The calmodulin fraction responsible for contraction in an intestinal smooth muscle

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Freeze-dried fibers of smooth muscle from *Taenia coli* were used to determine the concentration of calmodulin responsible for contraction. About 10% of the total intracellular calmodulin (12.6 μmol/kg wet wt) is directly involved in initiation of smooth muscle contraction.

Calmodulin *Myosin light chain kinase* *Smooth muscle* *Contraction control* Taenia coli

1. INTRODUCTION

The phosphorylation of myosin light chain-2 by myosin light chain kinase (MLCK) is thought to trigger smooth muscle contraction. MLCK is present in smooth muscle at a concentration of 1 μmol/kg wet wt [1] or 2.7 μM and is activated by calmodulin (CaM) in the presence of Ca^{2+}. In vitro experiments indicated that the [Ca^{2+}] required to activate either MLCK [2] or contraction of skinned smooth muscle fibers [3] is inversely correlated with the [CaM]. In vitro activation of smooth muscle MLCK is further controlled by cAMP-dependent phosphorylation of MLCK, the modification of which increases the [CaM] required to activate the enzyme half maximally from 3 to 50 nM [4]. The [CaM] of bovine uterus smooth muscle is about 37 μM [5], a concentration at which the activation of MLCK and the initiation of contraction should occur at [Ca^{2+}] below 0.1 μM. In contrast, contraction of living smooth muscle fibers has not been observed at such low Ca^{2+}-concentrations [6]. This discrepancy can be resolved if only part of the total CaM participates in the activation of MLCK. Using smooth muscle fibers from *T. coli* – which were skinned by freeze-drying [7] – we found that the fibers contain 12.6 μmol/kg wet wt or 34 μM CaM, but about 3–4 μM CaM is freely available for the activation of MLCK and the initiation of contraction. This value indicates that functionally no excess of CaM over MLCK exists in smooth muscle.

2. METHODS

2.1. *Preparation of fibers and measurement of contraction*

Smooth muscle fibers from guinea pig *T. coli* were shock frozen and freeze-dried at −20°C as in [7]. Freeze-dried fibers, approximately 0.5 cm in length and 0.1–0.2 mm in diameter, were attached at one end to an AME force transducer and at the other end to an adjustable micrometer drive. Fibers were then rehydrated for 1.5 min in an ATP-free salt solution containing 5 mM EGTA (rigor solution), followed by an incubation for 10 min (●) or 45 min (■) in relaxing solution (20 mM imidazole, 4 mM EGTA, 10 mM $MgCl_2$, 7.5 mM ATP, 1 mM NaN_3, 2 mM DTE, 10 mM creatinephosphate, 380 units/ml creatine phosphokinase, Boehringer, pH 6.7, $T = 20$°C), thereafter they were exposed to the [CaM] indicated. Contraction was initiated by raising the free Ca^{2+} to 1.6 μM [3]. Tension development (fig.1, table 1) was measured after 3 min and expressed as a percentage of the maximum contraction obtained

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in the presence of 30 μM Ca^{2+}.

2.2. *Determination of calmodulin*

Freeze-dried fibers (10 mg dry wt) were incubated at 20°C in 250 μl ATP-free salt solution containing 4 mM EGTA (rigor solution). This solution was replaced after 1.0 min by relaxing solution. Fibers were next incubated for 10 min or 60 min. Thereafter, fibers were homogenized in 350 μl extraction solution containing 1% (w/v) Lubrol WX [8]. Samples were further processed as in [8] and CaM concentration was determined by activation of a bovine heart phosphodiesterase using pig brain CaM as standard. The molecular mass of CaM was taken as 16.7 kDa.

3. RESULTS

3.1. *Fibers skinned by freeze-drying*

In initial experiments the basic properties of the freeze-dried fibers were determined. Rehydrated fibers relaxed completely in the presence of Mg-ATP and a free Ca^{2+} of less than 0.1 μM. They responded like other types of skinned fibers rapidly and reversibly to micromolar Ca^{2+} (fig.1, inset). Contraction was not elicited by the addition of caffeine suggesting that intracellular calcium stores were non-functional. Contraction was initiated at threshold concentrations of free Ca^{2+} of about 1 μM. Maximal tension development of freshly hydrated fibers amounted to 0.5–1.0 kg/cm^2 at a free Ca^{2+} of 30 μM.

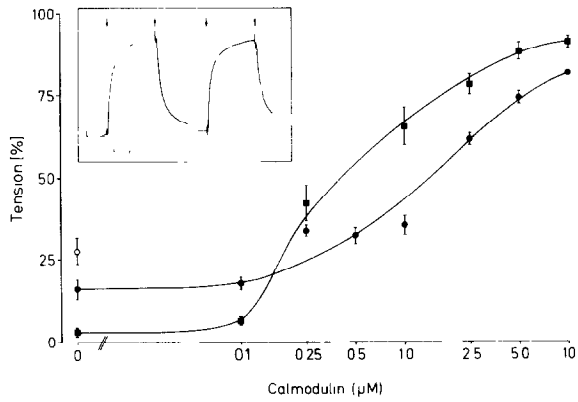

Fig.1. Effect of added calmodulin on the contractile response in freeze-dried smooth muscle fibers. Fibers were extracted for 0.5 min (○), 10 min (●) and 45 min (■). The contractile response was then measured in the absence of added calmodulin (CaM) (○) or in the presence of the indicated concentration of CaM (●, ■). The tension developed by the fibers in the absence of CaM (○) was significantly ($p < 0.05$) higher than that obtained after 10 min extraction in the absence of CaM. Each point represents $\bar{x} \pm$ SE for 7–10 different fibers and each fiber was used only for one [CaM]. Statistical analysis was performed by the use of a Student's t-test. Inset: reversibility and time course of isometric contraction cycles elicited by 30 μM Ca^{2+} (at arrow ↓).

Isometric contractile responses of freeze-dried fibers initiated by 1.6 μM Ca^{2+} decreased with time and could no longer be elicited when fibers were preincubated for 45 min in the relaxing solution

Table 1

Contraction of fibers is abolished by preincubation

Ca^{2+} (μM)	Extraction time (min)		
	10	45	45 + CaM
	(% of maximal contraction)		
1.6	21.5 ± 6.0 (4)	0.2 ± 2 (5)	20.3 ± 7.7 (5)
3.5	56.7 ± 5.7 (5)	1.9 ± 4 (5)	53.7 ± 14.9 (5)

Freeze-dried fibers were treated as described in section 2. They were incubated for 10 or 45 min in relaxing solution. A third set of fibers was first incubated for 45 min in relaxing solution and then transferred for another 10 min to a fresh relaxing solution containing 0.5 μM CaM. After the extraction period indicated, all fibers were immersed in a solution containing the indicated free [Ca^{2+}]. Each fiber was used once and values are given as $\bar{x} \pm$ SE with the number of individual fibers in parentheses

(table 1) before challenging with Ca^{2+}. Tension development in the presence of a maximal Ca^{2+} of 30 μM was not affected. Original contraction responses were restored after incubation of the fibers for a few minutes in the presence of 0.5 μM CaM. This suggested that during the preincubation period a part of CaM necessary for contraction was removed from the fibers by diffusion into the bath medium. The effect of added CaM on contraction was again reversed by incubation of these fibers in a CaM free relaxing solution for 10 min. This suggests that the freeze-dried fibers were freely permeable to proteins of a molecular mass of 20 kDa but retained larger proteins such as MLCK.

3.2. *Biochemical assays*

In the next series of experiments we therefore determined the total concentration of CaM and that part of CaM which exchanged rapidly with the fiber bath medium in the presence of a low free Ca^{2+}. Total CaM was 12.6 μmol CaM/kg wet wt which corresponds to an intracellular concentration of CaM of 34 μM if one assumes that CaM is only distributed in the intracellular water compartment (table 2). This value is similar to that reported previously for uterine smooth muscle [5] and probably describes the total CaM of *T. coli* correctly. About 50% of the total CaM was extracted from the fibers during a 60 min incubation period indicating that one half of the total CaM is tightly bound to non-diffusible structures in the presence of low Ca^{2+}. Incubation of the fibers for 0.5 min in rigor solution extracted 1% of the total CaM. Fibers incubated for an other 10-min period in relaxing solution lost 8% of their CaM which corresponds to an intracellular CaM of 2.7 μM. In a different set of experiments it was determined that 10–13% (n = 8) of the total CaM was lost during a 1–2 min incubation period. These findings indicated – together with the result that the effect of added CaM was reversed within 10 min – that only the freely exchangeable part of CaM was extracted during the first 10 min. This, therefore, suggests an intracellular free CaM of about 3 μM or a tenth of the total CaM.

3.3. *Bioassay using skinned fibers*

In freeze-dried skinned fibers extracted for either 10 or 45 min in relaxing solution to remove endogenous calmodulin, we have established the relationship between the concentration of added calmodulin and the relative force evoked at 1.6 μM Ca^{2+}. Fig.1 shows that a calmodulin concentration of 0.3–1 μM is required to develop the same tension as the force (27.6 ± 3.9%; n = 10) evoked by

Table 2

The concentration of intracellular calmodulin

Extraction		Calmodulin extracted			Calmodulin concentration (μM)	
Medium	Time (min)	μg/mg dry wt	%	μmol/kg wet wt	Extraction medium	Intracellular water
Total	–	1.24 ± 0.08 (6)	100	12.6[a]	[b]	34.0[c]
Rigor	0.5	0.015 ± 0.003 (7)	1.2	0.15	0.036	0.4
Relaxation	10	0.10 ± 0.02 (3)	8.1	1.0	0.23	2.7
Relaxation	60	0.57 ± 0.15 (3)	46.0	5.8	1.3	15.6

[a] The [CaM]/kg wet wt was calculated from the CaM/dry wt by correcting for the lost water (83%, n = 3)

[b] The [CaM] in the extraction medium was calculated from the CaM/dry wt by correcting for the used dry wt of fibers (10 mg) and the extraction volume (250 μl)

[c] Intracellular [CaM] was calculated from the [CaM]/kg wet wt using an intracellular water space of 37% of wet wt [9,10]. This last value assumes that the total amount of CaM is only distributed in the intracellular water space. This assumption is certainly not correct since about 50% of the total [CaM] was only extracted in the presence of detergent, suggesting that this part was largely bound to membranes (see also [8])

All values are corrected for buffer blanks. Original values are given as $\bar{x}$ ± SE with the number of fibers in parentheses

Ca^{2+} in a very briefly rehydrated fiber in the absence of exogenous calmodulin. This means that in freeze-dried fibers rehydrated for only 1.5 min in relaxing solution, the endogenous 'free' calmodulin available for eliciting contraction had a concentration of about 0.3–1 μM. If appropriate corrections are made for diffusional losses during the brief rehydration (table 1) an upper value of about 3–4 μM may be estimated for intracellular concentration of the free calmodulin responsible for contraction in living smooth muscle. At this calmodulin level a Ca^{2+} concentration of 1.5 μM would evoke a nearly maximal (i.e., 80%) contraction.

4. CONCLUSIONS

The results shown in fig.1 and table 2 strongly indicate that both the free intracellular CaM and that part of the intracellular CaM directly involved in contraction control are identical and are about 4 μM. This CaM is very similar to that of MLCK if the value given in [1] is corrected for the intracellular water space [9,10]. This suggests that functionally both proteins are present in approximately equimolar concentrations. In the presence of calcium some part of the free CaM may be bound to 'caldesmon' [11]. This protein has been identified in gizzard muscle and it has been suggested that complexation of caldesmon by Ca^{2+} and CaM is necessary for the initiation of contraction. It is not known if intestinal smooth muscle contains caldesmon and what function, if any, is regulated by this protein.

Nine tenths of the total amount of smooth muscle CaM is not directly involved in the control of tension development. The function of this large amount of CaM is unknown. It certainly represents a compartment which binds Ca^{2+} without eliciting contraction. From table 2 it can be calculated that at least 50 μmol Ca^{2+}/kg wet wt need to be released during contraction to saturate the 4 Ca^{2+} binding sites of CaM and thereby to activate the MLCK completely. This value is within the range of that amount of Ca^{2+} which is released from intracellular stores during contraction of smooth muscle [12]. It is, therefore, conceivable that the large excess of CaM represents a sink of Ca^{2+} which prevents tension development by small amounts of released Ca^{2+}. This consideration together with the relatively low concentration of free CaM available for the initiation of contraction suggests a high calcium requirement for smooth muscle contraction.

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