FLASH PHOTOLYSIS STUDIES WITH CAGED CALCIUM AND CAGED ATP*

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The pre-myosin light chain (MLC\textsubscript{20}) phosphorylation components of the lag phase ($t_\text{d}$) of contractile activation were determined in permeabilized smooth muscles activated by photolytic release of ATP from caged ATP and/or Ca\textsuperscript{2+} from 4-(2-nitrophenyl)-EGTA (NP-EGTA). Calmodulin (CaM) shortened the $t_\text{d}$ (470 ms at 0 added CaM) that followed Ca\textsuperscript{2+} release, but its effect ($t_\text{d} = 200$ ms) saturated at 40 \textmu M. Photolysis of caged ATP following pre-equilibration with identical [Ca\textsubscript{C},CaM] shortened $t_\text{d}$ to 41 ms. The rate of phosphorylation was very fast ($3.5 \text{s}^{-1}$ at 22°C in the presence of 5 \textmu M exogenous CaM) following photolysis of caged ATP, and, following Ca\textsuperscript{2+} release, phosphorylation was accelerated by CaM. Simultaneous photolysis of caged ATP and NP-EGTA was followed by a $t_\text{d}$ of 194 ms at 5 \textmu M CaM and a rate of MLC\textsubscript{20} phosphorylation intermediate between these parameters following photolysis of, respectively, NP-EGTA and caged ATP. In the presence of the normal, total endogenous CaM content (37 ± 4 \textmu M) of portal vein smooth muscles $t_\text{d}$ was 565 ms.

Steady state maximum force at pCa 5.5 was increased by much lower (100 nm) exogenous [CaM] than was required (>2.5 \textmu M) to shorten the $t_\text{d}$. We estimate the endogenous CaM available under steady state conditions in vivo to be approximately 0.25 \textmu M and probably less during a rapid Ca\textsuperscript{2+} transient.

We conclude that the [CaM] dependence of the kinetics of MLC\textsubscript{20} phosphorylation and force development ($t_\text{d}$, and $t_\text{f}$) initiated by Ca\textsuperscript{2+} reflects the recruitment of a slowly diffusible component of total CaM. The relatively long duration of $t_\text{d}$ (197 ms) at saturating [CaM] suggests the contribution to $t_\text{d}$ of an additional component, possibly a prephosphorylation activation/isomerization of the CaM myosin light chain kinase complex (To¨ro¨k, K., and Trentham, D. R. (1994) Biochemistry 33, 12807-12820). The relatively short delay (108 ms in the presence of 40 \textmu M CaM) following simultaneous photolysis of NP-EGTA and caged ATP suggests that preincubation with ATP (prior to photolysis of NP-EGTA) may inhibit the formation of a preactive Ca\textsubscript{2+}CaM myosin light chain kinase complex.

A long lag phase between the binding of an excitatory agonist to its receptor and contraction is a characteristic property of smooth muscle, evident even when diffusional delays are eliminated through photolysis of caged agonists (Somlyo et al., 1988a; Muralidharan et al., 1993; Walker et al., 1993). A major component of the delay (1–2 s in guinea pig portal vein smooth muscle stimulated with phenylephrine at room temperature; Somlyo et al. (1988a)) is due to pharmacomechanical Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (reviewed by Somlyo and Somlyo (1990)). Of the remaining delay, only a small fraction (<30 ms; Somlyo et al., 1992) is due to the time elapsed between the rise in inositol 1,4,5-trisphosphate and Ca\textsuperscript{2+} release; its major component occurs after the rise in cytoplasmic Ca\textsuperscript{2+}, whether the latter is the result of inositol 1,4,5-trisphosphate-induced Ca\textsuperscript{2+} release, electrical stimulation, or Ca\textsuperscript{2+} spikes during spontaneous electrical activity (Somlyo et al., 1988b, 1992; Yagi et al., 1988; Himpen and Somlyo, 1988). We had suggested (Somlyo et al., 1988b) that this lag, which is much longer than the few ms delay between the rise in [Ca\textsuperscript{2+}] and contraction in striated muscle (Ellis-Davies and Kaplan (1994); reviewed by Ashley et al. (1991)), could be the result of prephosphorylation reactions and/or the time course of phosphorylation of myosin light chains that activates contraction in smooth muscle. The purpose of the present study was to determine the relative contributions of these two components to the kinetics of activation in smooth muscle. To rapidly and synchronously activate contraction with Ca\textsuperscript{2+}, we used laser flash photolysis of a new caged Ca\textsuperscript{2+} (NP-EGTA)\textsuperscript{1} that combines the advantages of a 4-order of magnitude decrease in affinity for Ca\textsuperscript{2+} ($K_\text{D} = 80$ nM versus 1 mM) upon photolysis with a low affinity for Mg\textsuperscript{2+} ($K_\text{D} = 9$ mM; Ellis-Davies and Kaplan (1994)). Varying the calmodulin (CaM) concentration in permeabilized smooth muscle activated by photolysis of the NP-EGTA-Ca\textsuperscript{2+} complex allowed us to obtain a precise measure of both the delay and its dependence on calmodulin concentration and to relate, through rapid freezing, the mechanical to the biochemical events of MLC\textsubscript{20} phosphorylation. Comparison of the kinetics of contraction and MLC\textsubscript{20} phosphorylation initiated through photolysis of, respectively, NP-EGTA or caged ATP (the latter in smooth muscles

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\textsuperscript{1}The abbreviations used are: NP-EGTA, 4-(2-nitrophenyl)-EGTA; CaM, calmodulin; MLC\textsubscript{20}, 20-kDa myosin light chain; caged ATP, P\textsuperscript{3}-1-(2-nitrophenyl)-ethyl ATP; PIP\textsubscript{ES}, piperazine-N,N’-bis(2-ethanesulfonic acid); AP\textsubscript{A}, P\textsuperscript{3’,5’}-diadenosine 5’-pentaphosphate; ATP-S, adenosine 5’-O-(3-thiotriphosphate); CaM kinase II, calcium calmodulin-dependent protein kinase II; KN-62, 1-[N-O-bis(3-isoquinolinolsulfanyl)-N-methyl-L-tyrosyl]-4-phenylpiperazin; HPLC, high pressure liquid chromatography; MLCK, myosin light chain kinase.
proequlibrated with Ca\(^{2+}\)-calmodulin) provided strong evidence of the contribution of one or more significant kinetic steps, attributable to prephosphorylation reactions, to the delay during the in vivo activation of smooth muscle by Ca\(^{2+}\). A preliminary report of some of these findings has been published (Zimmermann et al., 1995).

MATERIALS AND METHODS

Tissue Preparation—The portal anterior mesenteric vein was dissected from New Zealand White rabbits anesthetized with halothane and killed by exsanguination through the carotid artery as approved by the Animal Care and Use Committee. After removal of the adventitia in Krebs solution, small strips (150–200 \(\mu\)m wide and 2 mm long) were cut out along the direction of the longitudinal muscle layer. Both ends of the tissue strips were strung with silk threads.

Solutions—The composition of commonly used solutions is shown in Table I. Unless otherwise noted, [MgATP] and [Mg\(^{2+}\)] were 4.5 mM and 1 mM, respectively. The ionic strength of all solutions was adjusted to 0.2 M with potassium methanesulfonic acid (Table I).

Krebssolution,smallstrips(150–200
\(\mu\)m and 1 mm, respectively. The intracellular [Ca\(^{2+}\)] was in the range of 0.05 to 0.1 
\(\mu\)M. The free Ca\(^{2+}\) concentrations in these as well as in the solutions with various [Ca\(^{2+}\)] were measured with Ca\(^{2+}\)-sensitive microelectrodes. Electrodes were calibrated with EGTA-buffered calibration solutions (calcium calibration kit with magnesium I; Molecular Probes Inc., Eugene, OR).

Steady State Experiments—The methods used in the steady state experiments have been described in detail (Horiuti et al., 1989, Kitazawa et al., 1989). Briefly, single muscle strips were connected to a force transducer and mounted in a well on a temperature-controlled plate. After measurement of contractions induced in HEPES-force transducer and mounted in a well on a temperature-controlled solution, the strips were permeabilized with 50
\(\mu\)M DMSO for 30–45 min at 30°C.

Photolysis Experiments—The methods used in the photolysis experiments were identical to those described by Nishiye et al. (1995). Briefly, single muscle strips were connected to a force transducer and mounted in a well on a temperature-controlled plate. After measurement of contractions induced in HEPES-force transducer and mounted in a well on a temperature-controlled solution, the strips were permeabilized with 50
\(\mu\)M DMSO for 30–45 min at 30°C.

Photolysis Experiments—Strips were mounted in a quartz capillary (inner diameter, 0.55 mm), placed above the objective lens (10 of an inverted microscope (Olympus IMT2) equipped with epifluorescence optics and a monochromator (Thorn EM, 9924B15), connected to a strain gauge (AE801, Akers) as described by Somlyo et al. (1992), and stretched by 20–30%. The setup allowed continuous superfusion of the entire muscle strip. All photolysis experiments were carried out at room temperature (22-23°C). The system for data collection in the majority of the experiments was identical to that described by Nishiye et al. (1993). Later experiments were performed using an AD-10–16 AD conversion card at an acquisition rate of 500 Hz in combination with the data acquisition software Labview 4.0 (National Instruments) on a PC. Force transients were fitted using the software SigmaPlot (Jandel Scientific).

Caged Ca\(^{2+}\)—Permeabilized preparations were washed in G0 and relaxed by incubating them for 12 min in photolysis solution containing

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\begin{align*}
\text{G0} & : 5.82 & 5.88 & 10 & 0.50 & 0.50 \\
\text{G0.5} & : 5.82 & 5.90 & 10 & 0.50 & 0.50 \\
\text{G10} & : 5.78 & 6.24 & 10 & 0.50 & 0.50 \\
\text{pCa 6.0} & : 5.78 & 5.96 & 10 & 2.10 & 7.90 \\
\text{pCa 4.5} & : 5.86 & 5.89 & 10 & 10.00 & 4.490 \\
\text{GOR} & : 1.00 & & & & 136.74 \\
\text{G0.5R} & : 1.02 & & & & 135.21 \\
\text{G10R} & : 1.25 & & & & 106.22 \\
\text{pCa 5.5R} & : 1.02 & & & 0.77 & 9.23 \\
\text{G4.5R} & : 1.00 & & & 0.06 & 9.94 \\
\end{align*}
\]

*a Potassium methanesulfonic acid.*
Fig. 1. Force records illustrating the experimental protocol used for activation by photolysis of NP-EGTA and caged ATP. A, after the control contraction elicited by depolarization with 143 mM K+, the strips were permeabilized with β-escin, incubated for 10 min in A23187, washed in G0, and relaxed for 12 min in photolysis solution, followed by photolysis of NP-EGTA with a single laser pulse (arrow). B, the sequence of incubations for the photolysis of caged ATP consisted of permeabilization and incubation in A23187 (not shown), relaxation of the strips in G10, removal of ATP in G10R, and an increase of [Ca\(^{2+}\)]\(_{free}\) in pCa 5.5R. The preparations were finally incubated for 3 min in caged ATP containing photolysis solution and activated by photolytic liberation of 2.7 ± 0.2 mM ATP (arrow).

 identical to those used in the photolysis experiments described above. To avoid evaporative cooling in air, the strips were exposed to a continuous stream of humidified air at room temperature. Frozen samples were freeze-substituted in 10% trichloroacetic acid in acetone, subjected to two-dimensional gel electrophoresis and stained with colloidal gold as described previously (Kitazawa et al., 1991). MLC\(_{cp}\) phosphorylation was quantified by densitometry with a Bio-Rad GS-670 imaging densitometer. The degree of thiophosphorylation of MLC20 was measured in strips frozen with liquid nitrogen inside the quartz capillaries and subjected to the same procedure.

Measurement of Tissue Calmodulin Content—Muscle strips of defined size were homogenized in 1% SDS, 15% glycerol, 15 mM dithiothreitol, 62.5 mM Tris, pH 7.5, and subjected to SDS-polyacrylamide gel electrophoresis together with known amounts of purified CaM from bovine brain (Sigma). Subsequently, proteins were transferred onto polyvinylidene difluoride membranes in 25 mM potassium phosphate buffer, pH 7.0, and fixed with 0.2% glutaraldehyde in the same buffer. After blocking of nonspecific antibody binding sites with 5% milk in phosphate-buffered saline, 0.05% Tween 20, the membranes were incubated with a monoclonal antibody binding sites with 5% milk in phosphate-buffered saline, 0.05% Tween 20, the membranes were incubated with a monoclonal antibody (Cappel, West Chester, PA) and in horseradish peroxidase-conjugated goat anti-mouse IgG (1:1000 dilution, Amersham Corp.). Calmodulin was finally quantified by densitometric analysis using a Bio-Rad GS-670 scanning densitometer. We calculated the cellular concentrations of CaM based on a thickness of our preparations of 47 μm and a cellular fraction of 0.56 (values obtained by morphometric analysis of electron micrographs).

Statistical Analysis—Statistical comparisons were made by independent Student’s t test, using two-tailed values. p values <0.05 were considered significant. Data are expressed as mean ± S.E.

RESULTS

The Effects of Exogenous CaM on Steady State Tension

In order to compare the CaM dependence of the delay with the steady state response to Ca\(^{2+}\), we first determined the effects of exogenous CaM on steady state tension in strips permeabilized with β-escin (Kabayashi et al., 1989; Iizuka et al., 1994), by cumulatively increasing [Ca\(^{2+}\)]\(_{free}\) in the presence of variable [CaM]. Fig. 2 shows the following: 1) 100 nM exogenous CaM was sufficient to significantly increase the calcium sensitivity of force, as compared to the Ca\(^{2+}\)-sensitivity in the presence of 10 nM CaM, and 2) further increasing the concentration of CaM resulted in an increase of Ca\(^{2+}\) concentration curves to lower [Ca\(^{2+}\)]\(_{free}\), as previously shown in taenia coli permeabilized with Triton X-150 (Sparrow et al., 1981). The effect of CaM on the Ca\(^{2+}\)-force relationship showed no sign of saturation within the range of CaM concentrations tested.

Increasing, at pCa 4.5, the exogenous CaM concentration from 10 nM to 40 μM did not change the steady state level of tension, indicating that, although exogenous CaM increased the sensitivity for Ca\(^{2+}\) (Fig. 2), at this high free [Ca\(^{2+}\)] it had no effect on maximum isometric force.

we also determined the steady state Ca\(^{2+}\)-sensitivity of force in the presence of the endogenous CaM concentration, by permeabilizing smooth muscles with Staphylococcus aureus α-toxin. The pores produced by this toxin (1-2 nm) retain molecules (M, > 600-1000) like CaM but are permeable to low molecular weight solutes (Cassidy et al., 1979; Kitazawa et al., 1989, 1991). The pCa-tension curves of muscles permeabilized with α-toxin lay between the pCa-tension curves obtained in the presence of 0.1 and 1 μM exogenous CaM in α-toxin-permeabilized strips (Fig. 2; pCa\(_{so}\) = 6.2, n = 4), corresponding to [CaM] = 0.25 μM. In contrast, the total [CaM] determined in intact preparations was 37 ± 4 μM (n = 4).

Photolysis of NP-EGTA

The Effects of CaM Concentration on the Kinetics of Force Development Initiated by Photolytic Release of Ca\(^{2+}\)—Both the preflash concentration of Ca\(^{2+}\)\(_{free}\) and the amount of Ca\(^{2+}\) photoreleased from NP-EGTA by a laser pulse of given duration and energy (photon flux) are proportional to the Ca\(^{2+}\)-loading of the photosensitive chelator, i.e. the Ca\(^{2+}\)\(_{total}\)NP-EGTA ratio (Ellis-Davies and Kaplan 1994). In order to liberate appreciable amounts of Ca\(^{2+}\) yet retain it at low resting levels prior to photolysis, we adjusted this ratio so that [Ca\(^{2+}\)].% of [Ca\(^{2+}\)]\(_{free}\) was near the threshold for the initiation of contraction. Once determined, this ratio was kept constant in all experiments. Under
these conditions \([Ca^{2+}]_{\text{free}}\) measured with fluo-3 before photolysis, was \(0.10 \pm 0.01 \mu M\) or pCa 7.0 \(\pm 0.08\) (n = 8). The final \([Ca^{2+}]_{\text{free}}\) reached after a single laser flash, was \(3.0 \pm 0.36 \mu M\) (pCa 5.5 \(\pm 0.16\), n = 8). We determined these values in the absence of tissue, since during preincubation the strips slowly accumulated the dye; this could have introduced an error into the Ca\(^{2+}\) measurements. Comparison of the fluorescence signals obtained in the presence and absence of the preparation, however, revealed no qualitative differences.

To evaluate the efficiency of photolysis, we calculated the concentration of photoproducts according to Zucker and Steinhardt (1978) using the dissociation constants determined by Ellis-Davies and Kaplan (1994) and the initial and final \([Ca^{2+}]_{\text{free}}\) measured by us. This calculation revealed that approximately 45% of the total NP-EGTA was photolyzed.

As could be expected from the steady state Ca\(^{2+}\)-tension relationship, in the presence of Ca\(^{2+}\)-loaded (pCa 7.0) NP-EGTA, \([Ca^{2+}]_{\text{free}} > 10 \mu M\) led to prephotolysis tension that, in the presence of 100 \(\mu M\) CaM, reached up to 30–60% of \(F_{\text{max}}\) (maximum force at pCa 4.5). After photorelease of Ca\(^{2+}\), isometric tension developed sigmoidally with an initial lag phase or delay (Fig. 3, Table II). Comparison with the tension reached in EGTA-buffered pCa 4.5 solution, recorded after the photolysis experiments, showed that the plateau tension reached after photolysis was at least 90% of maximum tension, except for the experiments performed in the absence of exogenous CaM. In the latter case, force usually reached variably lower plateau levels, consistent with the steady state results at pCa 5.5 (Fig. 2). The time required to attain 50% of the plateau tension (\(t_{50}\)) was a function of the exogenous CaM concentration (Fig. 4A), decreasing from 7.4 \(\pm 0.6\) s (0 added CaM, n = 5) to 4.3 \(\pm 0.8\) s (100 \(\mu M\) CaM, n = 5). This acceleration of force development by CaM is sufficient to have been detected by (diffusion rate-limited) activation of smooth muscle by increasing the \([Ca^{2+}]_{\text{1n}}\) level to be an important finding that also bears on the interpretation of the effects of CaM on the rate of force development (see "Discussion"). In the presence of 40 \(\mu M\) CaM, phosphorylation increased significantly within the first 100 ms of activation.

Photolysis of Caged ATP—To relate the kinetic data obtained by photorelease of Ca\(^{2+}\) (Scheme III, "Discussion") to the reaction steps preceding myosin light chain phosphorylation (Scheme II), we photoreleased ATP (2.7 \(\pm 0.2\) mm, n = 6) in the presence of Ca\(^{2+}\) (pCa 5.5) during low tension rigor (Figs. 1 and 6A). In the absence or presence of exogenous CaM (40 \(\mu M\)) the delay was 48 \(\pm 4\) ms (n = 6) and 41 \(\pm 6\) ms (n = 6), respectively. Surprisingly, at pCa 4.5 the delays were longer, and tension developed significantly more slowly, with delays of 122 \(\pm 13\) ms at 0 CaM (n = 7) and 76 \(\pm 5\) ms (n = 8) at 40 \(\mu M\) CaM. Following photolysis of caged ATP, exogenous CaM had no effect on the rate of tension development expressed as \(t_{50}\), at either concentration of free Ca\(^{2+}\) tested (see Table II).

To exclude the possibility that contaminant ATP caused MLC\(_{20}\) phosphorylation during the period of preincubation in the photolysis solution, we also performed experiments adding 18 units/ml apyrase to all rigor solutions and to the photolysis solution. This treatment caused the force response to become transient upon photolysis, as the result of ATP hydrolysis by apyrase, but had no significant effect on the delay (n = 2, CaM\(_{\text{max}}\) = 40 \(\mu M\); data not shown). Furthermore, the phosphorylation levels before, respectively, photolysis of caged ATP and NP-EGTA, both in the absence of exogenous CaM, were not significantly different (data not shown).

The time course of MLC\(_{20}\) phosphorylation initiated by photolysis of caged ATP was faster, even in the absence of exogenous CaM, than after activation by photolysis of NP-EGTA in the presence of 40 \(\mu M\) CaM\(_{\text{max}}\) (Fig. 6B, Table III). The fastest apparent rate constant of MLC\(_{20}\) phosphorylation, obtained by fitting the data to a single exponential function, was 3.5 \(s^{-1}\). The rate of phosphorylation following photolysis of caged ATP, much like the simultaneously monitored kinetic parameters of force development, was independent of exogenous CaM (Tables II and III). However, the final phosphorylation level (41%) was lower in the absence of exogenous CaM, corresponding well to the lower levels of peak force. At \([CaM]_{\text{max}} = 5 \mu M\), in spite of their different initial rates (Table III), the final levels of phosphorylation were not significantly different following activation by photolysis of caged ATP (56%) or NP-EGTA (57%).

Thiophosphorylation of MLC\(_{20}\) with ATP-yS, prior to photol-
Calmodulin Dependence of Smooth Muscle Activation Kinetics

Table I

| pCa | 0 μM CaM<sub>exog</sub> | 40 μM CaM<sub>exog</sub> |
|-----|------------------------|-------------------------|
|     | delay | t<sub>1/2</sub> | delay | t<sub>1/2</sub> |
| NP-EGTA | 5.5 ± 0.15 | 470 ± 32 | 7.4 ± 0.6 | 201 ± 9 | 3.9 ± 0.4 |
| NP-EGTA + caged ATP | 6.1 ± 0.01 | 188 ± 26 | 2.7 ± 0.6 | 108 ± 10 | 2.5 ± 0.4 |
| caged ATP | 5.5 | 48 ± 4 | 2.0 ± 0.2 | 41 ± 6 | 2.2 ± 0.2 |
| caged ATP/ATP<sub>γ</sub>S | 5.5 | ND<sup>a</sup> | ND | 19 ± 2 | 1.0 ± 0.2 |
| caged ATP | 4.5 | 122 ± 13 | 3.8 ± 0.4 | 76 ± 5 | 3.9 ± 0.4 |
| caged ATP/ATP<sub>γ</sub>S | 4.5 | ND<sup>a</sup> | ND | 32 ± 5 | 1.1 ± 0.1 |

<sup>a</sup> ND, not determined.

Fig. 4. CaM dependence of the reciprocal of t<sub>0.1</sub> of contractions induced by photolysis of NP-EGTA in muscles permeabilized with β-escin. B, dependence of the delay (t<sub>P</sub>) on exogenous CaM following permeabilization with β-escin and photolysis of NP-EGTA. Micromolar concentrations of CaM reduce the t<sub>P</sub> of contraction, but this effect of CaM saturates at approximately 40 μM with an EC<sub>50</sub> of 14 μM.

Analysis of caged ATP, resulted in the fastest rate of force development by shortening both t<sub>P</sub> and t<sub>0.1</sub>. (Fig. 7; Table I). t<sub>P</sub> decreased to 19 ± 2 (n = 6) and 32 ± 5 ms (n = 6) at pCa 5.5 and 4.5, respectively. We note that the shortest delay (19 ms) sets an upper limit on the possible contribution of the series elastic element to the lag phase (Horiuti et al., 1989) when contraction is initiated from rigor. The time course of tension development was well fitted, from t = 0.1 to t = 5 s, with two exponentials with rate constants k<sub>1</sub> = 2.6 ± 0.10 s<sup>-1</sup> and k<sub>2</sub> = 0.4 ± 0.02 s<sup>-1</sup> for experiments at pCa 4.5 and k<sub>1</sub> = 3.6 ± 0.29 s<sup>-1</sup> and k<sub>2</sub> = 0.5 ± 0.02 s<sup>-1</sup> at pCa 5.5, respectively. The relative amplitudes of the faster components were 0.4 ± 0.03% and 0.4 ± 0.02%. These t<sub>P</sub> and k<sub>1</sub> values at, respectively, pCa 4.5 and 5.5 are significantly different (p < 0.05).

Fig. 5. Dependence of the kinetics of phosphorylation induced by photolysis of NP-EGTA on exogenous CaM. The solid lines represent mathematical fits of the experimental data to a single exponential function (n = 3–6). The apparent rate constants of MLC<sub>20</sub> phosphorylation derived from these fittings are 1.5 ± 1.0 s<sup>-1</sup> (○, no added CaM), 0.9 ± 0.1 s<sup>-1</sup> (■, 5 μM exogenous CaM), and 2.9 ± 0.3 s<sup>-1</sup> (▲, 40 μM exogenous CaM).

Table II

| [CaM]<sub>exog</sub> (μM) | % phosphorylation | time (s) |
|------------------------|-------------------|----------|
| 0.01 | 0.6 ± 0.2 | 400 |
| 0.02 | 2.2 ± 0.4 | 400 |
| 0.05 | 6.1 ± 0.3 | 400 |
| 0.10 | 10.8 ± 0.6 | 400 |
| 0.20 | 22.2 ± 1.1 | 400 |

Simultaneous Photolysis of NP-EGTA and Caged ATP: Kinetics of Force Development and MLC<sub>20</sub> Phosphorylation

In order to investigate the possible influence of the different initial cross-bridge states (relaxed and rigor) on activation kinetics in the two groups of experiments described above, we also activated strips from low tension, low Ca<sup>2+</sup> rigor by simultaneously liberating Ca<sup>2+</sup> and ATP from their respective caged precursors. Photolysis of NP-EGTA and caged ATP caused [Ca<sup>2+</sup>]<sub>free</sub> to increase from 0.11 ± 0.004 μM (pCa 7.0 ± 0.02; n = 8) to 0.87 ± 0.02 μM (pCa 6.1 ± 0.01; n = 8), a smaller change than observed in the presence of NP-EGTA as the only caged compound. The amount of photoliberated ATP, however, was not different from the respective experiment performed in the absence of NP-EGTA. To our surprise, the delays observed under these conditions were significantly shorter than following activation with Ca<sup>2+</sup> (photolysis of NP-EGTA) in the presence of MgATP (Fig. 8A). The delays measured in the absence of exogenous CaM and after addition of 40 μM CaM were, respectively, 188 ± 26 ms (n = 4) and 108 ± 10 ms (n = 5) and, therefore, CaM-dependent, but shorter than in the case of activation by photolysis of NP-EGTA in the presence of MgATP (Table II). The respective t<sub>0.1</sub> were 2.7 ± 0.6 s (n = 4) and 2.5 ± 0.4 s (n = 5), also significantly shorter than following photolysis of NP-EGTA alone, and only slightly but not significantly longer than the t<sub>0.1</sub> following photolysis of caged ATP alone (in the presence of Ca<sub>3</sub>CaM). Reduction of the caged ATP concentration to 7.5 mM and the addition of 2.5 mM ATP to the photolysis solution restored the slower kinetics of tension (data not shown). Similarly, addition of 1 mM CTP, a poor substrate for myosin light chain kinase (MLCK), before photolysis of caged ATP, also prolonged t<sub>P</sub> and t<sub>0.1</sub> (data not shown). Increasing the [Ca<sup>2+</sup>]<sub>free</sub> to > 10 μM (concentrations saturating the fluo-3 signal) by increasing the intensity of the laser pulse, however, did not shorten t<sub>P</sub> to less than 100 ms (at [CaM]<sub>exog</sub> = 40 μM; data not shown), indicating that the contractile responses to simultaneous NP-EGTA/caged ATP photolysis were not Ca<sup>2+</sup>-limited.
Weber and Murray (1973), Goldman (1988b); for striated muscle, see Hartshorne (1987)). Scheme I represents experiments in which the muscles were stably thiophosphorylated (Cassidy et al., 1979), and contraction was initiated from rigor by photolytic release of ATP (Fig. 7 and Horiuti et al. (1989)), bypassing MLC20 phosphorylation and the steps preceding it. Under these conditions, the release of ATP causes rapid detachment of thiophosphorylated rigor cross-bridges followed by reattachment and force development (Somlyo et al., 1988b; Horiuti et al., 1989). Scheme II represents the case of muscles equilibrated with Ca2CaM to permit completion of prephosphorylation reactions between Ca2+, CaM, and MLCK prior to photolysis; the release of ATP then causes phosphorylation and contraction from an initial state of dephosphorylated rigor bridges. Scheme III represents the delay that follows the rise in [Ca2+]i, during physiological activation and includes Ca2+ bind-

**Table III**

|            | 0 μM CaM<sub>exog</sub> | 5 μM CaM<sub>exog</sub> | 40 μM CaM<sub>exog</sub> |
|------------|------------------------|------------------------|------------------------|
|            | delay (s) | k' (s<sup>-1</sup>) | P<sub>50</sub> (%) | delay (s) | k' (s<sup>-1</sup>) | P<sub>50</sub> (%) | delay (s) | k' (s<sup>-1</sup>) | P<sub>50</sub> (%) |
| NP-EGTA    | 470 ± 32 | 7.4 ± 0.6 | 1.1 ± 0.4 | 34 ± 2 | 412 ± 19 | 5.7 ± 0.6 | 0.9 ± 0.1 | 57 ± 3 | 201 ± 9 | 3.9 ± 0.4 | 2.9 ± 0.8 | 62 ± 3 |
| caged ATP* | 48 ± 4 | 2.0 ± 0.2 | 2.6 ± 0.4 | 41 ± 2 | 51 ± 11 | 2.1 ± 0.8 | 3.5 ± 1.3 | 56 ± 3 | 41 ± 6 | 2.2 ± 0.2 |
| NP-EGTA/caged ATP | 188 ± 26 | 27 ± 0.6 | 194 ± 25 | 3.3 ± 0.5 | 1.7 ± 0.1 | 36 ± 3 | 108 ± 10 | 2.5 ± 0.4 |
| caged ATP/ATPγS* |                 |                        |                 |                |                      |                 |                |                |

**Fig. 6.** A, comparison of the activation kinetics after liberation of, respectively, Ca2+ from NP-EGTA and ATP from caged ATP. Contractions induced by activation with ATP from rigor are faster than those induced by liberation of Ca2+. Both the t<sub>1/2</sub> and the delay are shorter. Contractions were induced successively in the same preparation at [CaM]<sub>exog</sub> = 40 μM. Curves are normalized to peak tension. Laser pulse at t = 0. B, time courses of MLC20 phosphorylation after activation by photolysis of caged ATP in the presence of [CaM]<sub>exog</sub> = 0 μM or 5 μM. Note the faster time course than observed after photolysis of NP-EGTA. Rate constants of phosphorylation obtained from single exponential fits (solid lines) are 2.6 s<sup>-1</sup> (no added CaM) and 3.5 s<sup>-1</sup> (5 μM exogenous CaM) (n = 3–4).

We also measured the rates of MLC20 phosphorylation to determine whether the faster contractile kinetics following simultaneous photolysis of NP-EGTA and caged ATP than of NP-EGTA alone were due to the different initial states of cross-bridges (rigor and detached, respectively) and/or to different rates of MLC20 phosphorylation. In the presence of 5 μM CaM, MLC20 phosphorylation increased from 1.6 to 36% with a pseudo-first-order rate constant of 1.7 ± 0.1 s<sup>-1</sup> (Fig. 8B). This rate was significantly faster than found after photolysis of NP-EGTA alone (0.9 ± 0.1 s<sup>-1</sup>), indicating that the acceleration of force development was not solely due to the mechanical effects of cooperative activation of nonphosphorylated by rigor cross-bridges (Somlyo et al. (1988b); for striated muscle, see Weber and Murray (1973); Goldman et al. (1984)).

**DISCUSSION**

The major findings of our study are the following: 1) the delay (or lag phase) preceding force development is signifi-

![Image](https://via.placeholder.com/150)

**Fig. 7.** Photolysis of caged ATP after thiophosphorylation of MLC20 with ATPγS. Satisfactory fitting of the initial 5 s of contraction requires the sum of two exponential functions (dotted lines) with mean rate constants of k<sub>1</sub> = 3.6 s<sup>-1</sup> and k<sub>2</sub> = 0.5 s<sup>-1</sup> at pCa 5.5. The fitted curve coincides with the experimentally obtained curve. [CaM]<sub>exog</sub> = 40 μM. The experimental trace is normalized to peak tension. Laser pulse at t = 0.
Calmodulin Dependence of Smooth Muscle Activation Kinetics

According to this second-order rate constant, at pCa 5.5 in the presence of 40 μM CaM (Ca_{5.5}CaM = 0.8 μM; average K_{5.5} – 5 μM; Maune et al. (1992)), Ca_{5.5}CaM will bind to MLCK at a rate of approximately 80 s^{-1}, too fast to make a significant contribution to the long (200 ms) lag phase. Even slow diffusion hindered by binding (Rüegg et al., 1984; Tansey et al., 1994), is unlikely to account for the entire delay, because increasing the CaM concentration between 40 and 100 μM caused very little or no shortening of t_d (Fig. 4B).

The CaM Dependence of the Delay and of Steady State Force Development—The next question to be examined is the dependence of the kinetics of activation on “available” [CaM] and [Ca^{2÷}]1, particularly since increasing [CaM] above 100 nM does not increase the amplitude of steady state force at pCa 5.5 (Fig. 2).

Exogenous CaM (~5 μM) shortened the delay (t_d) (Fig. 4B), but this effect saturated (197 ms) at 40 μM compared with its value (470 ms) at 2.5 μM CaM and in α-toxin-permeabilized preparations (560 ms) containing endogenous [CaM]. This is consistent with the conclusion that even under steady state conditions only a fraction of total endogenous CaM (30–40 μM) is available for contractile activation (Rüegg et al., 1984; Tansey et al., 1994; present study). The lack of effect of less than 2.5 μM CaM on t_d (Fig. 4B) contrasts with the increased Ca^{2÷}1 sensitivity and amplitude of steady state force caused by 0.1 μM CaM (Fig. 2) and suggests that steady state experiments overestimate the endogenous [CaM] available for rapid activation by Ca^{2÷}1. Indeed, at pCa 5.5 the effect of 1 μM CaM on steady state tension was saturating or near saturating (Fig. 2), whereas it was insufficient to affect t_d. These results and the long t_d following photolysis of NP-EGTA suggest that the [CaM] dependence of t_d reflects the recruitment of CaM that is not readily available at physiological [CaM] and/or some rate-limiting step(s) in the activation of MLCK by Ca^{2÷}1.

The CaM Dependence of the Delay and of Steady State Force Development—The next question to be examined is the dependence of the kinetics of activation on “available” [CaM] and [Ca^{2÷}]1, particularly since increasing [CaM] above 100 nM does not increase the amplitude of steady state force at pCa 5.5.

Diffusional recruitment is unlikely to contribute to the lag phase at saturating [CaM]. Therefore, the relatively long t_d (~200 ms at 40–100 μM [CaM]), particularly when contrasted with the short t_d following photolysis of caged ATP, suggests additional, nondiffusional contributions to t_d. The possibility that following photolysis of caged ATP the t_d was short (48 ms at pCa 5.5) because the series elastic elements were already extended by the initial, rigor tension can be excluded, because 1) there was a long delay (197 ms) after photolysis of NP-EGTA even when prephotolysis tension was high (30–40% of F_{max}; see "Results") due to very high (100 μM) [CaM], and 2) the initial rate of phosphorylation, which is not affected by mechanical delays, was also significantly faster following photolysis of caged ATP (Table III and Figs. 5 and 6B). Furthermore, delays were significantly different (48 versus 470 ms) when the photolysis levels of phosphorylation were identical, in the absence of exogenous CaM (Figs. 5 and 6B). Therefore, we attribute the short delay following photolysis of caged ATP at least in part to the formation of active and available Ca_{5.5}CaM-MLCK complexes during incubation with Ca^{2÷}1 and CaM prior to photolysis (shown in Scheme II), thereby shortening t_d by an amount attributable to these prephosphorylation reactions. The significant delay (about 200 ms) following photolysis of NP-EGTA at saturating [CaM] suggests the contribution of

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\[ \text{Ca}^{2+} \text{to CaM, diffusion and binding of Ca}_{5.5}\text{CaM to MLCK, activation of the Ca}_{5.5}\text{CaM-MLCK complex, and MLCl}_{20}\text{ phosphorylation and contraction.} \]

Of the steps represented in Scheme III the photolytic release of Ca^{2÷}1 (~88,000 s^{-1}) and Ca^{2÷}1 binding to CaM (t_{50} ~2 ms; Kasturi et al., 1993)) are very fast, as is the second-order rate constant (10^{3} M^{-1} s^{-1}) of Ca_{5.5}CaM binding to MLCK (Török and

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additional significant kinetic step(s), other than diffusion of CaM, to \( t_0 \). Isomerization of smooth muscle Ca\(_2\)CaM-MLCK at approximately 1 s\(^{-1}\) (Töörk and Trentham, 1994) could account for, or at least contribute to, the nondifussional component of \( t_0 \), but it is yet to be established whether this isomerization is on the activation pathway.

Several of our results (Table II) could not be fitted to the model (Hirori, 1979, cited in Horiuti et al., 1989), according to which the lag phase reflects the kinetics of two sequential reactions, MLCK\(_{20}\) phosphorylation ("slow") and transition into force-generating states ("fast"), resulting in a delay that approximates the \( t_0 \) of the faster of the two processes, estimated from the rate of contraction of smooth muscle containing thiophosphorylated MLCK\(_{20}\) (Horiuti et al., 1989). However, fitting to this two-step model is appropriate only when one of the two reactions is much faster than the other, whereas in the present study the rates of MLCK\(_{20}\) phosphorylation (in the presence of 40 \( \mu \)M CaM) and force development by thiophosphorylated cross-bridges were, in some cases, similar (Tables II and III). It is apparent that, depending on experimental conditions, the quantitative contributions of different processes to \( t_0 \) are variable.

The Rapid Rate of MLCK\(_{20}\) Phosphorylation and Its Correlation with Force Development—The very high rate of in situ MLCK\(_{20}\) phosphorylation (up to 3.5 s\(^{-1}\)) is a new finding, revealed by very rapid, synchronous activation by flash photolysis and rapid freezing at high (100-ms) time resolution. Considering the \( Q_{0.9} \) (about 2) of MLCK, even our slowest rate (1.1 s\(^{-1}\) at 22 °C), initiated by photolysis of NP-EGTA in the absence of exogenous CaM, is faster than the previously reported value of 1.1 s\(^{-1}\) at 37 °C (bovine trachealis; Kam and Stull, 1986) that was, probably, also rate-limited by the slower rise in [Ca\(^{2+}\)] that achieved by neural stimulation than by photolysis of NP-EGTA (present study). The fastest rate of MLCK\(_{20}\) phosphorylation (3.5 s\(^{-1}\) at 22 °C) was reached following photolysis of caged ATP (Table III). Extrapolating (for a \( Q_{10} \) of 2), this corresponds to a rate of about 9 s\(^{-1}\) at 37 °C. Considering the concentrations of MLCK (~4 \( \mu \)M; Adelstein and Klee, 1981, Ngi et al., 1984, Rüegg et al., 1984, Gong et al., 1992) and of myosin (approximately 60–80 \( \mu \)M; Cohen and Murphy, 1979, Butler et al., 1989) in smooth muscle, the highest initial rates observed by us (11–12 \( \mu \)mol of P\(_{i}\) transferred/min/mg of enzyme at 22 °C) are comparable with the \( V_{\text{max}} \) of the isolated enzyme in solution (Adelstein and Klee, 1981; Pereschini and Hartschorne, 1983). Therefore, it is obvious that the \( V_{\text{max}} \) of MLCK is not rate-limiting the cross-bridge cycle in smooth muscle, and that the slow physiological rates of phosphorylation are largely due to the very small fraction of CaM available for MLCK.

The rate, but not necessarily the final level, of phosphorylation correlated well with the rate of force development. For example, both rates were faster following photolysis of caged ATP than of NP-EGTA in the presence of 5 \( \mu \)M CaM, although the plateau levels of MLCK\(_{20}\) phosphorylation were not significantly different. Furthermore, increasing [CaM] increased the rates of both contraction and MLCK\(_{20}\) phosphorylation (Fig. 5) without necessarily increasing their maximum amplitudes. These results do not support the suggestion (Kühn et al., 1990) that the acceleration of contraction by CaM in the absence of increased steady state MLCK\(_{20}\) phosphorylation is mediated by a phosphorylation-independent mechanism.

Following simultaneous photolysis of caged ATP and NP-EGTA, the duration of the lag phase was intermediate (\( t_0 = 188 \text{ ms} \) with 0 added CaM, 108 ms with 40 \( \mu \)M CaM) between the values obtained after photolysis of either caged compound alone. The initial rate of phosphorylation was also faster (1.7 s\(^{-1}\) versus 0.9 s\(^{-1}\)) following simultaneous (NP-EGTA/caged ATP) photolysis than following photolysis of NP-EGTA alone at identical (5 \( \mu \)M) [CaM]. These unexpected findings may have been due to an inhibitory process during incubation with ATP, prior to photolysis of NP-EGTA, that would be absent when ATP is released simultaneously with Ca\(^{2+}\). For example, ATP could inhibit, directly or through phosphorylation of CaM, formation of a putative preactive Ca\(_2\)CaM-MLCK complex (Klee and Haietch, 1980). Photophosphorylation of CaM reduces its activity in vitro (Sacks et al., 1987; Quadroni et al., 1994). However, the use of a spinach CaM (40 \( \mu \)M) that lacks several phosphorylation sites (Thr-26 \( \rightarrow \) Cys, Tyr-99 \( \rightarrow \) Phe, Thr-146 \( \rightarrow \) Met; Watters et al., 1980; Lukas et al., 1984) did not accelerate contractions initiated by photolysis of NP-EGTA (data not shown). Inhibition of CaM kinase II by KN-62 (20 \( \mu \)M) to prevent inhibitory phosphorylation of MLCK by CaM kinase II (Tansey et al., 1994) also had no significant effect.

A second unexpected observation was that increasing [Ca\(^{2+}\)] from pCa 5.5 to pCa 4.5 prolonged both the lag phase and the \( t_0 \) of force developed following ATP release (Table II). This slowing effect of high [Ca\(^{2+}\)] was reversible and, therefore, not attributable to Ca\(^{2+}\)-activated proteases. Inhibition of MLCK could also not account for the slowing of the first phase of force development (\( k_0 = 2.5 \text{ s}^{-1} \) at pCa 4.5 versus \( k_0 = 3.6 \text{ s}^{-1} \) at pCa 5.5) in smooth muscles in which MLCK\(_{20}\) was thiophosphorylated. [Ca\(^{2+}\)] in smooth muscle normally does not rise to pCa 4.5 (reviewed in Somlyo and Himpens, 1989), and the physiological significance and mechanism, whether through binding to MLCK or action on thin filament-associated protein(s), of the inhibitory effects of high [Ca\(^{2+}\)] remain to be determined.

The major contributor to the lag phase of activation of intact smooth muscle by an agonist is the generation of inositol 1,4,5-trisphosphate preceding the rise in [Ca\(^{2+}\)] (Somlyo et al., 1988a; Miller-Hance et al., 1988; Somlyo et al., 1992). We now conclude that the delay that follows the increase in [Ca\(^{2+}\)] (Somlyo and Somlyo, 1994) is largely due to the combination of two mechanisms: 1) recruitment of Ca\(_2\)CaM from a slowly available CaM pool (Rüegg et al., 1984; Tansey et al., 1994; present study) and 2) activation of the Ca\(_2\)CaM-MLCK complex, possibly its isomerization (Töörk and Trentham, 1994). We also find that the rapidly available [CaM] is lower, that the rate of phosphorylation of MLCK\(_{20}\) in situ can be significantly faster than previous estimates, and that ATP may reduce (slow) the availability of CaM for contractile activation.

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Note Added in Proof—Western blots of tissues obtained under our experimental conditions (45 min following permeabilization with \( \beta \)-escin) showed no detectable loss of endogenous calmodulin, compared with the content of nonpermeabilized muscles.

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