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Modulation of calcium sensitivity in guinea pig taenia coli: skinned fiber studies

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1. Introduction

It is now widely accepted that – as in the case of skeletal muscle – smooth muscle excitation evokes contraction via an increase in intracellular free calcium. Thus following stimulation of smooth muscle, the myoplasmic calcium-levels rise[30, 33] and the force of 'isolated contractile structures' can be shown to be calcium-dependent, in much the same way as that of demembranated skeletal muscle fibers[11]. For these reasons it is generally held that the intracellular free calcium ion concentration is the major determinant of contractile activity in smooth muscle and part of the final common pathway leading to smooth muscle contraction. In recent years, however, it has become increasingly clear that the relationship between calcium ion concentration and contraction can no longer be regarded as unique; rather it can be varied over a wide range[30].

The conditions which alter the calcium sensitivity, i.e. the relation between contraction and myoplasmic calcium ion concentration may be studied in smooth muscle fibers which have been made hyperpermeable by membrane skinning. In skinned fibers, the contractile structures within the cell can be exposed to and equilibrated with a given calcium ion concentration which has been stabilized with the calcium buffer Calcium-EGTA which at neutral pH, has a stability constant of about $10^{6\ 50}$. Since the skinned fibers rapidly lose soluble substances, the bathing medium must be complemented with ATP as an energy source, magnesium ions and a pH-buffer. Typically, the responsiveness of these preparations depends also on the type of skinning used. With saponin for instance, internal membrane structures are left intact, so that the preparation may also be used to study the release of calcium from internal stores[10, 19]. Skinning procedures involving the treatment with the detergent triton X-100 on the other hand[14, 49] destroy internal membrane structures so that the sarcoplasmic reticulum and other calcium stores are no longer functional. Therefore, calcium uptake and release processes no longer interfere with the effect of added calcium on the contractile structures. Using these preparations it could be shown that calmodulin increases calcium sensitivity while calmodulin antagonists decrease it. A similar decrease in sensitivity is also brought about by addition of exogenous myosin phosphatase and by cyclic nucleotides.

2. Calmodulin influences calcium sensitivity

Increasing the calcium ion concentration produces a graded activation of skinned guinea pig taenia coli, leading to an increase in both the rate and extent of isometric tension development[47]. In addition the unloaded shortening velocity is also increased after raising the free calcium, but its calcium dependency is different from that of force activation[37] but resembles that of ATPase activation[2]. Thus, force development requires less calcium for half maximum activation and saturates at lower free calcium levels, than unloaded shortening velocity. With time, the calcium responsiveness decreases, but it can be restored by the addition of exogenous calmodulin[47]. Increasing the calmodulin concentration further reduces the calcium ion concentration required for 50% activation (EC_{50}) thus rendering the preparation more calcium sensitive. Such calcium sensitizing effects could be observed not only in the case of taenia coli, but also in skinned fibers prepared from chicken gizzard, guinea pig tracheal smooth muscle[48], rabbit ileum[8], coronary smooth muscle[39], carotid arteries[40] and rat tail arteries from normotensive and hypertensive animals. Since the calcium responsiveness depends critically on the free calmodulin concentration, saturating at about 5 μM calmodulin it is important to know the level of smoooth muscle calmodulin available for the initiation of contraction. The total calmodulin concentration of the smooth muscle cell is rather high, of the order of 50 μM, if it were dissolved in the cell water (chicken gizzard) or 30 μM (uterus or guinea pig taenia coli[15]. A large fraction of this calmodulin is, however, bound to membranes or calmodulin binding proteins. We attempted to determine the free calmodulin responsible for contraction, using the skinned fibers approach[41]. Guinea pig taenia coli fibers which are made hyperpermeable by freeze-drying and which are then briefly (for 1.5 min) rehydrated in an ATP containing relaxing solution are quite calcium sensitive, since they contain most (90%) of the endogenous calmodulin. Further extraction with relaxing solution for another 10–30 min, however, drastically reduces calcium sensitivity, since under these conditions a larger portion of calmodulin becomes extracted. However, equilibration with a solution containing 0.5-1 μM calmodulin restored responsiveness, suggesting that the calmodulin concentration within the skinned fibers responsible for calcium activation must have been initially of the order of 0.5–1 μM.

However, we should consider that these skinned fibers contain about 4 nmoles/ml less calmodulin than fresh taenia coli, since – as already mentioned – about 10% of the calmodulin may have diffused away during the brief period required for rehydration of the freeze-dried fibers. Therefore, the calmodulin concentration responsible for smooth muscle contraction may be as high as about 4–5 μM, which is still a small fraction of the total smooth muscle calmodulin. As a very rough estimate one may argue that only about a tenth of the smooth muscle calmodulin is responsible for smooth muscle contraction and therefore determining its calcium sensitivity and its responsiveness to c-AMP and c-GMP (cf. section 3). The

remainder is possibly involved in the activation of other enzymic processes and may act as a calcium sink, thus presumably binding most of the calcium (150–200 nmoles/ml cell water) which is known to be released from the sarcoplasmic reticulum during activation[5].

Since calmodulin is required for physiological calcium responsiveness we would expect that contraction of skinned smooth muscle is inhibited by calmodulin antagonists. Suitable inhibitors are trifluoperazine[47] and W 7 as well as the calcium antagonists fendiline[39] and prenylamine[28]. The latter antagonists have been shown to bind to calmodulin in micromolar concentration and to inhibit the activation by calmodulin of myosin light chain kinase[20]. In conjunction, all these studies suggest that it is the concentration of the calcium calmodulin complex rather than the free calcium ion concentration alone which determines contraction.

The mode of calcium activation may then be visualized as shown in figure 1. After being occupied by four calcium ions per molecule[23] calmodulin activates the myosin light chain kinase which phosphorylates the 20 KD myosin light chain. The unphosphorylated light chain inhibits actin myosin interaction while the phosphorylated species allows myosin to react with actin and to form contractile linkages, thus activating the actomyosin ATPase and contraction. It is possible that this activation is associated with a conformational change of myosin from a kinked into an elongated species[18]. According to this scheme the degree of phosphorylation and hence the extent of actomyosin activation would depend on the balance of activities of myosin light chain kinase and myosin phosphatase[16]. Thus, an increase in the amount or activity of phosphatase within the fibers inhibits contraction[4], and factors which inhibit kinase ought to inhibit contraction and promote relaxation as well. One such factor might be identified with c-AMP dependent protein kinase, which according to Adelstein and Hathaway[1] phosphorylates the myosin light chain kinase whereby inhibiting it.

3. *c-AMP-induced relaxation*

According to Adelstein and Hathaway[1] c-AMP-dependent protein kinase may inhibit smooth muscle contraction by phosphorylating and inhibiting the myosin light chain kinase. This – they suggested – may be the mechanism of β-adrenergic relaxation. Though controversial, it is generally held that relaxation induced by β-agonists is mediated via an increase in the level of cyclic AMP within the smooth muscle tissue[24]. c-AMP is known to activate the c-AMP dependent protein kinase by releasing the catalytic subunit from the enzyme moiety[35]. Following the administration of β-agonists activity levels of c-AMP dependent protein kinase are increased[45]. The protein targets of the phosphorylating kinase are probably manifold and may involve – as mentioned already – the myosin light chain kinase but additionally also proteins associated with calcium transport. For instance, calcium uptake into the sarcoplasmic reticulum[32], active calcium extrusion through the cell membrane[6] or calcium sodium exchange[43] may be enhanced while calcium influx through calcium channels may be inhibited[25]. These effects on calcium transport mechanisms would all result in a reduced intracellular calcium ion concentration and would hence be expected to cause relaxation.

Smooth muscle fibers from which the outer cell membranes had been removed by skinning procedures provide an excellent model to study the role of the sarcoplasmic reticulum and of myosin light chain phosphorylation in c-AMP mediated relaxation. In saponin skinned fibers, the internal membrane systems and the contractile machinery are left largely intact. In these fibers c-AMP-dependent protein kinase causes an increased calcium uptake into the sarcoplasmic reticulum or other calcium stores. The increase in the stored calcium is indicated by the observation that after c-AMP treatment, caffeine induces the release of more calcium and causes a larger contractile response than in the case where c-AMP was not present[19].

Evidence for an effect of c-AMP-dependent protein kinase on the actomyosin contractile system has also been obtained. Thus supercontraction and Mg-dependent Ca-activated ATPase activity of smooth muscle actomyosin is depressed by c-AMP-dependent protein kinase[31,44]. This inhibition is coupled with a phosphorylation and inhibition of myosin light chain kinase and associated with a partial dephosphorylation of myosin[44]. The acto-

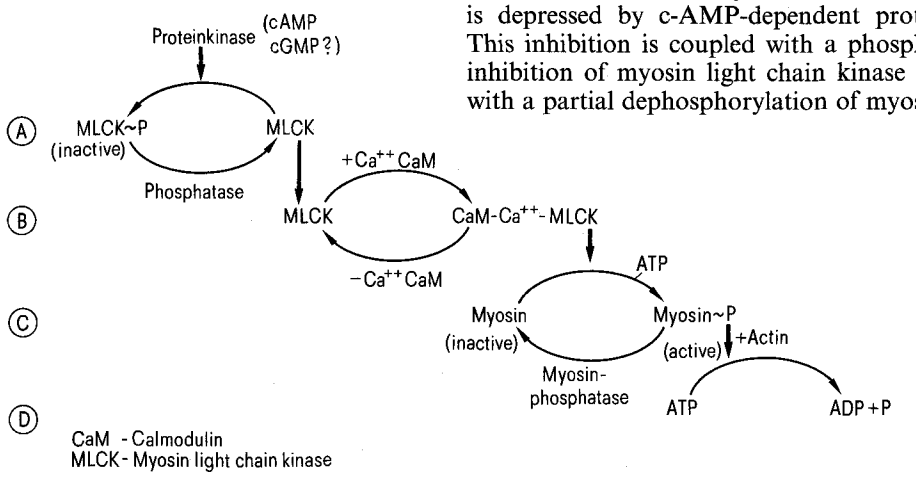

Figure 1. Activation of smooth muscle via phosphorylation of myosin by myosin light chain kinase. *A* Myosin light chain kinase can exist in a phosphorylated and a non-phosphorylated form. *B* Only non-phosphorylated myosin light chain kinase (MLCK) forms an active ternary complex with calcium and CaM (calmodulin), which catalyzes (C) the conversion of non-phosphorylated to phosphorylated myosin. *D* Phosphorylated myosin is activated by actin and catalyzes the hydrolysis of ATP to ADP and P_i thus providing the energy for contraction.

myosin contractile system can also be studied in situ in intact structured fibers from which the cell membrane had been removed by skinning with triton X-100. Using this experimental model it could be shown that the contractile system of various smooth muscles including chicken gizzard[22], guinea pig taenia coli[42], coronary arteries[39] and hog carotid arteries[40] become less calcium sensitive following exposure to c-AMP or c-AMP -dependent protein kinase. Thus, force development was inhibited in fibers which were half maximally activated with calcium, but not in those which were maximally activated, so that the force-pCa relationship was shifted to the right. c-AMP induced relaxation of skinned fibers was much more effective at low concentration of calcium and calmodulin than at high concentrations[26]. For under these conditions c-AMP-dependent protein kinase phosphorylates the myosin light chain kinase only at one site and does, therefore, not inhibit it[9] whereas at low calcium ion concentration two sites becomes phosphorylated.

The objection has been raised that these 'direct' effects of c-AMP and c-AMP-dependent protein kinase are usually (but cf.[26]) only observed at high concentrations and under rather special conditions. By improved methods however, it can be shown that c-AMP may induce relaxation of skinned fibers at micromolar concentrations[26]. It remains to be shown that c-AMP-induced relaxation of skinned fibers is associated with dephosphorylation of light chains and phosphorylation of myosin light chain kinase. We have addressed these problems as follows:

Skinned guinea pig taenia coli fibers were incubated in a relaxing solution at low calcium ion concentration and were made to contract by raising the free calcium to 10^{-5} M. After lowering the calcium ion concentration to 1.3 μM the fiber relaxed partially, reaching a plateau-tension after 30 min. Addition of c-AMP (2 μM) increased the rate and extent of relaxation (fig. 2).

Table 1 compares the relaxing effects induced by 2 μM c-AMP at different calcium ion concentrations. As already shown by Meisheri and Rüegg[26] these c-AMP effects are more pronounced when the calcium ion concentration is reduced and they can be mimicked by the addition of the catalytic subunit of c-AMP dependent protein kinase.

To find out whether the relaxing effects of c-AMP are associated with a decreased phosphorylation of myosin light chains the skinned fibers were fixed with ice-cold TCA during various stages of c-AMP-induced relaxation and during control relaxations in the absence of c-AMP, in order to determine the relative proportions of phosphorylated and non-phosphorylated light chains according to Gagelmann et al.[13]. Fibers were redissolved using urea and SDS and then subjected to two dimensional gel electrophoresis. The bands corresponding to the light chains and its satellites were stained with coomassie blue and the staining intensity determined by densitometry. Consistently, an increase in calcium ion concentration did not only increase the light chain phosphorylation but also caused the appearance of phosphorylated light chain satellites[12]. Phosphorylation was reduced to 41 ± 4% after reducing the calcium ion concentration to 1·3 μM and it was further reduced to 27 ± 5% 45 min after addition of c-AMP which also reduces tension from 71% to 23% of the maximal isometric force (fig. 3). No reduction in phosphorylation and force was observed in c-AMP-free control fibers during this period of time.

The reduction in myosin phosphorylation is probably due to a reduction in the activity ratio of myosin light chain kinase and myosin phosphatase, resulting from a decomposition of the enzymatically active ternary complex of myosin light chain kinase with calcium and cal-

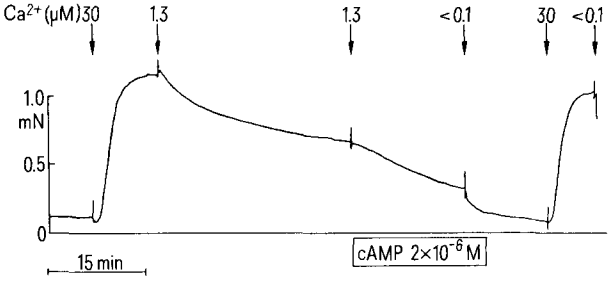

Figure 2. c-AMP promotes relaxation of skinned guinea pig taenia coli. Isometric contraction is induced by 30 μM Ca^{++} followed by partial relaxation when Ca^{++} is lowered to 1.3 μM. Addition of c-AMP (2 μM) causes relaxation which is further enhanced after lowering Ca^{++} to 0.1 μM. After raising Ca^{++} to 30 μM the fiber redevelops the same force as before and relaxes completely (not shown) after immersing into Ca-free relaxing solution containing: imidazole, 20 mM, EGTA, 4 mM, $MgCl_2$, 10 mM, ATP, 7.5 mM, NaN_3, 1 mM, creatinephosphate, 10 mM, DTE, 2 mM, creatinephosphokinase, 152 U/ml, phosphate, 6 mM, calmodulin 0.05 μM, 10^{-4} M IBMX, pH 6.7 (T = 20 °C).
In contraction-solution Ca^{++} is adjusted by CaEGTA buffers, assuming an apparent Ca dissociation constant of 1.3 μM.

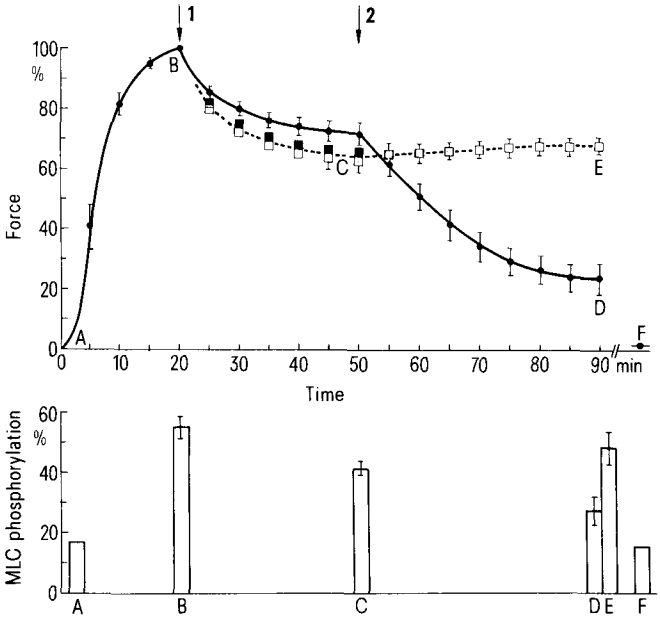

Figure 3. c-AMP induces relaxation (upper panel) coupled with dephosphorylation of 20 KD myosin light chain (lower panel). At (A) fibers suspended in relaxing solution (cf. fig. 2): Myosin light chains are slightly phosphorylated (17%). Fibers contracted with 30 μM Ca^{++} (B). After lowering Ca^{++} to 1.3 μM (at 1) they relaxed partially and more completely after addition of c-AMP (10^{-4}M), applied at 2 —●—.
Control fibers (curves —■— and —□—) relaxing after lowering Ca^{++} to 1.3 μM in absence of c-AMP. Note that force eventually reaches a plateau (E), with myosin light chains in fibers phosphorylated to 63 ± 9%. (F), Fibers in relaxing solution.
A, B, C, D, E: timepoints at which fibers were fixed with trichloroacetic acid to determine phosphorylation of MLC.

modulin, when the kinase becomes phosphorylated at two sites (fig. 4a).

A decrease in myosin light chain phosphorylation was also observed in intact smooth muscle during β-adrenergic relaxation[46] and myosin light chain kinase was found to be inhibited[36]. These findings, however, remained controversial[17,29]. It is possible, however, that the effect of c-AMP on the activity of myosin light chain kinase is comparatively small, so that the activity change cannot readily be detected. Let us consider below how a comparatively small activity change might be amplified to induce a large change in force. A possible mechanism is futile substrate cycling (fig. 4b).

4. The futile cycle of myosin phosphorylation and dephosphorylation

Suppose that before activation of c-AMP-dependent protein kinase both force and myosin phosphorylation are in a steady state resulting from a balance of phosphorylating and dephosphorylating processes. These form part of a futile substrate cycle as defined by Newsholme[34] causing a net ATP hydrolysis without any change in the reactants. For example, in the case of a small reduction, say by 10% of the myosin light chain kinase activity, only 90% of the phosphatase activity would be required to balance the phosphorylating activity of myosin light chain kinase while 10% would be free to cause a net dephosphorylation of myosin. This might be followed by a conformational change[18], crossbridge detachment and relaxation. Clearly, the rate of net dephosphorylation and relaxation would, under these circumstances, depend on the cycling rate of the futile cycle. If there was an initially high but balanced activity of myosin light chain kinase and phosphatase activity, a change by 10% might cause a dramatic effect. An increase of c-AMP concentration and hence a greater inhibition of myosin light chain kinase would further accelerate relaxation. Conversely, a small increase in myosin light chain kinase activity by increasing the calmodulin activity might lead to a fast increase in phosphorylation and force, if the activity of the futile cycle is high. If it were low the effect would be negligible. In conclusion then, the kinase-phosphatase futile cycle may be instrumental in increasing the responsiveness of the contractile system to c-AMP or small changes in the concentration of calmodulin or calcium (incidentally, without the need of having at the same time a low calcium treshold). A futile cycle involving the continued splitting of ATP (pseudoATPase) is a high price for maintaining the continued responsiveness of smooth muscle. During prolonged contraction, however, myosin phosphorylation may be reduced to a low resting level[3], a process which presumably implicates an inhibition of continued phosphorylation dephosphorylation processes. This reduction may – in conjunction with the observed reduction in crossbridge cycling rate – be one of the means by which the energy costs for maintaining tension may be reduced during prolonged tonic tension maintenance[7]. Additionally we should take into account however that the rate of relaxation following a tonic contraction may also be very low. This would allow tension support with very little energy expenditure as short periods of activity might be followed by long periods of slow relaxation (catch or latch state) in a repetitive sequence.

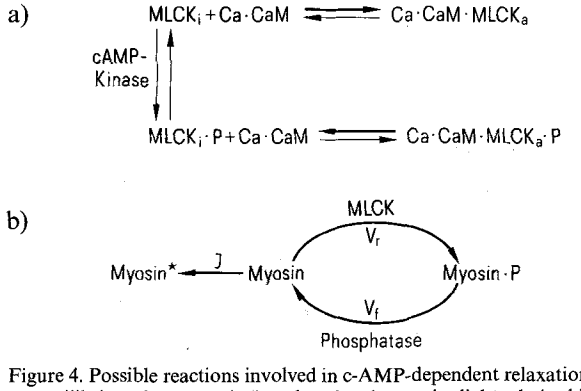

Figure 4. Possible reactions involved in c-AMP-dependent relaxation: *a* Equilibrium between dephosphorylated myosin light chain kinase (MLCK), MLCK phosphorylated at two sites and ternary complexes (Ca-CaM-MLCKa) of 4 Ca^{++}, MLCK and calmodulin (CaM) in presence and absence of c-AMP-dependent protein kinase. The non-phosphorylated ternary complex can be phosphorylated at one site only (not shown). Addition of c-AMP-dependent protein kinase would cause the phosphorylation of free MLCK which, in turn, would favor the dissociation of the enzymically active ternary complex of MLCK. At the same time, rather little phosphorylated ternary complex would be formed since the affinity of phosphorylated MLCK for calmodulin is much reduced leading to a partial inhibition of myosin light chain kinase activity. *b* Futile substrate cycle of myosin phosphorylation (V_r) and dephosphorylation (V_f) catalyzed by MLCK and myosin phosphatase. J: net dephosphorylation, resulting in a conformational change to myosin*. Further explanation see text.

Contractile force (%) Conditions	Control	with c-AMP
1.7 μM Ca^{++}	73.3 (2)	47.9 (2)
1.3 μM Ca^{++}	55.8 (1)	24.6 (1)
1.1 μM Ca^{++}	41.8 ± 5.1 (4)	14.5 ± 3.5 (4)

Effect of c-AMP (2 μM) on contractile force at different [Ca^{++}]. Values (in % of maximal contraction at 30 μM Ca^{++}): $\bar{x}$ ± SE with number of fibers tested in ( ). Solutions as in figure legend 2.

5. Conclusion

In summary then, skinned fiber experiments have provided evidence showing that the relationship between force development and free calcium ion concentration may be variable. The cyclic-nucleotides c-GMP (cf. 38) and c-AMP in particular might be important in modulating calcium sensitivity of skinned fibers possibly via an alteration of myosin phosphorylation. Additionally, the coupling between myosin phosphorylation and the ATPase activity of actomyosin or tension generation[27] may itself be modulated in actomyosin contractile systems. The recognition of the physiological relevance of these modulating mechanisms however must await experiments in which the relationship between force development, free calcium ion concentration and myosin phosphorylation is studied in intact fibers. With the advent of more sophisticated calcium measuring techniques, such information is now available[30].

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