SIMPLE MODEL OF SMOOTH MUSCLE MYOSIN PHOSPHORYLATION AND DEPHOSPHORYLATION AS RATE-LIMITING MECHANISM

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ABSTRACT A simple mathematical treatment of the model proposed by others in which a dynamic balance between Ca^{++}-dependent phosphorylation and Ca^{++}-independent dephosphorylation of myosin controls the activation of smooth muscle contractility is presented. The parameters of the model can be computed from the experimentally observed stable force-$[Ca^{++}]$ relationship. A simple extension of the model to the case of time-dependent activation yields an expression that quantitatively predicts the measured dependence of the rate of isometric tension development on the activating free $[Ca^{++}]$. The parameters of the mechanical model, which are derived from the rate constants for phosphorylating and dephosphorylating enzyme activities, are in reasonable agreement with the constants measured directly in purified protein systems. In addition, the model predicts values for several parameters that have not yet been experimentally measured, such as the ratio of kinase and phosphatase activities, the maximum extent of myosin phosphorylation, and the kinase turnover number.

INTRODUCTION

Work of the past several years on systems of isolated contractile proteins from a variety of smooth muscles has shown that Ca^{++}-dependent myosin light chain phosphorylation is an important regulatory process in the control of smooth muscle contraction (1, 2). Although exact details of the mechanisms involved remain unresolved and many apparent discrepancies exist, it is generally agreed that Ca^{++}-dependent myosin phosphorylation is a necessary condition for the initiation of actin-myosin interaction (cf. 3 for review), as judged by the Ca^{++}-dependent actin-activated myosin Mg-ATPase (4–7) and superprecipitation (8). This mechanism has been elucidated primarily with systems of purified isolated contractile proteins. It is of concern in such systems whether all proteins and enzymes that have roles in the physiological activation process are present in representative quantities and are functional in the isolated system. Also, geometrical considerations could be of importance. For example, enzymes that normally, by spatial considerations, have no access to certain proteins can perhaps modify these proteins when spatial barriers are removed (for instance, after extraction).

The quantitative and temporal relationship between the degree of myosin phosphorylation and tension development in contractile systems is in dispute. Very low values of myosin phosphorylation have been reported by Kerrick et al. (0.05–0.20 mol Pi/mol light chain) for maximally Ca^{++}-activated detergent-skinned smooth muscles (9). In contrast, Barron et al. (10) have reported high levels of myosin phosphorylation (0.8–1.2 mol Pi/mol light chain) during stable pharmacological activation of intact arterial smooth muscle, but as measured against high resting levels (0.55–0.59 for arteries relaxed in normal saline). Driska et al. (11) have reported that in intact arterial smooth muscle, myosin phosphorylation first increases somewhat more rapidly than tension development and reaches maximum values of 0.6–0.7 mol Pi/mol light chain, but then falls rapidly to values not different from the initial resting myosin phosphorylation level (~0.05) with no consequent decline in tension maintenance. The measurements of myosin phosphorylation in systems that retain contractility are subject to large potential errors, due to incomplete extraction of myosin, continued enzymatic activities during extraction, or perhaps differential extractability of phosphorylated and dephosphorylated myosins (12).

We have addressed this problem indirectly by mechanical experiments on chemically skinned smooth muscles, using a modified glycerination method that yields substantially improved contractile responses (13). The method is, in addition, more likely to retain a nearly full complement of normally active enzymes than detergent treatments. With skinned smooth muscles and the methods of "Ca jump" (14), the internal activating Ca^{++} concentration can be varied rapidly and held constant, so that the rate of Ca^{++} entry into the preparation is not rate-limiting to tension development. We present here a simple mathematical model, derived from the results with isolated proteins described above, in which a dynamic balance between

Ca^{++}-activated phosphorylation and Ca^{++}-independent dephosphorylation determines the steady-state level of myosin light chain phosphorylation. In line then with the observed correlation between Ca^{++}-activated actomyosin ATPase and myosin phosphorylation, the fractional degree of myosin phosphorylation is taken as directly proportional to the fractional degree of Ca^{++}-activated isometric tension development. The numerical parameters of the model can be determined from the experimentally observed relation between stable isometric tension and free $[Ca^{++}]$. Simply taking the time derivative of the model leads to a quantitative prediction of the way in which the rate of isometric tension development will depend on the activating free $[Ca^{++}]$. The prediction describes precisely the observed experimental dependence, including several features that would not otherwise be obvious. The data suggest that in guinea pig taenia coli, the maximum Ca^{++}-activated phosphorylating activity exceeds the phosphatase activity by a factor of 10, that maximum myosin phosphorylation in the contractile system is ~90%, and that the myosin light chain kinase turnover number is <10 s^{-1} at 37°C. This rate is only slightly faster than that measured directly by Mrwa and Hartshorne (15) for myosin phosphorylation by myosin light chain kinase isolated from chicken gizzard.

MATERIALS AND METHODS

Tissue Preparation

Guinea pigs were killed by a sharp blow to the head and sections of taenia coli were excised (typically 0.2 mm × 2 mm × 2 cm long), allowed to equilibrate for 1 h at 37°C in a normal physiological saline, and pinned at rest length to a wax-covered cork block. The method of "freeze-glycerination" was followed, as described elsewhere (13, 16). In summary, the blocks were immersed in cool (~5°C) glycerinating solution for 5–10 min then placed in a freezer at −25°C. After 1 d, tissues were sliced with a razor blade longitudinally into three to four strips and stored frozen in glycerinating solution for up to 3 mo. After removal from storage and mounting in an isometric tension apparatus, tension development was wholly dependent upon externally provided Ca^{++} and MgATP. No detectible alterations in properties such as maximum force generation or the force-$[Ca^{++}]$ relationship were noted as a function of storage. Some taenia coli pieces were not freeze-glycerinated, but were left intact for physiological measurements that were performed within a few hours of excision.

Tension Measurements

Small segments, typically 0.8–0.9 cm long and 1.0–1.5 mg wt/wt, were mounted horizontally using a small amount of fast water-setting cyanoacrylate glue between a semiconductor force transducer (AME 801 Aksjeselskapet Mikiro-Elektronikk, Horteu, Norway) and a fixed bent glass rod. The position of the force transducer was controlled so that tissue length could be adjusted. All measurements of tension development were performed at a length where passive tension was ~5% of the maximal active tension. Tissues were incubated in small (0.9 ml) thermostated glass cups of the appropriate bathing solutions, which could be changed rapidly and were rotated at ~1 Hz to provide stirring. Because tissues contracted and relaxed rather quickly, usually five or six contractions per tissue segment could be performed. Under these conditions, maximum tension generation was found to decline by ~20%/h, so that 2 h was considered a practical limit for the experiments.

Solutions

Glycerinating solution consisted of a 1:1 mixture of glycerol and normal relaxing solution (vol/vol) with 2mM dithiothreitol added. Normal relaxing solution contained 20 mM imidazole, 5 mM EGTA, 5 mM $MgCl_2$, 5 mM Na_2ATP, and, as ATP-regenerating system, 5 mM K-phosphoenolpyruvate with 20 U/ml of pyruvate kinase (type III; Sigma Chemical Co., St. Louis, Mo.). pH was adjusted to 7.0 at 37°C with KOH and KCl added to final ionic strength 0.16 M. The normal contracting solution consisted of the above with some fraction of the EGTA replaced by Ca-EGTA and KCl adjusted to constant ionic strength. ATP-γ-S [adenosine 5'-0-(3-thiotriphosphate); Boehringer Mannheim GmbH, F.R.G.] solutions were made up with or without Ca^{++} or ATP (as described in text) to constant ionic strength (KCl), pH (KOH), and free $[Mg^{++}]$ ($MgCl_2$).

Ca Jump Methods

In similar experiments with skinned hog carotid artery segments (13), it was found that abrupt replacement of EGTA by Ca-EGTA led to rather slow tension development. These activation rates were apparently limited by the rate of Ca^{++} entry into the preparation and the high Ca^{++} buffering capacity of the intracellular environment. Ashley and Moisescu (14) have developed a technique by which step changes in internal free $[Ca^{++}]$ can be attained rapidly by first incubating tissues in very low EGTA relaxing solutions to reduce Ca^{++} buffering capacity, then "jumping" to very highly buffered Ca^{++} solutions. In skinned skeletal muscle fibers, step changes in internal $[Ca^{++}]$ could be attained in ~200 ms. In these experiments with skinned taenia coli, "Ca jumps" from 100 μM EGTA to 10 mM Ca-EGTA were found to maximize the rate of isometric tension development. To assure that Ca^{++} diffusion was not rate-limiting, the jump conditions were increased, which did not further increase the rate of isometric tension development. Jump-relaxing solution contained 10–20 μM EGTA and Ca jump-activating solutions contained 20 mM EGTA/Ca-EGTA made to the desired free $[Ca^{++}]$. The total tetraacid concentration in all solutions was maintained at 20 mM by the addition of 1,6-diaminohexan-*N,N,N',N'*-tetraacetic acid (HDTA) (17), an EGTA analogue that does not bind Ca^{++}. After activations in Ca jump solutions, tissues were first relaxed in 20 mM EGTA solutions, then transferred to jump-relaxing solutions after tension had returned to near the Ca^{++}-free base-line tension. Free $[Ca^{++}]$ and ionic strength were calculated from the data of White and Thorson (18); $pCa = -\log[Ca^{++}]$.

RESULTS

Ca^{++}-dependent Thiophosphorylation

Fig. 1 presents experimental data in support of the premise that Ca^{++}-activated phosphorylation is a necessary and sufficient condition for tension generation in skinned taenia coli. Starting with the relaxed preparation (pCa > 8) in the presence of 5 mM Mg-ATP, the addition (pCa 5) and removal (pCa 8) of Ca^{++} activates a readily reversible contraction of skinned taenia coli that was ~80% of the maximum tension developed by similarly prepared taenia coli segments that were not freeze-glycerinated and activated with 10^{-5} M acetylcholine. Relaxation upon Ca^{++} removal was rapid and complete. The ATP analogue ATP-γ-S has been shown to be a substrate for myosin light chain kinase, whereas thiophosphorylated light chains are

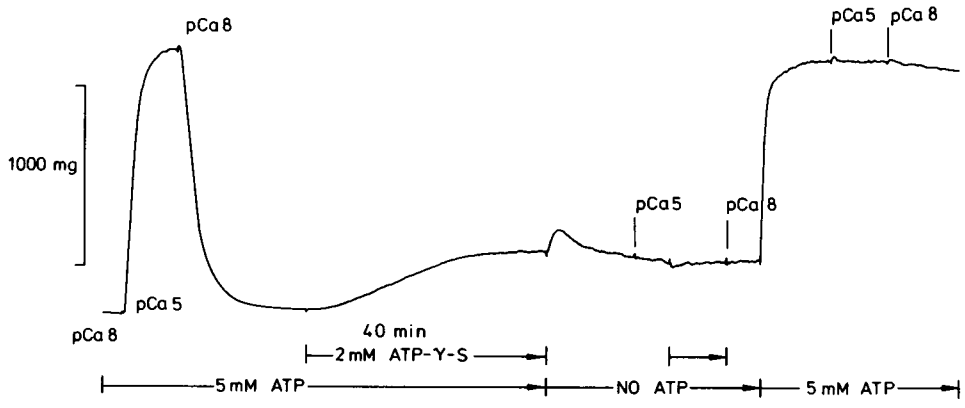

FIGURE 1 A normal Ca^{++}-activated contraction is compared with the effects of Ca^{++}-dependent thiophosphorylation in a segment of skinned guinea pig taenia coli. The details are provided in text.

a poor substrate for the phosphatase activity (19, 20), thus leading to an irreversible thiophosphorylation. Hoar et al. (21) have shown in skinned smooth muscle that treatment with ATP-γ-S in the presence of Ca^{++} leads to an incorporation of thiophosphate into the myosin light chains. As seen in Fig. 1, a 40-min exposure of skinned guinea pig taenia coli to 2 mM Mg-ATP-γ-S in the absence of Ca^{++} and the presence of ATP leads to only a small and slow activation of tension. To determine then whether Ca^{++} was needed to stimulate thiophosphorylation, the ATP was first removed so that a normal Ca^{++}-activated contraction would not result from the addition of Ca^{++}. Allowing 15 min for both ATP-γ-S and ATP to wash out, activating Ca^{++} (pCa 5) was then added and allowed 6 min to equilibrate, which stimulated no tension generation in the absence of ATP. Mg-ATP-γ-S (2 mM) was then added in the presence of Ca^{++} and the absence of ATP. No tension developed as a result of this treatment. After 9 min with ATP-γ-S and Ca^{++}, both were washed out (pCa 8), all still in the absence of ATP. After allowing 5 min for Ca^{++} removal (sufficient time to relax the normally activated preparation), 5 mM Mg-ATP was re-added to the Ca^{++}-Mg-ATP-γ-S treated tissue. Tension development in the absence of Ca^{++} was rapid and identical to that of the initial activation in the presence of Ca^{++}. Subsequent addition (pCa 5) and removal (pCa 8) of Ca^{++} had no effect on this Ca^{++}-dependent-thiophosphorylation-induced Ca^{++}-free contracture. Apparently, in the skinned guinea pig taenia coli, once Ca^{++}-activated phosphorylation has occurred, tension generation is activated regardless of the Ca^{++} concentration, so long as the phosphatase activity is blocked.

Dependence of Isometric Force on $[Ca^{++}]$

Fig. 2 shows the average measured dependence of stable isometric force on free $[Ca^{++}]$ for four skinned taenia coli segments in normal contracting solutions. Maximum tension development was typically 1 kgwt (10 N)/cm^2. The solid line drawn is the fit by linearized least-squares to the functional form:

$$\frac{\Delta P(Ca^{++})}{\Delta P_{max}} = \frac{1}{1 + (K/[Ca^{++}])^n},$$

where $\Delta P(Ca^{++})/\Delta P_{max}$ is the active tension developed at various free $[Ca^{++}]$ expressed relative to the maximum active tension observed at pCa 4.5. This form is derived from models of cooperative multiple-ligand binding and is frequently used to fit force-$[Ca^{++}]$ relations in muscle. In principle, the parameter n, which determines the steepness of the relation, sets a minimum on the number of Ca ions cooperatively bound in activating tension; that is, n ions binding with 100% cooperativity or more than n ions binding but with less than 100% cooperativity. The value K determines some average apparent binding constant. Although this description cannot be taken literally because the model may not strictly apply, the values of n and K so

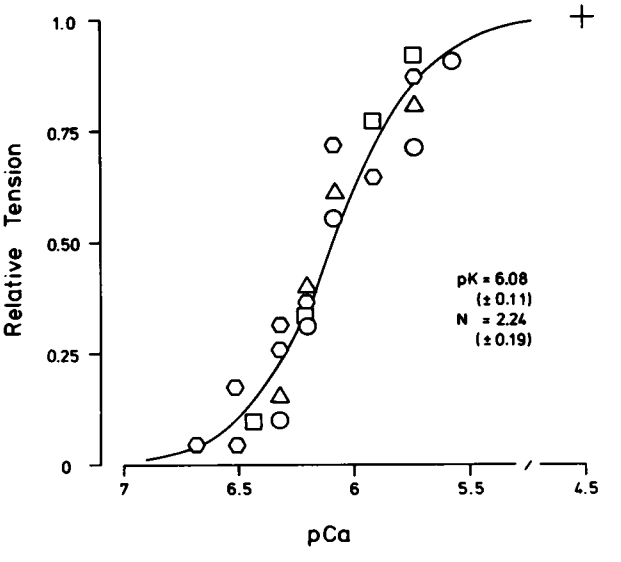

FIGURE 2 Active stable isometric tension developed at various free $[Ca^{++}]$ is expressed as a fraction of the maximal Ca^{++}-activated tension at pCa 4.5 and plotted as a function of $[Ca^{++}]$.

determined nonetheless adequately describe the functional form of the observed force-[Ca^{++}] relationship. The values for n (2.24 ± 0.19) and K ($10^{-6.08\pm0.11}$) obtained from the data of Fig. 2 are similar to those obtained by Gordon (22) in detergent-treated rabbit taenia coli.

Model

On the basis of three assumptions which are, in essence, the model proposed from biochemical experiments on isolated actomyosin (cf. 2, 4), a mathematical model can be readily formulated that makes quantitative predictions regarding the dependence of the rate of isometric tension development on the activating free Ca^{++} concentration.

(*a*) Ca^{++}-calmodulin-activated myosin light chain kinase (MLCK) activity (ECa) leads to an increased steady state level of myosin (M) phosphorylation by a simple mass-action balance with a Ca^{++}-independent phosphatase activity.

$$E \text{ (inactive)} + Ca^{++}\text{-calmodulin} \rightleftharpoons E\text{Ca (active)} \quad (1)$$

$$M \underset{k_d}{\overset{k_p}{\rightleftharpoons}} MP_i, \text{ where } k_p = k\,[E\text{Ca}]$$

$$M_o = M + MP_i, \text{ the total myosin}$$

$$E_o = E + E\text{Ca, the total MLCK.} \quad (2)$$

k is a rate constant per unit of active MLCK and is therefore Ca^{++} independent; as is k_d, the rate constant for the phosphatase activity.

(*b*) A phosphorylated myosin head (MP_i) can interact with actin filaments to produce active tension (ΔP) without the need of further intervention by Ca^{++}.

(*c*) Ca^{++}-dependent tension development relative to the maximum Ca^{++}-activated tension is directly proportional to the Ca^{++}-dependent degree of myosin phosphorylation.

$$\frac{\Delta P(Ca^{++})}{\Delta P_{max}} = \frac{MP_i(Ca^{++})}{MP_{i\,max}}. \quad (3)$$

At a sufficiently high Ca^{++} concentration to activate essentially all available MLCK (ECa $\rightarrow E_o$), maximum force is achieved. Solving the mass balance relation 2 with $k_p = k \cdot E_o$ for the steady state yields that the maximum degree of phosphorylation is

$$\frac{MP_{i\,max}}{M_o} = \frac{\lambda E_o}{1 + \lambda E_o}, \quad (4)$$

where $\lambda = k/k_d$. Hartshorne et al. (23) have observed a typical maximum phosphate incorporation with myosin isolated from visceral smooth muscles on the order of 0.9; λE_o is therefore expected to be on the order of 9 or greater. For submaximal Ca^{++} concentrations, we can again solve the mass balance Eq. 2 which, with Eqs. 3 and 4, leads to an expression that relates relative Ca^{++}-dependent tension development to MLCK and phosphatase activities:

$$\frac{\Delta P(Ca^{++})}{\Delta P_{max}} = \frac{E\text{Ca}}{E_o} \cdot \frac{1 + \lambda E_o}{1 + \lambda E\text{Ca}}. \quad (5)$$

That is, tension as a function of [Ca^{++}] is given by the relative degree of MLCK activation times a weighting factor which includes the relative phosphatase activity.

The functional fit to the force-[Ca^{++}] relation given by the data of Fig. 2 allows then an explicit solution for ECa as a function of [Ca^{++}]. Setting Eq. 5 equal to $1/\{1 + (K/[Ca^{++}])^n\}$ yields

$$E\text{Ca}(Ca^{++}) = \frac{E_o}{1 + (K'/[Ca^{++}])^n}, \quad (6)$$

where $K' = K(1 + \lambda E_o)^{1/n}$. It should be noted that the apparent binding constant K' for the MLCK is shifted from that observed for the force-[Ca^{++}] relation by an amount that depends on the relative phosphatase activity. That is, higher [Ca^{++}] would be needed to activate the same isometric tension if the phosphatase activity is increased. Both K and n are known with some statistical certainty from the fitting parameters of the observed force-[Ca^{++}] relationship.

We can now explicitly calculate the dependence of the initial rate of isometric tension development on the activating [Ca^{++}] by differentiating Eq. 3 with respect to time. Since at time $t = 0$, $M = M_o$ and the forward rate constant for phosphorylation is given by $k_p = k \cdot E\text{Ca}(Ca^{++}, t = 0)$, then the initial rate of phosphorylation [given that Ca^{++} binding is very much faster than the enzymatic activity, so that $E\text{Ca}(Ca^{++}, t = 0)$ equals $E\text{Ca}(Ca^{++})$, cf. 24] is

$$\frac{d}{dt}[MP_i(Ca^{++}, t = 0)] = k \cdot E\text{Ca}(Ca^{++}) \cdot M_o. \quad (7)$$

Substituting Eq. 7 into the time derivative of Eq. 3, substituting $MP_{i\,max}$ from Eq. 4, using the expression for steady state $\Delta P(Ca^{++})$ [equivalent to $\Delta P(Ca^{++})_{final}$ in the time-dependent case] from Eq. 5, and substituting Eq. 6 for $E\text{Ca}(Ca^{++})$ yields that the initial rate of isometric tension development expressed relative to the final steady tension achieved at that activating [Ca^{++}] is

$$\frac{\frac{d}{dt}(\Delta P(Ca^{++}, t = 0)}{\Delta P(Ca^{++})_{final}} = k_d\left\{1 + \frac{\lambda E_o}{[1 + (K'/[Ca^{++}])^n]}\right\}. \quad (8)$$

This expression makes several predictions about the dependence of the initial rate of isometric tension development on the activating free [Ca^{++}]. (*a*) At low [Ca^{++}], the initial rate of tension development is directly proportional to the final tension to be reached; that is, the ratio of initial rate to final tension to be reached is a constant (k_d) determined by the phosphatase activity. (*b*) The apparent pK of the rate-[Ca^{++}] dependence will be shifted to the

right relative to the observed force-[Ca^{++}] pK by an amount that reflects the relative MLCK and phosphatase activities; $\Delta pK = (1/n)\log\ (1 + \lambda E_o)$. (*c*) Tension development rate will show a sigmoidal dependence on [Ca^{++}] with a similar power dependence (n), provided that there are no additional interactions with Ca^{++} other than those provided for in the model. (*d*) The ratio of the fastest relative rate (high Ca^{++}) to the slowest relative rate (Ca^{++} going to 0) is given by $(1 + \lambda E_o)$. In other words, the ratio of maximum phosphorylating activity to phosphatase activity ($k_{p\,max}/k_d = kE_o/k_d = \lambda E_o$) determines uniquely the maximum degree of phosphorylation, the pK shift of the rate-[Ca^{++}] relation relative to the force-[Ca^{++}] relation, and the maximum extent by which the rate of tension development relative to the final force may be accelerated by Ca^{++}.

Dependence of the Rate of Isometric Tension Development on [Ca^{++}]

Fig. 3 (upper portion) illustrates the influence of free [Ca^{++}] on the rate of isometric tension development for three rapid "Ca jump" contractions in a single skinned taenia coli segment. In the lower panel, the time-courses of the three isometric contractions above have been normalized by expressing tension (as a function of time) relative to the final steady tension attained at each [Ca^{++}]. The contraction at pCa 5.4 is nearly 10 times more rapid in its onset than that at pCa 6.2, even under these strong Ca jump conditions where the entry of Ca^{++} is not rate-limiting. Similar experiments were performed with eight taenia coli segments from three animals over a wide range of pCa. The data from those experiments are normalized as in Fig. 3 and shown in Fig. 4. The maximal initial rates of isometric tension development seen at pCa 4.5 averaged 1.46% (±0.05) final tension per second. For low free-Ca^{++} concentrations (pCa > 6.0), the relative speed of tension development becomes constant, showing no tendency to decline below ~0.13%/s as [Ca^{++}] is further reduced to pCa 6.5. This was prediction (*a*) of the model and, from Eq. 8, is the rate constant for the phosphatase activity, k_d. Using these values for the maximum and minimum initial rates of tension generation, we compute $\lambda E_o = 10.2$, which gives a computed maximum degree of phosphorylation of 91%. Also, from this value of λE_o and assuming the expected value of n (2.24) measured from the force-[Ca^{++}] relation of Fig. 2, the value of pK' can be calculated from Eq. 6 to be 5.62. The solid curve drawn in Fig. 4 is simply Eq. 8 with the computed values of K' ($10^{-5.62}$) and λE_o (10.2) and n = 2.24 substituted. The fit to the experimentally observed dependence is very good, which suggests that the assumptions on which the model is based are not unreasonable.

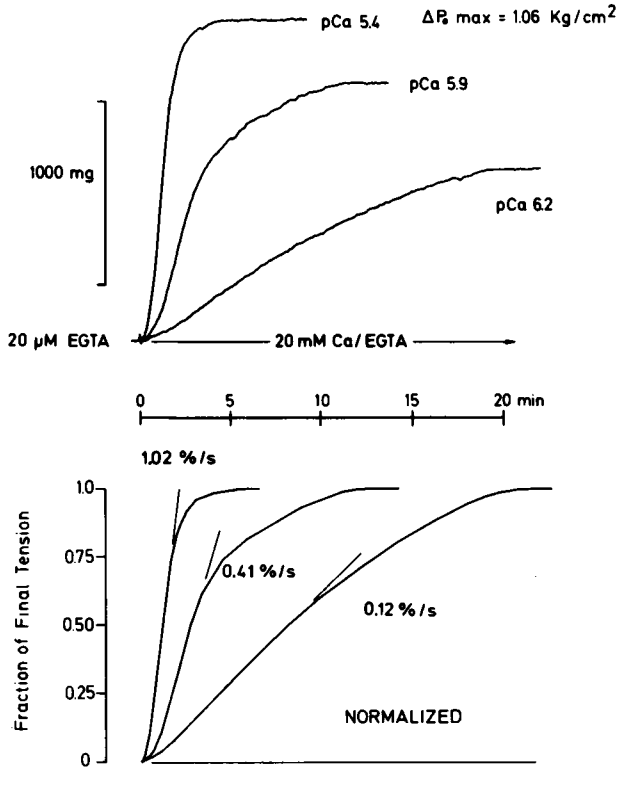

FIGURE 3 The influence of [Ca^{++}] on the rate of isometric tension development is illustrated by an experiment in a single skinned taenia coli segment. Force is shown in gram weight (1 gwt ~0.01 N). The three traces were superimposed at the small solution-change artifact. In the lower panel, the tension records are simply divided by the final tension attained at each pCa and replotted. The lines indicate the values of initial tension development rate taken from the early linear portion of the tension development record.

DISCUSSION

Using a simple model derived from biochemical experiments with isolated smooth muscle actomyosin in which a

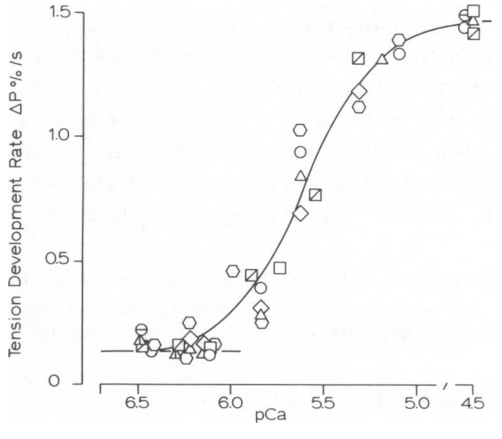

FIGURE 4 The relative rate of isometric tension development is plotted against pCa for experiments with eight taenia coli segments skinned by "freeze-glycerination." The solid line is the fit of the model to the observed data, using the parameters measured in Fig. 2.

dynamic balance between Ca^{++}-dependent phosphorylation and Ca^{++}-independent dephosphorylation of myosin controls the extent of tension development, the detailed dependence of the relative rate of isometric tension development on activating free $[Ca^{++}]$ can be accurately predicted. The model goes on then to suggest some interesting correlations between the various regulatory enzyme activities and mechanical behavior. The mechanical relation between the rate of tension development and free $[Ca^{++}]$ predicts, if the model is correct, that the phosphatase activity rate constant is on the order of 0.13%/s, where the percent sign refers to dephosphorylation of the total phosphorylated myosin. The maximum initial rate of myosin phosphorylation (kE_o) is on the order of 1.3%/s, where the percent sign refers to the total myosin content. At maximum isometric tension, about 90% (according to the model) of the myosin is phosphorylated.

The myosin content of visceral smooth muscles is, in general, on the order of 15 mg/g smooth muscle tissue (25). Mrwa and Hartshorne (15) derived a total yield of MLCK from gizzard of ~0.02 mg/g tissue, without including losses during purification. Assuming molecular weights of 250,000 and 105,000, respectively, one computes a myosin light chain content of ~0.12 μmol light chain/g and 0.2 nmol MLCK/g. The estimated maximum rate of myosin phosphorylation (1.3%/s) gives an apparent turnover number for MLCK then of ~8 s^{-1}. The isolated MLCK had a turnover number with whole myosin as substrate of ~3 s^{-1} when adjusted to 37°C. The turnover number is affected by ionic strength and the physical state of the substrate (15), so that these numbers are actually in reasonably good agreement. An actual MLCK content about two to three times higher than that estimated above could also account for the difference.

The model presented above further allows us to compute from the mechanical data that the apparent Ca^{++} binding constant (pK') for the Ca^{++} activation of MLCK (Eq. 6) is 5.62. The activation of MLCK is known to be mediated by Ca^{++} binding to calmodulin. Robertson et al. (24) have determined the apparent binding constant of calmodulin for Ca^{++} to be 5.62. The exact numerical agreement is probably fortuitous, but the general agreement nonetheless suggests that the affinity of calmodulin for Ca^{++} is the prime determinant of the force-$[Ca^{++}]$ and rate-$[Ca^{++}]$ relations observed in skinned guinea pig taenia coli.

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