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Energetics and regulation of crossbridge states in mammalian smooth muscle

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Key words. Smooth muscle energetics; light chain phosphorylation; crossbridges; calcium.

We have been studying the relationships between mechanical output and chemical energy input in smooth muscle in order to gain an understanding of the mechanisms by which energy derived from high energy phosphates is transduced into mechanical work. We have done this by determining the mechanical characteristics and associated energy utilization in smooth muscles under different mechanical conditions including rest, during an isometric tetanus, relaxation and isovelocity stretch. We have also attempted to learn how contractile activity may be regulated, specifically with respect to the role of phosphorylation of the 20,000-dalton light chain of myosin. The results to be summarized here represent a synthesis of work we have done in recent years and our current views on the existence and regulation of crossbridge states in smooth muscle.

Methods

The taenia coli muscle of immature virgin female rabbits was isolated and divided into three segments about 15 mm long and weighing about 15 mg. The segments from each muscle were subjected to similar or different experimental designs and compared internally. All of the methods for dissection, the apparatus for measurement of isometric tension, isovelocity stretch and maximum velocity of shortening, procedures for measurement of high energy phosphate usage and degree of myosin light chain phosphorylation have been described in detail previously[12,14,15,32,33] and will not be repeated here.

Briefly, the muscles were bathed in plexiglass chambers containing flowing, oxygenated Krebs bicarbonate solution (in mM/l, NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; $CaCl_2$, 1.9; and glucose, 11; bubbled with a mixture of 95% O_2, 5% CO_2, pH 7.4) at 21 °C, and were allowed to equilibrate for 2 h at 2×10^{-2} Newton prior to any experimental maneuvers. The muscles were stimulated supramaximally in a transverse field with platinum-platinum chloride electrodes and 10 V rms 60 Hz AC pulses. For measurement of high energy phosphate usage, recovery reactions of glycolysis and respiration through which ATP synthesis occurs were blocked by treatment of the muscles in a Krebs solution lacking glucose and containing 0.5 mM iodoacetic acid and 5.0 mM sodium fluoroacetate under anaerobic conditions. The validity of this method has been described previously[12,32].

According to the particular experimental design, the muscles were quickly freeze-clamped at liquid nitrogen temperature, and the frozen tissues were extracted with 0.5 N $HClO_4$, neutralized and analyzed by liquid chromatography for ATP and ADP and spectrophotometrically for phosphocreatine (PCr) and total creatine (Ct) (see Butler et al.[12] for details). High energy phosphate usage ($-\Delta \sim P$) is calculated as the sum of the changes in PCr and ATP contents and is expressed relative to the total creatine content which is 2.7 μmoles/g wet wt[12].

The degree of myosin light chain phosphorylation was determined from tissues frozen and extracted in a manner identical to that used for metabolites. The perchloric acid insoluble material was dissolved in a solution containing 9.0 M urea, 5% (wt/vol.) 2-mercaptoethanol, 1.5% pH 5–7 and 0.5% pH 7–9 ampholines (LKB Instruments), and subjected to isolectric focussing followed by electrophoresis in sodium dodecyl sulfate in the second dimension[14]. The second dimension gel was stained with Coo-

massie blue, scanned at 570 nm and the relative areas of the peaks containing the phosphorylated and unphosphorylated forms of the 20,000-dalton light chain were determined. Measurements of myosin light chain phosphorylation were made on both untreated muscles and those treated with metabolic inhibitors with no significant differences between these groups.

Results and discussion

A) The resting state

It is well known that when smooth muscles are stretched they show an increase in force, or resistance to stretch, which is followed by a decay of force to some new level, or stress relaxation. Some years ago[30] we investigated this phenomenon in a variety of visceral and vascular smooth muscles at 21 °C and in all cases found that the responses to stretch were calcium-dependent. That is, in the absence of calcium in the bathing medium, the resistance to stretch and stress relaxation were markedly reduced, and could be restored upon return of calcium. We also found that the magnitude of the responses to stretch depended on initial muscle length, and that the calcium-dependent component had the same length dependence as active force development. The calcium-independent component varied with length in the same way as passive tension, that is, it coincided with the passive tension curve of the muscle, thereby representing the viscoelastic properties of passive, non-contractile elements in the muscle (fig. 1). We also found that the resistance to stretch and stress relaxation could be restored after treatment in calcium-free media by induction of the rigor state in the absence or presence of calcium[11]. On the basis of these results we hypothesized that in atonic, resting smooth muscles, the resistance to stretch and stress relaxation were due to the strain of attached crossbridges and their subsequent reattachment in an unstrained configuration at a new length following the length change. We hypothesized further that these attached crossbridges could maintain, but not generate a net force, and probably did not cycle.

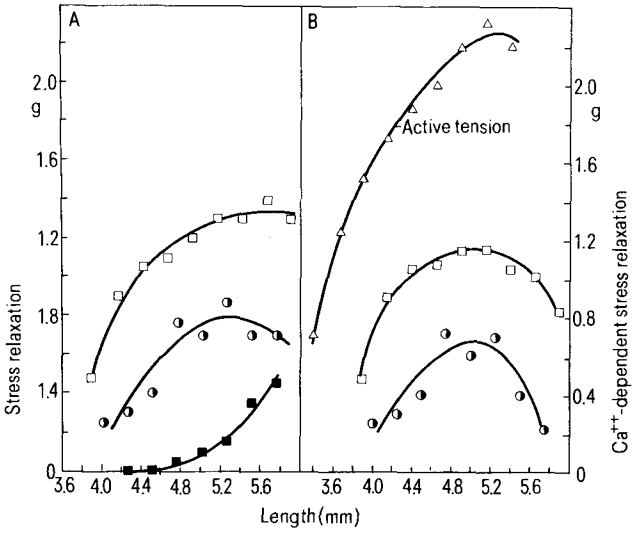

Figure 1. *A* Stress relaxation resulting from 0.5 mm stretch at 1 mm/sec (~ 2 L_o/sec) to length shown on abscissa in Krebs solutions containing 1.9 mM Ca^{++} (□), then 0 mM Ca^{++} (2mM EGTA) for 0.5 h (■), and finally 4.0 mM Ca^{++} for 1 h (◑). Data for 0 mM Ca^{++} treatment coincide with passive tension of the muscle at each length. *B* Calcium-dependent stress relaxation calculated as difference between stress relaxation in 1.9 mM Ca^{++}-Krebs (□), 4.0 mM Ca^{++}-Krebs (◑), and 0 mM Ca^{++}-Krebs (2 mm EGTA) solution based on values shown in (A). Active tension curve is from tetanically stimulated muscle in 1.9 mM Ca^{++}-Krebs solution (△). Stress relaxation is calculated as the difference between peak and subsequent steady-state tension reached 15 min after stretch. Temperature 21 °C (from Siegman et al.[30]).

In more recent experiments we tested the latter hypothesis by measuring the steady-state rate of energy usage from steady-state lactate production under anaerobic conditions where all ATP production must come from glycolysis and lactate production. Muscles were bathed in the presence or absence of calcium, conditions which would lead to attachment or detachment of crossbridges, respectively. There was no significant difference in the steady-state rate of lactate production by tissues bathed in the presence of absence or calcium, for the ratio of $J_{Lactate\text{-}Ca^{++}}$: $J_{Lactate\text{-}Ca^{++}\text{-}free}$ was 0.98 ± 0.05 (n = 11). Therefore, in the absence of a measurable difference in energy usage under the two conditions, we concluded that those crossbridges which are attached in resting smooth muscles do not undergo significant cycling at the expense of ATP.

We were also interested in learning whether the attachment of crossbridges in the resting state required the phosphorylation of myosin light chains. In the same type of experiments as those just described, the degree of myosin light chain phosphorylation in muscles bathed in calcium-free media was 8.4 ± 1.0% (n = 12) and was not significantly different from that of muscles bathed in a medium containing 1.9 mM Ca^{++}, 9.2 ± 1.5% (n = 8).

Taken together the results led us to suggest[30] that at very low calcium concentrations, there is no interaction between actin and myosin in resting smooth muscles, as shown by a loss of resistance to stretch. At the intracellular calcium concentrations normally prevailing at rest, there is marked resistance to stretch, suggesting that there is attachment of crossbridges, but without myosin light chain phosphorylation. As a result of activation of the muscle, intracellular calcium increases, actin-activated myosin ATPase activity increases with cycling of crossbridges and, based on current knowledge, this is probably preceded or accompanied by phosphorylation of the light chains of myosin.

B) Active muscle

The high energy phosphate utilization during an isometric tetanus was measured directly from changes in the ATP and PCr contents of muscles in which recovery reactions of glycolysis and oxidative phosphorylation were inhibited. We found that at 18 °C in muscles bathed in a medium containing 1.9 mM Ca^{++} at L_o, the average rate of energy usage ($-\Delta$[ATP/Ct + PCr/Ct]/sec) during isometric force development was some four times higher than for maximum isometric force maintenance (Siegman et al.[32]).

The apparent high economy of force maintenance in the smooth muscle, 700 (N/cm)/(μmol ~ P/g·s^{-1}), is 100-fold higher than skeletal muscle of the frog which contains about three times more myosin per gram wet wt of muscle. This can be explained, in part, on the basis of a longer myosin filament in smooth muscle[7] which would put

more crossbridges mechanically in parallel, and by a slower crossbridge cycle. An estimate of the time it takes each myosin to split ATP can be obtained be dividing the myosin content by the rate of energy usage during force maintenance for each muscle type. Assuming that each myosin head is splitting ATP then each crossbridge cycle would take 50 msec in skeletal compared to 7.5 sec in the smooth muscle. These values would be decreased, of course, to the extent that not all myosins are splitting ATP and increased to the extent that some energy usage occurs through ATPases other than myosin. The high economy of the anterior byssus retractor muscle (ABRM) in the catch state is an order of magnitude greater than that of the taenia coli[8], but after accounting for differences in myosin filament length (ABRM, 30 μm[26]; mammalian smooth muscle, 2.2 μm[7]), the economy of force maintenance by individual crossbridges in the two muscles is similar, assuming similarity in other structural and metabolic relationships. Therefore the ability of some smooth muscles to show catch may not be a unique ability of the crossbridges to maintain force economically, but rather an ability to vary economy according to stimulation parameters and to have a relatively large V_{max} for shortening compared to other smooth muscles[32].

We also determined the effects of calcium on force output and high energy phsophate usage during a 60 sec tetanus. In the taenia coli, active force can be graded by varying the calcium concentration of the medium in which the muscle is bathed. Force increases significantly when the external calcium is increased in the range 0–1.0 mM, reaching a maximum which is maintained even when the calcium concentration is raised to 6.0 mM. We found that as the calcium concentration of the bathing medium was increased from 0 to 0.1, 0.25, 0.5 and 1.0 mM, both force output and energy usage increased proportionately, as would be expected if the number of activated crossbridges increased; in 1.0 mM and 1.9 mM Ca^{++} media, the energy usage was not significantly different, nor was the force output. In calcium-free medium, force output upon stimulation was zero and high energy phosphate usage was not significantly different from basal energy usage (fig. 2). Interestingly, in this range of calcium concentrations, it would appear that those crossbridges that generate force split ATP such that the economy is constant. However, when the calcium concentration was increased from the normal 1.9 mM to 4.5 mM, there was a large increase in suprabasal high energy phosphate usage although force did not change significantly. In other words, there was a constant economy of force production at calcium concentrations up to 1.9 mM and a marked decrease in the economy of force production when the calcium concentration was increased to 4.5 mM. In studies on skinned smooth muscles it was found that maximum force generation occurred at a lower calcium concentration than did maximum ATPase activity[6,22].

More useful information could be gained by examining the energy usage during the force development and force maintenance phases of the tetanus (for experimental designs see Siegman et al.[32]). We found that at all calcium concentrations studied, the average rate of energy usage was greater during force development than when force was maintained during the plateau of the tetanus.

A major question is whether differences in the rates of energy usage during force development and maintenance reflect differences in the rates of crossbridge cycling? One approach to answering this important question was to determine the effects of isovelocity stretch on the energy usage during the two phases of the tetanus. On the basis of work done on skeletal muscle, it is known that slow isovelocity stretch during stimulation results in a reduction in the high energy phosphate usage attributable to actin-activated myosin ATPase under isometric conditions to the extent that the energy usage is similar to the activation energy of the muscle[1,2,13,18,24,25,27]. If the same obtains in smooth muscle, we would predict that stretch would be most effective in reducing energy usage at a time when the crossbridge cycling rate is high.

Because of the dramatic increase in energy usage when the calcium concentration was elevated from 1.9 to 4.5 mM, we determined the effects of isovelocity stretch (0.12 L_o/min) during the periods of force development and force maintenance of an isometric tetanus in muscles bathed in media containing these calcium concentrations. As shown in figure 3, in both calcium media stretch

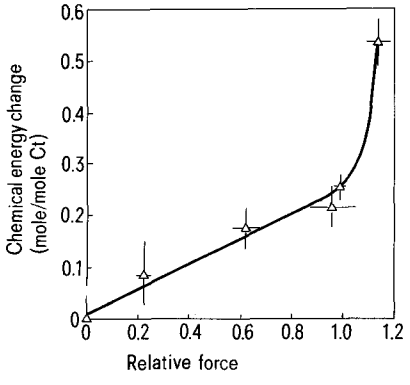

Figure 2. Relationship between isometric force and high energy phosphate usage during a 60-sec tetanus in the rabbit taenia coli at L_o at 18 °C, when force is graded by varying the Ca^{++} concentration of the bathing medium. The Ca^{++} concentration of the Krebs-bicarbonate solution was increased from (left to right) 0 to 0.1, 0.25, 0.5, 1.0, 1.9 and 4.5 mM. Chemical energy change is the sum of the changes in PCr and ATP contents relative to the total creatine content.

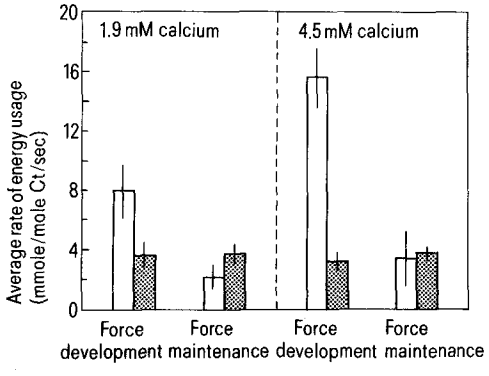

Figure 3. Average rate of high energy phosphate usage during isovelocity stretch and during isometric conditions in 1.9 and 4.5 mM Ca^{++}-containing media. The open bars represent isometric conditions and are taken from Siegman et al.[33]. The stippled bars show the energy usage when the muscle was stimulated and stretched at 0.12 L_o/min. The force development period was the first 25 sec and the force maintenance period was the next 35 sec of stimulation (from Butler et al.[15]).

during force development markedly reduced the rate of high energy phosphate usage, as would be expected if the rate of crossbridge cycling under isometric conditions had been high. In contrast, stretch had little, if any, effect on the rate of energy usage during force maintenance, where the rate of crossbridge cycling under isometric conditions was thought to be low. The results also suggest that elevation of the calcium concentration from 1.9 to 4.5 mM resulted in a further increase in the rate of crossbridge cycling during the two phases of the tetanus. These results are consistent with the idea that stretch during stimulation reduces the actin-activated myosin ATPase, and that under these conditions crossbridges can maintain force with little energy usage[19].

A more direct measure of the apparent time and calcium dependent changes in crossbridge cycling rate can be derived from determinations of the maximum velocity of shortening (V_{max}), and this was accomplished by using the slack test method of Edman[21]. The results are shown in figure 4B, together with the data for high energy phosphate usage. Two main effects are noted: first, there is a time-dependent change in V_{max}, such that the rate is high during the period of force development and then slows markedly during the period of force maintenance; second, in the presence of 4.5 mM Ca^{++}, V_{max} increases significantly compared to all of the corresponding time points in the 1.9 mM Ca^{++} medium. Time-dependent changes in V_{max} have been noted in other smooth muscles[20,23,34] as have calcium-dependent changes[3,4,5,29]. Importantly, the changes in V_{max} for shortening in the taenia coli are in very good agreement with the changes in rates of high energy phosphate usage.

Are the observed time- and calcium-dependent changes in crossbridge cycling rate related to the degree of myosin light chain phosphorylation? The time course of the degree of myosin light chain phosphorylation was determined in muscles that had been bathed in 1.9 or 4.5 mM Ca^{++}-Krebs solution and the results are shown in figure 4C. In both cases the degree of myosin light chain phosphorylation increases from about 10% at rest to about 32% at 25 sec and then decreases slightly but significantly with continued stimulation. However, there are no significant differences in the degree of phosphorylation in the 1.9 and 4.5 mM Ca^{++}-treated muscles.

Therefore, there can be a marked time-dependent decrease in the rate of crossbridge cycling (measured by the rate of energy usage and/or maximum velocity of shortening) during the course of a tetanus with only a minimal decrease in the degree of myosin light chain phosphorylation. For example, after 5 sec of stimulation, V_{max} is about 4 times faster than at 60 sec, but the degree of myosin light chain phosphorylation at these times differs by only 4%. These results stand in contrast to those of Murphy and co-workers and their hypothesis[20] that the time-dependent decrease in velocity of shortening results from the dephosphorylation of myosin light chains and the formation of slowly or non-cycling crossbridges, or 'latchbridges' which comprise an internal load against which cycling, phosphorylated crossbridges must operate; accordingly, velocity would depend on the relative number of dephosphorylated 'latch' and phosphorylated cycling crossbridges.

Are the results of our experiments consistent with the 'latch' hypothesis? The number of phosphorylated crossbridges, as reflected by the degree of myosin light chain phosphorylation, remained fairly constant, although V_{max} decreased. However, there could be an increase in the number of 'latchbridges' in the absence of a net decrease in myosin light chain phosphorylation if dephosphorylation occurred with a concomitant phosphorylation of previously unphosphorylated, not latched crossbridges. This could account for the decrease in V_{max} with no net change in phosphorylation and be consistent with the 'latchbridge' hypothesis. If the 'latchbridge' causes a slowing of V_{max} strictly because of a resistance it poses to the shortening of phosphorylated cycling crossbridges, then we would expect that under isometric conditions the

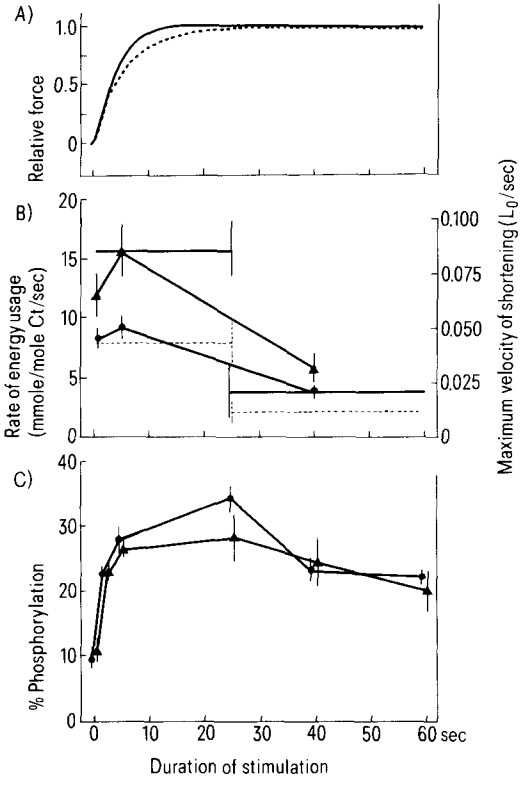

Figure 4. Effect of calcium concentration of the bathing medium on the time course of force output, chemical energy usage, maximum velocity of shortening (V_{max}) and degree of myosin light chain phosphorylation during an isometric tetanus in the rabbit taenia coli at L_0 at 18 °C. *A* Time course of an isometric tetanus for muscles bathed in Krebs solution containing 1.9 mM Ca^{++} (dotted line) and 4.5 mM Ca^{++} (solid line). *B* The horizontal dotted line shows the average rate of energy usage for muscles treated with 1.9 mM Ca^{++}-Krebs solution for the periods shown. The solid line shows the same for muscles incubated in 4.5 mM Ca^{++}-Krebs solution for 1 h prior to stimulation. The standard errors of the means are shown as vertical bars (n = 9–20). Maximum velocity of shortening (V_{max}) values at 0.5, 5.0, and 40.0 sec are plotted as (●) for muscles bathed in 1.9 mM Ca^{++}-Krebs and as (▲) for the same muscles after treatment for at least 1 h in 4.5 mM Ca^{++}-Krebs solution (n = 6). *C* The degree of myosin light chain phosphorylation in muscles bathed in 1.9 mM Ca^{++}-Krebs solution (●) and in muscles bathed in 4.5 mM Ca^{++}-Krebs solution for 1 h (▲). The values at 25 and 60 sec are from the same muscles in which chemical energy usage (B) was determined (n = 6–24), and all others are from muscles not treated with metabolic inhibitors. All values are means ± SEM. The values obtained for muscles bathed in 4.5 mM Ca^{++}-Krebs are displaced slightly because many overlapped those obtained in 1.9 mM Ca^{++}-Krebs (from Siegman et al.[33]).

'latchbridge' would offer no such resistance since no shortening is occurring.* Further, the energy usage under isometric conditions should depend only on phosphorylated crossbridges; we found large decreases in the rate of high energy phosphate usage and V_{max} at times when the number of phosphorylated crossbridges was relatively high and not changing.

Because of the inconsistencies discussed above, the results cannot be explained by a 'latchbridge' mechanism. Rather, it appears that the cycling rate of phosphorylated crossbridges can change as a function of time during a tetanus and as a function of intracellular calcium concentration. Such changes in the cycling rate may be mediated through a calcium-regulatory mechanism independent of or in addition to myosin light chain phosphorylation (fig. 5). The possible interrelationships of the two regulatory mechanisms, if any, need to be elucidated. A biochemical correlate that might explain these results is derived from observations of a calcium-dependent ATPase activity of phosphorylated smooth muscle myosin at low free magnesium concentrations[16,28].

C) Relaxation

The decline of force during isometric relaxation is generally thought to be the result of a progressive termination of crossbridge cycling and actomyosin ATPase activity as the intracellular free calcium concentration diminishes; mechanically, crossbridges detach and filament overlap decreases to the extent that elastic elements passively recoil. Bozler[9] noted the similarity of the time course of isometric relaxation to that of stress relaxation of resting muscle and suggested that both are passive processes due to crossbridge detachment. In view of the evidence for the existence of attached, but non-cycling crossbridges in resting smooth muscles, we thought it would be interesting to determine the energetics of relaxation and to correlate this with mechanical events[13,14,31,32]. We determined the relative energy utilization associated with maximum force maintenance during stimulation and relaxation by comparing paired muscles that developed and maintained maximum force (60-sec stimulation) with those that also developed maximum force but were then allowed to relax (25-sec stimulation followed by 45 sec of relaxation). These parameters were chosen because the average tension-time integrals for the two groups were the same. The total chemical energy change, after correction for the 10-sec difference in observation time for the two groups, was greater for muscles maintaining maximum force than for those allowed to relax, or -0.0615 ± 0.0241 mol ~ P/mol Ct ($n = 18$, $p < 0.02$). These results showed that during relaxation there is a change in the relationship between energy usage and force maintenance; force can be exerted with a lower expenditure of energy during relaxation than when maximum force is maintained during stimulation. Isometric relaxation, then, is not simply a proportional turning-off of both force generation and energy utilization.

In order to learn whether all of the force maintained during relaxation is attributable to crossbridge cycling, we determined the maximum force redeveloped following a quick-release at the peak of the tetanus and at intervals following cessation of stimulation. During relaxation, the ability to redevelop force decays more rapidly than does the force maintained by the muscle. This suggests that not all of the force maintained during relaxation from a tetanus can be attributed to crossbridge cycling. Also, the small energy usage during relaxation correlated well with the extent to which force maintenance due to crossbridge cycling occurs during relaxation.

It is interesting that the time course of crossbridge cycling during relaxation from an isometric tetanus follows closely the decrease in relative degree of light chain phosphorylation[14]. It is not known whether there is a causal relationship between the degree of light chain phosphorylation and the cycling of crossbridges and associated energy usage during relaxation.

There is, then, an 'extra' force maintained during relaxation. This may be due to the return to a condition where force is maintained by attached, but non-cycling crossbridges[30,32] or perhaps by the back-rotation of crossbridges by the recoil of the series elasticity with eventual detachment of the strained crossbridges[10].

During relaxation, the following scheme may operate: calcium removal from the contractile filaments results in gradual cessation of crossbridge cycling and associated energy input possibly through net dephosphorylation of the myosin light chains. As this occurs, the crossbridges revert to the attached state, in which force is transiently maintained. Force maintained during relaxation, then, may be determined primarily by the time course of calcium removal and secondarily by stress relaxation of attached, but non-cycling crossbridges.

Conclusion

On the basis of measurements of the high energy phosphate usage associated with different mechanical states, as well as the degree of myosin light chain phosphorylation and mechanical properties, information has been

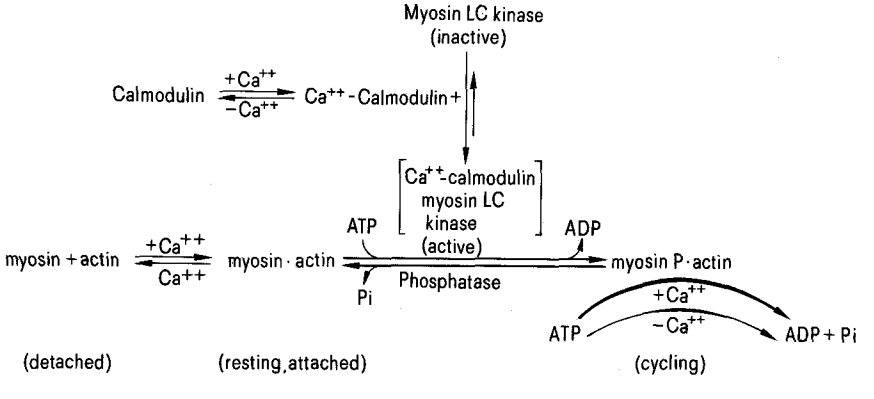

Figure 5. Operational scheme showing crossbridge states in smooth muscle. See 'conclusion' for description.

gained concerning the existence and regulation of different crossbridge states in smooth muscle. Although incomplete, a general operational scheme is shown in figure 5. At very low intracellular calcium concentrations, actin and myosin are dissociated, as shown by a loss of resistance to stretch in resting muscles. At somewhat higher intracellular calcium concentrations in atonic, resting muscles, crossbridges can attach and be manifest mechanically as an increased resistance to stretch without ATP-driven crossbridge cycling and active force production. When the muscle is activated, intracellular calcium increases further, the light chains of myosin are phosphorylated through the calcium-calmodulin activation of myosin light chain kinase, actin-activated myosin ATPase activity increases and crossbridges cycle. Calcium also appears to modulate the ATPase activity and the rate of cycling of the phosphorylated crossbridge. The crossbridge cycling rate is highest during force development and slows with time as maximum isometric force is maintained reflecting a change in the rate at which phosphorylated crossbridges cycle. This may result from a decrease in the intracellular free calcium concentration with continued stimulation. During relaxation, the intracellular calcium concentration decreases, there is net dephosphorylation of the myosin light chains, the rate at which phosphorylated crossbridges cycle slows further with a gradual return to the attached, but non-cycling state or the detached state.

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* During consecutive contractions in the taenia coli force redevelopment occurs with a small percentage of the original light chain phosphorylation and a very slow rate of crossbridge cycling, conditions consistent with a "latch" state. However, energetics measurements on shortening muscles show no evidence for an internal load under such conditions. (Siegman et al., Biophys. J. *47* (1985) 297a, abstract).

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