Pflügers Arch – Eur J Physiol (1997) 434:534–542

ORIGINAL ARTICLE

Kerstin Albrecht · Alexandra Schneider
Claudia Liebetrau · J. Caspar Rüegg
Gabriele Pfitzer

Exogenous caldesmon promotes relaxation of guinea-pig skinned taenia coli smooth muscles: inhibition of cooperative reattachment of latch bridges?

Received: 25 February 1997 / Received after revision: 29 May 1997 / Accepted: 2 June 1997

Abstract In smooth muscle, the state of prolonged contraction (latch state) is associated with very slow energy turnover and cycling of crossbridges that are dephosphorylated. A similar state may be reproduced in skinned fibres when the calcium-induced contraction is terminated by calcium removal with ethylenebis(oxonitrilo)tetraacetate (EGTA) and, during the slow relaxation that follows, force is maintained by dephosphorylated crossbridges that cycle slowly or not at all and may cooperatively reattach after detachment (Khromov et al. 1995, Biophys J 69:2611–2622). In guinea-pig skinned taenia coli that has been pretreated by prolonged incubation with caldesmon (5 μM), the rate of relaxation is approximately 1.6 times greater than in untreated controls, with half-times of relaxation being 1.3 and 2.1 min, respectively. In contrast, preloading the fibres with calponin does not accelerate relaxation. Preloading the fibres with caldesmon also accelerates the relaxation of skinned fibres from the state of rigor contraction when the latter is terminated by immersion into an ATP-containing relaxing solution or, in the presence of inorganic phosphate (Pi), also by flash-photolytic release of ATP from caged-ATP. Even in the latter case, relaxation is comparatively slow, possibly because of cooperative reattachment of dephosphorylated crossbridges which delays *net* crossbridge detachment and hence relaxation. We propose that by inhibition of cooperative reattachment caldesmon accelerates relaxation, even in the presence of Pi, and that the latch-like state of skinned fibres is supported by dephosphorylated cooperatively attaching crossbridges and may be regulated by the activity of caldesmon in the smooth muscle cell.

K. Albrecht · A. Schneider · C. Liebetrau · J.C. Rüegg (✉)
II. Physiologisches Institut, Universität Heidelberg,
Im Neuenheimer Feld 326, D-69120 Heidelberg, Germany

G. Pfitzer
Institut für Vegetative Physiologie, Universität Köln,
Robert-Koch-Strasse. 39, D-50931 Cologne, Germany

Key words Skinned smooth muscle · Caldesmon · Calponin · Latch crossbridges · Caged-ATP

Introduction

In smooth muscle, contraction is initiated by the activation of the calcium-calmodulin myosin light chain kinase complex that phosphorylates the regulatory light chains, while dephosphorylation causes relaxation (see [37] for review). If, however, dephosphorylation occurs while crossbridges are attached, smooth muscles fail to relax quickly, and instead a remaining contraction (catch-like state or latch state) results, in which force is maintained by dephosphorylated, slowly cycling or non-cycling crossbridges (as explained in Fig. 1A; [7]). Active state, as determined by the quick release method, is low, as in the catch state of lamellibranch smooth muscle [30, 36]. Originally, it was suggested that latch bridges may only form from dephosphorylation of attached crossbridges (Fig. 1A, pathway 3; see [12]). However, more recent schemes also consider latch bridge formation by cooperative reattachment of dephosphorylated detached bridges (reaction 5; see also [29, 38, 44]). Since the latter are slowly detaching, the latch bridge cycle is also very slow.

In the latch state, myoplasmic free calcium levels, the extent of myosin light chain phosphorylation and the rate of oxygen consumption are low (see [27] for review). As in the catch state of molluscan muscle [35], crossbridges may be in a "locked" actomyosin-ADP (AM-ADP) state, as suggested by Rüegg [31] and by Somlyo and colleagues [9, 25]. However, this catch-like state may be terminated by withdrawal of stimulation, as in the case of the smooth muscle from chicken gizzard [8], or by interventions that cause an increase in cAMP levels [16], which presumably result in an increased rate of *net* crossbridge detachment. This occurs if the crossbridge detachment rate is increased and exceeds that of crossbridge reattachment or if reattachment is inhibited. As ADP has a high affinity for smooth muscle crossbridges

[9, 25], crossbridge detachment is said to be rate-limited by the rate of dissociation of the AM-ADP complex (see Fig. 1B). Thus, in the latch state the crossbridge detachment rate of smooth muscle may be low because of the slow dissociation of ADP from AM and it may therefore be increased by interventions that increase the rate of ADP dissociation or that lower the ADP concentration in the myoplasm [9, 18]. However, *net* detachment of latch bridges and hence relaxation may also be accelerated by inhibiting cooperative reattachment of dephosphorylated bridges (via pathway 5, see Fig. 1A). It has long been suggested that cooperative crossbridge attachment might occur in smooth muscle [2, 5, 29, 38] and might be inhibited by caldesmon [13]. Here we tested this hypothesis by investigating the effect of caldesmon on relaxation of skinned, i.e. permeabilized, smooth muscle.

A latch-like state may be reproduced in skinned fibres (see [45]), in particular if a calcium-induced contraction is terminated by the removal of calcium with ethylenebis(oxonitrilo)tetraacetate (EGTA), e.g. in skinned fibres of guinea-pig taenia coli [20] and rabbit femoral artery [18]. Here, relaxation induced by lowering the free calcium concentration may be much slower than dephosphorylation of light chains. Force is then maintained by dephosphorylated latch bridges in a passive manner. Thus, ATPase activity is inhibited and there is no recovery after quick release [20] and crossbridge detachment may be very slow [10]. Crossbridges are probably in a strongly bound AM-ADP state [18]. According to Khromov et al. [18], the low rate of tension decay may then be accounted for by cooperative reattachment of crossbridges, but also by the low rate of ADP dissociation from the crossbridges, as ADP prolongs the lifetime of dephosphorylated bridges. Thus, relaxation is accelerated by lowering the concentration of MgADP in the relaxing solution [18]. Yet relaxation may also be greatly accelerated by the addition of inorganic phosphate (Pi) [10, 34]. This is because Pi reverses some steps of the crossbridge cycle (in particular reaction 5 in Fig. 1B) thereby forming AM-ADP-Pi which dissociates into myosin-ADP-Pi and actin by a process of "reverse attachment" (reaction 4 in Fig. 1B). By this pathway the lifetime of dephosphorylated crossbridges that are attached in the AM-ADP state is greatly abbreviated and hence the rate of relaxation is enhanced. However, reverse attachment and hence relaxation is counteracted by the process of reattachment of crossbridges that are in the M-ADP-Pi state and interact with actin to form AM-ADP-Pi. One would expect, therefore, that interventions that inhibit the cooperative attachment of crossbridges would increase the rate of relaxation. Inhibition of cooperative attachment of crossbridges could account for the enhanced relaxation rate at basal levels of myosin light chain phosphorylation observed in the intact chicken gizzard fibre bundles after termination of stimulation [8]. This suggests that the rate of relaxation is regulated independently of dephosphorylation of myosin light chains, the potential regulatory proteins being caldesmon and calponin. It had been shown by Horiuchi and Chacko [13] that caldesmon, unlike calponin, inhibits cooperative tropomyosin-dependent attachment of crossbridges and the activation of AM ATPase, and also crossbridge attachment in skinned skeletal muscle [4, 41]. Moreover, a peptide derived from the actin-binding region of caldesmon induced contraction in permeabilized smooth muscle at very low, constant concentrations of calcium, implying that endogenous caldesmon inhibits force production in resting smooth muscle [17]. Here we show that caldesmon, rather than calponin, will accelerate net crossbridge detachment and hence the decay of tension during relaxation of skinned smooth muscle preparations, presumably by inhibiting cooperative attachment of dephosphorylated crossbridges.

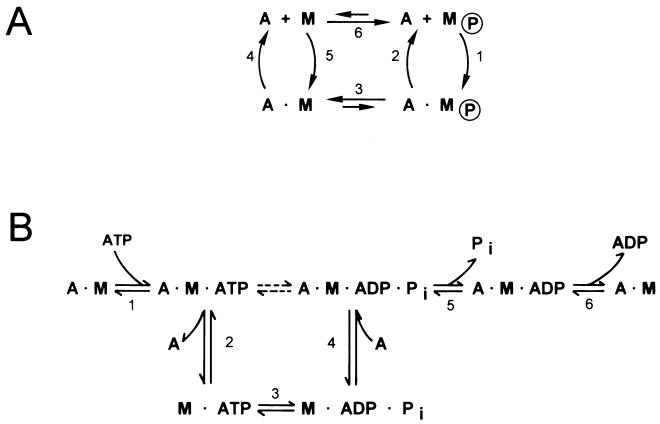

Fig. 1 **A** Schematic representation of a four-state crossbridge model (see [29]). Formation of contractile linkages (AM_P) by attachment of phosphorylated crossbridges (M_P) to actin (A) via reaction 1. Latch bridges (AM) formed by dephosphorylation of phosphorylated crossbridges (AM_P) via reaction 3 or by cooperative reattachment (reaction 5) of dephosphorylated myosin crossbridges (M). Latch bridges detach slowly (reaction 4), so that the latch bridges cycle at a slow rate. Contractile linkages (AM_P) may be formed from dephosphorylated crossbridges via reaction 6 and 1 or by reversal of reaction 3. Relaxation of smooth muscle in latch probably depends on the rate of *net* crossbridge detachment depending on reactions 4 and 5. [M_P Phosphorylated myosin crossbridges]. **B** Schematic scheme of actomyosin ATPase following photolytic release of ATP (see [2]). (A Actin, M myosin, $AM \cdot ADP \cdot Pi$ actomyosin with bound ADP and phosphate). Upon flash photolysis of caged-ATP, released ATP dissociates rigor crossbridges (AM), i.e. it detaches myosin crossbridges from actin. Myosin reattaches (via reaction 4) to form weakly binding, then strongly binding crossbridges (via reactins 5 and 6). Caldesmon may inhibit reaction 4 (see text)

Materials and methods

Guinea-pig chemically skinned taenia coli and rabbit femoral artery preparations were prepared essentially as described by Sparrow et al. [39]. Fibre strips were dissected and incubated for 20 min in a solution containing (in mM): EGTA 5, KCl 50, sucrose 150, dithioerythritol (DTE) 2 and imidazole 20 (pH 7.4). Subsequently, they were immersed for 4 h at 4°C in the same solution containing in addition 1% (v/v) Triton-X-100. Thereafter, Triton X-100 was washed out and the fibres were stored at –20°C in 50% glycerol and 50% of a solution containing (in mM): EGTA 4, $MgCl_2$ 10, Na_2ATP 7.5, NaN_3 1, DTE 2, imidazole 20 (pH 6.7). β-Escin-skinned taenia coli and chicken gizzard were prepared as

Table 1 Half-time of relaxation ($t_{1/2}$) elicited by EGTA jumps (see Materials and methods) terminating a calcium-induced isometric contraction. Triton-X-100-skinned fibres from taenia coli and ileum (guinea-pig), arteria femoralis (rabbit) and chicken gizzard

Tissue	$t_{1/2}$ (min)	*n*
Taenia coli	1.76±0.12	10
Arteria femoralis	1.93±0.19	8
Gizzard	0.72±0.04	8
Ileum	0.77±0.15	6

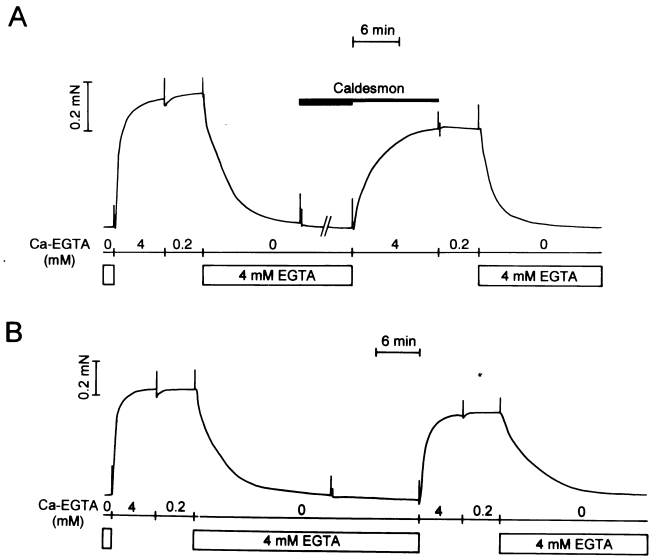

Fig. 2 A, B Effect of caldesmon on relaxation of Triton-X-100-skinned, guinea-pig taenia coli. **A** Isometric contraction elicited by calcium-EGTA (4 and 0.2 mM, respectively; pCa 4.3) followed by relaxation induced by immersion into relaxing solution containing 4 mM EGTA (EGTA jump). Thereafter, the preparation was incubated (for 30 min) in relaxing solution containing 5 μM caldesmon and a second contraction was elicited by immersion into contraction solution also containing 0.5 μM caldesmon. Relaxation was induced by reimmersion into (caldesmon-free) relaxing solution ("EGTA jump"). **B** Control experiment, in the absence of exogenous caldesmon, but using otherwise the same experimental procedure as in experiment **A**. Note the marked increase in the rate of relaxation induced by caldesmon

described elsewhere [32, 33]. Strips of 4–7 mm in length and of width and thickness each of approximately 0.1 mm were mounted horizontally for mechanical studies on an AME 801 force transducer (SensoNor, Horten, Norway) using a nitrocellulose-based glue; they were slightly stretched in relaxing solution until resting tension was just noticeable (0.01 mN).

The composition of the relaxing solution was (in mM): K^+ 21, Na^+ 36, $MgCl_2$ 10, EGTA 4, ATP 7.5, Na azide 1, phosphocreatine 10, creatine phosphokinase (CPK) 140 U/ml, calmodulin 2 μM, DTE 2, imidazole 20 (pH 6.7), 22–23°C. The contraction solution was identical except for the replacement of EGTA with Ca-EGTA, thereby increasing the free calcium concentration. Free calcium was calculated according to Andrews et al. [1] and ionic strength was adjusted to about 0.11 M with KCl.

During a calcium-induced contraction, rapid relaxation was induced by using an "EGTA jump" technique (see also [18]) to minimize diffusional delays: first the contraction solution was replaced by a low-EGTA contraction solution in which 4 mM Ca-EGTA was replaced by 0.2 mM Ca-EGTA. After about 5 min the fibres were then immersed in standard relaxing solution containing 4 mM EGTA. These experiments were also carried out using fibres that were preloaded with caldesmon [by incubation in caldesmon-containing (5 μM)-relaxing solution prior to contraction]. In the subsequent contraction, the exogenous caldesmon concentration was reduced (to 0.5 μM) and then in the relaxation that followed caldesmon was not present in the relaxing solution. Despite this, the relaxation is accelerated because the fibre has been preloaded with caldesmon. Preloading of fibres does increase the caldesmon content, as shown by Malmqvist et al. [22].

In another series of experiments, the isometrically contracted fibres were immersed in a rigor solution also containing 4 mM Ca-EGTA, hexokinase (100 U) and glucose (10 mM) to remove traces of ATP. After 10 min this solution was replaced by a standard rigor solution containing (in mM): $MgCl_2$ 3, EGTA 4, KCl 50, imidazole 25 (pH 6.7) for 30 min. In this solution force declined to about 30% of the initial force in the preceding contraction. In some experiments the rigor solution also contained caldesmon or calponin, which was kindly donated by Dr. J.M. Chalovich.

Relaxation from rigor was induced either by immersion into standard relaxing solution or by flash photolysis of caged-ATP [adenosine 5'-triphosphate, P^3-1-(2-nitrophenyl)ethyl ester, disodium salt, obtained from Calbiochem] which was added to the rigor solution as described in Arner et al. [2]. In these experiments, thin fibre bundles of skinned taenia coli were teased out and mounted horizontally between a fixed pin and the arm of an extended AME 801 (SensoNor) force transducer in an apparatus similar to that described previously [2]. The muscles were stretched in relaxing solution until the resting tension was just noticeable. The experiments were performed at 22°C. The fibres were induced to contract in contraction solution and then immersed into ATP-free rigor solution as described above. The fibres held in 1-ml baths in solution were transferred to a 10-μl trough, equipped with a quartz window, prior to photolysis. Photolysis was performed using rigor solutions which also contained 7.5 mM caged-ATP, 10 mM $MgCl_2$ and 10 mM DTE at pCa ($-\log_{10}$ [Ca]) 9.0 (for details see legend to Fig. 7 and Table 5). In some cases, this solution was pretreated for 30 min with 2.5 U/ml apyrase to remove traces of ADP. Subsequently, apyrase was removed by filtration through a Millipore filter and 3 min was allowed for the photolysis solution to diffuse into the muscle fibre bundle. Thereafter the preparation was illuminated with a strong ultraviolet light flash from a xenon flash lamp (G. Rapp Optoelektronik, Hamburg, Germany) through a UG11 filter. Photolysis causes the conversion of about 15% of the caged-ATP into ATP (see [2]) under the conditions used. The force transients after release of ATP were monitored on a digital oscilloscope Nicolet explorer 1090A, stored on a personal computer and printed using a pen recorder.

To determine myosin light chain phosphorylation, fibre strips were fixed in a rigor solution containing 15% trichloroacetic acid and 4% pyrophosphate precooled to 4°C. The strips were processed for determination of myosin light chain phosphorylation as described previously [33]. In brief, the strips were homogenized in urea buffer, and subjected to isoelectric focusing within a pH range of 4.5–5.4. Phosphorylated myosin light chains were resolved on a 15% polyacrylamide gel in two dimensions and quantitated densitometrically as described elsewhere [33]. Statistics: all values are given as means ± SEM.

Results

Contraction and relaxation may be induced in skinned taenia coli by rising and lowering the calcium concentration. The half-time of relaxation of guinea-pig skinned taenia coli was similar to that of rabbit femoral artery (Table 1; see also [18]), but much longer than that of phasic smooth muscle such as chicken gizzard and guinea-pig ileum. Pi (10 mM), which was included in the relaxing medium, accelerated relaxation (data not shown) as shown previously [10, 13]. Here we show (Fig. 2A)

Table 2 Effect of caldesmon and calponin on the rate of relaxation (EGTA jump). Values give relative half-times of relaxation ($t_{1/2}$ of second relaxation as a % of the $t_{1/2}$ of the first relaxation

Skinning agent	$t_{1/2}$ (%)		
	+ Control buffer	+ Caldesmon	+ Calponin
Triton-X-100	125.0±5.9	77.3±10.4	144.7±5.8
β-escin	127.5±14.75	129.6±8.72	–

Table 3 Relaxation induced by EGTA jump. Values indicate half-times of relaxation ($t_{1/2}$ in min±SEM; n = 5 experiments)

Preparation	1st relaxation ($t_{1/2}$ min)	2nd relaxation ($t_{1/2}$ min)
Triton-X-100	1.74+0.17	2.14+0.14 (control)
Triton-X-100	1.78+0.19	1.32+0.12 (+ caldesmon)
β-Escin-skinned	0.96+0.07	1.22+0.09 (control)
β-Escin-skinned	0.84+0.08	1.06+0.04 (+ caldesmon)

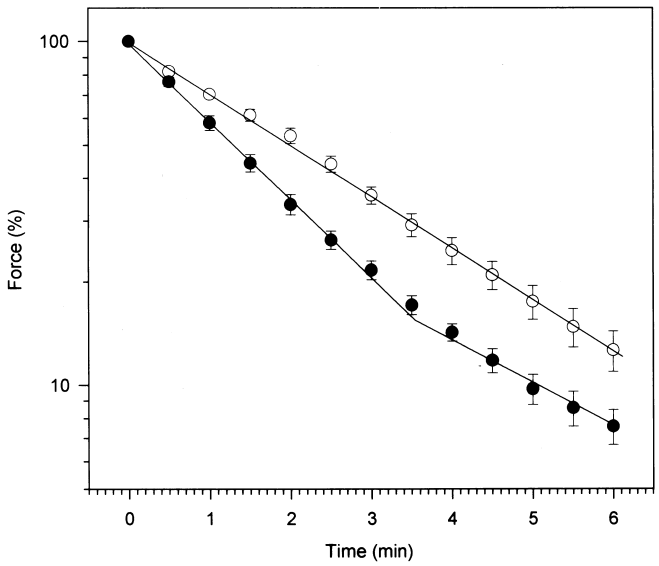

Fig. 3 Time course of force decay during relaxation induced by EGTA jump. In caldesmon-treated skinned fibres (*closed circles*) and in control fibres (*open circles*). Values are means ± SEM of n = 6 experiments. For details of experimental procedure see Fig. 2

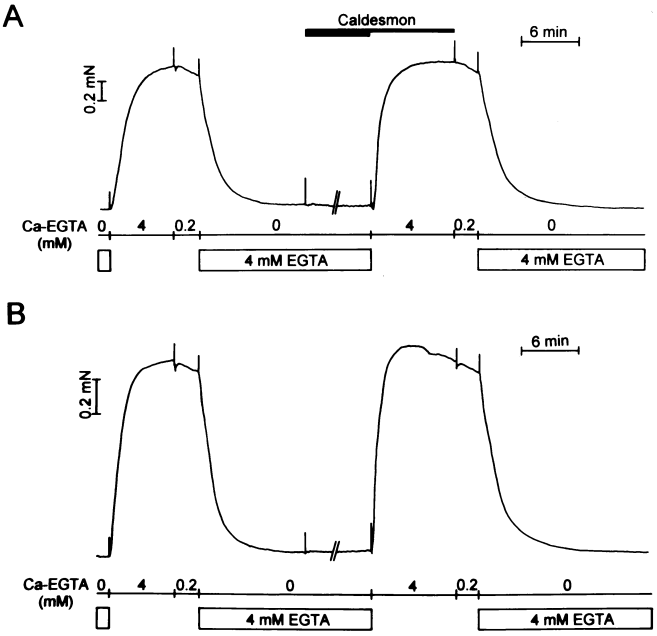

Fig. 4A, B Effect of caldesmon on contractile responses of guinea-pig taenia coli skinned with β-escin. **A** Contraction cycle before and after incubation in caldesmon. Experimental procedure as in Fig. 2. **B** Control experiment in the abscence of caldesmon. Note that even prolonged incubation in caldesmon-containing relaxing solution (for 30 min) did not affect contractile force and rate of relaxation

that relaxation was also much faster after loading the fibre with additional exogenous caldesmon prior to relaxation. In these experiments, a control contraction was elicited first by increasing the free calcium concentration. Thereafter, the fibre was relaxed by the EGTA jump technique and it was then incubated for at least 30 min in relaxing solution that also contained 5 μM caldesmon in order to load the fibre with this regulatory protein. Subsequently, a second contraction was elicited using a contraction solution also containing caldesmon and relaxation was induced by immersion of the caldesmon-loaded fibre into (caldesmon-free) relaxing solution (EGTA jumps). Note that the half-time of relaxation of the second contraction was much shorter than that of the first contraction/relaxation cycle prior to caldesmon loading (see Table 2). In contrast, in control experiments of the type shown in Fig. 2B, the relaxation of the second contraction was slower (by 25%, see Table 2) than that of the first. As shown in Fig. 3 and tabulated in Table 3, the rate of relaxation of the second contraction was about 1.6 times higher in caldesmon-treated than in untreated fibres, the half-times of relaxation being 1.32 ± 0.12 and 2.14 ± 0.14 min, respectively. However, when skinned fibres were loaded with calponin (5 μM) rather than caldesmon, the rate of relaxation was not enhanced (Table 2), whereas calcium-elicited force was inhibited by 50 ± 7% (n = 8).

Unlike Triton-X-100-skinned fibres, in guinea-pig taenia coli fibres that were permeabilized with β-escin caldesmon treatment had no effect on the rate of relaxation, as illustrated in Fig. 4 and documented in Table 2. Note that the second relaxation was always slower than the relaxation following the first contraction (by 25%), bothin the presence and absence of caldesmon (Table 2). Thus, half-times of relaxations were 0.96 ± 0.07 and 1.22 ± 0.09 min before and after treatment (for 30 min) with caldesmon. By comparison, relaxation half-times were 0.84 ± 0.08 and 1.06 ± 0.04 min in control experiments before and after incubation in (caldesmon-free) buffer. Note also that the rates of relaxation were much higher in β-escin-skinned than in Triton-X-100-skinned fibres, and, in contrast to the latter, the contractile force did not deteriorate during repeated contraction cycles (Table 3).

Table 4 Effect calcium and ATP depletion (rigor) on extent of LC20 phosphorylation in Triton-X-100-skinned, guinea-pig taenia coli. (pCa=$-\log_{10}$ [Ca^{2+}])

Conditions	Extent of LC_{20} phosphorylation (%)	*n*
Isometric contraction (pCa 4.3)	48±1.75	3
relaxation (pCa > 8)	<6	3
rigor (pCa 8)	<6	3

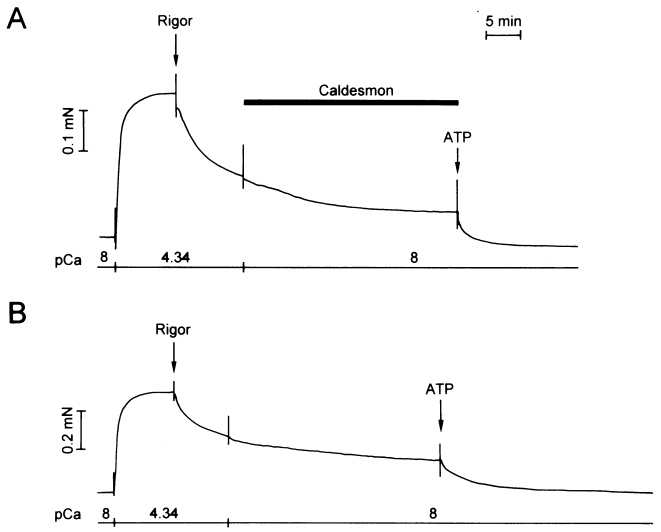

Fig. 5 A, B Skinned guinea-pig taenia coli: relaxation from rigor is affected by caldesmon. **A** Contraction solution (pCa 4.3) elicits an isometric contraction. At *arrow* (*Rigor*) the fibre is immersed into ATP-free rigor solution, also containing glucose, hexokinase and 4 mM calcium-EGTA (see Materials and methods), and then into calcium-free rigor solution containing 5 μM caldesmon. At *arrow* (*ATP*) the fibre bundle is immersed into relaxing solution. **B** Control experiment (experimental procedure as in **A**, but without exogenous caldesmon). Note that relaxation from rigor is slower than in **A**

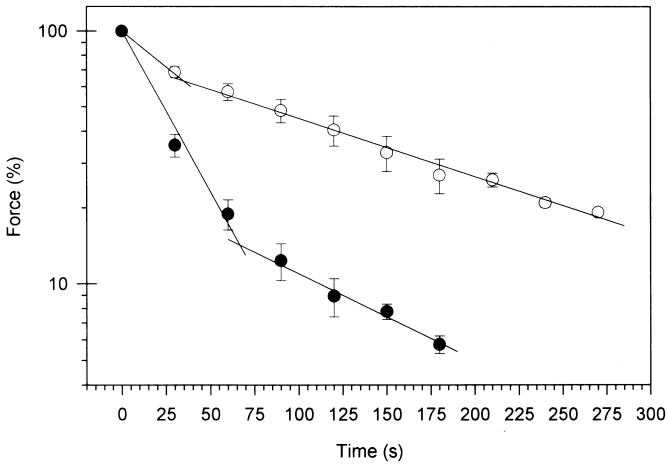

Fig. 6 Time course of ATP-induced relaxation from rigor in caldesmon-treated fibres (*closed circles*) and in non-treated controls (*open circles*). Values represent means ± SEM of $n = 6$ experiments

During calcium-induced contraction the regulatory light chains (LC_{20}) were 50% phosphorylated, but almost completely dephosphorylated in relaxation (Table 4). We have previously shown that dephosphorylation precedes relaxation in guinea-pig skinned taenia coli [20]. To find out whether caldesmon promotes net detachment of *dephosphorylated* crossbridges, we immersed contracted skinned fibres into an ATP-free solution that caused a rigor state. After 30 min in rigor myosin light chain phosphorylation reached basal levels (Table 4), while about 30% of the initial force was still maintained. After immersion into an ATP-containing relaxing solution, the fibres relaxed as shown in Fig. 5 with a quasi-exponential time course that may be accelerated by Pi [20] or by pretreatment of the fibres with caldesmon. In these experiments, caldesmon (5 μM) was present in the rigor solution (to load the fibres with caldesmon), but not in the ATP-containing relaxing solution. Figure 6 compares the time course of relaxation from rigor in caldesmon-treated fibres with that of untreated controls. The time constant of the slow relaxation phase in the absence of Pi and caldesmon is about 2.6 min; it is much larger (by a factor of 1.6) than that in the presence of caldesmon and it is also much greater than the lifetime of attached crossbridges (see below). Thus, during the slow relaxation, tension is presumably supported by dephosphorylated crossbridges that detach but reattach cooperatively even at very low levels of calcium (pCa > 8). In skinned fibres from rabbit femoral arteries, relaxation from rigor was also accelerated by caldesmon treatment. The half-time of relaxation was 0.65 ± 0.14 min as compared to 1.1 ± 0.25 min ($n = 6$) in controls. We confirmed that, like caldesmon, Pi also accelerated relaxation from rigor (see [20]).

We further investigated the effect of caldesmon on cooperative reattachment of dephosphorylated crossbridges during relaxation from rigor induced by flash photolysis of caged-ATP rather than by immersion into an ATP-containing relaxing solution. It had been shown previously [2, 38] that in skinned smooth muscle fibres in rigor ATP (0.5–1 mM) that was liberated from caged-ATP by flash photolysis may cause a transient increase in force followed by relaxation, and the kinetics of relaxation indicated cooperative attachment of crossbridges. Here we studied the relaxation from rigor induced by flash photolysis of caged-ATP under a variety of conditions (Table 5): as shown in Fig. 7, there was an initial fast phase followed by a slower exponential phase presumably due to cooperative crossbridge attachment. We also confirmed previous results [2] showing that relaxation was much faster in the presence of Pi (Table 5). At high $MgCl_2$ concentrations, relaxation was slow, as found previously [2], but could be accelerated by preincubation of the fibres with caldesmon, at least in the presence of Pi (Fig. 7). Thus, the half-times of relaxation (at high Mg^{2+} concentrations) were 17.2 ± 1.9 s, but only 3.15 ± 0.36 s ($n = 7$) and 5.49 ± 0.28 s ($n = 6$) after addition of Pi (10 mM) in caldesmon-treated and untreated fibres, respectively (see Fig. 8 and Table 5). Compared to

Table 5 Relaxation from rigor induced by flash photolysis of caged-ATP. Basic solution composition (mM): KCl 50; imidazole (pH 6.7) 25; dithioerythritol (DTE) 10; EGTA 4 (T = 22–23 °C). For further additions (mM) see table. Prior to incubation in caged-ATP solution, fibres were held in rigor solution (made ATP and ADP-free by treatment with apyrase). Values indicate half relaxation time ($t_{1/2}$ in s±SEM; n = 6 experiments). Tension in rigor was about 30% of that of the preceeding Ca^{2+}-induced contraction.

ATP (caged)	$MgCl_2$	Pi	ADP	CaD* (μM)	$t_{1/2}$ (s)
12.5	3	–	–	–	5.10±0.87
12.5	3	10	–	–	2.15±0.16
12.5	3	–	–	5	6.63±0.51
12.5	3.5	–	0.5	–	8.80±0.36
12.5	3.5	–	0.5	5	8.23±1.04
12.5	3.5	10	0.5	–	4.88±0.41
7.5	10	–	–	–	17.25±1.9
7.5	10	–	–	5	19.75±3.2
7.5	10	10	–	–	5.49±0.28
7.5	10	10	–	5	3.15±0.36

* Caldesmon (*CaD*) was present in the rigor solution, but not in the caged-ATP solution

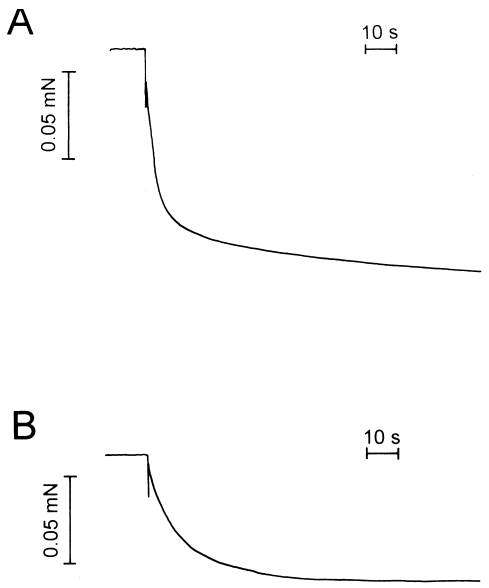

Fig. 7 A, B Relaxation from rigor induced by flash photolysis of caged-ATP. In caldesmon-treated (**A**) and untreated fibres (**B**), respectively. Experimental conditions and treatment with caldesmon as in Fig. 5A. Fibres were held in rigor solution under tension (30% of maximally calcium-activated force) and caged-ATP (7.5 mM) was diffused into the fibre in a rigor solution containing 7.5 mM caged-ATP, 10 mM $MgCl_2$, 10 mM DTE, 25 mM imidazole

controls, in caldesmon-treated fibres the rate of relaxation from rigor following flash photolysis of caged-ATP was found to be larger by a factor of 1.6. In contrast, an increase in the concentration of MgADP (0.5 mM) increased the half-time of relaxation (Table 5), as already found by Fuglsang et al. [9]. During the slow relaxation following the flash photolysis of caged-ATP, force is supported by dephosphorylated crossbridges, since, as mentioned already, preincubation in rigor solution for 30 min causes almost complete light chain dephosphorylation by endogenous phosphatase (Table 4). Obviously, caldesmon inhibited force generation by cooperatively attaching dephosphorylated crossbridges but, interestingly, this effect was observed only at high $MgCl_2$ concentrations and in the presence, but not in the absence, of Pi or at low $MgCl_2$ concentrations (Table 5).

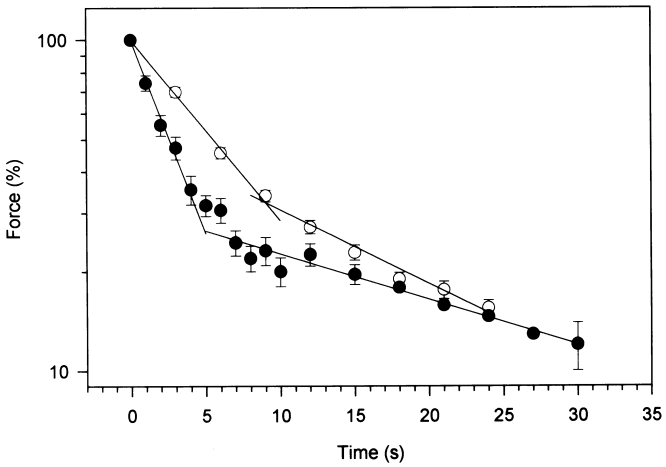

Fig. 8 Time course of tension decay during relaxation from rigor induced by flash photolysis of caged-ATP in guinea-pig skinned taenia coli treated with exogenous caldesmon (*closed circles*, 5 μM) and in control fibres (*open circles*). Values are means ± SEM of n = 6 experiments

Discussion

The time course of contraction and relaxation elicited by the rise and fall of free calcium is slower in tonic than in phasic smooth muscle skinned fibres, as found by Khromov et al. [18]. The very slow relaxation that occurs in α-toxin-treated skinned femoral artery [18] or in Triton-X-100-skinned, guinea-pig taenia coli [20] is said to be related to the latch- or catch-like state of smooth muscle. As in the latch state, dephosphorylation of myosin precedes relaxation. Thus, during relaxation at low calcium, force is maintained by dephosphorylated crossbridges that cycle slowly or not at all, as indicated by the low ATPase activity [10] and the absence of an active state [20]. Crossbridge detachment also appears to be very slow [10] and may be rate-limited by the process governing the ADP release from crossbridges (see [18]). Additionally, however, and perhaps alternatively, cooperative reattachment of detached dephosphorylated crossbridges might also be responsible for the slowness of relaxation in permeabilized fibres [18]. Cooperative attachment of crossbridges as well as the cooperative activation of smooth muscle AM ATPase is tropomyosin dependent [5] and may be inhibited by caldesmon [13]. Thus, we studied the effect of caldesmon on the latch-like state in the absence of calcium and showed that preloading skinned fibres with caldesmon accelerated the decay of force during relaxation. Preloading was achieved by incubating Triton-X-100-skinned fibres in

relaxing solution containing caldesmon (5 μM), a procedure which was previously shown [22] to increase the caldesmon content of fibres. However, when skinned fibres were preloaded with calponin rather than with caldesmon, there was no acceleration of relaxation, though both regulatory proteins inhibit the actin–myosin interaction [23, 26]. However, calponin, unlike caldesmon, is said not to inhibit the tropomyosin-dependent cooperative activation of the contractile system [13]. Thus, it seems likely that the described effect of caldesmon on the rate of relaxation induced by lowering the free calcium concentration may in fact be due to an inhibition of tropomyosin-dependent cooperative attachment of dephosphorylated crossbridges during the relaxation phase in the absence of calcium.

In further experiments, smooth muscle fibres (of taenia coli and femoral artery) in rigor were relaxed by sudden immersion into an ATP-containing relaxing solution. This relaxation from rigor was considerably faster than the relaxation that followed the termination of calcium-induced contraction with EGTA. Like the latter, it was accelerated by Pi [20], but also by preloading the fibre with caldesmon. Since phosphorylation of the regulatory light chains is basal in the rigor state, we conclude that tension is supported by cooperatively attaching crossbridges (see [2, 38]), rather than by "latch-bridges" generated by dephosphorylation of attached phosphorylated crossbridges according to the four-state model of Murphy [12]. Caldesmon could accelerate the tension decay by either inhibiting cooperative reattachment or – alternatively – by enhancing the rate of detachment of "latch-bridges". However, in the latch state, crossbridges most likely are in an ADP-bound state, and since caldesmon has no effect on the rate of ATP hydrolysis [42] it is thus unlikely that caldesmon accelerates detachment of "latch-bridges". Rather as a competitive inhibitor of the binding of actin to myosin [6, 43], it may inhibit cooperative reattachment of crossbridges. The acceleration of relaxation by Pi might be due to a different mechanism, presumably the reversal of the crossbridge step involving transition from weakly bound to strongly bound crossbridges (AM-ADP state) followed by reverse attachment (see reaction 4 in Fig. 1B), as previously suggested [2, 38].

Relaxation from rigor followed by cooperative crossbridge reattachment of detaching crossbridges was also induced by ATP that is rapidly released by flash photolysis of caged-ATP [2, 38]: Thus, following the photolytic liberation of ATP from caged-ATP, rigor crossbridges detach immediately, but rapidly reattach in a cooperative manner as explained in Fig. 1B. Thereafter, tension decays comparatively slowly, as crossbridges become detached with a time course governed by the rate of ADP release [9, 25]. We confirmed previous studies [2, 9, 25] showing that ADP and Mg^{2+} slowed, while Pi accelerated, relaxation from rigor. The force maintained during this tension decay must have been supported by dephosphorylated crossbridges, since preincubation in ATP-free solution to induce rigor also caused complete dephosphorylation of myosin. When fibres were preloaded with caldesmon in the rigor phase, relaxation following flash photolysis of caged-ATP was much faster, at least in the presence of Pi suggesting that caldesmon interferes with cooperative crossbridge attachment.

However, it is quite surprising that in the flash photolysis experiments caldesmon accelerated relaxation in the presence, but not in the absence, of Pi. This is presumably because in the absence of phosphate cooperative crossbridge attachment is induced by nucleotide-free crossbridges (rigor linkages) rather than by strongly bound crossbridges in the AM-ADP state. As rigor bridges are reported to displace caldesmon from actin (see [13]), caldesmon has no inhibitory effect on crossbridge attachment and the rate of subsequent crossbridge detachment and tension decay is then governed by the rate of ADP release (see [9]). In contrast, added Pi interacts with strongly bound crossbridges in the AM-ADP state to form crossbridges in the AM-ADP-Pi state that become rapidly detached (reaction 4 in Fig. 1B, see [2]) but will probably partly reattach (by reversal of reaction 4) by a cooperative mechanism. Cooperative attachment may be induced by cycling crossbridges (see [11]) that are (even in the presence of Pi) attached in the strongly bound AM-ADP state rather than in the rigor state (AM state). Under these conditions, i.e. in the absence of rigor linkages, caldesmon will be bound by actin [13] and is thus in a position to inhibit the cooperative reattachment, thereby enhancing relaxation. Caldesmon-sensitive cooperative attachment induced by strongly bound crossbridges in the AM-ADP state is also expected to occur when crossbridges are slowly cycling in the latch-like state of skinned fibres, as described above, and may play a role at submaximal levels of calcium activation [22, 40] or at submaximal light chain phosphorylation [28]. However, it is still controversial whether caldesmon inhibits cooperative crossbridge attachment under these conditions by inhibiting the formation of weakly bound crossbridges (as suggested by Brenner et al. [4] and Velaz et al. [43]) or strongly binding crossbridges (see [24]).

Another matter of controversy is the physiological in vivo function of caldesmon. The finding that addition of exogenous caldesmon ([28, 40], present studies) as well as exogenous calponin [15] causes inhibition of force generation in skinned fibres has been controversially discussed (see [14]), as *endogenous* calponin and caldesmon are still present in Triton-X-100-extracted smooth muscle fibres. However, the hypothesis that caldesmon is inhibitory in vivo is supported first by experiments by Katsuyama et al. [17], showing that a non-inhibitory actin-binding peptide derived from caldesmon competitively antagonizes the endogenous caldesmon action thereby inducing contraction and, secondly, by experiments [4, 41] showing that caldesmon inhibited contraction in skinned skeletal muscle fibres which are, of course, devoid of caldesmon. In this respect, it is also worth noting that, unlike calponin, actin-bound caldesmon is not homogeneously distributed among thin filaments in smooth muscle cells

[21], as it is located only in the contractile domain of the cell, but not in the cytoskeletal domain. Triton-X-100 skinning procedures may even cause a redistribution of caldesmon within the smooth muscle tissue [19], most likely from the contractile domain to the β-actin filaments of the cytoskeletal domain. This could be taken to mean that, due to the redistribution, some α-actin filaments of the contractile domain become caldesmon depleted, or that endogenous caldesmon may even be partly extracted to be reconstituted by exogenous caldesmon [22]. Thus, after caldesmon extraction, contractile force and calcium sensitivity were partly increased at an intermediate free calcium concentration, but could be normalized to some extent by reconstituting the extracted fibres with exogenous caldesmon (see [22]). In a similar vein, the effects of caldesmon reported in this paper may also be accounted for by the reconstitution of partly caldesmon-depleted thin filaments with exogenous caldesmon. In contrast, caldesmon was without any effect on the contractile force and relaxation rate of guinea-pig taenia coli muscle that had been skinned with β-escin rather than with Triton X-100, despite the fact that both types of fibres were found to be permeable to caldesmon. In fact, we found in preliminary experiments that both in Triton-X-100-skinned and in β-escin-skinned fibres the strips could be homogeneously loaded with exogenous caldesmon, as verified by confocal microscopy using rhodamin-labelled caldesmon (kindly donated by Dr. J.M. Chalovich, data not shown). Thus, it would be interesting to find out whether, unlike in Triton-X-100-skinned fibres, caldesmon does not redistribute within the β-escin-skinned fibres.

In conclusion, relaxation of skinned fibres induced by rapid calcium chelation with EGTA probably corresponds to a latch-like state, as suggested by Khromov et al. [18], and may be due to crossbridges that become dephosphorylated while being attached. These dephosphorylated latch crossbridges (that are in the AM-ADP state, see Fig. 1B and [9]) may cycle slowly because of slow dissociation of bound ADP and because of cooperative reattachment of dephosphorylated crossbridges. In a two-state crossbridge model, force (F) depends at any one time on the rate constants of latch bridge detachment and reattachment ($g_{app.}$ and $f_{app.}$), respectively, according to the relationship $F = k \times f$ app. / ($f_{app.} + g_{app.}$) (see [3]). Thus, latch force may decrease by increasing either $g_{app.}$ (e.g. by lowering the ADP affinity or concentration; see [18]) or by decreasing $f_{app.}$. We suggest that caldesmon inhibits cooperative attachment of dephosphorylated crossbridges in the latch- or catch-like state of guinea-pig skinned taenia coli – possibly by decreasing $f_{app.}$ – and in this way it may decrease force and abolish latch crossbridges, thereby accelerating relaxation.

In intact smooth muscle cells, the inhibitory activity of caldesmon is probably regulated. As reviewed by Horowitz et al. [14], it may be reversed either by calcium-calmodulin or, alternatively, by caldesmon phosphorylation catalysed by MAP kinase which is itself controlled by the low-molecular-weight GTPase, ras p^{21}, and by protein kinase C. Both ras p^{21} and protein kinase C have been shown to modulate smooth muscle contraction [14, 32]. Therefore, a latch-like state might be induced by activation of MAP kinase that causes phosphorylation of caldesmon and it may be terminated by caldesmon dephosphorylation or also perhaps by lowering the free calcium to very low levels. If this did occur, latch crossbridges would be abolished since interaction of caldesmon with calcium-calmodulin would give way to its interaction with actin, thereby inhibiting cooperative reattachment of dephosphorylated crossbridges.

Acknowledgements This work was supported by a grant from the Deutsche Forschungsgemeinschaft to G. Pfitzer. We thank A. Ebling for assistance in the preparation of the manuscript, M. Troschka for technical help in the estimation of myosin light chain phosphorylation and R. Wojciechowski for the construction of the skinned fibre apparatus. We also thank Dr. J.M. Chalovich for the generous gift of calponin, caldesmon and rhodamin-labelled caldesmon and Dr. H. Sauer for help with confocal microscopy of skinned fibres.

References

1. Andrews MAW, Maughan DW, Nosek TM, Godt RE (1991) Ion-specific and general ionic effects on contraction of skinned fast-twitch skeletal muscle from the rabbit. J Gen Physiol 98:1105–1126
2. Arner A, Goody RS, Rapp G, Rüegg JC (1987) Relaxation of chemically skinned guinea pig Taenia coli smooth muscle from rigor by photolytic release of adenosine-5'-triphosphate. J Muscle Res Cell Motil 8:377–385
3. Brenner B (1988) Effect of Ca^{2+} on crossbridge turnover kinetics in skinned single rabbit psoas fibers: implications for regulation of muscle contraction. Proc Natl Acad Sci USA 85:3265–3269
4. Brenner B, Yu LC, Chalovich JM (1991) Parallel inhibition of active force and relaxed fiber stiffness in skeletal muscle by caldesmon: implications for the pathway to force generation. Proc Natl Acad Sci USA 88:5739–5743
5. Chacko S, Eisenberg E (1990) Cooperativity of actin-activated ATPase of gizzard heavy meromyosin in the presence of gizzard tropomyosin. J Biol Chem 265:2105–2110
6. Chalovich JM, Yu LC, Brenner B (1991) Involvement of weak binding crossbridges in force production in muscle. J Muscle Res Cell Motil 12:503–506
7. Dillon PF, Aksoy MO, Driska SP, Murphy RA (1981) Myosin phosphorylation and the cross-bridge cycle in arterial smooth muscle. Science 211:495–497
8. Fischer W, Pfitzer G (1989) Rapid myosin phosphorylation transients in phasic contractions in chicken gizzard smooth muscle. FEBS Lett 258:59–62
9. Fuglsang A, Khromov A, Török K, Somlyo AV, Somlyo AP (1993) Flash photolysis studies of relaxation and cross-bridge detachment: higher sensitivity of tonic than phasic smooth muscle to MgADP. J Muscle Res Cell Motil 14:666–677
10. Güth K, Junge J (1982) Low Ca^{2+} impedes cross-bridge detachment in chemically skinned Taenia coli. Nature 300:775–776
11. Güth K, Potter JD (1987) Effect of rigor and cycling crossbridges on the structure of troponin-C and on the calcium affinity of the calcium specific regulatory sites in skinned rabbit psoas fibers. J Biol Chem 262:13627–13635
12. Hai CM, Murphy RA (1988) Cross-bridge phosphorylation and regulation of latch state in smooth muscle. Am J Physiol 254:C99–C106
13. Horiuchi KY, Chacko S (1989) Caldesmon inhibits the cooperative turning-on of the smooth muscle heavy meromyosin by tropomyosin-actin. Biochem 28:9111–9116

14. Horowitz A, Menice CB, Laporte R, Morgan KG (1996) Mechanisms of smooth muscle contraction. Physiol Rev 76: 967–1003
15. Horowitz A, Clement-Chomienne O, Walsh MP, Tao T, Katsuyama H, Morgan KG (1996) Effects of calponin on force generation by single smooth muscle cells. Am J Physiol 270: H1858–H1863
16. Kamm KE, Stull JT (1985) The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. Annu Rev Pharmacol Toxicol 25:593–620
17. Katsuyama H, Wang CLA, Morgan KG (1992) Regulation of vascular smooth muscle tone by caldesmon. J Biol Chem 267: 14555–14558
18. Khromov A, Somlyo AV, Trentham DR, Zimmermann B, Somlyo AP (1995) The role of MgADP in force maintenance by dephosphorylated cross-bridges in smooth muscle: a flash photolysis study. Biophys J 69:2611–2622
19. Kossmann T, Fürst D, Small JV (1987) Structural and biochemical analysis of skinned smooth muscle preparations. J Muscle Res Cell Motil 8:135–144
20. Kühn H, Tewes A, Gagelmann M, Güth K, Arner A, Rüegg JC (1990) Temporal relationship between force, ATPase activity, and myosin phosphorylation during a contraction/relaxation cycle in a skinned smooth muscle. Pflügers Arch 416:512–518
21. Lehman W, Craig R, Lui J, Moody C (1989) Caldesmon and the structure of smooth muscle thin filaments: immunolocalization of caldesmon on thin filaments. J Muscle Res Cell Motil 10:101–112
22. Malmqvist U, Arner A, Makuch R, Dabrowska R (1996) The effects of caldesmon extraction on mechanical properties of skinned smooth muscle fibre preparations. Pflügers Arch 432:241–247
23. Marston SB, Huber PAJ (1996) Caldesmon. In: Bárány M (ed) Biochemistry of smooth muscle contraction. Academic, San Diego, pp 77–90
24. Marston SB, Fraser IDC, Huber PAJ (1994) Smooth muscle caldesmon controls the strong binding interaction between actin-tropomyosin and myosin. J Biol Chem 269:32104–32109
25. Nishiye E, Somlyo AV, Török K, Somlyo AP (1993) The effects of MgADP on cross-bridge kinetics: a laser flash photolysis study of guinea-pig smooth muscle. J Physiol (Lond) 460: 247–271
26. Obara K, Szymanski PT, Tao T, Paul RJ (1996) The effects of calponin on isometric force and shortening velocity in permeabilized taenia coli smooth muscle. Am J Physiol 270: C481–C487
27. Paul RJ (1990) Smooth muscle energetics and theories of crossbridge regulation. Am J Physiol 258:C369–C375
28. Pfitzer G, Zeugner C, Troschka M, Chalovich JM (1993) Caldesmon and a 20-kDa actin-binding fragment of caldesmon inhibit tension development in skinned gizzard muscle fiber bundles. Proc Natl Acad Sci USA 90:5904–5908
29. Rembold CM, Murphy RA (1993) Models of the mechanism for crossbridge attachment in smooth muscle. J Muscle Res Cell Motil 14:325–333
30. Rüegg JC (1971) Smooth muscle tone. Physiol Rev 51: 201–248
31. Rüegg JC (1992) Calcium in muscle contraction. Cellular and molecular physiology, 2nd edn. Springer, Berlin Heidelberg New York
32. Satoh S, Rensland H, Pfitzer G (1993) Ras proteins increase Ca^{2+}-responsiveness of smooth muscle contraction. FEBS Lett 324:211–215
33. Schmidt US, Troschka M, Pfitzer G (1995) The variable coupling between force and myosin light chain phosphorylation in Triton-skinned chicken gizzard fibre bundles: role of myosin light chain phosphatase. Pflügers Arch 429:708–715
34. Schneider M, Sparrow M, Rüegg JC (1981) Inorganic phosphate promotes relaxation of chemically skinned smooth muscle of guinea-pig Taenia coli. Experientia 37:980–982
35. Sohma H, Inoue K, Morita F (1988) A cAMP-dependent regulatory protein for RLC-a myosin kinase catalyzing the phosphorylation of scallop smooth muscle myosin light chain. J Biochem (Tokyo) 103:431–435
36. Somlyo AP (1967) Discussion remarks to Twarog BM. The regulation of catch in molluscan muscle. J Gen Physiol [Suppl] 50:157–169
37. Somlyo AP, Somlyo AV (1994) Signal transduction and regulation in smooth muscle. Nature 372:231–236
38. Somlyo AV, Goldman YE, Fujimori T, Bond M, Trentham DR, Somlyo AP (1988) Cross-bridge kinetics, cooperativity, and negatively strained cross-bridges in vertebrate smooth muscle. A laser-flash photolysis study. J Gen Physiol 91: 165–192
39. Sparrow MP, Mrwa U, Hofmann F, Rüegg JC (1981) Calmodulin is essential for smooth muscle contraction. FEBS Lett 125:141–145
40. Szpacenko A, Wagner J, Dabrowska R, Rüegg JC (1985) Caldesmon-induced inhibition of ATPase activity of actomyosin and contraction of skinned fibres of chicken gizzard smooth muscle. FEBS Lett 192:9–12
41. Taggart MJ, Marston SB (1988) The effects of vascular smooth muscle caldesmon on force production by “desensitised” skeletal muscle fibres. FEBS Lett 242:171–174
42. Velaz L, Hemric ME, Benson CE, Chalovich JM (1989) The binding of caldesmon to actin and its effect on the ATPase activity of soluble myosin subfragments in the presence and absence of tropomyosin. J Biol Chem 264:9602–9610
43. Velaz L, Chen Y, Chalovich JM (1993) Characterization of a caldesmon fragment that competes with myosin-ATP binding to actin. Biophys J 65:892–898
44. Vyas TB, Mooers SU, Narayan SR, Witherell JC, Siegman MJ, Butler TM (1992) Cooperative activation of myosin by light chain phosphorylation in permeabilized smooth muscle. Am J Physiol 263:C210–C219
45. Zhang Y, Moreland RS (1994) Regulation of Ca^{2+}-dependent ATPase activity in detergent-skinned vascular smooth muscle. Am J Physiol 267:H1032–H1039