CALMODULIN IS ESSENTIAL FOR SMOOTH MUSCLE CONTRACTION

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1. Introduction

Calmodulin (CaM) is the regulatory component of the myosin light chain kinase (MLCK) which in smooth muscle phosphorylates the regulatory light chain of myosin, a prerequisite for actin-activated myosin ATPase activity [1–3]. Ca^{2+} at μM levels bind to CaM and the complex activates the MLCK [4]. This Ca^{2+}-dependence of actin-activated myosin ATPase activity is lost after thiophosphorylation of myosin light chain by ATPγS [5]. The activation of MLCK can be partially inhibited by phosphorylation with cAMP-dependent protein kinase [6]. Ca^{2+}-dependent phosphorylation of myosin light chain has now been demonstrated in membrane-skinned [7] and in intact smooth muscle where myosin phosphorylation correlates closely with contraction [8,9]. Direct evidence that CaM is required for Ca^{2+}-activated tension development is lacking.

Membrane-skinned smooth muscle can be used to investigate regulation of contraction because the fibre bundles relax and contract in response to μM Ca^{2+} [10] and the tension developed is comparable to the living muscle [11]. Accordingly we investigated the influence of CaM on tension development and Ca^{2+}-sensitivity of detergent skinned fibres of guinea pig taenia coli. We report that CaM at nM levels strikingly increased the Ca^{2+} sensitivity of muscle fibre, increased the rate of contraction and the maximum tension developed and also greatly maintained the mechanical viability of the smooth muscle, so that reproducible and consistent responses could be obtained for many hours. Catalytic subunit of cAMP-dependent protein kinase reversibly inhibited contraction to threshold $[Ca^{2+}]$ only in the absence or at low [CaM]. Trifluoperazine at 0.01–0.1 mM completely and reversibly inhibited tension to maximum $[Ca^{2+}]$ in the presence of CaM but if CaM was absent the inhibition became irreversible. Loss of Ca^{2+} dependence occurred after thiophosphorylation using ATPγS with activation of tension, that could be enhanced a further 11% by CaM. It is concluded that CaM is essential for Ca^{2+} regulation of skinned smooth muscle. Its action is compatible with stimulation of MLCK to phosphorylate myosin light chain.

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2. Methods

2.1. *Skinned fibre preparation*

Taenia coli from mature guinea pigs was skinned essentially as in [11] with the following modifications. Short lengths were incubated in 20 mM imidazole (pH 7.4), 5 mM EGTA, 50 mM KCl and 150 mM sucrose for 2 h at 4°C, then 1% (v/v) Triton X-100 and dithioerythritol (DTE) 0.5 mM were added for a further 4 h at 4°C. After rinsing for 15 min in this solution without Triton X-100, they were stored in 20 mM imidazole, 4 mM EGTA (ethyleneglycol bis-aminoethylether *N,N*-tetraacetic acid), 10 mM $MgCl_2$, 7.5 mM ATP, 1 mM NaN_3, 0.5 mM DTE (pH 6.7) with 50% glycerol at −20°C for up to 10 days. Bundles of fibres ~3 mm long and 40–70 μm diam. were teased out for measurement of force development [12]. Absolute force production by the fibre bundles was 1–2 mNewtons (mN).

2.2. *Solutions and procedures*

Fibres were bathed at 21–23°C in a 'relaxing solution' containing 20 mM imidazole, 4 mM EGTA, 10 mM $MgCl_2$, 7.5 mM ATP and 1 mM NaN_3 adjusted

to pH 6.7 with KOH. Contraction was induced by increasing the free $[Ca^{2+}]$ by varying the ratio of EGTA/CaEGTA [13]. All solutions contained 6 mM phosphate because it was essential for relaxation, which otherwise was slow and incomplete. Contractions were elicited regularly at 20 or 25 min intervals, generally giving 10–15 min exposure to the contracting solution until a plateau had been attained and then changing to the relaxing solution. With CaM present at 0.3–0.5 μM, consistent responses to maximum or supramaximum $[Ca^{2+}]$ of 21 μM could be elicited for 4–6 h. The maximum tension developed with each contraction became smaller with successive contractions but in the first 3 h the decrease was <10%. The Ca^{2+} threshold for initiation of tension was detected by increasing the $[Ca^{2+}]$ at 5 min intervals, until an increase in tension of 0.05 mN was observed. Cumulative $[Ca^{2+}]$/tension response curves (fig.2a) were obtained by increasing the $[Ca^{2+}]$ stepwise for 10 min by which time the fibre had developed >90% of its response to that $[Ca^{2+}]$. Usually 7 cumulative curves could be obtained from one fibre bundle. A 25 min equilibration period with added CaM was given. Diffusion of Ca^{2+} was not rate limiting in tension development of the fibres as shown in some contractions induced by the 'Ca^{2+}-jump' technique, which permits a very rapid increase in the $[Ca^{2+}]$ within the fibre [14]. Purified CaM was prepared from beef heart according to [15]. Purified catalytic subunit of cAMP-dependent protein kinase, freshly prepared from rat skeletal muscle [16], was obtained from Dr M. Gagelmann. Trifluoperazine was a gift from Dr C. Metzger, Hoechst AG, Frankfurt. ATP and ATPγS were obtained from Boehringer, Mannheim.

3. Results

Calmodulin increased the speed of contraction induced by Ca^{2+}, the maximum tension developed, and lowered the threshold $[Ca^{2+}]$ at which the skinned fibre responded to Ca^{2+}. Fig.1a shows that 0.5 μM CaM increased the speed of isometric contractions resulting in a quicker attainment of plateau, a feature that was more pronounced in the fibre stored >2 days (fig.1b). The fibres contracted immediately in response to 21 μM Ca^{2+} in contrast to the delay of 26 ± 4 s (mean ± SEM 6 fibres) consistently observed in the absence of CaM. The tension developed to maximum or supramaximum $[Ca^{2+}]$ in the presence of CaM was increased by 13 ± 1%. The action of exogenous CaM was rapid; its full effect was observed within <5 min of prior exposure. When CaM was introduced simultaneously with 21 μM Ca^{2+} the delay in contraction was abolished and the maximum tension achieved was the same as with pre-incubation for 5 min but the initial rate of contraction was in general slightly slower.

Table 1
Threshold $[Ca^{2+}]$ required for initiation of tension in the presence of added calmodulin

Calmodulin (μM)	Threshold $[Ca^{2+}]$ (μM)
No addition	3.0 –6.6
0.012	0.8 –1.8
0.048	0.34–0.8
0.24	0.19–0.34
0.48	0.19–0.34
2.40	0.08–0.19

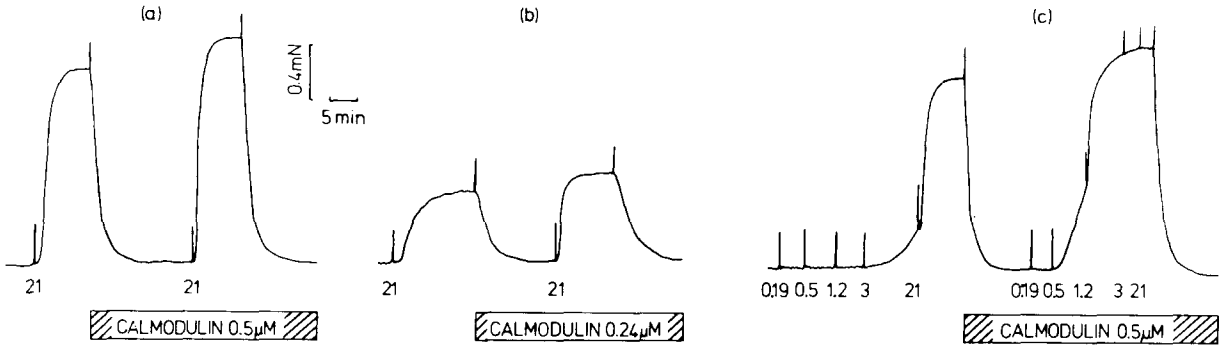

Fig.1. The effect of calmodulin on contraction of skinned smooth muscle fibre bundles induced by Ca^{2+}. (a) Isometric tension to 21 μM Ca^{2+} developed by fibres 1 day after skinning before and after exposure to CaM. (b) 3 days after preparation. (c) The threshold $[Ca^{2+}]$ at which tension is elicited, is shifted from 3–0.5 μM Ca^{2+} in the presence of CaM. Numbers below the flush artefact indicate the free $[Ca^{2+}]$ (μM) of the CaEGTA/EGTA buffer. Relaxation was induced by replacing the CaEGTA with EGTA to restore the free $[Ca^{2+}]$ to $<10^{-8}$ M.

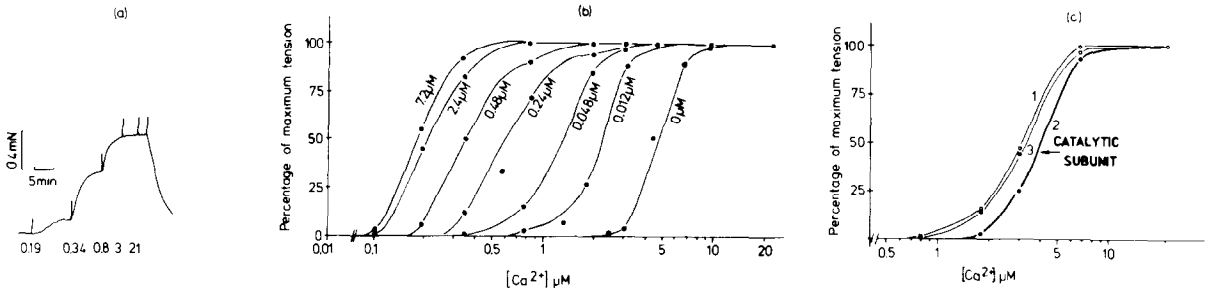

Fig.2. (a) The cumulative tension response of a smooth muscle bundle to stepwise increase in $[Ca^{2+}]$ in the presence of 0.48 μM CaM. (b) The effect of exogenous CaM on the force development/$[Ca^{2+}]$ relationship of skinned smooth muscle. Curves were plotted from cumulative tension responses as in (a). The curve at the far right was elicited in the absence of added CaM. Subsequent cumulative curves were elicited after 25 min equilibration with added CaM. In the absence of CaM the threshold $[Ca^{2+}]$ for initiation of tension was variable and increased with the time of storage of the fibres (1.8–6.6 μM Ca^{2+}). (c) The effect of the catalytic subunit of cAMP-dependent protein kinase on tension/Ca^{2+} relationship in the presence of constant 8 nM CaM. After a control cumulative tension response had been elicited (curve 1) catalytic subunit 4 μg/ml was incubated with the fibre for 20 min, then Ca^{2+} was introduced. The Ca^{2+} threshold for tension initiation was shifted to the right (curve 2) but at 7 and 21 μM Ca^{2+} tension was unaffected. The effect was reversed after washing out the catalytic subunit for 30 min (curve 3).

The effect of CaM was washed out within 20 min in EGTA relaxing solution. CaM lowered the threshold at which the skinned muscle fibre responded to Ca^{2+}. Fig.1c shows a fibre in which the threshold $[Ca^{2+}]$ was decreased 6-fold from 3–0.5 μM Ca^{2+} when 0.5 μM CaM was present. Table 1 shows that 200-fold increase in [CaM] decreased the $[Ca^{2+}]$ required for threshold tension >80-fold. The same phenomenon was observed for $[Ca^{2+}]$ required for maximum tension, and fig.2 shows this shift to the left in the relationship between $[Ca^{2+}]$ and tension development. Saturation to CaM occurred at ~7 μM. These curves were plotted from the cumulative tension responses to increasing $[Ca^{2+}]$ as shown in fig.2a (see section 2).

The catalytic subunit of cAMP-dependent protein kinase inhibited tension responses to threshold $[Ca^{2+}]$ in the absence of CaM or when it was present in low concentrations, but at high $[Ca^{2+}]$ or [CaM] no inhibition occurred. Fig.2c shows the shift to the right of the lower region of the curve relating $[Ca^{2+}]$ and force development in the presence of 8 nM CaM. The threshold $[Ca^{2+}]$ for initiation of tension was shifted from 0.8–1.8 μM Ca^{2+} and the half-maximum activation with Ca^{2+} was achieved with 3 μM Ca^{2+} before and with 4 μM Ca^{2+} after preincubation with catalytic subunit of 4 μg protein kinase/ml. Treatment with catalytic subunit caused an inhibition of contraction of 49% at 3 μM Ca^{2+}. In the fibres not exposed to CaM, catalytic

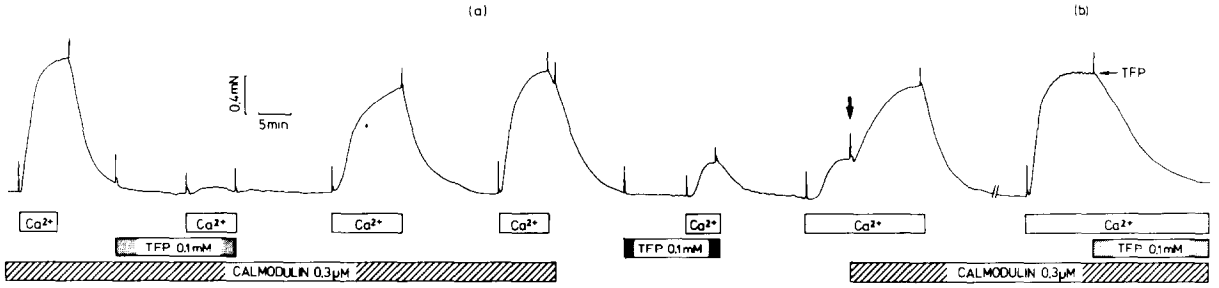

Fig.3. The effect of trifluoperazine (TFP) on contractions of skinned smooth muscle induced by 21 μM Ca^{2+}. (a) In the presence of CaM in the bathing solution the inhibition by 0.1 mM TFP was reversible. When CaM was absent the effect was not reversible. Addition of CaM (↓) reversed this inhibition (Ca^{2+} present). (b) TFP also relaxed the fibre contracted at constant $[Ca^{2+}]$. The fibre was stimulated with 21 μM Ca^{2+} and after tension had plateaued, TFP was added and relaxation ensued. In the absence of TFP the fibre remained fully contracted.

subunit (4 μg/ml) caused >90% inhibition of the tension response to threshold [Ca^{2+}]. Higher concentrations of the enzyme had no further effect. This inhibition could be rapidly overcome by addition of 0.25 μM CaM.

Trifluoperazine, an inhibitor of CaM [4], was very effective in inhibiting tension responses elicited by threshold and maximal [Ca^{2+}] at 0.01–0.1 mM TFP. In 6 fibres the average inhibition by 0.1 mM TFP was 87.5 ± 4%. Fig.3 shows that 0.1 mM TFP reversibly inhibits contractions elicited by 21 μM Ca^{2+} when 0.3 μM CaM was present. If absent, then inhibition by TFP became irreversible until CaM was replaced. Furthermore, the TFP relaxed the fibre contracted in the presence of 21 μM Ca^{2+} with 50% relaxation occurring in 9.2 ± 1.0 min [4]. This was not as rapid as the relaxation produced by reducing Ca^{2+} to $<10^{-8}$ M, when the fibre bundle relaxed 50% in 2.6 ± 0.2 min [1]. The above evidence argues strongly that CaM is an essential activator for Ca^{2+}-dependent contraction in smooth muscle. This activation may be mediated largely, but not necessarily entirely via phosphorylation of myosin light chains. Using ATPγS the light chains can be irreversibly phosphorylated [5,17], which causes the fibres to contract when ATP is added even at very low [Ca^{2+}] with no additional contraction occurring at maximal [Ca^{2+}] of 21 μM (fig.4). However, the addition of CaM produced, in the presence of 21 μM Ca^{2+} a further increase in force of 11% which could be reversed after washing out the CaM and restored again by replacing it. This finding may suggest the existence of additional phosphorylation-independent but CaM-dependent calcium regulation of activation. The extent of force development on addition of ATP was not increased by prolonging the thiophosphorylation up to 30 min in the presence of maximal [Ca^{2+}].

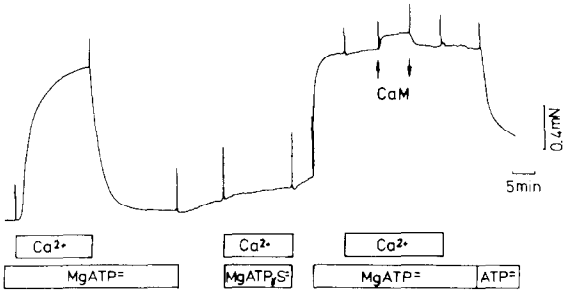

Fig.4. The effect of thiophosphorylation on the Ca^{2+}-dependence of skinned smooth muscle. After control isometric tension responses to 21 μM Ca^{2+} had been elicited, the fibre was exposed to ATP free relaxing solution for 5 min, when 2 mM ATPγS was used to replace the ATP and the free [Ca^{2+}] increased to 21 μM for 10 min. A further wash in ATP free relaxing solution for 2 min followed, then ATP was replaced. The fibre contracted very rapidly and addition of 21 μM Ca^{2+} had no additional effect. Calmodulin 2 μM (↑) produced a further 11% increase in the tension and this effect was reversible (↓) and could be repeated. The fibre could be relaxed by removing Mg^{2+}.

4. Discussion

These experiments show that CaM plays an essential role in the contraction of smooth muscle. The striking effect of added CaM in low concentrations in increasing Ca^{2+} sensitivity of the fibres and the speed of contraction suggests that some endogenous CaM is either lost from the fibre or inactivated during skinning treatment and storage. The rapidity with which added CaM acts suggests that it readily penetrates the skinned smooth muscle preparation. An inverse relationship was observed between [Ca^{2+}] and [CaM] required for threshold tension development. This is compatible with a Ca^{2+}/CaM complex required for activation of MLCK. The more of this Ca^{2+}/CaM complex, the more active MLCK is formed and therefore the phosphorylation of myosin light chain occurs faster as shown [18] using purified rabbit MLCK to phosphorylate MLC. As the [CaM] was increased the [Ca^{2+}] required for half-maximum activation of MLCK activity was decreased. The complete inhibition of tension development at maximal activating [Ca^{2+}] by TFP which binds CaM [4,18] strongly supports such a function. The complete reversibility of this inhibition after 15 min in relaxing solution ($Ca^{2+} < 10^{-8}$ M) further indicates that this intracellular CaM is exchangeable with the exogenous CaM in the bath. If absent, then the inhibition by TFP was irreversible until CaM was restored. Furthermore, the relaxation of the fibre contracted at maximum [Ca^{2+}] by TFP is again consistent with CaM regulation of MLCK. An inactivation by TFP binding would lead to a reduction in phosphorylation rate with net dephosphorylation of MLCK through phosphatase activity.

Further support for regulation of contraction by phosphorylation of myosin was obtained:

(i) by the action of catalytic subunit of cAMP-dependent protein kinase which inhibited tension responses at threshold [Ca^{2+}]. This is consistent

with the finding that phosphorylation of MLCK by the catalytic subunit reduces the activity of the enzyme [6]. Additional Ca^{2+} or CaM reversed this inhibition.

(ii) Thiophosphorylation with ATPγS produced near but not complete tension development which was independent of Ca^{2+}. This effect has now been shown to be due to irreversible phosphorylation of myosin in chemically-skinned rabbit ileum [17] and mechanically-disrupted chicken gizzard [7]. Nevertheless, the ability of CaM to produce a further small but consistent Ca^{2+}-dependent tension increase after thiophosphorylation (fig.4) suggests that CaM can act also via an additional pathway. In skinned smooth muscle, the possibility remains that the magnitude of this effect may be greatly diminished through loss of essential factors (cf. [19–21]) during skinning and preparation of the smooth muscle bundles.

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References

[1] Dabrowska, R., Sherry, J. M. F., Aromatorio, D. K. and Hartshorne, D. J. (1978) Biochemistry 17, 253–258.
[2] Sobieszek, A. (1977) Eur. J. Biochem. 73, 477–483.
[3] Mrwa, U., Troschka, M., Gross, C. and Katzinski, L. (1980) Eur. J. Biochem. 103, 415–419.
[4] Klee, C. B., Crouch, T. H. and Richman, P. G. (1980) Annu. Rev. Biochem. 49, 489–515.
[5] Sherry, J. M. F., Gorecka, A., Aksoy, M. O., Dabrowska, R. and Hartshorne, D. J. (1978) Biochemistry 17, 4411–4418.
[6] Adelstein, R., Conti, M. A. and Hathaway, D. R. (1978) J. Biol. Chem. 253, 8347–8350.
[7] Hoar, P. E., Kerrick, W. G. L. and Cassidy, P. S. (1979) Science 204, 503–506.
[8] de Lanerolle, P. and Stull, J. T. (1980) J. Biol. Chem. 255, 9993–10000.
[9] Barron, J. T., Bárány, U., Bárány, K. and Storti, R. V. (1980) J. Biol. Chem. 255, 6238–6244.
[10] Filo, R. B., Bohr, D. F. and Rüegg, J. C. (1965) Science 147, 1581–1583.
[11] Gordon, A. R. (1978) Proc. Natl. Acad. Sci. USA 75, 3527–3530.
[12] Peterson, J. W. (1980) Biochem. Biophys. Res. Commun. 95, 1846–1853.
[13] Jewell, B. R. and Rüegg, J. C. (1966) Proc. Roy. Soc. B 164, 428.
[14] Griffiths, P. J., Güth, K., Kuhn, H. J. and Rüegg, J. C. (1980) Pflügers Arch. 387, 167–173.
[15] Wolf, H. and Hofmann, F. (1980) Proc. Natl. Acad. Sci. USA 77, 5852–5855.
[16] Gagelmann, U., Pyerin, W., Kübler, D. and Kinzler, V. (1979) Anal. Biochem. 93, 52–59.
[17] Cassidy, P., Hoar, P. E. and Kerrick, W. G. L. (1979) J. Biol. Chem. 254, 11148–11153.
[18] Stull, J. T. (1980) Adv. Cyclic Nucl. Res. 13, 39–93.
[19] Mikawa, T., Nonomuwa, Y. and Ebashi, S. (1977) J. Biochem. 82, 1789–1791.
[20] Walters, M. and Marston, S. B. (1981) 9th Eur. Congr. Muscle and Motility, Salzburg, Austria, in: J. Musc. Res. Cell Motil. in press.
[21] Persechini, A., Mrwa, U. and Hartshorne, D. J. (1981) submitted.