Kinetics of Contraction Initiated by Flash Photolysis of Caged Adenosine Triphosphate in Tonic and Phasic Smooth Muscles

KEISUKE HORIUTI, AVRIL V. SOMLYO, YALE E. GOLDMAN, and ANDREW P. SOMLYO

From the Pennsylvania Muscle Institute and the Department of Physiology, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19104

ABSTRACT Laser flash photolysis of caged adenosine triphosphate (ATP), in the presence of Ca^{2+}, was used to examine the time course of isometric force development from rigor states in glycerinated tonic (rabbit trachealis) and phasic (guinea-pig ileum and portal vein) smooth muscles. Photolytic liberation of ATP from caged ATP initiated force development, at 20°C, with half-time ($t_{1/2}$) of 5.4 s in trachealis and 1.2–2.2 s in the phasic muscles. Prior to photolysis, some muscles were phosphorylated with ATP plus okadaic acid (an inhibitor of myosin light-chain phosphatase) or thiophosphorylated with ATPγS to fully activate the regulatory system, before turning on the contractile apparatus. In these prephosphorylated muscles, force development, after caged ATP photolysis, was more rapid than in the unphosphorylated muscles, but the $t_{1/2}$ values for trachealis (0.8–1.1 s) were still longer than for ileum and portal-vein muscles (0.20–0.25 s). The results suggest that both the contractile machinery and the regulatory system are slower in the tonic than in the phasic smooth muscles. The time course of force development for each muscle type was sigmoidal, with an initial delay (t_d) of ~10% of the $t_{1/2}$ value. Some possible chemical and mechanical origins of the delay are discussed.

INTRODUCTION

Smooth muscles can be classified into two major subtypes: fast-contracting phasic and slow-contracting tonic smooth muscles (Somlyo and Somlyo, 1968*a*). The basis of this classification, at the level of electrophysiological properties of surface membrane, is well established. Tonic smooth muscles respond to excitatory transmitters with graded depolarization without action potentials, whereas phasic muscles normally generate action potentials (Somlyo and Somlyo, 1968*b*). In addition, in response to depolarization with high K^+ solutions, tonic smooth muscles tend to

Address reprint requests to Dr. Keisuke Horiuti and Dr. Avril V. Somlyo, Department of Physiology, Box 449, Medical Center, University of Virginia School of Medicine, Charlottesville, VA 22908.

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exhibit slow, sustained K^+ contractures, whereas phasic smooth muscles tend to show a fast transient phase (Somlyo and Somlyo, 1968*a*).

It is generally accepted that the activation level of smooth muscle contraction is controlled by phosphorylation of the myosin regulatory light-chain. There is a balance between phosphorylation of the light-chain by a calcium-calmodulin regulated kinase and dephosphorylation by phosphatases (Hartshorne, 1987). According to this hypothesis, the different rates of force development in various smooth muscles could be controlled by the rates of myosin light-chain phosphorylation, as well as by the intrinsic reaction rates of the contractile machinery.

The main purpose of the present experiments was to compare both the light-chain phosphorylation rate and the reaction rate of the contractile machinery of, respectively, tonic and phasic smooth muscles. A previous study (Somlyo et al., 1988) indicated that these two reaction rates could be distinguished by activating muscle via photolysis of caged ATP (Kaplan et al., 1978; Goldman et al., 1984*a*). In glycerinated portal-vein smooth muscles, the rate of contraction initiated by photolysis of caged ATP, in the presence of Ca^{2+}, was accelerated 10-fold by prior thiophosphorylation of the myosin light-chain (Somlyo et al., 1988). This result suggested that the onset of contraction of muscles that had not been previously phosphorylated was rate-limited by myosin light-chain phosphorylation, whereas the fast contraction of thiophosphorylated muscles more closely reflected the reaction rate of the contractile machinery.

Because the effect of thiophosphorylation with ATPγS in the previous study could be different from that of normal phosphorylation with ATP, it was also desirable to examine contractions in muscles that were prephosphorylated with normal (not sulfur-substituted) inorganic phosphate. A recently characterized inhibitor of myosin light-chain phosphatase, okadaic acid (Tachibana et al., 1981; Bialojan et al., 1988), was suitable for this purpose, as it would maintain high phosphorylation levels even in the absence of ATP before caged ATP photolysis.

Our results suggested that myosin light-chain phosphorylation and the intrinsic contractile reaction are both several times faster in phasic than in tonic smooth muscles, and also supported the hypothesis that phosphorylation rate limits force development in muscles that are not prephosphorylated. A preliminary report of this study has been presented (Horiuti et al., 1989).

METHODS

The experimental procedures and setup were as in a previous study (Somlyo et al., 1988; Goldman et al., 1984*a*) with minor modifications. Smooth muscle preparations were dissected from guinea-pig ileum and portal-anterior mesenteric vein as examples of phasic muscle, and from rabbit trachea as tonic muscle. The tissues were immersed in a Hepes-buffered Krebs solution (Himpens and Somlyo, 1988).

Longitudinal smooth muscle strips of portal vein were prepared in Krebs' solution, as described previously (Somlyo et al., 1988). The longitudinal muscle layer of the ileum (thickness ~50 μm) was peeled away from segments of the gut with serosa on it (Himpens and Somlyo, 1988), and then transferred to a relaxing solution containing 2 mM EGTA (*G2*; Horiuti, 1988), where it was split into 0.3–0.4-mm-wide strips.

For the dissection of bundles of trachealis muscle, the connective tissue around the trachea was first removed, and the trachea was opened longitudinally in the cartilaginous region. The

tissue was transferred from Krebs' solution to the relaxing solution. The mucosa and the adventitia of the membranous portion were removed, leaving the trachealis muscle (thickness ~0.1 mm) attached to the cut ends of the cartilages. Bundles of fibers could be seen and were cut or teased with a fine needle into small strips 0.2–0.3 mm wide.

To permeabilize the surface membranes of the smooth muscle cells, we treated the muscles with 50% glycerol in a relaxing solution (*Relax;* see below) at −18°C for at least 1/4 h, but not more than 2 d. The relaxing solution also contained 40 mM glutathione as a thiol protecting agent. After glycerol treatment, the muscle strips were cut to short lengths and attached to the force measurement apparatus by aluminum foil T-clips. Clip-to-clip length of the preparations was 1–2 mm for trachealis and 2–3 mm for ileal and portal-vein muscle.

Experimental Protocol

The muscle was mounted in the experimental apparatus and stretched to 1.2 times its resting length while in a relaxing solution (*Relax;* in millimolar: 2 Na_2ATP, 5.7 $MgCl_2$ for 1.5 free Mg^{2+}, 20 EGTA, 40 glutathione, pH 7.1, adjusted with KOH and buffered with 30 Pipes, and ionic strength 200 adjusted with 21.4 Na_2-creatine phosphate [CP]). The muscle was then

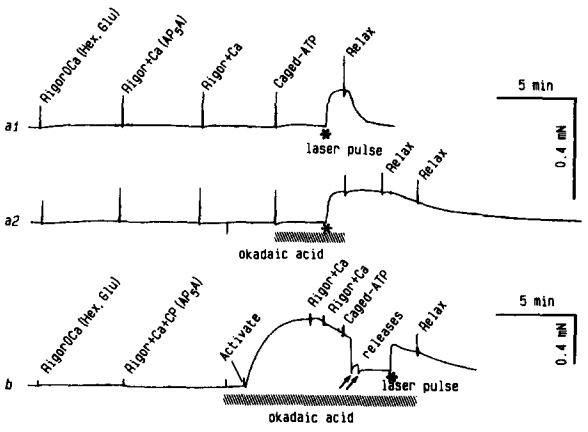

FIGURE 1. Force records on a slow time base to show the experimental protocols. (*a1*) Not prephosphorylating; (*a2*) not prephosphorylating, but with okadaic acid present in the caged-ATP solution; (*b*) prephosphorylating with Ca^{2+} and okadaic acid. See Methods for details. *a1* and *a2* are consecutive runs with a trachealis specimen, but *b* is from a different trachealis. The thiophosphorylating protocol has been shown previously (Somlyo et al., 1988).

activated with a Ca^{2+} solution (*Activate;* 20 mM-CaEGTA, ~3×10^{-5} M-free Ca^{2+}) to test the force-generating capacity of the muscle. The Ca^{2+} was washed out before force reached a plateau to minimize damage to the preparation by a maximal contraction. All of the experiments were carried out at room temperature (19–21°C).

Fig. 1 shows strip chart recordings of force development during several of the experimental protocols. Before application of caged ATP (Fig. 1 *a1*), the muscle was put into rigor by washing out ATP with an ATP-free solution (*Rigor OCa;* HDTA, instead of CP, for adjustment of ionic strength). To ensure ATP depletion, 20 U/ml hexokinase and 5 mM glucose were added to the rigor solution. After 5 min incubation in this solution, the muscle was further incubated with Ca^{2+}, still without ATP (*Rigor +Ca;* 20 mM-CaEGTA), for 5 min. To inhibit possible production of ATP from ADP by intrinsic myokinase in the muscle, an inhibitor, 50 μM-diadenosine pentaphosphate (AP_5A), was added to this solution.

When thiophosphorylation of myosin light-chains was intended, the muscle was further incubated with 2 mM-ATPγS still in the presence of AP_5A (*Thio*) for 9 min (see Fig. 13 *A* in Somlyo et al., 1988, for illustration of this protocol). Force usually developed in the ATPγS

solution, and, therefore, some muscles were released to a lower force level after the washout of ATPγS, to reduce possible effects of stretching out the series elastic elements on the rate of force development.

AP_5A or AP_5A plus ATPγS was washed out of the muscle by incubation in the plain *Rigor +Ca* for 4 min, and then the preparation was transferred to a solution containing 10 mM caged ATP (P^3-1-[2-nitrophenyl]ethyladenosine-5′-triphosphate; solution *Photolysis +Ca* in Somlyo et al., 1988; labeled *Caged-ATP* in Fig. 1). After a period of 2.5–3 min for diffusion of caged ATP throughout the preparation, a 50-ns pulse of 347 nm light from a frequency-doubled ruby laser was applied to the muscle chamber to liberate ~1 mM ATP exponentially at 120 s^{-1} (Fig. 1 *) and initiate contraction. When force reached a plateau, the muscle was transferred to *Relax* and the force returned toward baseline. In some runs with ATPγS, caged ATP was dissolved in a Ca^{2+}-free solution (*Photolysis OCa*), and in this case the preceding rigor solution was also Ca^{2+}-free (*Rigor OCa*). Occasionally, an ATP regenerating system was included in the caged ATP solution (*Photolysis +Ca +CP;* 10.5 mM-creatine phosphate).

When we examined effects of the phosphatase inhibitor, okadaic acid, on muscles that were not prephosphorylated (Fig. 1 *a2*), 10 μM of the toxin was added to both the cage and the preceding rigor solutions.

In some experiments (Fig. 1 *b*) the myosin light-chains were prephosphorylated with ATP and, using okadaic acid, kept phosphorylated until photolysis pulse. For those experiments the muscle was put into *Rigor OCa* with hexokinase and glucose, followed by *Rigor +Ca +CP* with AP_5A. 10 μM okadaic acid was added to this and all subsequent solutions. The muscle was next maximally activated with ATP and Ca^{2+} (*Activate*), ATP was then removed by applying *Rigor +Ca* for 3–5 min and then *Photolysis +Ca* (labeled *Caged-ATP* in Fig. 1). Approximately half the maximum active force remained in the last solution, so some muscles were released (Fig. 1 *b*, *arrows*) to a lower force level just before the photolysis.

Calmodulin (5 μM) was added to all Ca-containing solutions. 50 U/ml creatine phosphokinase was added to all solutions containing creatine phosphate. All solutions except *G2* used for dissection also contained protease inhibitors, 1 μM leupeptin, and 1 mM phenylmethylsulphonyl fluoride (PMSF), and a mitochondrial blocker, 1 μM carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP). Solutions with okadaic acid contained 0.1% dimethyl sulfoxide (DMSO) as a vehicle. This concentration of DMSO had no detectable effect on the rate of force development. For details of solution composition, see the previous paper (Somlyo et al., 1988).

Only one, two, or occasionally three experimental trials were carried out with each muscle strip, because after permeabilization, there was a decrease in the maximum force observed when trials were repeated or prolonged. The measured half-time ($t_{1/2}$; see below) for force development was probably not seriously affected within a single contraction by the trial-to-trial rundown, because the time course of the force decline was always some 10 times slower than that of the main rising phase of contraction. Assuming that both force deterioration and development occurred exponentially with 10-fold different time constants, the measured half-time of force development would be truncated by only ~25% due to the force deterioration.

For measurement of phosphorylation level of myosin light-chain, muscle specimens were rapidly frozen with liquid nitrogen or copper hammers cooled to that temperature. The hammer device was mounted on the photolysis apparatus and allowed the freezing of prephosphorylated muscles under identical conditions to those used for photolysis experiments. The frozen specimens were stored in liquid nitrogen. The phosphorylation levels were measured by Dr. T. M. Butler, with two-dimensional gel electrophoresis as described previously (Butler et al., 1983).

Data Collection and Analysis

The time course of isometric force development after photolysis of caged ATP was displayed on a pen recorder and also stored digitally on video tape via a modified digital audio processor (Bezanilla, 1985). The force signal was sampled and digitized to be stored at 44 kHz and 16-bit resolution. We wanted to analyze the initial delay of the force development with millisecond resolution, while also recording the whole time course of force development that occupied up to 50 s. To achieve this, the data on the video tape were restored with logarithmic time compression by a microcomputer. Approximately 50 s of the force response starting 0.1 s before the laser pulse were redigitized (12-bit resolution) at an interval of 1 ms. The 50×10^3 samples generated were reduced to 640 samples by averaging intervals of 10 ms before the laser pulse and intervals ranging smoothly from 1 ms near the pulse to ~0.5 s at the end of the record.

When the 640 data points in the compressed form were plotted on a linear abscissa, the axis was nearly proportional to the logarithm of (time after photolysis + 100 ms). The time from the photolysis to half the plateau force level ($t_{1/2}$) was measured on this logarithmic display. When the initial part of force development was replotted using a linear time axis (Fig. 2), the traces were appreciably sigmoidal, indicating a lag in force development. A line was

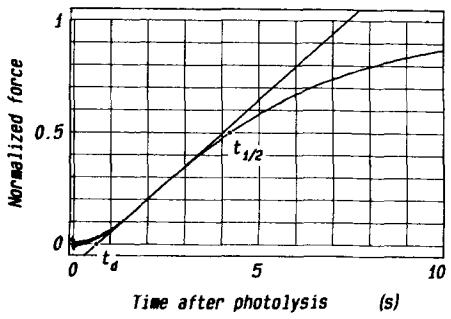

FIGURE 2. Measurement of the delay (t_d) and the half-time ($t_{1/2}$) in force development upon photolysis of caged ATP. Laser pulse for photolysis of the caged compound occurred at time 0 on the abscissa. The force was normalized to the amplitude of its response, from the force level immediately after the photolysis to the peak. The muscle was a trachealis not prephosphorylated. 10 μM okadaic acid was present.

fitted to the steepest portion, chosen by eye, of each force trace, and the time (t_d) from the laser pulse to the baseline intercept of this line (Fig. 2) was measured as an index of the delay.

RESULTS

Time Course of Force Development in Trachealis Muscle

After liberation of ATP by photolysis of caged ATP, force development by not prephosphorylated (Fig. 1 *a1*) trachealis muscles was slow (Fig. 3 *n*), the average half-time ($t_{1/2}$) being 5.4 s. Although there was appreciable variation from preparation to preparation (Table I; not prephosphorylated, without okadaic), this $t_{1/2}$ was significantly longer ($P < 0.02$) than the 2.2-s $t_{1/2}$ of the phasic ileum and portal-vein muscles measured under the same conditions (Table II). The 20-kD myosin light-chain in trachealis frozen in *Rigor OCa* migrated as only one spot, localized to the region of nonphosphorylated light-chain, on two-dimensional electrophoresis gels ($n = 2$), indicative of minimal (< 5%) phosphorylation of the light-chains.

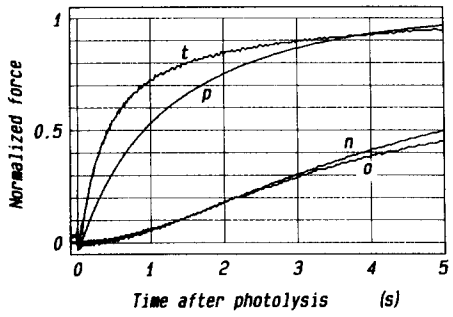

FIGURE 3. Force responses of trachealis muscles after photolysis of caged ATP. (*t*) Muscle thiophosphorylated before photolysis; (*p*) muscle prephosphorylated using ATP and okadaic acid; (*n*) muscle not phosphorylated before photolysis; (*o*) another not prephosphorylated muscle, but okadaic acid (10 μM) was added to the photolysis solution. *t* and *n* are records obtained from the same strip, *o* from another one from the same animal, and *p* was obtained from a different animal. Each force record was normalized to its amplitude of response. An HPLC analysis revealed that the amount of liberated ATP was 1.4 mM in the run *t*, 1.0 mM in *p*, 1.5 mM in *n*, and 1.4 mM in *o*.

Thiophosphorylation of trachealis muscle with ATPγS accelerated contraction, shortening $t_{1/2}$ by a factor of ~7 (Fig. 3 *t*; Table I, thiophosphorylated). This shorter $t_{1/2}$ was not significantly altered by the absence of Ca^{2+} during photolysis (Table I), suggesting that thiophosphorylation is sufficient for full activation, as reported by others (Cassidy et al., 1979). Gel electrophoresis carried out on separate muscle preparations ($n = 2$) showed almost complete (one spot) thiophosphorylation of the light-chain.

We also prephosphorylated (Fig. 1 *b*) the light-chains with ATP, rather than ATPγS, and attempted to maintain the elevated level of phosphorylation during ATP withdrawal by washing ATP out in the presence of Ca^{2+} and the phosphatase inhibitor, okadaic acid (Fig. 1 *b*). The trachealis muscles pretreated in this way con-

TABLE I

Half Time ($t_{\frac{1}{2}}$) of Force Development in Trachealis Muscle

Not prephosphorylated			Thiophosphorylated		Prephosphorylated (using ATP and okadaic acid)*	
Without okadaic		With okadaic*	With Ca^{2+}	Without Ca^{2+}		
11	2.2‡	5.8	0.84	1.93	0.95	1.0¶
2.9	8.6	6.4	1.02	0.51	0.81	0.92
6.1	3.9	4.3	0.37	0.52	1.02	1.08¶
5.4	3.4‡	4.0	0.54¶	0.92	1.1	2.0
2.4	4.2	4.3		0.30	0.88	
4.1	3.3§	3.6				
5.9	13‖	9.4				
4.1	6.4‖	4.2				
5.4 ± 0.77 (16)		5.3 ± 0.68 (8)	0.77 ± 0.17 (9)		1.08 ± 0.12 (9)	

Values are given in units of seconds. Last row represents mean ± SEM (number of observations).

*10 μM okadaic acid.

‡AP_5A was omitted from the rigor solution.

§Calmodulin was increased from 5 to 50 μM in the cage solution.

‖An ATP backup system was added to the cage solution.

¶The muscle was released to reduce its force and to prevent elongation of the series elastic before the time of photolysis.

tracted with $t_{1/2}$ values of ~1.1 s (Fig. 3 *p*; Table I, prephosphorylated), which were comparable with those in thiophosphorylated muscles and significantly shorter ($P < 0.01$) than those in control muscles (not prephosphorylated). Gel electrophoresis carried out on identically treated trachealis indicated 60–80% ($n = 3$) light-chain phosphorylation in those muscles.

Okadaic acid at 10 μM, a supramaximal concentration (Bialojan et al., 1988), had no appreciable effect on the $t_{1/2}$ of the trachealis muscles that were not prephosphorylated (Figs. 1 *a2* and 3 *o;* Table I, not prephosphorylated, with okadaic),

TABLE II

Half-time ($t_{1/2}$) and Delay (t_d) of Force Development

	$t_{1/2}$	t_d
Trachealis muscle		
Not prephosphorylated		
Without okadaic	5.4 ± 0.8 (16)	0.30 ± 0.05 (15)
With okadaic	5.3 ± 0.7 (8)	0.51 ± 0.08¶ (8)
Prephosphorylated*	1.08 ± 0.12 (9)	0.05** (2)
Thiophosphorylated‡	0.77 ± 0.17 (9)	0.05 ± 0.014 (9)
Ileum longitudinal muscle		
Not prephosphorylated		
Without okadaic	2.2 ± 0.4 (7)	0.26 ± 0.07 (8)
With okadaic	1.2 ± 0.2 (9)	0.24 ± 0.06 (8)
Thiophosphorylated§	0.25 ± 0.03 (8)	0.032 ± 0.003 (8)
Portal vein longitudinal		
Not prephosphorylated		
Without okadaic	2.2‡‡ ± 0.5 (4)	0.23 ± 0.04 (4)
With okadaic	1.8 ± 0.1 (3)	0.27 ± 0.02 (3)
Thiophosphorylated‖	0.20§§ ± 0.02 (16)	—

Values are given as mean ± SEM (number of observations) in units of seconds.

*Prephosphorylation with Ca^{2+} and ATP in the presence of 10 μM okadaic acid.

‡Photolysis carried out in the presence of Ca^{2+} in four runs, and in its absence in five runs. Results were not significantly different (Table I).

§Six runs in the presence of Ca^{2+}, two runs in its absence.

‖11 runs with Ca^{2+}, five without it.

¶Out of nine observed t_ds one outlier was rejected ($P < 0.025$).

**Only a preliminary analysis was possible, for most force data could not be recorded at a high sampling rate.

‡‡Note that this $t_{1/2}$ is quite consistent with the rate constant (0.3 s^{-1}) extracted in Somlyo et al. (1988).

§§$t_{1/2}$ was calculated from the rate constants extracted in Somlyo et al. (1988).

though the same sample of the toxin enhanced force development at low Ca^{2+} concentrations (e.g., 0.1 μM; data not shown), and slowed the rate of relaxation induced by removing Ca^{2+} from the bathing medium (cf. Fig. 1, *a1* vs. *a2, Relax*), as shown by Bialojan et al. (1988).

Force development had an initial slow phase, the overall time course being sigmoidal (Figs. 2 and 3). Delay times (t_d), measured as in Fig. 2, are listed in Table II. The t_d values in not prephosphorylated trachealis were ~0.3–0.5 s and those in thiophosphorylated trachealis 0.05 s ($P < 0.001$).

Comparison of the Phasic Muscles with Trachealis

Similar experiments were carried out also in the phasic muscles from ileum and portal-vein. The $t_{1/2}$ and t_d values extracted from the data are compiled in Table II, together with those from trachealis. The prephosphorylation protocol with okadaic acid was not applied to the phasic muscles. Data on thiophosphorylated portal-vein muscle are from a previous study (Somlyo et al., 1988), and the $t_{1/2}$ values were estimated from $\ln(2)/k$, where k is the rate constant obtained in that study by fitting an exponential curve to the main rising phase of the force recording. The t_d for thiophosphorylated portal-vein could not be extracted reliably from the data in that study, because the data sampling interval (10 ms) was too long. Gel electrophoresis carried out on thiophosphorylated ileum specimens ($n = 2$) showed almost complete (one spot) thiophosphorylation of the light-chains.

The $t_{1/2}$ of the phasic muscles was clearly shorter than that of trachealis, whether they were prephosphorylated or not. In not prephosphorylated muscles, okadaic acid appeared to shorten $t_{1/2}$, but this effect was statistically significant ($P < 0.05$) only in the ileum.

DISCUSSION

Our experiments, carried out under conditions where neither release nor diffusion of Ca^{2+} and substrates were limiting the initial rate of contraction, suggest that both the regulatory mechanism and the contractile machinery are slower in tonic than in phasic smooth muscle.

We confirmed that thiophosphorylation with ATPγS accelerates the force response of smooth muscle to photolysis of caged ATP. Furthermore, experiments with okadaic acid in trachealis muscle showed that the acceleration is not an effect specific to ATPγS, but it also occurs when ATP is the phosphate donor for prephosphorylation. Therefore, the more rapid development of force seems to be a direct consequence of myosin light-chain phosphorylation. This supports the notion (Kamm and Stull, 1986; Somlyo et al., 1988; Yagi et al., 1988) that slow light-chain phosphorylation, rather than the intrinsic contractile reaction, limits the contraction rate of smooth muscle under physiological (not prephosphorylated) conditions.

Effect of Okadaic Acid on Not Prephosphorylated Muscles

Addition of a phosphatase inhibitor to the photolysis solution of the not prephosphorylated muscles did not appreciably affect the time course of force development by trachealis and portal vein. The net rate of myosin light-chain phosphorylation, after liberation of ATP within the muscles, should depend on both the rates of phosphorylation by kinase and dephosphorylation by phosphatases. However, if the rate of the phosphatases is low compared with that of the kinase, inhibition of the slower reaction would have no appreciable effect on the overall rate. The results thus suggest that, under our experimental conditions with high concentrations of Ca^{2+} and calmodulin, the kinase rate predominates in trachealis and in portal vein muscle. Okadaic acid decreased $t_{1/2}$ in ileal muscle ($P < 0.05$), suggesting a possibly larger contribution of the phosphatase activity to the observed kinetics of this muscle.

Delay of Force Development

The delay in the onset of force developed by thiophosphorylated muscles (~50 ms with trachealis, ~30 ms with ileal muscle) was longer than could be attributed to the rate (120 s^{-1}; Goldman et al., 1984*a*) of the photochemical dark reactions leading to liberation of ATP. Some delay is expected from the mechanical effects of series compliance inevitably present within the muscle and at the ends. For example, even if the contractile machinery were to be turned on instantaneously by ATP, it would have to shorten by some length to elongate the elastic component, resulting in a delayed force output (Singer and Murphy, 1987). Given a shortening velocity of 0.2 s^{-1} (muscle length), a delay of ~50 ms would be required to take up a length change in the series compliance equivalent to 1% of the muscle length. (See also the next section and Fig. 4 for estimated effects of the compliance.)

The delay in the not prephosphorylated muscles was much longer (~0.4 s in trachealis and ~0.25 s in ileum and portal-vein preparations), corresponding to ~10% of the $t_{1/2}$ value for the main rising phase. This does not necessarily indicate an appreciable delay between photolysis and significant phosphorylation, because the kinetics of the contractile machinery can also influence the length of the delay.

Consider, for example, the following two-step, sequential, pseudo–first order reaction scheme:

$$\underset{\text{myosin}}{\text{Inactive}} \underset{\text{ATP}}{\overset{\tau_1}{\rightleftharpoons}} \underset{\text{myosin}}{\text{Phosphorylated}} \underset{\text{ATP}}{\overset{\tau_2}{\rightleftharpoons}} \underset{\text{cross-bridge}}{\text{Active}}$$

where τ_1 and τ_2 are time constants (inverse of the sum of forward and backward rate constants), respectively, for phosphorylation of the light-chains and for the cross-bridge reaction leading to force generation. In this scheme, the time course of build-up of the active cross-bridge population after application of ATP to inactive, nonphosphorylated myosin should be sigmoidal, with a delay followed by a nearly exponential rising phase. Generally, the delay is more closely related to the faster of the two reactions, the time constant of the rising phase being the slower (cf. Hiromi, 1979). For $\tau_1 \gg \tau_2$ in our case, t_d is nearly equal to τ_2.

Assuming a fully activated regulatory system in thiophosphorylated muscles, the $t_{1/2}$ values in those experiments (0.8 s in trachealis and 0.2–0.25 s in the phasic muscles) also provide an indication of the intrinsic time constants (τ_2) of active cross-bridge formation. Indeed, in each type of muscle, those $t_{1/2}$ values roughly approximate t_d for the not prephosphorylated muscles. This result is consistent with the explanation of the sigmoidal force development in terms of the above two-step reaction scheme, although it does not exclude other possible mechanisms, including a slow reaction leading to light-chain phosphorylation (cf. Yagi et al., 1988) and mechanical effect of the series elastic element (see below).

The Effects of Series Compliance

It could be argued that under certain assumptions, force development would be slow in not prephosphorylated muscles even with a fast phosphorylation, due to further effects of the compliance mechanically in series with the contractile apparatus. After liberation of ATP from caged ATP, if a possible fast kinase reaction quickly

brings phosphorylation to a low steady level and the speed of active shortening at this submaximal activation level is markedly slower than at full activation (Gerthoffer and Murphy, 1983), then the time required for the contractile apparatus to stretch series compliance would delay the onset of force. This potential effect of the series compliance might be less significant in prephosphorylated muscles, because the presumed faster velocity of the contractile apparatus would enable it to stretch the series compliance more rapidly.

We consider this alternative possibility unlikely, because (*a*) phosphorylation levels measured in permeabilized muscles are fairly high (e.g., 50–80%, Moreland et al., 1988) at full activation, and (*b*) okadaic acid, which increases the phosphorylation level (Bialojan et al., 1988, in skinned taenia; Erdödi et al., 1988, in aortic homogenate), did not prominently accelerate the rate of force development (this study).

The effect of the series compliance on the rate of force development in the pres-

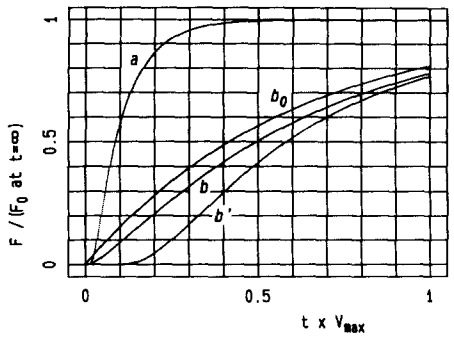

FIGURE 4. Calculated force development of a system with the Hill type contractile component and series elasticity, responding to an exponential increase in activation level. (*a*) Force development with instantaneous, full activation at $t = 0$; (b_0) time course of an activation with a time constant of 2.1 s; (*b*) force development at this activation with a constant V_{max}; (*b'*) similar to *b* but with assumption that V_{max} increases with the activation, from 0 to the steady level. Time on the abscissa is expressed as a fraction of the inverse of V_{max} (in *b'*, this V_{max} is that at $t = \infty$). The force and activation level on the ordinate is normalized to unity. See text for details.

ent experiments can be estimated by a calculation. We assume that (*a*) the stress (F)–strain (x) relation in the series elastic component is $F/F_0 = (x/x_0)^n$, where F_0 is the isometric force at full activation, $x_0 = 0.05$ (muscle length) and $n = 4$, thus the stiffness $d(F/F_0)/dx$ at $F = F_0$ is $n/x_0 = 80$ (muscle length)$^{-1}$ (cf. Warshaw et al., 1988), (*b*) the force (F)–velocity ($V \equiv dx/dt$) relation of the contractile component follows Hill's equation, $V/V_{max} = (a/F_0)\ (1 - F/F_0)/(F/F_0 + a/F_0)$, where $a/F_0 = 0.16$, V_{max} (at full activation) = 0.29 (muscle length) s^{-1} in accordance with Arhenden et al. (1988), and (*c*) either F_0 or both F_0 and V_{max} increase exponentially to the asymptotic value(s) with a certain time constant ($1 - \exp - t/\tau$). A numerical integration (Euler's method) gives the predicted time courses of normalized force ($F/[F_0$ at $t = \infty]$) development in Fig. 4. The curve *a* is that predicted with instantaneous activation ($\tau = 0$), and has a $t_{1/2}$ of 0.30 s, which is comparable with the observed $t_{1/2}$ for the thiophosphorylated phasic muscles (Table II). Curve b_0 is an exponential time course of the activation level at $\tau = 2.1$ s set comparable with the

observed $t_{1/2}$ for not prethiophosphorylated phasic muscles. Curves b and b' are simulated time courses of force development including effects of the series compliance; in b only F_0 increases exponentially at $\tau = 2.1$ s, and in b' both F_0 and V_{max} increase at the same rate. The $t_{1/2}$ values for b_0, b, and b' are 1.4, 1.7, and 2.0 s, respectively. It should be noted that these estimates are inversely proportional to the adopted V_{max} value.

Although the force curves b and b' lag behind the activation level b_0, the half-time of activation is shorter than that of the force development by only a factor of ~0.7–0.8. This ratio is much closer to unity than the observed ratio of $t_{1/2}$ values of prephosphorylated to nonphosphorylated muscles, which is ~0.1–0.2. The observed large difference similar to that between curve a and curve b or b' is, thus, unlikely to be due to series compliance. However, the onset of force development is liable to be delayed to some extent by the compliance (cf. a, b, and b').

Comparison of Trachealis with Phasic Muscles

According to our hypothesis, the $t_{1/2}$ for force development in thiophosphorylated muscle represents the time constant of the intrinsic reaction of the contractile machinery (τ_2 in the above scheme), and was three to four times longer in trachealis (0.8 s) than in ileal (0.25 s; $P < 0.05$) and portal vein (0.2 s; $P < 0.001$) muscle. This difference was not due to different levels of thiophosphorylation, that were comparable (~100%) in both types of muscle. Unloaded shortening velocity has also been reported to be some four times slower in tonic (hog carotid) than in phasic (guinea pig taenia) smooth muscle (Paul et al., 1983). The steady-state shortening velocity is probably related to steps near the end of the working stroke of the cycle where the cross-bridges detach, and it also critically depends on the number of interacting myofilaments functionally in series in the muscle preparation (Huxley, 1957). The $t_{1/2}$ of isometric force development measured in this study in prephosphorylated preparations may be more closely related to steps of attachment into force-generating states nearer the beginning of the working stroke (Goldman et al., 1984*b*; Brenner and Eisenberg, 1986), although it could also depend on properties of the series elasticity and the shortening velocity as discussed above. In any event, the overall reaction rate in the contractile machinery seems to be slower in tonic than in phasic muscles. It is interesting to note that the velocity of myosin-coated beads moving on *Nitella* actin also varies according to the source of myosin (smooth muscle) (Umemoto et al., in press).

The longer $t_{1/2}$ of the not prephosphorylated muscle is probably due to relatively slow phosphorylation of myosin light-chain (longer τ_1 in the above scheme). If this is the case, then the difference in those $t_{1/2}$ values between trachealis (~5 s) and the phasic muscles (~2 s) probably indicates a slower phosphorylation in trachealis. In keeping with this interpretation are the observations that phosphorylation peaks at ~5 s at 37°C after electrical stimulation in bovine trachealis (Kamm and Stull, 1986) and at ~3 s in ileum muscle depolarized with high K^+ at 24°C (Himpens et al., 1988).

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