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Creatine kinase binding and possible role in chemically skinned guinea-pig taenia coli

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The activity and role of creatine kinase (CK) associated with contractile proteins of smooth muscle have been investigated using skinned guinea-pig taenia coli fibers. Total CK activity was 163 ± 22 IU/g (ww) and agarose electrophoresis showed BB, MB, and MM isoforms (BB-CK being the predominant isoenzyme). After skinning for 1 h with Triton X-100, BB-CK was specifically associated with the myofibrils, representing 22% of the preskinned CK activity. When relaxed fibers were exposed to p*Ca* 9 in the presence of 250 μM ADP, 0 ATP and 12 mM PCr, tension was not significantly different from resting tension, but changing to p*Ca* 4.5 caused the fibers to generate 59.1 ± 5.2 percent of maximal tension. When a high-tension rigor state was achieved (250 μM ADP, 0 ATP, 0 PCr, and p*Ca* 9), the addition of 12 mM PCr effected significant relaxation. These observations implicate an endogenous form of BB-CK, associated with the myofilaments and capable of producing enough ATP for submaximal tension generation and significant relaxation from rigor conditions. It was also shown that ADP is bound to the myofibrils and available for rephosphorylation by BB-CK. These results suggest co-localization of ATPase, MLCK and CK on the contractile proteins of the taenia coli. This enzymic association may play a role in the compartmentation of adenine nucleotides in smooth muscle.

Introduction

Creatine kinase (CK) catalyzes the reversible exchange of high-energy phosphates between phosphocreatine (PCr) and ATP. The reaction is:

$$PCr + ADP + H^+ \leftrightarrow ATP + Cr.$$

Four major isoforms of CK have been characterized. Mitochondrial CK is found on the outside of the inner mitochondrial membrane [1]. The other three isoforms are dimers composed of B and M monomers. MM-CK is the isoenzyme found in mature skeletal muscle while MB-CK (a heterodimer) is an isoenzyme found predominantly in heart muscle. Significant BB-CK activity can be found in brain and smooth muscles. The isoenzyme profiles and activity vary from muscle to muscle, depending upon muscle type and function. White skeletal muscle contains a relatively high CK activity and PCr concentration; nearly all CK activity comes from the MM isoenzyme. Red muscle and heart contain a lower activity but present a variety of CK isoenzymes. In white muscle CK is mainly a cytosolic enzyme while the different isoforms in red muscle are bound to sites of energy production (mitochondrial) and energy utilization (membranes and myofibrils) [2].

In skeletal and heart muscle it has been demonstrated that CK is bound to the myofilaments. Its location [1], as well as role and isoenzyme specificity, has also been determined for these muscles [3,4]. Wallimann et al. [3,4] found MM-CK bound to the M-line of chicken skeletal muscle and BB-CK bound to the Z-line of chicken heart while MM-CK is associated with the M-line in mammalian heart [1,2]. Ventura-Clapier et al. [5] proposed a role for CK in force development of skinned rat papillary muscle fibers. This was suggested after observing that CK is bound to the skinned myofibrils, that endogenous CK is able to relax rigor tension, and induce maximal tension with a normal cross bridge cycling rate. It was also observed that exogenous CK can be reapplied to the skinned myofibrils and induce submaximal tension. CK bound

Abbreviations: PCr, phosphocreatine; Cr, creatine; CK, creatine kinase; FDNB, fluoro-1-dinitro-2,4 benzene; MM-CK, muscle CK; BB-CK, brain CK; MLCK, myosin light chain kinase; IU, international units.

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to the myofibrils was postulated as being more efficacious than soluble CK in rephosphorylating ADP [3]. Thus, ADP produced by myosin ATPase could be rephosphorylated by CK located in the myofibrils of heart and skeletal muscle.

Actomyosin isolated from smooth muscle has a low ATPase activity compared to skeletal muscle [6,7]. In smooth muscle, an increase in intracellular Ca^{2+} concentration causes stimulation of myosin light chain kinase (MLCK), resulting in phosphorylated myosin light chains (MLC-P) which can then interact with actin and produce tension by the formation of actin-myosin (A-M) crossbridges. Tension generation is thus dependent upon calmodulin and myosin light chain phosphorylation [8,9]. Tension maintenance, however, does not parallel Ca^{2+} concentration or MLC-P [6,9,10,11].

In spite of the low [PCr], ATPase and CK activity [12,13,14], the porcine carotid artery is able to maintain sustained contraction and buffer its ATP concentration [12]. The predominant isoenzyme of CK found in the smooth muscle is the BB isoform, ranging from 42 to 100% [15]. For example three non mitochondrial isoenzymes of CK have been found in carotid artery in approximately a 1 : 1 : 1 ratio [16]. Because smooth muscle has neither a well-defined Z line nor M line, it remains possible that the role and localization of CK may be different from that of striated muscle. It also remains to be determined whether there are differential functions for the different isoenzymes within the smooth muscle; currently, it is not known what, if any, role CK has in contraction and relaxation. Moreover, if CK is present in the myofibrils, which isoenzyme is utilized and where it is localized is unknown.

This study was performed to investigate the possible presence and role of CK and the CK system in the contraction and relaxation of skinned guinea-pig taenia coli. The results implicate an endogenous form of CK associated with the taenia coli myofilaments after skinning and contributing to relaxation from rigor conditions as well as submaximal tension generation.

A preliminary report of these data appeared in abstract form at the XIXth European Conference on Muscle Contraction and Cell Motility of the European Muscle Congress, September 10–13, 1990, Brussels, Belgium.

Methods

Tissue preparation

Guinea pigs ($N = 7$) weighing 300–500 g were anesthetized with an intraperitoneal injection of pentobarbitol. While under the anesthetic, the animal was exsanguinated. All research animals were housed and treated humanely and in accordance with local and national guidelines. The taenia coli was collected and rinsed in a Krebs solution containing (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 0.6 and glucose 5 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. Skinning was accomplished by the addition of 1% Triton-X 100 to this solution for 1 h at 21°C with continuous stirring [9].

Taenia coli fibers were isolated by blunt dissection and mounted onto stainless steel hooks for experimental measurements [5]. To change the bathing solutions as desired, eight 2.5 ml chambers were arranged around a disk that could be rotated under the muscle fibers. The disk was mounted on a magnetic stirrer and solutions vigorously and continuously stirred at > 1000 rpm to facilitate diffusion of Ca^{2+}, EGTA and substrates into and out of the muscle fiber.

Fibers were stretched to 120% of the slack length and allowed to equilibrate to a stable baseline in Control Relaxing Solution (Table I). Fiber length and diameter were measured via a binocular microscope fitted with a micrometer. Mechanics measurements were acquired using an AE801 force transducer (Aksjeselskapet Mikroelectronik, Horten, Norway) with a band width of 2 kHz.

TABLE I

Solution compositions

Solution letter designations correspond to those used in previous publications from our laboratory [5]. Solution A is the Control Relaxing Solution, B is the Control Stimulating Solution, E is the CK-Relaxing Solution, F is the CK-Stimulating Solution, H is the Rigor Solution + Ca^{2+}, and G is the Rigor Solution − Ca^{2+}.

Solutions	A	B	E	F	G	H
p*Mg*	2.7	2.7	2.7	2.7	2.7	2.7
p*MgATP*	2.5	2.5	[a]	[a]	[a]	[a]
p*Ca*	9	4.5	9	4.5	9	4.5
PCr (mM)	12	12	12	12	0	0
Imidazole (mM)	30	30	30	30	30	30
ADP (mM)	0	0	0.25	0.25	0.25	0.25
EGTA (mM)	5	5	5	5	5	5
DTT (mM)	0.3	0.3	0.3	0.3	0.3	0.3
pH	6.8	6.8	6.8	6.8	6.8	6.8

[a] Zero *Mg*ATP added to the solution.

Solutions

All solutions (Table I) were calculated using the program of Fabiato [17]. They were calculated to contain 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 [18,19]. Control Stimulating (p*Ca* 4.5) and Relaxing (p*Ca* 9) Solutions also contained 3.16 mM MgATP and 12 mM PCr. CK-Relaxing and CK-Stimulating Solutions, designed to check the CK effi-

cacy, contained 250 μM MgADP, 12 mM PCr with no ATP. Rigor Solutions contained no ATP or PCr.

EGTA was obtained from Sigma Chemicals (St. Louis, MO, USA). PCr (Neoton, Schiapparelli Farmaceutica, Turin, Italy) was a generous gift of Prof. E. Strumia.

CK activity

Tissue samples were prepared by separating the taenia coli from surrounding circular muscle and adventitia. For total CK activity and isoenzyme fractionation, tissue samples were homogenized in a solution containing 100 mM KH_2PO_4, 1 mM EGTA and 15 mM N-acetyl cysteine (NAC) at a pH of 8.5 and 4°C (100 mg of tissue/ml). CK activity was also determined in Triton X-100-treated fibers. The fibers were thoroughly rinsed after Triton X-100 treatment prior to homogenization and were assayed.

CK activity was determined using the coupled enzyme assay of glucose-6-phosphate dehydrogenase (G6PDH) and hexokinase producing NADPH. NADPH production was measured spectrophotometrically at 340 nm (Gilford Spectrophotometer, Corning, NY). The CK activity was assayed in a solution containing (mM) 25 Hepes, 5 $MgCl_2$, 0.5 DTT, 1 ADP, 10 PCr, 20 glucose, 0.6 NADP, 9 AMP (to inhibit adenylate kinase), and 2 IU/ml of hexokinase and G6PDH at a pH of 7.4 and 30°C.

Isoenzyme fractionation was 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) 50 Hepes, 10 magnesium acetate, 1 DTT, 20 glucose, 15 AMP, 10 ADP, 6 NADP, 100 PCr, 18 IU/ml hexokinase and 6 IU/ml G6PDH at a pH of 7.4. Individual isoenzyme bands can be visualized by observing the fluorescence of NADPH.

Statistics

Statistical methods employed were the Student's *t*-test for significance. Reported values are accompanied by the standard error of the mean.

Results

CK activity

Total CK activity in the taenia coli was 163 ± 22 IU/g (ww) ($N = 7$). After the fibers were skinned, the activity which remained was found to be 35 ± 10 IU/g (ww). Upon electrophoresis it was observed that the whole taenia coli contained MM and MB-CK, while the dominating band came from BB-CK activity. Moreover, only BB-CK activity remained with the fibers after the skinning process (Fig.1). The effect of this selectively bound BB-CK was evaluated with the mechanics experiments discussed below.

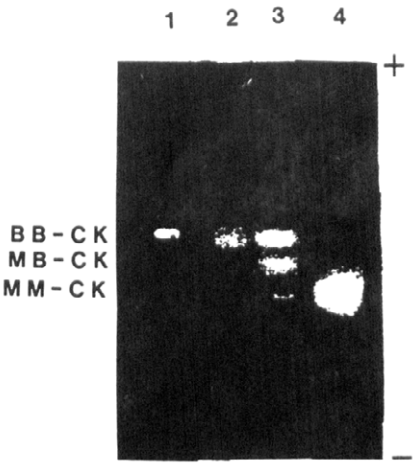

Fig. 1. Agarose electrophoresis of the creatine kinase isoenzymes from the guinea pig. Bands 1 and 4 are taken from the brain and skeletal muscle as controls for BB-CK and MM-CK, respectively. Band 2 was obtained from taenia coli exposed to the Triton X-100 skinning procedure and band 3 was produced by whole taenia coli.

Tension generation by Triton X-100 skinned taenia coli

Triton X-100-treated fibers developed maximal tension of 28.8 ± 2.9 mN/mm^2 in the presence of 12 mM PCr and pMgATP 2.5 at p*Ca* 4.5 (Table II). Increasing calcium concentration induced a stepwise increase in force. Data were fitted using the Hill equation: T (relative force) $= [Ca]^{n_H}/(K + [Ca]^{n_H})$. The mean p*Ca* for half maximal activation $pCa_{50} = (-\log_{10} K)/n_H$ found for the taenia coli was 5.70 ± 0.07 ($N = 5$). The Hill coefficient (n_H) was 4.08 ± 0.55. After Triton X-100 treatment, the essential enzymatic activities and regulatory functions of the contractile proteins are conserved.

CK and tension generation

To determine CK's role in skinned fibers, they were stimulated with a solution which would permit them to produce ATP only via bound and active myofibrillar CK (CK-Stimulating Solution, see Table I). This solution induced a substantial increase in force (Fig. 2). Maximal tension generation was again measured with the Control Stimulating Solution. The fibers were then re-exposed to the CK-Stimulating Solution resulting in

TABLE II

Characteristics of skinned taenia coli fibers

	Length (μm)	Diameter (μm)	Passive tension (mN/mm^2)	Active tension (mN/mm^2)
Taenia	1195	224	7.9	28.8
$N = 17$	± 102	± 18	± 1.3	± 2.9

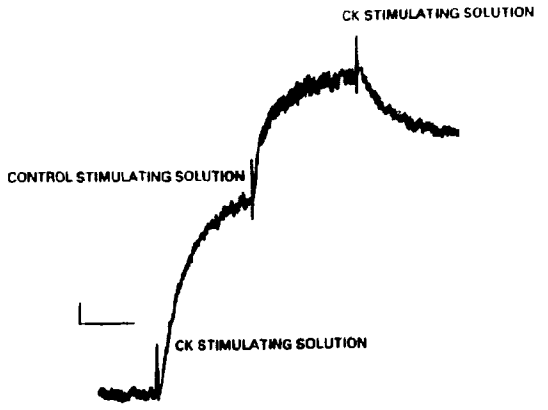

Fig. 2. Representative tension recording illustrating tension generation in the CK-Stimulating Solution. CK-activated contraction is achieved by transferring the muscle from a relaxing solution to an activating solution containing Ca^{2+}, PCr and ADP without ATP, followed by the Control Stimulating Solution with ATP and PCr. This was followed by re-exposure to the CK-Stimulating Solution. The vertical and horizontal bars correspond to 1 mN/mm^2 and 10 min, respectively.

a decrease in tension. In these conditions, tension in Control Stimulating Solution was 22.9 ± 6.2 mN/mm^2 ($N = 12$). The mean tension due to the CK-Stimulating Solution was 59.1 ± 5.2 percent of maximal.

In order to verify that this tension was in fact active (not rigor tension), the Rigor Solution + Ca^{2+} was applied to the muscle fibers prior to activation (Fig. 3). There was no significant increase in tension indicating that smooth muscle is not able to generate rigor tension prior to activation. Therefore, tension generated by the CK-Activating Solution is due to active tension generation. Tension in the CK-Stimulating Solution could not be attributed to ATP produced by adenylate kinase because no change in tension was observed when adenylate kinase was inhibited by P^1,P^5-di (adenosine-5')pentaphosphate (data not shown). Moreover, the fact that Rigor Solution + Ca, which contained ADP, was not able to induce tension further suggests that no adenylate kinase was present in these fibers.

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 fiber's response to the CK-Stimulating Solution. To inhibit endogenous CK, 10 nM fluoro-1-dinitro-2,4-benzene (FDNB), an irreversible CK inhibitor [20], was added to the CK-Relaxing Solution. After thorough rinsing, the fiber's response to the CK-Stimulating Solution was abolished. However, the fibers were still able to develop maximal tension in Control Stimulating Solution, thus demonstrating that neither MLCK nor myosin ATPase was inhibited by FDNB (Fig. 4). In another set of experiments, the fibers were subsequently incubated in Control Relaxing Solution with 500 IU/ml MM-CK (Sigma). Again, after thorough rinsing, the fibers were stimulated with the CK-Stimulating Solution, and they generated $85.0 \pm 6.6\%$ of the maximal tension (data not shown). These data demonstrated attachment of MM-CK to some as yet undefined binding sites on the myofibrillar proteins.

ADP and tension generation

Because ADP can inhibit MLCK 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 fibers were sequentially exposed to the CK-Stimulating Solution with 0, 10, 30, 50, 100, 250, 400, 500 μM MgADP (Fig. 5). Tension developed

Fig. 3. Representative tension recording illustrating the taenia coli's inability to generate tension in Rigor Solution + Ca prior to activation. Maximal contraction and relaxation are subsequently observed in Control Stimulating Solution and Control Relaxing Solution respectively. The vertical and horizontal bars correspond to 1 mN/mm^2 and 10 min, respectively.

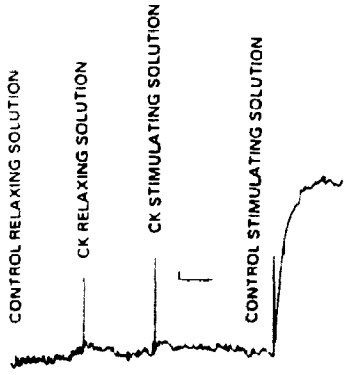

Fig. 4. Representative tension recording of taenia coli after inhibition of CK by FDNB. After inhibition, the fiber was rinsed in the Control Relaxing Solution followed by the CK-Relaxing Solution. Subsequent exposure to p*Ca* 4.5 in the presence of 12 mM PCr, 250 μM ADP and 0 ATP failed to produce tension. Again maximal tension generation is elicited by exposure to the Control Stimulating Solution. The vertical and horizontal bars correspond to 1 mN/mm^2 and 10 min, respectively.

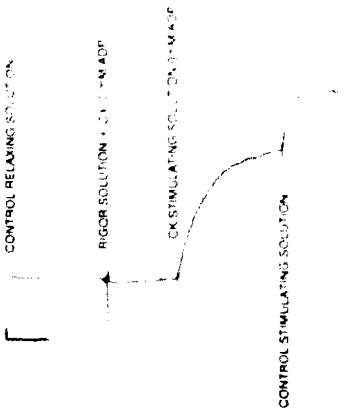

Fig. 6. A representative trace of the response to CK Stimulating Solution with 0 ADP after 30 minutes pre-incubation in the absence of nucleotides with p*Ca* 4.5 (Rigor Solution + Ca, 0 mM ADP). Maximal force was elicited with Control Stimulating Solution. Vertical and horizontal calibration bars are 1 mN/mm^2 and 20 min respectively.

a plateau at an ADP concentration of 250 μM and subsequently fell off at higher concentrations. It was also observed that the fibers can generate (and maintain for at least 15 min) 41% of the maximal tension with 0 mM ADP in the CK-Stimulating Solution. Similar results were obtained when the fibers were preincubated for 30 min in the complete absence of nucleotides (Fig. 6). 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.

PCr and contraction

To determine the effect of PCr on the contractile response of skinned fibers they were stimulated with the CK-Stimulating Solution using a PCr concentration

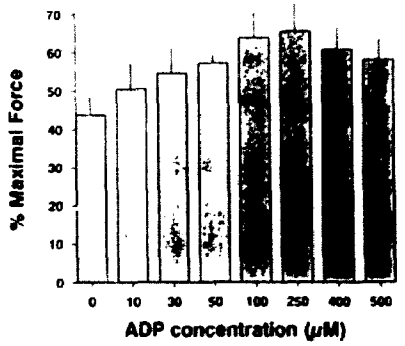

Fig. 5. Histogram of the tension responses to increasing ADP concentrations in the CK-Stimulating Solution, expressed as percent of maximal force in the Control Stimulating Solution. Error bars are standard error of the mean.

closer to that found in the intact taenia coli (0.5 and 1 mM). Fig. 7 shows the typical response to 0.5, 1.0 and 12 mM PCr in the CK-Stimulating Solution. It thus appears that physiological concentrations of PCr are able to elicit a force equivalent to that of 12 mM PCr.

CK and relaxation from rigor conditions

Since CK is able to generate submaximal tension in taenia coli, we attempted to characterize CK's ability to relax taenia coli from rigor conditions. Rigor tension

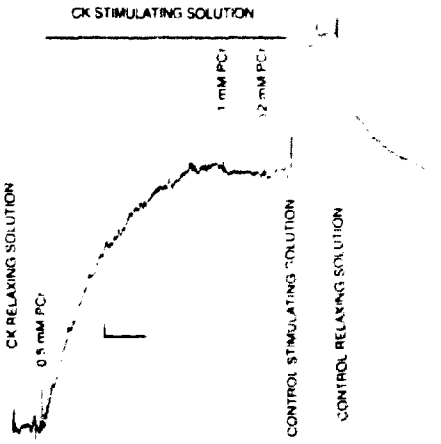

Fig. 7. Representative force recording of the response to varying PCr concentrations in the CK Stimulating Solution. The horizontal bar represents the duration of exposure to the CK Stimulating Solution before exposure to the Control Stimulating Solution. Vertical calibration bar represents 1 mN/mm^2 and horizontal calibration bar represents 20 min.

was produced after maximum tension generation in the Control Stimulating Solution because (as seen above) the taenia coli does 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: first ATP and PCr were removed and 250 μM ADP added in the presence of p*Ca* 4.5 (Rigor Solution + Ca^{2+}). This maneuver initiates a high-tension rigor state which remains when Ca^{2+} is reduced (Rigor Solution − Ca^{2+}) [30]. To produce relaxation from rigor with the CK reaction, 12 mM PCr was added to the bathing solution (CK-Relaxing Solution). Active CK in or on the skinned myofibrils and in the presence of PCr, relaxed the fibers utilizing ATP produced via the CK reaction. Finally, Control Relaxing Solution was added to demonstrate the extent of relaxation. In this study, relaxation produced after Control Relaxing Solution is referred to as maximal relaxation.

Fig. 8 is a representative recording obtained from taenia coli demonstrating CK's ability to relax the fibers from rigor conditions. As can be seen, rigor conditions produced a diminution in maximal tension due to detachment or slippage of some cross-bridges. After stabilization, CK-Relaxing Solution induced an acceleration in the rate of tension decrease. In this fiber the relaxation was nearly completed, since further addition of Control Relaxing Solution containing ATP did not induce further relaxation. As a mean, the extent of CK-induced tension fall was 51.2 ± 7.6 (N = 17) percent of the total tension decrease produced after the Control Relaxing Solution. A significant relaxation from rigor can thus be accomplished when the taenia coli is exposed to conditions where ATP production is entirely dependent upon the CK reaction. The second contraction observed in Fig. 8 demonstrates the viability of the fiber after the rigor protocol.

Discussion

In this study we investigated the possible presence and role of CK in the generation of tension and relaxation from rigor conditions in skinned guinea-pig taenia coli. The results suggest a contribution of CK to skinned taenia coli muscle mechanics. We presented evidence that BB-CK is specifically attached to the myofilaments and that it remains active throughout the skinning process. This active CK associated with the skinned myofilaments can supply ATP from endogenous ADP to generate submaximal tension. Relaxation from rigor tension was also observed with bound BB-CK.

In fast skeletal muscle, there is a high PCr concentration which is considered as a reservoir or buffer fo. the high-energy phosphates. Creatine kinase is present in high specific activity and is found mainly in the cytoplasm at or near equilibrium. It has thus been

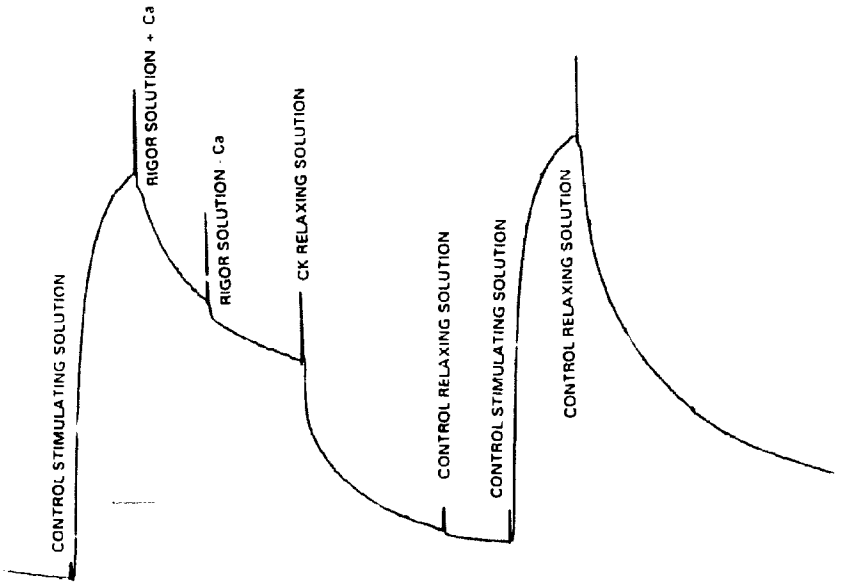

Fig. 8. Representative tension recording of taenia coli illustrating the ability of the CK-Relaxing solution to relax the tissue from rigor conditions. A high-tension rigor state is achieved by transfering the muscle from the Control Relaxing Solution to the Control Stimulating Solution, followed by removal of ATP and PCr in the presence of 250 μM ADP and subsequent removal of Ca. Relaxation is induced first by adding PCr (CK-Relaxing Solution) and finally by applying the Control Relaxing Solution. The second contraction and relaxation in Control Solutions illustrates tissue stability. The vertical and horizontal bars correspond to 1 mN/mm^2 and 10 min, respectively.

proposed that in spite of large diffusion distances, a mechanism of facilitated diffusion may explain spatial as well as temporal buffering of ATP in this muscle [21]. On the other hand, aerobic muscles, especially heart muscle, are characterized by different CK isoforms which are localized at the sites of excitation-contraction coupling (membranes, myofilaments and sarcoplasmic reticulum) and of energy production (mitochondria and glycolytic complexes). This organization has led to another proposed role for CK in production, transfer and utilization of energy [1,3].

In contrast to skeletal muscle, smooth muscle contractile activity is associated with only a 2- to 3-fold increase in ATP utilization and virtually no change in the ATP and PCr content because the rate of ATP breakdown matches its resynthesis rate. In this respect smooth muscle behavior is closer to that of cardiac muscle, even though smooth muscle is a glycolytic muscle compared to the aerobic heart muscle. However, it is also known that the rate of energy utilization during force maintenance is two orders of magnitude lower than skeletal muscle [22,23]. A large component of this difference appears to reside in the intrinsic differences between the actin-activated myosin ATPases of striated and smooth muscle [6,24,25]. Since PCr levels are very low in smooth muscle, ranging from 0.5 to 4 mM [22], the concept of a pool of PCr as a buffer for ATP may be less meaningful than in skeletal muscle [12,22,23,26]. On the other hand, if the role of CK in smooth muscle was more intimately involved in contraction and relaxation, then one might predict that CK would be specifically bound to the contractile filaments and associated with constituents of the cross bridge cycle.

The question that remains is to determine the role and significance of CK in smooth muscle. The first question to be addressed is regarding the activity of CK in smooth muscle. The results reported here show that in taenia coli total CK activity was 163 IU/g (ww), a value 3–4-times lower than in cardiac muscle and about 10-times lower than in skeletal muscle. This is higher than the value reported for carotid artery [16] or uterine muscle [13], but is consistent with other reported values of CK activity in smooth muscle [13]. The CK activity therefore seems to depend upon smooth muscle type. PCr concentration in rabbit taenia coli is about 3 mM [23,27]. These results are in agreement with Iyengar [13], who proposed that the concentration of PCr in a tissue is related to the CK activity.

The second question concerns the polymorphism of CK in smooth muscle. We found that, although BB-CK is the main isoform in the taenia coli, significant amounts of MB as well as MM-CK are present. In cardiac muscle, the significance of the different isoforms of CK was related to their intracellular localization rather than to kinetic differences among them [1,2]. In carotid artery, it has been shown that BB-CK has a high K_m for ATP, favoring ATP synthesis [16]. Nonetheless, such kinetic differences for BB-CK in smooth muscle could be related to the localization of CK near sites of energy utilization.

Thus, the third question to be addressed is the localization of the different isoforms of CK in smooth muscle. Prior to this study it had not been determined whether any CK isoenzyme was bound to the myofilaments, but the results presented here suggest that there is in fact some of the BB-CK associated with the myofilaments. We gave evidence above that a substantial amount of CK was bound to contractile structures in the skinned smooth muscle and that the isoform present was BB-CK. This myofibrillar CK represented 22% of total CK activity. The binding of BB-CK in myofibrils seemed to be specific for the reasons listed below. First, Triton X-100 treatment is known to solubilize all membrane structures, thus liberating membrane and cytoplasmic proteins [5]. Second, although there are different CK isoforms in fresh taenia coli, only BB-CK is present in skinned fibers. Third, CK activity is still present in the fibers 2 to 3 days after Triton X-100 treatment. Finally, although it was shown to be associated with the thin filament in skeletal and cardiac muscle, adenylate kinase (which is able to rephosphorylate ATP from ADP) was not present in skinned smooth muscle fibers since ADP, in the presence of calcium, was not able to induce tension. Also, no difference in tension was found in the presence of the CK-Activating Solution after inhibition of adenylate kinase, indicating that loosely bound enzymes are lost after Triton X-100 skinning in smooth muscle, as was reported for cardiac muscle [5]. These results strongly suggest that BB-CK is specifically associated with contractile structures in taenia coli.

In these experiments, we found that the CK which remained bound to the skinned fibers was unable to produce enough ATP to maintain and generate maximal tension. The tension caused by the CK-Stimulating Solution is therefore a reflection of the inability of bound CK to fully supply ATP to the contractile elements for maximal tension. Such a limitation of the ATP supply from CK could be due to a low specific activity of CK in the fibers and/or structuring of CK to some discrete location in or on the myofilaments. This could also contribute to the gradual increase in tension with the CK-Stimulating Solution. Additionally, we showed that MM-CK can attach to the taenia coli myofilaments and function to generate ATP. Thus other weak and/or nonspecific binding sites for MM-CK may be present in the fibers. These loose binding sites for CK may be physiologically significant because of the presence of MM-CK in the whole taenia coli. Finally, one may hypothesize that the other CK isoenzymes may be associated with intracellular structures

like the sarcoplasmic reticulum or sarcolemma. Evidence for clustering of the glycolytic enzymes [6,28] has been given previously. It is possible that CK would be associated with such glycolytic complexes for PCr generation [29].

The fourth and final question concerns the role of CK in contraction and relaxation. Our results show that bound BB-CK was able to sustain submaximal tension and to induce partial relaxation by rephosphorylating MgADP, in the complete absence of MgATP. Subsequent addition of MgATP induced maximal tension or accelerated relaxation from rigor conditions. The tension generated with the CK-Stimulating Solution was observed to be slower than in Control Stimulating Solution but was not due to rigor-bridges because, unlike heart muscle, smooth muscle in rigor solution is not able to generate rigor tension from resting conditions, even in the presence of calcium [18,30] (Fig. 3).

In smooth muscle, high-tension rigor state can be induced by removing high energy phosphates from preactivated fibers. In this case high-tension rigor is produced even in the absence of calcium. The addition of PCr, in the presence of MgADP, is able to partially relax rigor tension. This is very similar to the relaxation from rigor tension elicited in skinned smooth muscle by photolytic release of caged ATP [18,30]. Our results suggest a rapid rephosphorylation of ADP by active CK bound to the skinned fibers.

It appears that CK associated with the myofilaments in skinned taenia coli fibers is active but can only supply sufficient ATP from ADP to generate submaximal force. This is not due to inhibition of contraction by MgADP. Indeed, we have demonstrated that ADP has an optimum concentration of 250 μM for tension generation. Inhibition of CK, myosin ATPase and/or MLCK, may explain the fall in average tension at 400 and 500 μM MgADP. Tension due to the CK reaction was found to be identical between 0.5 and 12 mM PCr, indicating that physiological concentrations of PCr are able to produce qualitatively similar contractile responses as 12 mM PCr. Taken together, these results indicate that tension development via the CK reaction is limited by the action of CK and not by substrate (ADP and PCr) inhibition or availability. In intact smooth muscle, at physiological concentrations of ADP and PCr, bound myofibrillar BB-CK (and other putative loose binding sites) may participate in tension generation and relaxation.

Our results show that a substantial tension can be elicited in the presence of PCr and in the virtual absence of MgADP. This strongly suggests that endogenous ADP (or cycling ADP/ATP) is bound to the skinned taenia coli myofilaments and available to the enzymes of the crossbridge cycle. This binding is specific and is not due to residual nucleotides, since a 15 min pre-wash without nucleotides had the same effect. Butler et al. [31] using permeabilized rabbit portal vein have shown that in resting smooth muscle ADP is bound to myosin in the form of myosin-ADP-P_i complex and that phosphorylation of myosin and its subsequent interaction with actin, increases the rate of ADP release. They estimated 60 to 70 μM radiolabeled ADP present in the permeabilized fibers [32]. A pool of bound ADP therefore may be very important for smooth muscle regulation and we have shown that this ADP is available to CK for rephosphorylation. Nishimura and Van Breemen [33] proposed that ADP could affect the calcium sensitivity of smooth muscle fibers by increasing the number of A · M-ADP complexes. We believe that tension generation in the absence of exogenous ADP is evidence in support of CK acting as one of the effectors of the A · M-ADP complex by its ability to phosphorylate ADP. Nonetheless, the results presented here support a tightly bound ADP available for cross-bridge cycling in the skinned smooth muscle. This ADP forms a complex which should have a very low dissociation rate constant, since the response is maintained and tension does not decrease during a 30 min activation, both in the absence of added nucleotides. The fact that tension can be maintained under these conditions is supportive of functional compartmentation of some adenine nucleotides in smooth muscle. Indeed, functional compartmentation of the glycolytic cascade associated with the plasma membrane and of glycogenolysis linked to contractile filaments or cytoplasmic elements, has been shown in this tissue [6,28,34]. Recently, Ishida et al. [35] reported the presence of the mitochondrial type of CK, Mi-CK, in guinea-pig taenia caeci and proposed it to be the basis for the dependence of the PCr production on oxidative metabolism observed in guinea-pig taenia caeci. Indeed, as described in the heart, CK isoenzymes by their ability to bind to myofibrillar structures or mitochondria and to associate with glycolytic enzymes [29,36], may participate in production, transfer and utilization of high energy phosphates in smooth muscle. Such an efficacious arrangement of enzyme systems may allow for independent regulation of various energy-dependent cellular function in the smooth muscle [34]. We would like to propose that the results presented here could be explained by the presence of myosin-ATPase, CK and MLCK tightly bound to the myofilaments. Such an arrangement may be attributed to the co-localization of these enzymes in or on the contractile elements of the taenia coli.

One hypothesis we wish to put forward for smooth muscle, is that such an enzyme cluster may enable efficacious functioning of the contractile system in spite of the low concentration of ATP. More work needs to be done to determine the existence, precise location and role of this enzyme cluster.

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