A QUANTITATIVE MODEL OF MYOSIN PHOSPHORYLATION AND THE PHOTOMECHANICAL RESPONSE OF THE ISOLATED SPHINCTER PUPILLAE OF THE FROG IRIS

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ABSTRACT The time courses of isometrically recorded photomechanical responses of isolated sphincter pupillae of *Rana pipiens* can be accurately predicted by a set of differential equations derived from phosphorylation theory of smooth muscle contraction. We compared actual light-stimulated contractions with calculated ones over a wide range of stimulus intensities (56-fold) and durations (0.4–4.0 s). The hypothetical Ca^{++}–calmodulin–myosin light chain kinase cascade acts as a "valve" to control the flow of ATP through a phosphorylation–dephosphorylation cycle. When the rate of flow of ATP through the phosphorylation–dephosphorylation cycle is increased, the percentage of phosphorylated myosin increases. The time courses of the concentrations of phosphorylated myosin during different responses are seen to be functions of the time courses of the opening and closing of the coupling cascade "valve." The calculations predict experimentally measurable intermediate variables, which can aid the investigation of the application of quantitative phosphorylation theory to amphibian sphincter pupillae and to smooth muscle in general.

INTRODUCTION

This study makes direct comparisons of the time courses of photomechanical responses of the isolated sphincter pupillae and the equivalent predictions of a simple version of the myosin phosphorylation hypothesis of smooth muscle contraction.

The sphincter pupillae of the frog, *Rana pipiens*, contracts when illuminated with an action spectrum similar to the absorption spectrum of rhodopsin (1). The response, which is graded, occurs without any intervening change in the transmembrane potential or the presence of external calcium (1). This stimulus–response sequence is interesting from at least two different standpoints. First, by inference from the contraction itself, it is an example in a vertebrate system (smooth muscle) of the absorption of light by rhodopsin, causing a release of intracellular calcium. Second, since the contraction is a continuous function of the stimulus parameters, the photomechanical response provides a good system for testing quantitative hypotheses of smooth muscle contraction. It is the latter aspect we will explore in this paper. The sphincter pupillae, whose degree of contraction determines pupil size, is a small annular smooth muscle lying close to the pupillary margin. In amphibia, although the sphincter pupillae is innervated (2), the photomechanical response of the muscle is more important in controlling the pupil diameter than is the better known retinal reflex characteristic of pupillary control in mammals. Although it is weak, the direct photomechanical response does occur in a number of species of mammals.

As far as is known, the activation of contraction in all muscles requires the rise of intracellular calcium (3). In recent years, evidence has accumulated that indicates that calcium activates the contractile machinery of smooth muscle via calmodulin–myosin kinase cascade (4, 5). While this hypothetical scheme has been widely accepted, in some preparations there may be a second subsequent requirement for Ca^{++} for the contractile apparatus to maintain contractility (6, 7). The complication of a second Ca^{++} process has been studied in tonic contractions lasting many minutes, which were elicited by muscarinic agonists or high extracellular potassium solutions. Nonetheless, the initiation of contraction in all cases seems to be closely related to the phosphorylation of myosin via a Ca^{++}–calmodulin cascade. In the experiments reported here, the sphincter pupillae were stimulated with short flashes (<10 s) and the response durations were always <30 s. In the model we have investigated, myosin is phosphorylated when myosin light chain kinase (MLCK) is activated by calcium-activated calmodulin.

Previous theoretical analyses of the myosin phosphorylation models by Peterson (8) and Kato et al. (9) have been

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concerned largely with the responses of smooth muscles to step changes in internal calcium, mimicking experiments using skinned fibers. Peterson (8) made the simplifying assumptions that the phosphorylation and dephosphorylation of myosin is rate limiting for changes of tension (i.e., that all other steps in the stimulus–response sequence are essentially in equilibrium with free Ca^{++} and that active force is proportional to the amount of myosin phosphorylated). These assumptions allowed the algebraic calculation of steady-state tensions and maximum rates of tension development by freeze-glycerinated strips of guinea pig *Taenia coli;* these calculations corresponded well with experimental values. Peterson (8) showed that the apparent binding constant of Ca^{++} for activation of MLCK is similar to the apparent binding constant of calmodulin for Ca^{++} (i.e., pK ~5.62). Kato et al. analyzed the steady-state responses of a Ca^{++}–calmodulin-dependent activation of a network of MLCK and myosin phosphatase that control tension. In addition to steady-state solutions, Kato et al. calculated the time courses of tension as a result of simple arbitrary transients of calcium concentration, that of steps and ramps. Following Peterson (8), Kato et al. (9) implicitly assumed that all steps involving Ca^{++} binding and leading up to the activation of MLCK are infinitely fast and are always in equilibrium with the free calcium concentration.

The kinetic analysis described here differs from these earlier studies in two regards. First, the present model includes the stimulus to smooth muscle explicitly as the first step in the activation sequence. It makes no simplifying assumptions about steps or ramps, etc. in the level of internal calcium. This expansion of the analysis and its comparison to experiment is possible because of the special nature of the frog iris photomechanical response, i.e., the output is a continuous function of the input. Second, the present analysis makes no assumptions about rate-limiting reactions or that certain reactions are always at equilibrium, but instead the time courses of mechanical responses are calculated directly from the set of differential equations that describe the chemical steps related to (*a*) the movement of calcium in and out of intracellular stores, (*b*) the calcium–calmodulin cascade, and (*c*) the enzymatic phosphorylation and dephosphorylation of myosin.

The purpose of this paper is to examine quantitatively a tractable version of the myosin phosphorylation hypothesis to see how well it can predict real contractile responses. The testing of quantitative predictions of the myosin phosphorylation hypothesis, in this way, is complementary to experimental physiological and biochemical methods. The analytic solution of the equations describing even a simple chemical network is not feasible, therefore digitally computed numerical solutions will be used throughout.

EXPERIMENTAL METHOD

Rana pipiens were kept in the dark for several days at room temperature. All experimental procedures were carried out in light passed through Wratten II filters. After the animals were decapitated, the eyeballs were excised and pinned down in a Sylgard bottomed petri dish. The cornea was removed and the ring-shaped sphincter pupillae preparations were made by a circular cut in the iris about one-third of the way out from the pupillary margin. The resulting annular preparation was hung vertically between a hook in a small chamber and another hook suspended from the tension transducer. The tension transducers used were either a Grass Instrument Co. (Quincy, MA) FT-03 or the more sensitive Cambridge Instruments, Inc. (Monsey, NY) model 403. They gave equivalent results for larger responses. Some responses were taken from an earlier study (1) for comparison. The responses were recorded on a Grass Instrument Co. polygraph recorder and also stored digitally on floppy discs using a PDP-11 system. The solute concentrations of the physiological saline were NaCl, 110 mM; KCl, 2 mM; Tris maleate, 2.5 mM; NaH_2PO_4, 0.5 mM; Ca (gluconate), 1 mM; Na_2 succinate, 2 mM; glucose, 10 mM; and Na_2 EDTA, 0.1 mM. The solutions were saturated with air and adjusted to pH 7.3. The experiments were run at room ambient temperature, 22°–25°C.

White light stimuli were provided by imaging a 3,000°K tungsten filament on the preparation. Incident intensity was varied using Kodak neutral density filters.

The strength and speed of the photomechanical responses of preparations from different frogs varied somewhat with the size and physiological status of the preparations. In good preparations the amplitudes of photomechanical responses to flashes in the near linear range decreased by <25% in 4 h. The latent period, time-to-peak force, and the empirical time constant for decay of force at the end of a response all changed to a smaller degree, <10%. Typically, a muscle left overnight (12–18 h) would respond to equivalent stimuli with a third or more of original peak force. The latent period would change little but the time-to-peak and the decay time constant often increased as much as two or three times. The durations of experiments was typically <4 h.

DESCRIPTION OF THE MODEL

An important problem that must be faced if computer simulations are to be useful tests of biochemical models is the minimization of free parameters. Although there are literally no biochemical data available from the sphincter pupillae muscle, there is a sizeable collection of kinetic and compositional data available from other smooth muscles. Therefore, we were able to start with reasonable a priori estimates of most of the system parameters.

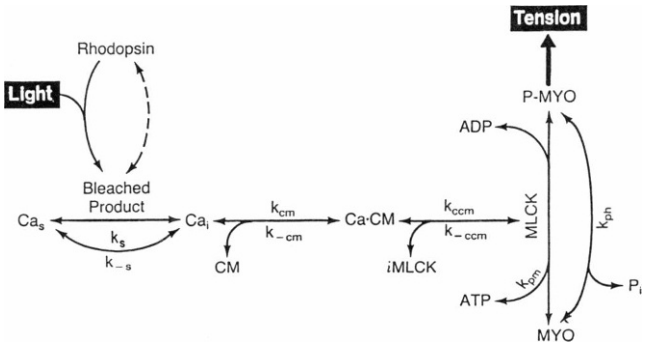

FIGURE 1 A myosin phosphorylation hypothesis describing the photomechanical response of the frog sphincter pupillae. This scheme shows the components and chemical rate constants used to calculate the photochemical responses to flashes of light varying in duration and intensity. Ca_s, intracellularly stored Ca^{++}; Ca_i, intracellular Ca^{++}; CM, calmodulin, Ca · CM, the calcium–calmodulin complex; iMLCK, inactive MLCK; ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, inorganic phosphate; MYO, myosin; and PMYO, phosphorylated myosin. Values of the parameters are given in Table I.

A final important aspect of this study results from the fact that the contractile response of the spincter pupillae is graded with respect to illumination over a wide range of light stimulus intensities and durations; therefore, the hypothetical activation sequence must predict the output relationships over a wide range. The behavior of the present generalized dynamic model is much more complex than the earlier quasi-equilibrium models. In the next portion of this paper before we relate our results we will (*a*) describe the set of biochemical reactions that constitute our physical model of the photomechanical response, (*b*) convert the equivalent biochemical equations into mathematical ones and estimate and rationalize the values of the system parameters, and finally (*c*) describe the computer technique we used to numerically describe our mathematical model.

A Hypothetical Activation Sequence for the Frog Sphincter Pupillae: The Physical Model

We have taken the main sequence of chemical steps in the activation process as shown in Fig. 1 from references 4 and 5. This simplified scheme does not include a role for a cAMP effect on MLCK. This was a tentative deletion whose justifications were the desire for simplicity and the observation that agents that increase or act like cAMP attenuate but do not change the time course of the photomechanical response.

There are several steps in the scheme of Fig. 1 that subsume a number of substeps. The first step is a good example of this. After the absorption of quanta, rhodopsin molecules quickly form metarhodopsin and decay through a well known series of dark reactions. Since the action spectrum of the frog iris approximates the main absorption peak of frog retinal rhodopsin and the dark adaptation curve of the iris is similar to the frog electroretinogram dark adaptation curve, we have had no reason to suspect the rhodopsin cycle in the iris is much different from that in the retina. Because it is most likely that the absorption of one photon causes the release of many Ca ions into the cytoplasm, we have indicated in the schema that some downstream result of bleaching will act on the rate at which Ca^{++} leaves its storage compartment and enters the cytosol. Although the most probable source of Ca^{++} might be thought to be a sarcoplasmic reticulum system (4) there are other possibilities; for example, that the triggering calcium is bound to some cytosolic component or that it is associated with an organelle whose membrane contains rhodopsin. If the rhodopsin in the iris is associated with G proteins, as is likely, inositol triphosphate may well be the second messenger between the light receptor and the release of intracellular calcium. In any case, the formalism of the present model (Fig. 1) simply stipulates that there is a storage depot that has a light-modulated calcium leak. In the dark there must be a steady state between the stored calcium (Ca_s) and the free cytoplasmic calcium (Ca_i). If the storage depot is assumed to be the sarcoplasmic reticulum, then it would follow that k_s is the empirical rate constant for the sarcoplasmic reticulum membrane leak and k_{-s} is the empirical Ca^{++} pump rate constant.

The next step in the activation pathway is the binding of Ca^{++} to calmodulin. The role of Ca^{++}–calmodulin complexes in modifying a variety of enzymatic activities and in particular, MLCK, is well established (10). However, although equilibrium binding studies show that calmodulin has four Ca^{++} binding sites, it is not yet agreed as to how many need to be occupied by Ca^{++} to allow activation of MLCK. Data from skinned smooth muscle (*Taenia coli*) yield empirical stoichiometric coefficients for Ca^{++} activation of slightly greater than two (8). Nonetheless, if for any of a variety of kinetic reasons the binding of one of the calcium ions is much more significant than the others (e.g., the binding site might be much slower), then a one Ca^{++} scheme would be appropriate.

After the Ca^{++}–calmodulin complex is formed, it in turn combines with an inactive form (iMLCK) of MLCK, converting the latter into the active MLCK (11). In this case, the binding stoichiometry is definitely 1:1 (12). Since the product of this step is an active enzyme rather than a reactant, this step would seem a priori to be an important regulation point. It may be that the activation of myosin requires both light chains to be phosphorylated, as has been inferred from the degree of phosphorylation as a function of MgATPase activity (13). Still, in the simple scheme (Fig. 1), a single phosphorylation step is used, which of course could be kinetically valid if the phosphorylation of one head was much faster than that of the other (14).

The last step in the control sequence is the one that turns off muscle activation, the dephosphorylation of p-myosin. There are apparently several enzymes with myosin light chain phosphatase activity in smooth muscles. Presently it is not possible to decide if there are or are not more than one involved in physiological relaxation (15–17), so that the k_{ph} of the model must be considered to be an "effective" rate constant.

The Steady State of the Sphincter Pupillae in the Dark

It is assumed that the fraction of myosin that is phosphorylated is equal to the fraction that the steady-state tension is of the maximum conceivable tension generated by the sphincter. In short, the fractional steady-state force is assumed to be proportional to the fraction of phosphorylated myosin:

$$T/T_m = [\mathrm{PMYO}]/M_{\mathrm{Total}}. \quad (1)$$

It is also assumed that [PMYO] is in a steady state in the dark and not at equilibrium with MYO. True equilibrium would require equilibration between [ATP], [ADP], and [P]. Because the phosphorylation and dephosphorylation

of myosin occur cyclically, the product of the equilibrium constants of these reactions must equal the equilibrium constant for the hydrolysis of ATP. In other words, the sum of the free energies of these reactions equals the energy of hydrolysis of ATP. If the free energy released when myosin is phosphorylated by the terminal phosphate of ATP is small enough, then the fraction of the myosin that can ever be phosphorylated due to activation of MLCK will be significantly different from 100%. If the free energy released when myosin phosphate is dephosphorylated is too small, then a significant concentration of myosin phosphate would exist in equilibrium with phosphate in the cytoplasm. It is assumed that neither of these cases occurs and that both steps are highly exergonic and thus for the steady state the back reactions can be ignored for both the phosphorylation and dephosphorylation steps, i.e., $k_{pm} \gg k_{-pm}$ and $k_{ph} \gg k_{-ph}$. Therefore, in the dark steady state,

$$k_{pm}\,[\text{MLCK}]\,[\text{MYO}] = k_{ph}\,[\text{P} - \text{MYO}]\,. \qquad (2)$$

Given that $[Ca_i]$ is constant in the dark steady state, then it and the two association/dissociation reactions leading to the formation of MLCK constitute a closed system (Fig. 1) and both steps are in equilibria described by their respective dissociation constants:

$$K_{Dccm} = k_{-ccm}/k_{ccm} = [\text{Ca} \cdot \text{CM}]\,[\text{iMLCK}]/[\text{MLCK}] \qquad (3)$$

and

$$K_{Dcm} = k_{-cm}/k_{cm} = [Ca_i]\,[\text{CM}]/[\text{Ca} \cdot \text{CM}]. \qquad (4)$$

Therefore from Eqs. 2–4, [PMYO] is a function of $[CA_i]$ as follows: define $C_T = ([\text{CM}] + [\text{Ca} \cdot \text{CM}] + [\text{MLCK}])$ (i.e., the total concentration of calmodulin) and $K_T = [\text{iMLCK}] + [\text{MLCK}]$ (i.e., the total concentration of MLCK). Since $K_T \ll C_T$, [MLCK] can be ignored relative to C_T (i.e., $C_T = ([\text{CM}] + [\text{Ca} \cdot \text{CM}])$). For any steady-state combination of Eqs. 2–4 elimination of CM and MLCK results in

$$[\text{PMYO}] = M_T[Ca_i]/(K_{Dcm}K_{Dccm}k_{ph}k_{pm}^{-1}C_T^{-1}K_T^{-1} + (K_{Dccm}k_{ph}k_{pm}^{-1}C_T^{-1}K_T^{-1} + k_{ph}k_{pm}^{-1}K_T^{-1} + 1)[Ca_i]). \qquad (5)$$

Thus, the parameter values chosen for calculations describe the steady-state as well as the photomechanical responses. Although there are many published measurements related to various constants in Eq. 5, there are no available data in the literature for several of them. Where they could not reasonably be determined from the steady state, they were left as free parameters in the calculations within certain constraints (vide infra). In attempts to calculate the behavior of other biochemical sequences it has not often been possible to use values determined in vitro for calculations of in vivo transients (18). The deviations of the best fit parameter values from those given in the literature are possibly due to differences between the physical and chemical conditions for a binding or reactions rate determination in a partially purified preparation from an organ homogenate, etc. and the conditions for the same determination in a living cell.

Therefore, values for the parameters of Table I were chosen by various compromises between (*a*) adherence to values reported in the literature, (*b*) compatibility with reasonable steady-state values, and (*c*) the goodness of fit of the photomechanical response. There are enough conflicts between these goals for the primary system parameters to prevent simultaneous satisfaction of each of all three criteria. To facilitate estimation of the primary parameters

TABLE I

Parameters	Value	Source or comment
Primary		
Ca_T	2×10^{-3} M	Reference 22
C_T	4×10^{-5} M	Reference 10
K_T	2×10^{-7} M	Reference 8
M_T	4×10^{-5} M	Reference 10
k_s	8.25×10^{-5} s^{-1}	Calculated from steady-state assumptions and reference 23
k_{-s}	33 s^{-1}	Calculated directly from reference 23
k_{cm}	8.25×10^{5} M^{-1} s^{-1}	Calculated from program "optimization" of $F_1 = k_{cm}C_T/k_{-s}$, i.e., setting $F_1 = 1$
k_{-cm}	8.25	Calculated from k_{cm} and $K_{Dcm} = 1 \times 10^{-5}$ M (19)
k_{ccm}	5×10^{7} M^{-1} s^{-1}	Calculated from program "optimization of $F_2 = k_{ccm}K_T/k_{-cm}$, i.e., setting $F_2 = 1.2121$
k_{-ccm}	5 s^{-1}	Calculated from k_{ccm} and $K_{Dccm} = 1 \times 10^{-7}$ M (19)
k_{pm}	4.29×10^{5} M^{-1} s^{-1}	Calculated from Eq. 8 and 14, see text
k_{ph}	0.1287	Calculated from Eqs. 8 and 14, see text
Secondary		
K_{Dcm}	1×10^{-5} M	The dissociation constant for calcium–calmodulin, reference 19
K_{Dccm}	1×10^{-7} M	The dissociation constant for (calcium–calmodulin) MLCK, reference 19
F_1	1	Model fitting, equal to $k_{cm}C_T/k_{-s}$, coupling efficacy of Ca_i
F_2	1.2121	Model fitting, equal to $k_{ccm}K_T/k_{-cm}$, coupling efficacy of Ca–calmodulin
F_3	0.6667	Steady-state considerations, equal to $k_{pm}K_T/k_{ph}$, maximum attainable ratio of PMYO/MYO
λ	0.142	Time constant for late decay of tension, assumed to be equal to $k_{ph} + k_{pm}[\text{MLCK}]_{dss}$

from physiological considerations, three secondary parameters, F_1, F_2, and F_3 were defined.

$$F_1 = k_{cm} C_T / k_{-s} \quad (6)$$

$$F_2 = k_{ccm} K_T / k_{-cm} \quad (7)$$

$$F_3 = k_{pm} K_T / k_{ph}. \quad (8)$$

F_1 and F_2 are the ratios of the maximum forward rate constants to the backward rate constants of the two binding steps involving calmodulin. They represent a kind of efficacy of the coupling sequence. After a first approximation of from 1 to 100, the values of these parameters were statistically fitted to obtain the best fits of the photomechanical calculations (see below). The third parameter, F_3, has a peculiar significance for the steady state since it is equal to maximum possible ratio of phosphorylated myosin to myosin, i.e., the ratio that would occur in the real muscle if [MLCK] equals K_T (Eq. 2). Thus as a free parameter, F_3 is an important determinant of maximum steady-state tension. It is also relatable to the steady-state $[Ca_i]$ under all circumstances by further consideration of Eq. 5. Of particular interest is the steady state of the unstimulated muscle in the dark. From Eq. 8 it may be inferred that it is unlikely that F_3 is less than one-half since that would mean that less than half the myosin will ever be turned on. In contrast, consideration of Eq. 9, obtained by combining Eqs. 8 and 5 for any steady state

$$M_T/[\text{PMYO}] = 1 + F_3^{-1} + F_3^{-1} K_{Dccm} C_T^{-1} + F_3^{-1} K_{Dccm} K_{Dcm} C_T^{-1} [Ca_i]^{-1}, \quad (9)$$

shows the interplay for any steady state between steady-state tension, steady-state $[Ca_i]$, and F_3. To evaluate the right-hand terms, the values of various other parameters must be fixed, preferably from the literature. In addition, the relative magnitudes of C_T, K_{Dccm}, K_{Dcm}, and $[Ca_i]$ must be such that $M_T/[\text{PMYO}]$ has a reasonable value. The physiological data indicate that perhaps as much as 20% of the maximum observable force is present in the dark steady state. Therefore, $M_T/[\text{PMYO}]$ was conservatively set to be 10 for the dark steady state. On the basis that concentration determinations are less likely to be in error by orders of magnitude than are kinetic measurements, the total amounts of calmodulin, MLCK, and myosin (Table I) were set as fixed parameters in the analysis. Given that C_T is at least 4×10^{-5} M (Table I), the third term on the right-hand side of Eq. 9 cannot contribute significantly to the right-hand sum because 10^{-7} M must be the upper bound for the dissociation constant, K_{Dccm}. The values given K_{Dccm} by Walsh and Hartshorne (10) are in the 10^{-9}–10^{-8} M range, but Demaille (19) gives data yielding a K_{Dccm} nearer 10^{-7} M. Phosphorylation of turkey gizzard MLCK increases K_{Dccm} some 20 times to $\sim 5 \times 10^{-8}$ M, according to Conti and Adelstein (20). For compatibility with reasonable intracellular Ca^{++} concentration values, K_{Dccm} was set equal to 10^{-7} M, which was as large as possible given the literature values (10, 19). By the same reasoning, K_{Dcm} was set equal to 10^{-5} M even though most physiological measurements put the calcium–calmodulin dissociation constant a factor of three smaller (8) (but see reference 21 also). Even with these extreme values for these parameters, including F_3 being equal to 0.667, the internal calcium concentration in the dark steady state is only 0.5×10^{-8} M. Since this value can only be made larger at the expense of awkward changes of the other parameters, the rather low estimate of the intracellular calcium concentration for an unstimulated smooth muscle (22) was accepted.

In summary, the biochemical parameters (Table I) of the hypothetical activation scheme (Fig. 1) were reconciled so that (*a*) the calculated maximum phosphorylation of myosin is 40%, (*b*) in the dark steady state, the myosin is 10% phosphorylated, and (*c*) the intracellular calcium concentration is 0.5×10^{-8} M. Although not absolutely fixed these values can be changed very little without making some other parameter take on unacceptable values or without compromising the model.

Estimation of the Remaining Parameters of the Model

The time courses of photomechanical responses of sphincter pupillae resulting from square wave illumination of different intensities and durations can be predicted from differential equations, which describe the steps of the hypothetical pathway. The following Eqs. 10–13 define the model.

For the relationship between the incident light intensity and the rate of release of Ca^{++} into the myoplasm, the simplest possible assumption, proportionality was chosen. From these assumptions and Fig. 1 one may write for the rate of change of the internal free calcium convention

$$d[Ca_i]/dt = K\, I(t, D) + k_s Ca_s + k_{-cm}[Ca \cdot CM] - (k_{-s} + k_{cm}[CM])[Ca_i], \quad (10)$$

where K is a constant, which includes the stimulating light intensity ($K = kI$). The units of K are mol s^{-1}. D is the stimulating light duration. $I(t, D)$ equals 1 for $t \leq D$ and equals 0 for $t > D$.

For the rate of change of concentration of the calcium–calmodulin complex

$$d[Ca \cdot CM]/dt = k_{-ccm}[MLCK] + k_{cm}[CM][Ca_i] - k_{-cm}[Ca \cdot CM] - k_{ccm}[iMLCK][Ca \cdot CM]. \quad (11)$$

Similarly, for the rate of change of concentration of MLCK

$$d[MLCK]/dt = k_{ccm}[iMLCK][Ca \cdot CM] - k_{-ccm}[MLCK]. \quad (12)$$

Finally for the rate of change of phosphorylated myosin

concentration

$$d[PMYO]/dt = k_{pm}[MLCK][MYO] - k_{ph}[PMYO]. \quad (13)$$

(Note both backreactions are ignored.)

Up to this point there are four free parameters, two from the binding steps (because only the dissociation constants K_{Dcm} and K_{Dccm} were fixed) and k_{pm} and k_{ph}. Consideration of Eq. 13 allows an additional constraint on the parameters of the system of equations. From the experimental records it was clear that the declining phase of a photomechanical response is an exponential and that the rate constant, λ, for the decline is essentially constant for a given muscle (1). For the muscle response simulated here $\lambda = 0.143\ s^{-1}$. This value was obtained using a multiexponential time constant extracting program, PEEL (written by Randall Stein and available on the University of Illinois Cyber 175). From this exponential relaxation it was inferred that [MLCK] is essentially at its dark steady-state value during relaxation and therefore, from Eq. (13)

$$\lambda = k_{ph} + k_{pm}[MLCK]_{dark\ steady\ state}. \quad (14)$$

Taking Eqs. 8 and 14 and the setting $F_3 = 0.667$, it was calculated that $k_{ph} = 0.1287\ s^{-1}$ and $k_{pm} = 4.29 \times 10^5\ M^{-1}s^{-1}$.

The values for k_{pm} and k_{ph} obtained in this way are similar to values available from the literature (8, 10).

The result of these approximations was to leave only two free parameters. In short, one rate constant of each of the two cascade binding steps still needed to be estimated. This problem was approached by considering the secondary parameters F_1 and F_2. These representative parameters are important in determining how effectively a light-evoked perturbation passes through the system from a change in $[Ca_i]$ to a final change in [PMYO]. Initial values between 1 and 100 were used for the fitting.

Computational Method

Computer-simulated photomechanical responses were obtained by numerically solving the set Eqs. 10–14. ACSL (Advanced Continuous Simulation Language) was used with the following options; (*a*) IALG = 2 (Gear's stiff algorithm), (*b*) simulation output interval 0.1 s, and (*c*) calculation interval of 1 ms. The computations were run on the University of Illinois CYBER 174–175 system. The values of F_1, F_2, and F_3 were adjusted to 1, 1.21212, and 0.66683, respectively. These values were accepted as reasonable for the biochemical requirements.

A nonlinear optimization procedure NONLSQ (solution of a nonlinear least squares problem) and a data interpolation procedure INTERPOLATE from the package PROTRAN combined with a procedure DGEAR (a differential equation solver for variable order using the Adams predictor corrector method or Gears method) from the package IMSL was used to estimate the free parameters F_1 and F_2. The main parameters in these procedures were: For DGEAR, N = 4 (input number of first-order differential equations); TOL = 10^{-4} (input relative error bound); H = 0.00001 (the step on the first call); METH = 2 (input basic method indicator. METH = 2 implies the stiff methods of GEAR are to be used); MITER = 1 (input interaction method indicator. MITER = 1 implies that the chord method is used with an analytic Jacobian).

The initial values of (F_1, F_2) (1.0, 1.21212) yielded estimation values (0.892646, 10.41300) with FF = 0.02227, where $FF = (D(t_i) - S(t_i))^2$, $D(t_i)$ is the experimental data value, and $S(t_i)$ is the simulation value at the moment t_i both normalized according to their own peak values.

For the initial guess (1.0, 1.21212), FF = 0.0376, which is still giving a quite good fit. If F_1 and F_2 are in the neighborhood of 1, the fit is always quite good and not very sensitive to F_1 and F_2.

Therefore, the values $F_1 = 1$ and $F_2 = 1.21212$ were used. The small loss of goodness of fit seen in Figs. 5 and 6 when taken one by one, F_1 and F_2 halved and doubled, demonstrates the stiffness of this system.

Calculation of the Photomechanical Responses

Fig. 2 shows how well the calculations fit the experimentally measured tension time courses obtained in response to 2-s flashes of light of different intensities. It has been known for some time (1) that over a considerable range of flash intensities, the time courses of the photomechanical responses to short flashes normalized to the response peak tension are virtually superimposable. The amplitudes of the responses increase monotonically with light stimulus intensity but their normalized shapes are the same, inde-

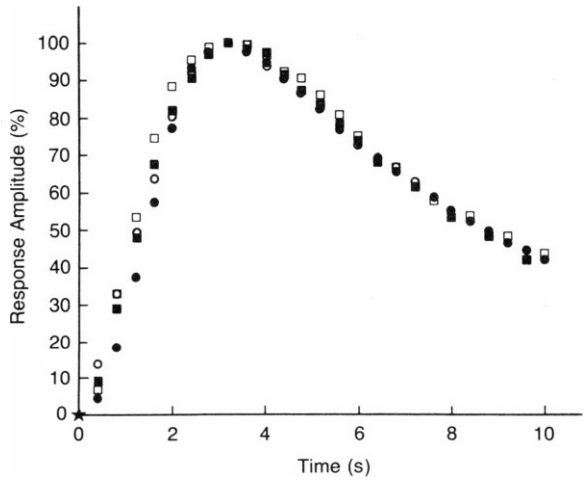

FIGURE 2 The normalized time courses of photomechanical responses to 2-s light flashes. The force generated during each response is expressed as a percentage of the peak force for that response. *Open circles* and *open squares*, experimental iridial sphincter force time courses after 2 s of exposure of the sphincter preparations to 0.74×10^3 and 41.2×10^3 lux, respectively (1). *Solid circles* and *solid squares*, calculated force time courses using K equal to 1.796×10^{-7} and 10^{-5} mol s^{-1}, respectively.

pendent of intensity. In Fig. 2 are shown two experimentally measured tension time courses resulting from two flashes that differed by a factor of 56× in intensity and two calculated tension time courses whose "theoretical light input" also differed by a factor of 56. When adjusted to the time of the rise of tension, they conform closely to each other.

The calculations also predict the change of amplitude of the photomechanical responses with flash intensity. Fig. 3 shows how the experimentally determined photomechanical response amplitudes compare with those resulting from simulating the responses to different strength stimuli. Experimental intensities were varied by a factor of 179 and theoretical intensities were varied by a factor of 1,000. The model parameter "k," which fixes the proportionality between light intensity and rate of rise of myoplasmic calcium, was not optimized by computer but merely adjusted to show the similarity of the dependencies of response amplitudes on light stimulus intensity for both the experimental and the calculated photomechanical responses.

Whereas the force–time waveform of responses does not change with light intensity, it certainly does change with flash duration. Longer flashes prolong the period of force generation, yielding larger peak forces and longer delays before reaching peak force. Longer flashes evoke responses of larger amplitude and longer duration. Fig. 4 shows the degree to which the simulated responses accurately predict the time courses of the experimentally measured photomechanical responses to flashes of different durations from 0.4 to 4 s. The preparation in this case was a different one, but the simulation parameters were not changed. The simulation parameters fitted to predict one of the experimental responses accurately result in accurate predictions of other responses due to either different intensities or durations of stimuli.

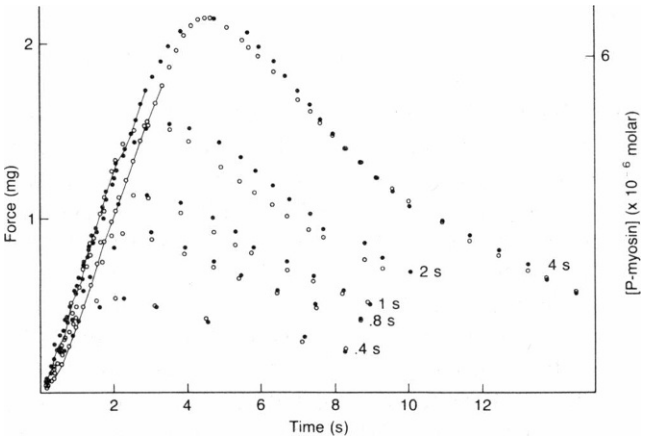

FIGURE 4 The time courses of experimental and calculated photomechanical responses evoked by flashes of different durations. *Open circles,* time courses of forces evoked by flashes of different durations but constant intensity (0.74×10^3 lux). *Solid circles,* time courses of the simulated photomechanical responses to "stimuli" of durations matching with the experimental ones.

The maximum rate at which the calcium–calmodulin complex can be formed per unit calcium concentration is $k_{cm}C_T$. Therefore the parameter F_1 (Eq. 6) gives the maximum ratio of formation of the complex to resequestration of any calcium released by photobleaching of rhodopsin. The response waveform changes shape when F_1 is changed away from its near an optimum value. Fig. 5 shows the effect on the time course of the photomechanical response of halving and doubling the value of F_1.

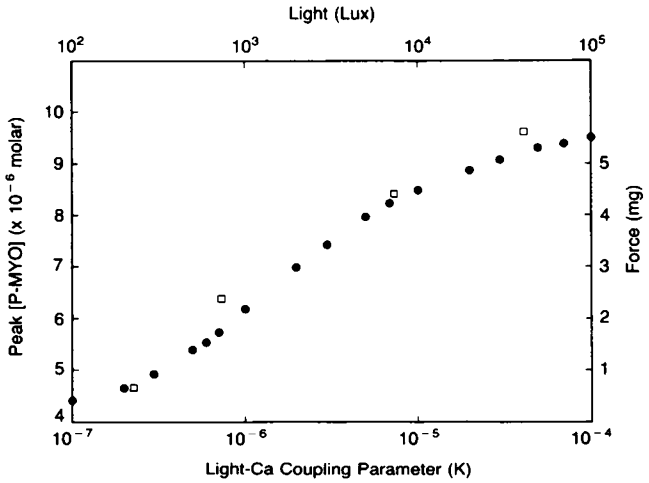

FIGURE 3 The variation of the peak force of photomechanical responses as a function of stimulating light intensity. *Open squares,* experimentally measured peak forces for four photomechanical responses to 2-s flashes of light of 0.23, 0.74, 7.28, and 41.2×10^3 lux, respectively. The upper and right-hand coordinate scales refer to the experimental data (1). *Solid circles,* time courses of responses that result from 2-s theoretical stimuli varying over a range of 1,000-fold in intensity.

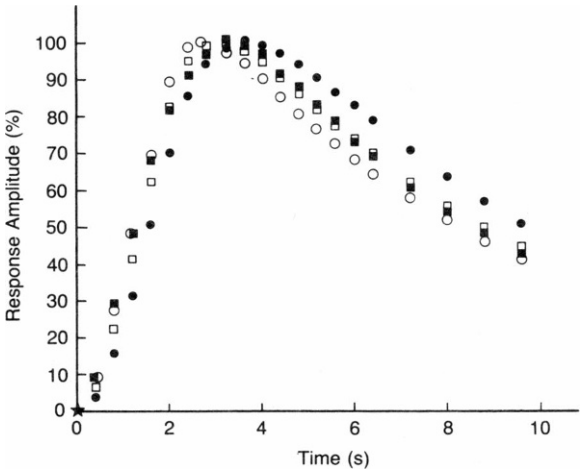

FIGURE 5 The effect of variation of parameter, F_1, on the normalized time course of simulated photomechanical response to 2-s flashes. The response amplitude was calculated as percent of peak for each response. The values for F_2 and F_3 used in these simulations were 1.21212 and 0.66683, respectively. *Solid squares,* experimental data points. *Open squares,* the simulated data points using $F_1 = 1$. *Open circles,* calculated data points obtained using $F_1 = 2$. *Solid circles,* calculation data points obtained using $F_1 = 0.5$.

Similarly, the manner in which the secondary parameter F_2 (Eq. 7) also influences the waveform of the photomechanical response indicates the influence of the rate of binding of the calcium–calmodulin complex to inactive MLCK relative to the rate of decay of the calcium–calmodulin complex back to Ca^{++} and calmodulin. Fig. 6 shows the change in waveform of the simulated iridial photomechanical response as a result of halving and doubling the value of F_2.

Variation of the value of the secondary parameter F_3 (Eq. 8) had a much smaller effect on the time course of the simulated photomechanical response. In contrast, it had a strong influence on both the predicted dark steady-state force and the simulated amplitude of the peak force generated. Fig. 7 shows the relationship between the dark steady-state force and F_3. The dark steady-state force goes up monotonically with F_3 following the curve of a rectangular hyperbola. The relationship between the peak force of a response to a short flash and F_3, however, shows a maximum as F_3 is increased. This reflects the fact that as F_3 and [MLCK] increase in the dark steady state, the MCLK available for a phasic activation must eventually decrease. The F_3 value 0.66683 is almost a factor of 10 smaller than the F_3 value, which gives the largest peak response amplitude. While the fraction of the myosin that is phosphorylated is an intensive property of a muscle, force is not. The bigger the muscle the bigger the force. Therefore to compare muscles we have used normalized forces and concentrations of phosphorylated myosin. Although we used a simulated flash of intermediate intensity for demonstrating how the response peak varies with

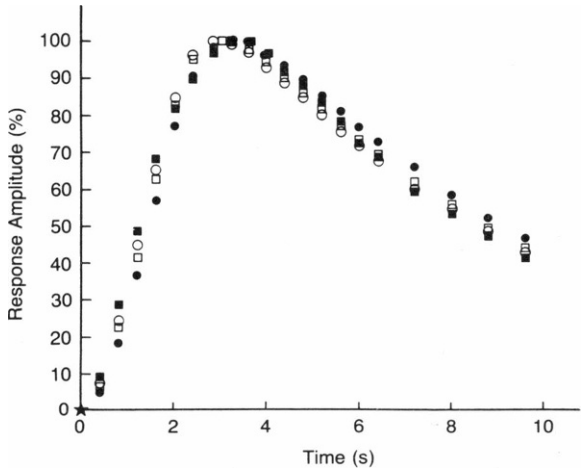

FIGURE 6 The effect of variation of the parameter, F_2, on the normalized time courses of calculated photomechanical responses to 2-s flashes. The response amplitude was calculated as percent of peak for each response. The values for F_1 and F_3 used in these calculations were 1.0000 and 0.66683, respectively. *Solid squares*, experimental data points. *Open squares*, calculated data points using F_2 = 1.21212. *Open circles*, calculation data points obtained using F_2 = 2.42424. *Solid circles*, calculation data points obtained using F_2 = 0.60606.

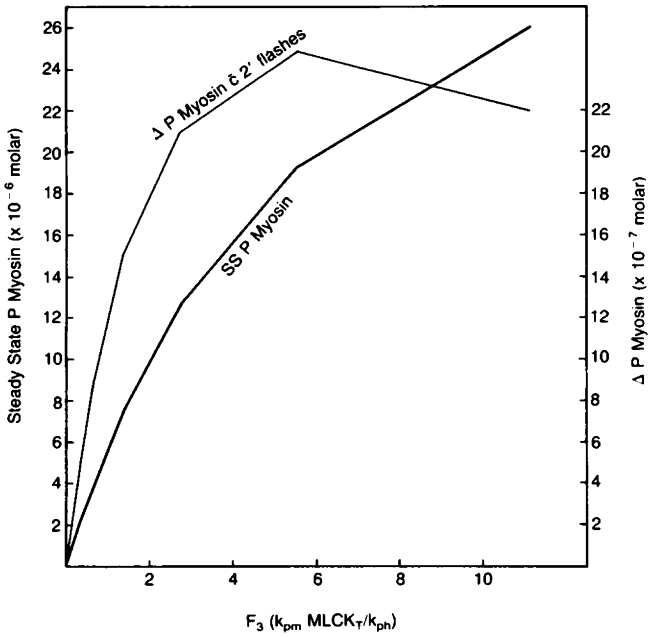

FIGURE 7 The variation of predicted dark steady-state P-myosin and the peak of the change of P-myosin concentration during photomechanical response with changes in the parameter F_3. The relationships between the steady-state myosin concentration (left-hand coordinate) the amplitude of the phosphorylated myosin concentration transient (right-hand ordinate) and F_3 are plotted. These data were obtained with best fit values for F_1 and F_2 and the stimulus parameters being 2-s and $K = 10^{-7}$. The latter is equivalent to an intermediate experimental light intensity. Increases in F_3, beyond 5.3, cause a decrease in light-elicited increase in [PMYO].

F_3, very large simulated stimuli result in closer approaches to maximum phosphorylation and asymptotic saturation type curves.

One of the most useful aspects of simulating reaction networks is the prediction of the time courses of the concentrations of the intermediates of the stimulus–response sequence. In Fig. 8 are shown (*A*) MLCK concentration transients and (*B*) phosphorylated myosin concentrations as functions of time after 2-s simulated stimuli of different intensities. For higher intensities the [MLCK] rises to a plateau in <0.25 s (Fig. 8 *A*). Clearly, earlier intermediates ([Ca_i] and [Ca · CM]) will approach a constant value even sooner. In this range of stimuli, the rise of [PMYO] and tension approach an exponential whose argument is dominated by k_{ccm} [MLCK]. For smaller simulated intensities the rise of [MLCK] does not reach a limit even in 2 s and the [PMYO] and tension curves are more complicated.

The return of the [MLCK] to dark steady-state levels is essentially complete in 5 s after the beginning of a 2-s simulated flash. This predicts that relaxation of the muscle, which has barely begun at 5 s, will be essentially an exponential thereafter as described by Eq. 14, dominated by $k_{ph} + k_{pm}$[MLCK].

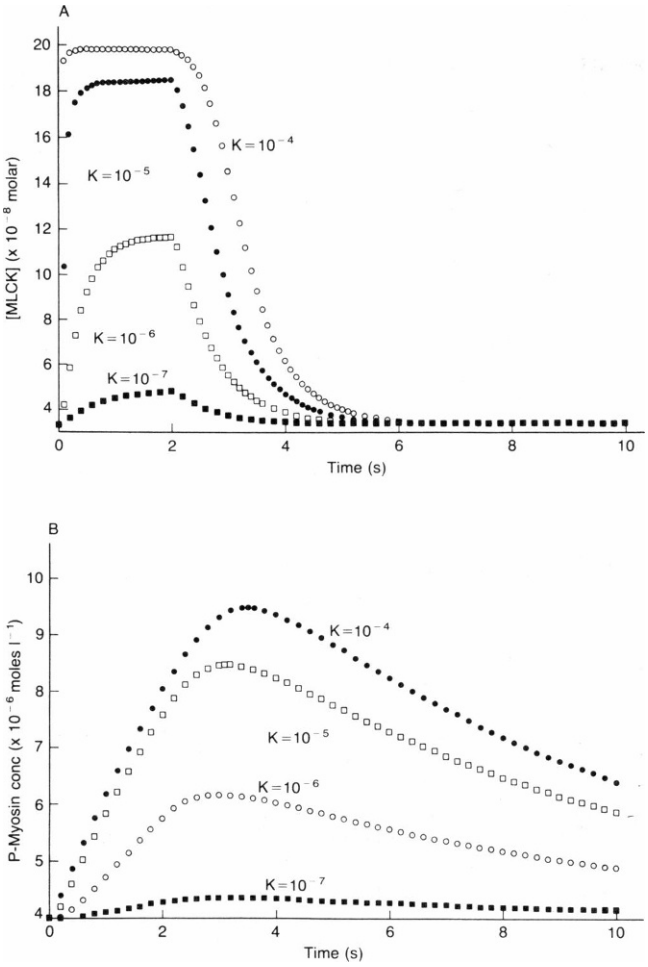

FIGURE 8 The calculated time courses of intermediates for light flashes of 2-s duration and differing intensities. (*A*) Time courses of the concentration of active MLCK for four different intensities of flashes 2-s long. (*B*) Time courses of the concentration of phosphorylated myosin for the same flashes as in *A*.

DISCUSSION

It has been demonstrated that equations derived from the myosin phosphorylation theory predict that the change in phosphorylated myosin concentration after a light flash follow the time courses of photomechanical responses of the sphincter pupillae within experimental error. The success of the model encourages experimental testing of its predictions of the time courses of the intermediates Ca^{++}, Ca^{++}–calmodulin, and MLCK. It should be noted, however, that increasing the hypothetical number of calciums bound to calmodulin will predict a latent period. Some of the successes of the model lead to tentative hypotheses about the muscle operation, for example, the goodness of fit during the relaxation phase does not lend support to the idea that a rise of cAMP-dependent protein kinase is participating in the relaxation process. Our steady-state results with the iris sphincter tend to corroborate Peterson's steady-state calculations for skinned guinea pig *Taenia coli* (7). They are also compatible with the steady-state results of Kato et al. (20). However, in this latter study, which simulated the change in tension of a generalized skinned muscle after a step change of bath [Ca], a series elastic component (SEC) was introduced while we did not do so. The present study also differs in the sense that we simulated a transient response of an intact muscle to a physiological stimulus, and so it is important that the extra complexity of an SEC is not needed to obtain accurate predictions of this muscle's behavior. The present model is equivalent to assuming a series elastic element that does not contribute because the change of activation of the muscle is slow enough that the series elastic component is always in force equilibrium with the force generator of the muscle. By this assumption we do not intend to dismiss the SEC as an important kinetic component of smooth muscle because we believe it is. That the simpler model predicts adequately, however, does require further study. In a recent report Kamm and Stull (14) have found that stiffness, phosphorylation, and force have latencies ~0.5 s in tracheal smooth muscle, although a rise of external force then lags behind the other variables. Another possible complication is the presence of other cytoplasmic proteins that might bind Ca-activated calmodulin. The simplest effect of such proteins would be to act as a buffer relative to activated complex. It does not seem necessary to invoke this complication as part of this first step of analysis.

The model can be improved by reevaluating the assumption of proportionality between the intensity of stimulating light and the rate of release of Ca^{++} from intracellular stores. One might expect a more complicated relationship since one would expect that the efficiency of quanta to bleach rhodopsin would fall off with stimulus intensity and duration. Yet as demonstrated by Figs. 2 and 3, the simulated phosphorylated myosin responses are virtually indistinguishable from the experimental photomechanical responses for flashes in the neighborhood of 1-s duration over a wide range of intensities. We neither expect nor does the model predict that the shape of responses to 2-s flashes will be independent of intensity over all intensities. However, the experimental data are not presently available to test all the simulations available. The simulation data point out the need to obtain experimental measurements at both much higher (Fig. 2) and much lower (Figs. 3 and 8 *B*) intensities.

Putting all the issues into balance, we conclude from this study that a simple kinetic scheme derived from the phorphorylation theory of smooth muscle gives rise to accurate enough quantitative predictions of a wide range of the photomechanical responses of the sphincter pupillae of *Rana pipiens* to warrant increased confidence in the essential correctness of that theory. This approach provides some first steps towards generating a quantitative theory of smooth muscle contraction.

It is tempting to make the analogy that the phosphoryla-

tion–dephosphorylation cycle works like a flow (of myosin) into a (P myosin) bucket controlled by a valve (MLCK), whose degree of opening is stimulus controlled and where the bucket also has a leak (P myosin phosphatase). The amount of myosin in the P myosin bucket would, in the analogy, be proportional to the number of active cross-bridges. Even though this view is too simple to be completely valid, it does predict that light intensity (determining the amplitude of the valve opening) would have a strong influence on response amplitude and a weaker effect on response time course. That the degree of quick turn on of the MCLK value varies with intensity can be seen from Fig. 8. For a 2-s flash during the time that MLCK is elevated but nearly constant, after ~1 s, force should rise exponentially. After MLCK falls back to unstimulated levels (after 4 s), the decay of force should follow an exponential time course determined by the phosphatase and unstimulated MCLK activities. This is exactly what is seen experimentally (1).

Fig. 3 shows that for short flashes and intermediate levels of light intensity, the amplitude of the photomechanical responses should be nearly proportional to the logarithm of light intensity. Thus, a direct way to estimate k, the proportionality constant between light intensity and rate of calcium release from storage of the model, would be to adjust it to provide the best fit of the model predictions to the experimental data. Because the amounts of melanin in and around the smooth muscle cells affect k strongly, this correlation will be more accurately made using albino animals.

The prediction from the biochemical schema of the relative influences of intensity and duration is a strong feature of the model. The strongest feature of the model is its ability to predict the response time courses for a range of flash intensities and durations after only two of 12 parameters have been optimized by fitting to a single response while the other 10 were fixed a priori from the biochemical literature. Finally, this is the first attempt to completely predict the mechanical response of a smooth muscle from a mathematical description of reasonable underlying biochemical events after physiological stimulation.

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