IU ml^{-1} G-6-PDH at a pH of 6·8 at 30 °C. Individual isoenzyme bands were visualized by observing the fluorescence of NADPH.

Immunoenzyme determination of mitochondrial creatine kinase. Antibodies against mitochondrial CK (0·3 μg), in 100 μl of phosphate-buffered saline (PBS) containing 10 mM NaH_2PO_4 (pH 7·4) and 150 mM NaCl, were placed into each of ninety-six cells of polystyrol microtitre plates and kept overnight at 4 °C. Then 100 μl of bovine serum albumin in PBS (1 mg ml^{-1}) was added, incubated for 1 h and then washed four times with 0·05 % Twin-20 in PBS. Supernatants of uterine tissue and mitochondria homogenate were added in different dilutions. For quantitative determination, mitochondrial creatine kinase was added in different concentrations ranging from 1 mg ml^{-1} to 1 ng ml^{-1} in PBS, containing 0·05 % of Twin-20. Into all cells, 50 μl of peroxidase-conjugated creatine kinase was added (conjugation was performed by a periodate method). The plate was incubated at room temperature and continuously stirred for 2 h and then washed with a solution of Twin-20 in PBS. For visualization of the antibody-bound conjugate, 100 μl of a solution containing 4 μM *O*-phenylendiamine and 0·015 % H_2O_2 in 20 mM sodium citrate buffer (pH 4·7) was added to the cells. After 15 min the reaction was terminated by adding 100 μl of a 50 % solution of H_2SO_4 and optical density was measured at 490 nm in a Microelisa autoreader. The data were plotted in co-ordinates $\ln\{(B/B_0)/(1-(B/B_0))\}$ *versus* $\ln C$, where B_0 is the optical density in the absence of purified non-conjugated creatine kinase, B is the optical density with added creatine kinase at a concentration C.

Statistical analysis

The results are presented as means ± S.E.M. Significant changes were assessed by Student's t test when appropriate. A value of $P < 0{\cdot}05$ was accepted as the level of significance.

RESULTS

Creatine kinase activity

Total CK activity of fibres increased significantly ($P < 0{\cdot}01$) during gestation from 74 ± 11 ($n = 8$) to 196 ± 39 IU (g wet wt)$^{-1}$ ($n = 6$). Myofibrillar CK activity

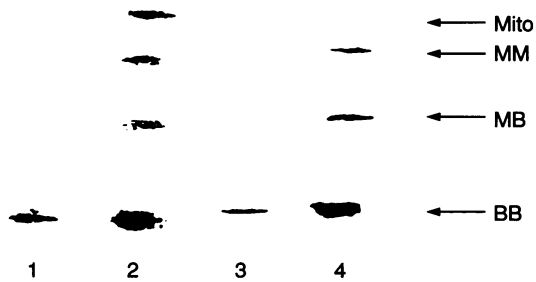

Fig. 1. CK isoenzymes pattern after electrophoresis with an agarose gel of extracts from skinned fibres of gravid guinea-pig myometrium (1), extract of gravid unskinned longitudinal guinea-pig myometrium (2), skinned fibres from control myometrium (3), extract of control unskinned longitudinal myometrium (4).

was measured as CK activity present in fibres after skinning with 1 % Triton X-100. Myofibrillar-bound CK increased ($P < 0{\cdot}05$) from $6{\cdot}7 \pm 1{\cdot}9$ ($n = 6$) in control to $44{\cdot}2 \pm 13{\cdot}3$ IU (g wet wt)$^{-1}$ ($n = 4$) in gestating uterus. This result is unlikely to be due to differential extraction of fibres from control or gravid uteri since electrophoresis clearly showed a complete extraction of soluble or mitochondrial form of CK after Triton X-100 skinning (see below).

The CK isoenzyme distribution pattern was determined using agarose gel electrophoresis (Fig. 1) in the presence of 15 mM AMP to inhibit adenylate kinase. Control as well as pregnant uteri contained different isoenzymes of creatine kinase. The two homodimers MM and BB as well as the heterodimer MB were present in control (lane 4) and in gravid (lane 2) uterus. In addition, the mitochondrial isoenzyme was present and observable in gravid uterus but was not detectable by this technique in control uterus. No fluorescence was observed when PCr was omitted from the staining solution, showing that fluorescence was specifically due to CK activity. In skinned fibres from control (lane 3) and gravid (lane 1) uteri only BB isoenzyme was present, showing specific binding of BB-CK to contractile proteins and providing evidence for complete extraction of soluble or mitochondrial CK by Triton X-100 skinning in uterine fibres from control as well as gravid guinea-pigs. Figure 2 shows the densitometer traces of the electrophoretograms of one control (*A*) and one gravid (*B*) uterus. In addition, an electrophoretogram of isolated mitochondria from gravid uterus (*C*) shows that mt-CK isoenzyme is associated with mitochondria. MM, MB and BB isoenzymes represented respectively $20{\cdot}3 \pm 2{\cdot}6$, $10{\cdot}3 \pm 4{\cdot}4$ and $72{\cdot}7 \pm 2{\cdot}2$ % of total activity in control

($n = 3$) and $21{\cdot}7 \pm 4{\cdot}1$, $8{\cdot}3 \pm 1{\cdot}7$ and $64{\cdot}7 \pm 3{\cdot}5$ ($n = 3$) in gravid uterus and the values were not statistically different. In addition, gravid guinea-pig uteri contained mitochondrial CK which represented $5{\cdot}3 \pm 1{\cdot}4$ % of total CK activity while no mt-CK was observed in control.

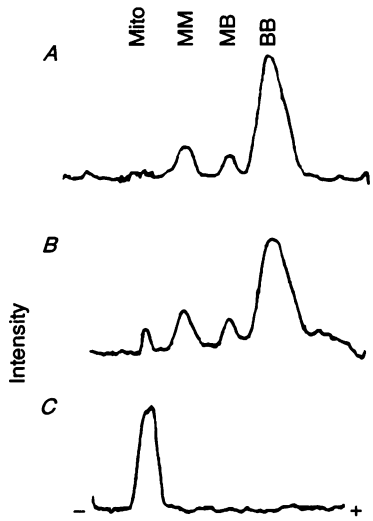

Fig. 2. Creatine kinase densitometer trace from electrophoretograms of control myometrium (*A*), gravid myometrium (*B*), and mitochondria extracted from gravid myometrium (*C*). Isoenzyme patterns were visualized by activity staining.

Direct immunoenzyme determinations were made using monoclonal antibodies against human mitochondrial creatine kinase purified by an affinity method. These antibodies were highly specific and reacted only with mitochondrial creatine kinase (Khuchua, Ventura-Clapier, Kuznetsov, Grishin & Saks, 1989). Guinea-pig uterine mitochondrial CK cross-reacted with the human mitochondrial CK antibodies. Under these conditions, trace amounts of mt-CK could be detected in control uterus. The amount of mitochondrial CK increased during gestation from $17{\cdot}5 \pm 8{\cdot}4$ μg (g wet wt)$^{-1}$ in control to 140 ± 22 μg (g wet wt)$^{-1}$ in gestating uterus. CK activity of isolated mitochondria from gravid uterus was 1·86 IU mg^{-1} mitochondrial proteins.

Mechanical properties of control and gravid uteri

No difference in length or diameter was observed in dissected fibres from both types of animals. The mean length was 1840 ± 147 μm for control ($n = 22$) and 1873 ± 85 μm ($n = 14$) for gravid guinea-pigs. The mean diameter was 495 ± 37 for control *versus* 476 ± 43 μm for gravid guinea-pigs.

Tension generation by Triton X-100-skinned uterus

Skinned fibres permit the study of intrinsic mechanical properties while controlling the level of activation. Resting tension in the absence of calcium was not significantly different in control and gestating uteri (Table 1). Triton X-100- treated

fibres developed maximal tension in the presence of 12 mM PCr and pMgATP 2·5 at pCa 4·5. Increasing calcium concentrations induced stepwise increases in force. At maximal activation, tension was significantly increased threefold during gestation. The observed maximal tension obtained in the control stimulating solution was $6{\cdot}06 \pm 0{\cdot}68$ mN mm^{-2} ($n = 22$) in control and increased to $19{\cdot}3 \pm 1{\cdot}9$ mN mm^{-2} ($n = 14$) in gestating uterus (Table 1).

TABLE 1. Mechanical parameters of Triton X-100-skinned fibres from control and gravid guinea-pig uteri

	Passive tension (mN mm^{-2})	Active tension (mN mm^{-2})	CK-stimulated tension with 250 μM ADP (%)	CK-induced relaxation with 250 μM ADP (%)
Control ($n = 22$)	$3{\cdot}3 \pm 1{\cdot}0$	$6{\cdot}1 \pm 0{\cdot}7$	$43{\cdot}3 \pm 4{\cdot}4$	$47{\cdot}3 \pm 4{\cdot}2$
Gravid ($n = 14$)	$2{\cdot}1 \pm 0{\cdot}4$	$19{\cdot}3 \pm 1{\cdot}9$***	$65{\cdot}4 \pm 3{\cdot}9$*	$60{\cdot}6 \pm 3{\cdot}2$*

n, number of fibres. * $P < 0{\cdot}05$; *** $P < 0{\cdot}001$ compared with values from control uteri by Student's t test. All values are means ± S.E.M.

CK and tension generation

To determine the role of creatine kinase in skinned fibres, fibres were stimulated with a solution which could produce ATP only via bound and active myofibrillar CK (CK stimulating solution, see Methods). This solution induced a substantial increase in tension (Fig. 3). Maximal tension generation was measured with the control stimulating solution. The mean tension due to the CK stimulating solution represented $43{\cdot}3 \pm 4{\cdot}4$% of maximal tension in control and $65{\cdot}4 \pm 3{\cdot}9$% in gestating uterus. The fibres were then re-exposed to the CK stimulating solution resulting in a decrease in tension. CK-stimulated tension was significantly higher in gestating uterus showing an increased participation of creatine kinase in tension generation in accordance with the higher CK activity of the fibres from gravid uterus (Table 1).

In order to verify that this tension was in fact active (not rigor tension), the rigor solution + Ca^{2+} was applied to the muscle fibres prior to activation (results not shown). There was no significant increase in tension indicating that uterine muscle is not able to generate substantial rigor tension prior to activation in agreement with what was found in taenia coli (Clark *et al.* 1992). Therefore tension generated by the CK activating solution is due to cycling cross-bridges. Tension in the CK stimulating solution could not be attributed to ATP produced by adenylate kinase because no change in tension was observed in the rigor solution + Ca^{2+} which contained ADP.

FDNB effects on tension generation

If CK is in fact responsible for the tension generation observed with the CK stimulating solution, then inhibiting CK should impair the fibre's response to the CK stimulating solution. To inhibit endogenous CK, 10 nM fluoro-1 dinitro-2,4 benzene (FDNB), an irreversible CK inhibitor was added to the CK relaxing

solution. After thorough rinsing, the fibre's response to the CK stimulating solution was abolished. However, the fibres were still able to develop maximal tension in control stimulating solution thus demonstrating that neither myosin light chain kinase nor myosin ATPase was inhibited by FDNB (data not shown).

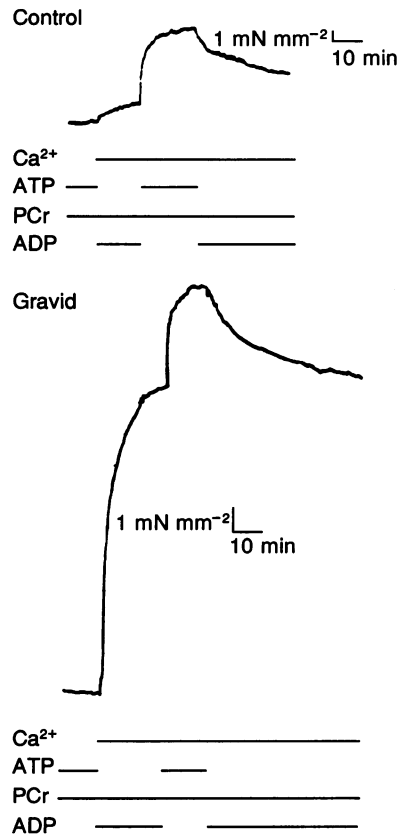

Fig. 3. Representative tension recordings of Triton X-100-skinned fibres from control and gravid guinea-pig myometrium illustrating tension generation via the CK system. CK activated tension is achieved by transferring the muscle from relaxing solution to CK stimulating solution containing 250 μM MgADP, 12 mM PCr and no MgATP, at pCa 4·5. The fibre is then transferred in the stimulating solution containing MgATP, followed by re-exposure to the CK stimulating solution. Horizontal bars represent: Ca^{2+}, pCa 4·5; ATP, pMgATP 2·5; PCr, 12 mM; ADP, 250 μM.

ADP and tension generation

Because ADP can inhibit myosin light chain kinase and myosin ATPase, we varied the ADP concentration of the CK stimulating solution to ensure that submaximal tension was not due to partial inhibition of these enzymes. We could then determine to what extent ADP affected tension development. After equilibration in CK relaxing solution, the fibres were sequentially exposed to the CK stimulating solution with 0, 50, 100, 250, 400 and 500 μM MgADP (Fig. 4).

Tension developed a plateau at an ADP concentration of between 100 and 250 μM and fell off slightly at higher concentrations. Control fibres could generate (and maintain for at least 15 min) 44·1 $\pm$ 3·0 % ($n = 4$) of the maximal tension with 0 mM ADP in the CK stimulating solution while the gestating uterine fibres could

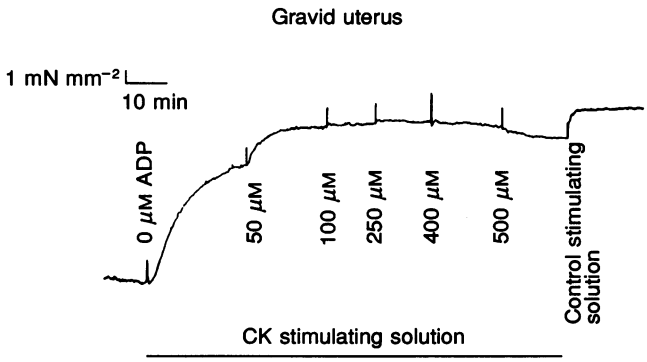

Fig. 4. Representative tension recording of a fibre from gravid guinea-pig myometrium illustrating MgADP dependency of CK-activated tension. The muscle is transferred from low-calcium rigor solution without ADP to CK stimulating solution containing no MgADP and 12 mM PCr. Then the fibre is transferred to CK stimulating solutions of increasing MgADP concentrations before control stimulating solution containing MgATP.

generate significantly higher tension (67·0 $\pm$ 5·1 % of maximal tension, $n = 4$). This may be due to the presence of ADP and/or ATP fixed to the myofibrils, and available for rephosphorylation by CK leading to active tension generation.

CK and relaxation from rigor conditions

Since CK is able to generate submaximal tension in the control and gestating uterus, we attempted to characterize the ability of creatine kinase to relax the uterine fibres from rigor conditions. Rigor tension was produced after maximum tension generation in the control stimulating solution because (as seen above) the fibres do not generate rigor tension without prior activation. Rigor tension can be abolished when ATP is supplied to the attached and non-cycling cross-bridges. Rigor conditions were generated by the following: after control activating solution, ATP and PCr were removed and 250 μM ADP added in the presence of pCa 4·5 (rigor solution + Ca^{2+}). This manoeuvre initiates a high-tension rigor state which remains when Ca^{2+} is reduced (rigor solution − Ca^{2+}). To produce relaxation from rigor with the CK reaction, 12 mM PCr was added to the bathing solution (CK relaxing solution). Endogenous CK on the skinned myofibrils relaxed the fibres by rephosphorylating ADP. Finally, control relaxing solution containing ATP was added to demonstrate the extent of relaxation. In this study, relaxation produced from the rigor state and following the control relaxing solution is referred to as total relaxation.

Figure 5 shows representative recordings obtained from control and gestating uterus demonstrating the differential ability of CK to relax the fibres from rigor

conditions. As can be seen, rigor conditions produced a diminution in maximal tension. Under these conditions, CK relaxing solution induced a significantly greater tension decrease in the gestating uterus compared to the control uterus. The extent of tension fall was $47{\cdot}3 \pm 4{\cdot}2\,\%$ ($n = 22$) of the tension decrease in

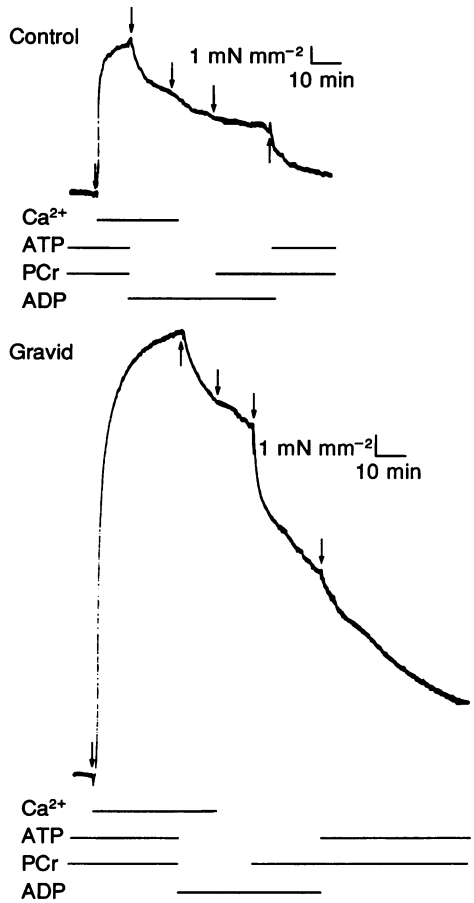

Fig. 5. Representative tension recordings of Triton X-100-skinned fibres from control and gravid guinea-pig myometrium illustrating relaxation from high-tension rigor conditions by the CK relaxing solution. A high-tension rigor state is achieved by transferring the muscle from the relaxing solution to the stimulating solution, followed by removal of ATP and PCr in the presence of 250 μM ADP and subsequent removal of calcium. Relaxation from rigor conditions is induced first by adding PCr (CK relaxing solution) and finally by applying the relaxing solution containing MgATP. Horizontal bars represent: Ca^{2+}, pCa 4·5; ATP, pMgATP 2·5; PCr, 12 mM; ADP, 250 μM.

control and $60{\cdot}6 \pm 3{\cdot}2\,\%$ ($n = 14$) in the gestating uterus, produced by the control relaxing solution (see Table 1). A significant relaxation from rigor can thus be accomplished when the uterine fibres are exposed to conditions where ATP production is entirely dependent upon the CK reaction.

Functional activity of mitochondrial creatine kinase

To study the changes in respiratory capacity of mitochondria and functional activity of mitochondrial CK during gestation, mitochondrial function was investigated in saponin-skinned fibres. This method is based on the fact that

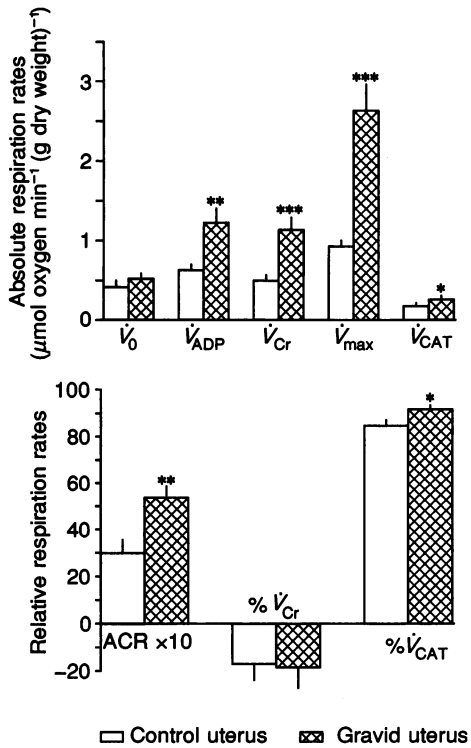

Fig. 6. Absolute and relative parameters of mitochondrial respiration rates in control and gravid guinea-pig myometrium. Oxygen consumption rates in the absence of MgADP ($\dot{V}_0$) and after subsequent addition of 100 μM ADP ($\dot{V}_{ADP}$), 20 mM creatine ($\dot{V}_{Cr}$), 1 mM ADP ($\dot{V}_{max}$), and finally 35 μM carboxyatractyloside ($\dot{V}_{CAT}$), % Cr, percentage of respiration rate change after addition of 20 mM creatine in the presence of 100 μM ADP; ACR, acceptor control ratio which is the ratio of maximal respiration rate in the presence of 20 mM creatine and 1 mM ADP to basal respiration rate; % $\dot{V}_{CAT}$, percentage of inhibition of respiration rate by carboxyatractyloside. * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$.

saponin perforates the sarcolemma because of a high amount of cholesterol, while intracellular membrane structures remain intact. It permits one to study the total mitochondrial population in its environment without isolation of these organelles.

Figure 6*A* shows a bar graph of the absolute respiration rates from saponin-skinned fibres of normal and gravid uteri. The basal respiration rate in the presence of glutamate and malate ($\dot{V}_0$) was accelerated by addition of 100 μM ADP ($\dot{V}_{ADP}$). No increase in respiration rate was observed after addition of 20 mM creatine ($\dot{V}_{Cr}$). The maximal rate of respiration ($\dot{V}_{max}$) was observed after further addition of 1 mM ADP whereas addition of carboxyatractyloside considerably depressed the

respiration rate ($\dot{V}_{CAT}$). Respiratory parameters (expressed per milligram of saponin-skinned fibres dry weight) were increased in gestating uterus as compared to control. Maximal respiration rate ($\dot{V}_{max}$) was increased from $0{\cdot}91 \pm 0{\cdot}05$ ($n = 9$) to $2{\cdot}61 \pm 0{\cdot}16$ μmol oxygen min^{-1} (g dry wt)$^{-1}$ ($n = 6$). This reflects an increased mitochondrial volume and/or increased respiratory capacities of mitochondria with gestation. When respiratory parameters were expressed in relative values, control and gestating uterus exhibited different behaviour. The acceptor control ratio (V_{max}/V_0) was significantly increased from $2{\cdot}93 \pm 0{\cdot}48$ to $5{\cdot}28 \pm 0{\cdot}32$ during gestation. Furthermore, the percentage of inhibition of respiration by carboxyatractyloside (% $\dot{V}_{CAT}$) was slightly but significantly higher in gestating uterus ($91{\cdot}2 \pm 1{\cdot}3$ *versus* $84{\cdot}4 \pm 1{\cdot}8$ %). In saponin-skinned fibres of cardiac tissue, in the presence of submaximal [ADP], the addition of 20 mM creatine stimulates respiration. This effect is due to coupling of the mitochondrial creatine kinase reaction and oxidative phosphorylation which decreases the apparent binding constant (K_m) of respiration for ADP (Veksler *et al.* 1987; Saks *et al.* 1991). However, we found that after addition of creatine, respiration was not increased either in control or gestating uterus. Changing creatine concentration from 10 to 50 mM did not change the response. This result cannot be attributed to lack of accessibility of creatine inside saponin-skinned fibres since electrophoresis showed that they were permeable to soluble forms of CK and since ADP and carboxyatractyloside had access to the mitochondrial compartment. In addition, mt-CK was active as its enzymatic activity could be detected on electrophoresis.

DISCUSSION

Besides the well-documented oestrogen-dependent increase in CK activity during gestation we have shown that the uterine CK system undergoes qualitative changes. Multiple isoenzymes of CK have been found in other smooth muscle tissues but the presence of all four isoenzymes of creatine kinase such as we have identified in the gestating uterus had not been found previously. We also found that the mitochondrial as well as the myofibrillar CK are increased during gestation, suggesting a shift from cytosolic to compartmentalized CK isoenzyme distribution. The results indicate that, in the gestating uterus, the CK system participates in high-energy phosphate production and utilization for contraction and relaxation. These events are closely associated with cell growth and maturation.

Creatine kinase isoenzyme pattern

Total creatine kinase activity doubled during pregnancy in the guinea-pig myometrium. However, the significance and role of creatine kinase is dependent not only upon total activity but also on CK isoforms and localization. Our results show that the four CK isoenzymes are present in the control and gravid uterus. BB-CK which is the predominating isoenzyme represented 70 % of the total CK activity while the heterodimer MB represented 10 % of total activity. The homodimeric form MM-CK was also present in control as well as in gravid guinea-pig uterus, representing 20 % of total CK activity. This electrophoretic band could not be due to adenylate kinase contamination since this enzyme was inhibited by 15 mM AMP and also because no fluorescence was detected in the absence of PCr.

Since the relative proportion of the MM, MB and BB isoenzymes did not significantly change during gestation, this suggests a general process of induction for M- and B-CK genes. Interestingly mt-CK, which was not detectable on agarose electrophoresis in control uterus, showed a comparatively greater increase in gravid uterus.

Mitochondrial function and creatine kinase

Selective perforation of sarcolemma by saponin has been used in cardiac fibres and allows skinned fibres to be obtained with morphologically and functionally intact mitochondria (Veksler *et al.* 1987). The same technique was applied here to longitudinal myometrial fibres. In pregnant uterus, the acceptor control ratio and percentage of inhibition of respiration by carboxyatractyloside were very similar to cardiac values (Veksler, Murat & Ventura-Clapier, 1991; Hoerter *et al.* 1991), showing that this method of investigation of mitochondria *in situ* is suitable for this tissue. Maximal oxygen consumption rate can thus be recorded under conditions of unlimited substrates or oxygen concentrations leading to high values (3·4 μmol oxygen min^{-1} (g dry wt)$^{-1}$ at 37 °C with a Q_{10} (temperature coefficient over a 10 °C temperature range) of 2·5 in non-pregnant uteri) compared to intact tissue. In intact mesenteric vein, Paul & Peterson (1975) reported values of 0·864 μmol oxygen min^{-1} (g dry wt)$^{-1}$ at 37 °C. In intact rat immature uteri, oxygen consumption rate was 1·5 μl (mg dry wt)$^{-1}$ h^{-1}, i.e. 1·1 μmol min^{-1} (g dry wt)$^{-1}$ at 36 °C (Degani *et al.* 1988). In addition, differences in protein content between skinned and intact preparations as well as species specificity may also be involved. The maximal respiration rate, however, was much lower in pregnant uterus compared to heart and skeletal muscle, reflecting the lower mitochondrial content of this tissue. In non-pregnant uterus, maximal respiration was even threefold lower, evidencing an increased mitochondrial content during gestation. Moreover, the significantly lower acceptor control ratio and percentage of inhibition by carboxyatractyloside in non-pregnant uterus suggests increased coupling between oxidation and phosphorylation of the mitochondria during gestation. A higher susceptibility to saponin treatment of the mitochondria in control fibres cannot be ruled out at this time.

These results show that mitochondrial CK content increased during gestation. This eightfold increase cannot be exclusively attributed to the increased mitochondrial content because the $\dot{V}_{max}$ increased only threefold. If we use $\dot{V}_{max}$ as an estimate of oxidative capacities of the muscle the greater increase in the mt-CK activity reflects an increase in the specific activity of the enzyme in mitochondria. Such an increase was not observed in the bovine uterus in an earlier stage of gestation (Iyengar *et al.* 1980). It may be that the mitochondria of the gestating uterus increase their content of mt-CK later during gestation in preparation for the energy demands of parturition.

We have found that mt-CK is present and active in the mitochondria but not functionally coupled to respiration under conditions where CK has been found to be coupled to respiration in the heart and skeletal muscle. CK coupling to respiration requires that it be associated with ATP synthesis, using ATP to phosphorylate creatine and producing ADP in the process, thus increasing the local ADP concentration and further stimulating respiration (Jacobus & Saks, 1982; Saks *et al.*

1991). It has been shown in intact cardiac mitochondria that, when mt-CK is detached from the inner membrane and soluble in the intermembrane space, the effects of coupling are lost (Kuznetsov, Khuchua, Vassil'eva, Medved'eva & Saks, 1989). In the uterus the presence of mt-CK but lack of coupling to respiration may be caused by one or a combination of reasons. Indeed, mt-CK from smooth muscle is not the same protein as in sarcomeric tissue since it is the product of a different gene (Hossle *et al.* 1988; Haas & Strauss, 1990); this protein may not have the properties needed to associate with the mitochondrial membranes; alternatively, it may be readily detached from the inner mitochondrial membrane due to specific sensitivity to saponin treatment, ionic or substrate environment. However, the increase in mt-CK during gestation will allow more PCr to be synthetized from oxidative phosphorylations and may participate in the increased CK fluxes observed following oestrogen administration (Degani *et al.* 1988; Dawson & Raman, 1990).

Creatine kinase and myofibrillar function

We observed a 3-fold increase in maximal force developed by skinned fibres during gestation. Absolute values of force were very close to the values reported for skinned fibres from non-pregnant and pregnant rat myometrium (Sparrow, Mohammad, Arner, Hellstrand & Ruegg, 1988) or human myometrium (Izumi, Ichihara, Uchiumi & Shirakawa, 1990). However, these values are substantially lower than in the intact preparation (Sparrow *et al.* 1988) and may be attributed to extraction of contractile proteins during skinning (Kossmann, Fürst & Small, 1987). Nevertheless, this increase in force is similar to the enhancement of force development of intact tissue and should be due to an increase in the density of contractile proteins. Actomyosin concentration and tension per unit cross-sectional area were shown to gradually increase until the termination of gestation in rabbit myometrium (Michael & Schofield, 1969). The contractile response during the period of pregnancy seems to be controlled by both the changes in Ca^{2+} sensitivity and in the amount of free calmodulin in uterine muscle cells (Maruyama, Ochiai, Tokutome, Hachiya & Umazume, 1986), while the presence and role of a myosin isoenzymic shift is still controversial (Sparrow *et al.* 1988; Paul, Hewett & Martin, 1991).

Our results show specific association of BB-CK with contractile proteins of uterine muscle. This creatine kinase is able to rephosphorylate enough ATP to sustain submaximal tension generation and to induce significant relaxation from rigor conditions. This suggests that myofibrillar CK participates in energy transduction at the myofilaments. It should be noted that, as in other muscles, the CK pathway is not an obligatory one since tension can be generated in the absence of PCr. The specificity of BB-CK (only one of the 4 isoenzymes found in the gravid uterus) binding to the skinned myofilaments may suggest that this isoenzyme plays a role in energy transduction at the contractile filaments. During pregnancy the activity of BB-CK in myofibrils increased 6 times while maximal developed tension exhibited a threefold increase. Thus, the specific activity of BB-CK was increased during gestation and its efficacy was greater in inducing contraction and relaxation, reaching values close to the ones determined for guinea-pig taenia coli (Clark *et al.* 1992). The results also demonstrate the presence of ADP in the

myofibrils, probably due to its binding to myosin ATPase at rest (Butler, Pacifico & Siegman, 1989), otherwise, diffusion effects of ATP/ADP away from the contractile apparatus in the absence of exogenous nucleotides would have precluded significant tension generation.

As in taenia coli (Clark *et al.* 1992), BB-CK is bound to the myofilaments in uterine muscle and the results could be explained by the co-localization of CK, myosin ATPase and myosin light chain kinase to produce contraction. In guinea-pig taenia coli, the absence of PCr causes a decrease in tension (Ishida & Paul, 1989) which supports the idea of a CK transducing system functioning at the contractile filaments. An interesting possibility is that if the BB-CK bound to the contractile filaments has the role of energy transducer (to produce ATP for contraction and relaxation) then one could predict that this enzyme might have a high affinity for PCr and a relatively low affinity for ATP. This may enable efficacious functioning of the contractile system in spite of the low concentration of ATP and PCr in smooth muscle. Indeed BB-CK of bovine myometrium has a lower K_m for PCr than MM-CK (Iyengar *et al.* 1982). PCr content of smooth muscle and myometrium is much lower than in skeletal muscle and this low K_m isoenzyme at energy-using sites will favour the rephosphorylation of ADP locally produced during contraction. More interestingly it has been shown recently that BB-CK can be phosphorylated (Quest, Soldati, Hemmer, Perriard, Eppenberger & Wallimann, 1990). Protein kinase C can phosphorylate BB-CK and this phosphorylation results in a decreased K_m for PCr (Chida, Tsunenaga, Kasahara, Kohno & Kuroki, 1990). At term in human myometrium, α-adrenergic agonists stimulate phospholipase C and hence the release of inositol phosphates (Breuiller, Doualla-Bell Kotto Maka, Geny & Ferré, 1991) and induce contractions. Carbachol and oxytocin stimulate the generation of inositol phosphates in the guinea-pig myometrium (Marc, Leiber & Harbon, 1984). Protein kinase C activation and possible phosphorylation of BB-CK together with a higher PCr content could be favourable events for increased ATP utilization in myofibrils during parturition.

Creatine kinase and gestation

While gestation was characterized by a threefold increase in maximal respiration and contractile force, and a twofold increase in total CK, a greater fraction of creatine kinase was found associated with intracellular structures (i.e. myofibrils and mitochondria) during gestation. In addition Iyengar *et al.* (1982) already reported that CK activity associated with microsomes also increased during gestation.

Uterine cell growth during gestation is accompanied by intracellular compartmentation of calcium movements, increased cellular excitability, well-developed intercellular communications, increased metabolic fluxes, increased contractility, and increased oxidative as well as glycolytic metabolism. Increased amount of contractile proteins per unit cross-section permit increased force of contraction. Increased mitochondrial volume allows an increase in metabolic fluxes and synthesis of oxygen-derived high energy phosphates. It is worth noting that in this context the creatine kinase system becomes compartmentalized by expression of specific isoenzymes and localization of these isoenzymes to the sites of ATP production and utilization.

The role of the creatine kinase system in smooth muscle is not known at present. The low concentration of PCr and CK in this tissue, the low contraction time as well as the small intracellular diffusion distances preclude a role of facilitated diffusion or intracellular buffer due to CK reaction at near equilibrium in smooth muscle, as was claimed for striated muscle (Meyer *et al.* 1984). Additionally, it has been shown in taeni coli that ATP and PCr do not distribute in a manner predicted by the equilibrium of the Lohmann reaction in a single uniform compartment (Ishida & Paul, 1989). On the other hand, in aerobic muscles having high energy fluxes, compartmentation of CK isoenzymes has been the basis for the proposed role for CK in energy transduction, transfer and utilization (Saks *et al.* 1978; Wallimann, Wyss, Brdiczka, Nicolay & Eppenberger, 1992). However, it is also known that the rate of energy utilization during force maintenance is much lower in smooth muscle than in heart. In spite of low PCr content and low ATPase and CK activities, smooth muscle is able to maintain sustained contraction and to buffer its ATP concentration although the metabolic flux through intermediary metabolism is increased two- to threefold. In smooth muscle, the rate of oxygen consumption appears correlated with the level of isometric force while lactate production is more closely correlated with the energy requirements of membrane-associated processes (Ishida & Paul, 1989, 1990); this functional compartmentation appears to reflect enzymatic compartmentation of the glycolytic cascade associated with the plasma membrane and of glycogenolysis linked to contractile elements. Recently, Ishida *et al.* (1991) reported the presence of mt-CK in guinea-pig taenia coli and proposed it to be the basis for the dependence of the PCr production on oxidative metabolism observed in this tissue. The major significance of such compartmentation may lie in the requirement for independent regulation of various energy-dependent cellular functions (Paul, 1989). In this respect, compartmentation of CK isoenzymes will specifically link oxidative metabolism and contraction through creatine and PCr. Due to the low phosphagen content, a quick signalling between energy production and demand is necessary in smooth muscle and could be provided by CK compartmentation. The presence of mt-CK in mitochondria and of BB-CK in myofibrils and their increased specific activity during pregnancy, will lead to better interrelation and coupling between oxidative metabolism and force of contraction during increased demand for parturition. Such a reorganization seems to be under hormonal control in gestating uterus.

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