Temporal aspects of excitation-contraction coupling in airway smooth muscle

GARY C. SIECK,1,2 YOUNG-SOO HAN,1 CHRISTINA M. PABELICK,1 AND Y. S. PRAKASH1

1Departments of Anesthesiology and 2Physiology and Biophysics, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Received 1 May 2001; accepted in final form 20 July 2001

Sieck, Gary C., Young-Soo Han, Christina M. Pabelick, and Y. S. Prakash. Temporal aspects of excitation-contraction coupling in airway smooth muscle. J Appl Physiol 91: 2266–2274, 2001.—In airway smooth muscle (ASM), ACh induces propagating intracellular Ca2+ concentration ([Ca2+]i) oscillations (5–30 Hz). We hypothesized that, in ASM, coupling of elevations and reductions in [Ca2+]i, to force generation and relaxation (excitation-contraction coupling) is slower than ACh-induced [Ca2+]i oscillations, leading to stable force generation. Therefore, in ASM cells, ACh-induced force generation depends on the global cellular [Ca2+]i, response to ACh, as established by the spatial and temporal integration of [Ca2+]i oscillations. Accordingly, the purpose of the present study was to establish the time frame of the intracellular cascade involved in excitation-contraction coupling in porcine ASM.

In contrast to the extremely short delay (on the order of a few milliseconds) (1, 4) between elevated [Ca2+]i; and force generation in striated muscle, smooth muscle is characterized by substantially longer temporal delays. Excitation-contraction coupling in smooth muscle involves a cascade of intracellular events (Fig. 1), including 1) mobilization of calmodulin (CaM) (delay 1); 2) binding of Ca2+ to CaM and CaM/Ca2+-dependent activation of myosin light chain kinase (MLCK) (delay 2); 3) phosphorylation of the regulatory myosin light chain MLC20 (delay 3); and 4) cross-bridge recruitment and cycling (delay 4) (reviewed by de Lanerolle, Ref. 3). Previous studies in other types of smooth muscle have demonstrated that both the binding of Ca2+ to CaM and the activation of MLCK (delay 2) are extremely fast and are not likely to contribute more than a few milliseconds to the total delay in the contractile response (19, 33). Accordingly, the delay between elevation of [Ca2+]i; and contraction most likely involves CaM recruitment, MLC20 phosphorylation, and actin-

Address for reprint requests and other correspondence: G. C. Sieck, Anesthesia Research, Mayo Clinic and Foundation, 200 First St. SW, Rochester, MN 55905 (E-mail: sieck.gary@mayo.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
myosin interactions. To explore excitation-contraction coupling delays in vascular smooth muscle, Zimmermann and colleagues (34) developed elegant techniques utilizing flash photolysis of caged compounds to discriminate the contributions of the various events intervening between elevated [Ca\(^{2+}\)], and contractile response. In one set of experiments, photolytic release of caged Ca\(^{2+}\) was used to rapidly elevate [Ca\(^{2+}\)]. The delay in the contractile response was then measured to determine the total delay in excitation-contraction coupling (Fig. 1). By adding exogenous CaM, these investigators (34) demonstrated that the recruitment of CaM from intracellular stores introduced the longest delay in excitation-contraction coupling in rabbit vascular smooth muscle. Photolytic release of caged ATP (in the presence of complexed Ca\(^{2+}\)-CaM and thus activated MLCK) was used to determine the delay introduced by MLC\(_{20}\) phosphorylation (delay 3) and cross-bridge recruitment and cycling (delay 4) (Fig. 1). Although significant, these combined delays were shorter than those introduced by CaM recruitment. In the present study, similar procedures were used to determine the contributions of the different components of the excitation-contraction coupling cascade in porcine ASM.

**METHODS**

**ASM Preparation**

Fresh porcine trachea was obtained from a local abattoir. The endothelial layer was removed from the underlying smooth muscle in Hanks’ balanced salt solution (HBSS) buffered with 10 mM HEPES (pH 7.4; Life Technologies, Rockville, MD) at room temperature. The smooth muscle layer was then excised along the direction of the longitudinal muscle layer, from which small strips (~300 μm wide and 3 mm long) were cut for force experiments. Single ASM cells were prepared as described previously (18, 27).

---

**Single Cell Imaging**

ASM cells were plated on collagen-coated coverslips, washed with HBSS, and incubated for 30–45 min at 37°C in 5 μM fluo 3-AM (Molecular Probes). The coverslip was then washed in HBSS, mounted on an open slide chamber (RC-25F, Warner Instruments) mounted on the stage of a Nikon Diaphot inverted microscope, and imaged by using an Odyssey XL real-time confocal system (Narus Company) as described previously (18, 27). Cells were exposed to 1 μM ACh, and the [Ca\(^{2+}\)] responses were recorded in real time at 30 Hz. The images were transferred to a processing software (ANALYZE, Mayo Biomedical Imaging Resource), in which the delay between elevation in [Ca\(^{2+}\)] (>10 gray level increase in pixel intensity from baseline) and the initiation of contraction was measured by detecting the edges of a myocyte and automatically tracking for movement of the edge. Measurements were made in regions of 1 μm × 1 μm to increase sensitivity.

**Force Measurements**

The technique for dissection and β-escin permeabilization of ASM strips has been previously published (15, 16). Briefly, 0.3–0.5 mm wide strips of ASM were mounted in a Gühr muscle research system (Scientific Instruments) (9). In this system, samples were mounted in a quartz tissue cuvette between length and force transducers by using stainless steel microforceps. Signals were recorded via a data acquisition board (National Instruments, AT-MIO-16-L9) and software (National Instruments, LabView) running on a personal computer. The strips were initially perfused at 1 ml/min with physiological saline solution aerated with 95% O\(_2\)-5% CO\(_2\).

**Solutions**

The relaxing and activating solutions containing known concentrations of different metals, ligands, and metal-ligand complexes were made with the use of a computer program described by Fabiato and Fabiato (5), with stability constants reported by Godt and Lindley (6). The solutions contained the following (in mM): 7.0 EGTA, 1.0 free Mg\(^{2+}\), 5.0 MgATP, 15.0 creatine phosphate, 64.0 imidazole, and sufficient potassium propionate to adjust ionic strength to 180 mM. The pH was adjusted to 7.00 with hydrogen propionate. The composition of the various solutions is shown in Table 1. Unless otherwise noted, 30 μM Mg\(^{2+}\) concentration was 1 mM. We prepared all solutions at room temperature and adjusted to pH 7.0 and to 0.20 M ionic strength with potassium propionate. Caged Ca\(^{2+}\) [1-(2-nitro-4,5-dimethoxyphenyl)-1,2-diaminoethane-N,N',N'-N'-tetraacetic acid (DM-nitrophen)] solution contained additional composition: the protease inhibitors phe- nylmethylsulfonyl fluoride (1 mM) and leupeptin (1 μM) and the mitochondrial blockers KCN (1 mM), carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (1 μM), or oligomycin (2 μg/ml). To remove contaminant ATP and ADP from the caged ATP solution, 100 μg/ml apyrase (grade V, Sigma Chemical) was used. pCa 4.0 rigor solution contained 50 P\(_1\), P\(_2\)-di(adenosine-5')pentaphosphate (Sigma Chemical), an inhibitor of myokinase activity. Creatine phosphokinase (50 units/ml) was added to all solutions containing creatine phosphate. The diazo-2 solution contained the same protease inhibitors and mitochondrial blockers as the caged Ca\(^{2+}\) (DM-nitrophen) solution.

**Flash Photolysis System**

A xenon lamp-based flash photolysis system (Rapp Opto-Elektronik) was used for these studies. Caged compounds...
were uncaged by using a 2-ms flash of ultraviolet light (347 nm) from a high-pressure xenon arc lamp (XBL2-B). The light was focused onto the tissue via a condenser and focusing lens. The focused flashlight was adjusted to cover the entire muscle strip. We have measured the power output of this system to be ∼85–110 mJ over the 2-ms exposure. We have also verified that this power output is sufficient to produce maximum uncaging.

Measurement of Temporal Delays in Force Generation

Excitation-contraction coupling after elevation of [Ca\textsuperscript{2+}], in ASM involves a cascade of intracellular events, which are summarized in Fig. 1. To explore excitation-contraction coupling delays in ASM, we adapted the elegant techniques developed by Zimmermann and colleagues (34) for vascular smooth muscle, utilizing flash photolysis of caged compounds to discriminate the contributions of the various events intervening between elevated [Ca\textsuperscript{2+}], and contractile response.

Delays 1 + 2 + 3 + 4. In the first set of experiments, photolytic release of caged Ca\textsuperscript{2+} (DM-nitrophen) was used to rapidly elevate [Ca\textsuperscript{2+}]. Each strip was first repeatedly contracted with 100 μM ACh, and muscle length was increased after each contraction until the maximum contraction was reached. Each strip was maintained at this optimum length for the duration of the experiment. The strip was then permeabilized for 45 min at pCa 6.0 with 50 μM β-escin, exposed to 10 μM Ca\textsuperscript{2+} ionophore A-23187 for 15 min, washed in pCa 9.0 for 10 min, and washed again in pCa 9.0 without EGTA for 10 min, relaxed for 5 min in photolysis solution (2 mM DM-nitrophen, 1.25 mM CaCl\textsubscript{2}, 25 mM hexamethylenediamine-N\textsubscript{2},N\textsubscript{2},N\textsubscript{2},N\textsubscript{2}-tetraacetate, 40 μM free Mg\textsuperscript{2+}, 5.0 mM Na\textsubscript{2}ATP, 15 mM creatine phosphate, 10 mM reduced glutathione, 70 mM TES at pH 7.1, protease inhibitors, and mitochondrial blockers (34)), followed by photolytic release of DM-nitrophen (without 40 μM calmodulin) using a single flash of ultraviolet light.

Delays 2 + 3 + 4. In the second set of experiments, the protocol described above was modified by adding 40 μM CaM to the photolysis solution before photolytic release of caged Ca\textsuperscript{2+}. The delay to the onset of contraction was then measured.

Delays 3 + 4. In the third set of experiments, permeabilized strips were relaxed in pCa 9.0 for 30 min and then washed in a rigor pCa 9.0 solution (without ATP) for 10 min, followed by a high-Ca\textsuperscript{2+} EGTA-buffered rigor solution (pCa 4.0 without ATP) for another 10 min. The strip was finally incubated for 3 min in 5.0 mM caged ATP [1-(2-nitrophenyl)-ethyl-ATP] with 40 μM CaM, followed by photolytic release of caged ATP. Delay 4. In the fourth set of experiments, the protocol described for caged ATP above was modified by thiophosphorylation of MLCK\textsubscript{20} via addition of 2 mM ATP\textsubscript{S} to the high-Ca\textsuperscript{2+} rigor solution before incubation with caged ATP and photolytic release of caged ATP.

Measurement of Temporal Delays in Force Relaxation

The same procedure was used as described above for delays 1 + 2 + 3 + 4, except the permeabilized strips were incubated for 5 min with a solution containing a pCa of 5.7 (sufficient to generate ∼95% maximum force) and 2 mM diazo-2 (22). The composition of solution for this experiment is shown in Table 1. Photolytic release of the Ca\textsuperscript{2+} chelator diazo-2 causes it to convert from a low-affinity (dissociation constant = 2.2 μM) to a high-affinity state (dissociation constant = 0.073 μM), thereby rapidly reducing [Ca\textsuperscript{2+}]. Photolytic release of diazo-2 was induced only after maximum steady-state force was achieved. After photolytic release of diazo-2, the delay to the onset of force relaxation (t\textsubscript{onset}) and the time for force to relax to 50% of initial force (t\textsubscript{50}) were measured.

Statistical Analysis

Comparisons were made by using independent Student’s t-tests with Bonferroni corrections for repeated comparisons. Statistical significance was established at P < 0.05.

RESULTS

ACh-Induced [Ca\textsuperscript{2+}], Oscillations in ASM Cells

Exposure of single ASM cells to ACh resulted in the initiation of propagating [Ca\textsuperscript{2+}], oscillations that typically initiated from one end at the long axis of the cell and propagated toward the other end (Fig. 2). Within a localized region of an ASM cell (e.g., ∼1 μm\textsuperscript{3}), the frequency of [Ca\textsuperscript{2+}], oscillations was initially higher and more fused (reduced peak-to-trough amplitude) compared with a subsequent steady-state phase (Fig.
3). The frequency of both transient and steady-state phases of the localized $[\text{Ca}^{2+}]_i$ oscillations depended on ACh concentration (Fig. 3). The propagation velocity of $[\text{Ca}^{2+}]_i$ oscillations through the ASM cell also depended on ACh concentration (Fig. 4). Within a localized region of a cell, there was relatively little change in peak amplitude of $[\text{Ca}^{2+}]_i$ oscillations with increasing ACh concentration (Fig. 5). In contrast, the peak-to-trough amplitude tended to decrease with increasing ACh concentration, reflecting an increase in baseline (Fig. 6). When the entire cell was circumscribed, it was observed that the global $[\text{Ca}^{2+}]_i$ increased with increasing ACh concentration (Fig. 5). These characteristics of ACh-induced $[\text{Ca}^{2+}]_i$ oscillations were comparable to those reported in our previous studies (17, 27, 28, 31).

Excitation-Contraction Delays in Single Cells

The ACh-induced elevation of $[\text{Ca}^{2+}]_i$ did not result in an immediate initiation of contraction (Fig. 2). At a resolution of 33 ms (30-Hz sampling rate), the first indication of contraction occurred at time points ranging from 367 to 500 ms ($450 \pm 43$ ms; $n = 6$). The first wave of $[\text{Ca}^{2+}]_i$ resulted in considerably greater shortening compared with subsequent waves (data not shown). Typically, after the first two to three waves, the contraction was maintained with continuing $[\text{Ca}^{2+}]_i$ waves.

Excitation-Contraction Delays in ASM Strips

In the first set of experiments, elevation of $[\text{Ca}^{2+}]_i$ after flash photolytic release of caged Ca$^{2+}$ in the absence of exogenous CaM resulted in the initiation of a force response after 800 ms (Figs. 7 and 8; $n = 5$). In contrast, addition of 40 $\mu$M CaM significantly shortened the delay between elevated $[\text{Ca}^{2+}]_i$ and force generation to 470 ms (Figs. 7 and 8; $n = 5$). In the presence of 100 $\mu$M extracellular free Ca$^{2+}$ and 40 $\mu$M CaM, flash photolysis of caged ATP resulted in force generation after 95 ms (Figs. 7 and 8; $n = 5$). Under the same conditions, but with prior thiophosphorylation of MLC$_{20}$, the delay between caged release of ATP and force generation was reduced to 55 ms (Figs. 7 and 8; $n = 5$).

The time required to reach 50% of maximum force ($t_{1/2}$ of force generation) as well as the time required to reach maximum steady-state force were also determined under each experimental condition (Fig. 9). After flash photolytic release of caged Ca$^{2+}$ in the absence of exogenous CaM, the $t_{1/2}$ of force generation was 16 s whereas the time required to reach steady-state force was 80 s. Providing exogenous CaM shortened these delays considerably ($7$ s for $t_{1/2}$ of force generation and $40$ s for maximum steady-state force). The shortest delays in force generation were observed under conditions in which MLC$_{20}$ was thiophosphorylated ($3$ s for $t_{1/2}$ of force generation and $15$ s for maximum steady-state force).

Force Relaxation in ASM Strips

After photolytic release of diazo-2, force relaxation occurred after a temporal delay of 1.16 ± 0.06 s ($t_d$; Fig.
10; \( n = 5 \). The half-time for force relaxation \( (t_{1/2}) \) was \( 57 \pm 11 \) s. The rate constant \( (k_r = 0.014 \pm 0.003 \text{ s}^{-1}) \) of force relaxation was estimated from \( \ln(2)/t_{1/2} \) (7).

**DISCUSSION**

The present study demonstrated that in porcine ASM cells ACh-induced propagating \([Ca^{2+}]_i\) oscillations lead to a force response that is nonoscillatory, considerably delayed, and sustained for the duration of ACh exposure. Compared with single cells, the delay between \([Ca^{2+}]_i\) elevation and initiation of contraction is longer in multicellular preparations, most likely reflecting the contribution of noncontractile elements of muscle. In these permeabilized multicellular preparations, recruitment (dissociation and diffusion from some internal binding site) of CaM after elevation of \([Ca^{2+}]_i\) provides the greatest contribution to the excitation-contraction delay. However, in intact ASM cells, it appears that there is sufficient cytosolic CaM to initiate contraction. The results of the present study also demonstrated a substantial delay between the reduction of \([Ca^{2+}]_i\) and force relaxation. Together, these results support the hypothesis that slower \(Ca^{2+}\)-

---

**Fig. 4.** Propagation velocity of \([Ca^{2+}]_i\) oscillations. The propagation velocity of \([Ca^{2+}]_i\) oscillations through ASM cells also increased with ACh concentration.

**Fig. 5.** Regional peak vs. global amplitudes of \([Ca^{2+}]_i\) oscillations. Within localized regions (1 \( \mu \text{m}^3 \)) of ASM cells, the peak amplitude of ACh-induced \([Ca^{2+}]_i\) oscillations did not depend on ACh concentration. In contrast, the global response of the entire ASM cell did increase with increasing ACh concentration.

**Fig. 6.** Peak-to-trough amplitude of \([Ca^{2+}]_i\) oscillations. Within a localized region, the peak amplitude of \([Ca^{2+}]_i\) oscillations did not depend on ACh concentration. In contrast, the peak-to-trough amplitude decreased with increasing ACh concentration, reflecting an increase in baseline.
Dependent processes in the excitation-contraction coupling cascade integrate local [Ca\textsuperscript{2+}]i oscillations leading to stable force production. The presence of propagating [Ca\textsuperscript{2+}]i oscillations reflects the spatial and temporal heterogeneity of [Ca\textsuperscript{2+}]i responses of ASM cells to agonist stimulation (31). Differences in processes that regulate [Ca\textsuperscript{2+}]i, including second messenger production, sarcoplasmic reticulum Ca\textsuperscript{2+} release and reuptake, and plasma membrane Ca\textsuperscript{2+} influx and efflux, may lead to this intercellular heterogeneity. Spatial heterogeneity in [Ca\textsuperscript{2+}]i regulation may also result within a cell from variations in the distribution of membrane receptors, production and/or diffusion of second messengers, and SR and membrane Ca\textsuperscript{2+} channels. Spatial heterogeneity in [Ca\textsuperscript{2+}]i regulation within cells is further evidenced by spontaneous localized [Ca\textsuperscript{2+}]i transients, termed Ca\textsuperscript{2+} sparks observed in several cell types (2, 8, 11, 21, 29), including porcine ASM cells (26), and by propagated agonist-induced [Ca\textsuperscript{2+}]i waves (18, 20, 25, 27, 28, 31). These spatial heterogeneities also give rise to temporal heterogeneities in [Ca\textsuperscript{2+}]i regulation, leading to nonsynchronized elevations in [Ca\textsuperscript{2+}]i in different parts of the cell. The results of the present study are consistent with these observations.

Fig. 7. Examples of the delays in force generation by porcine ASM strips after flash photolysis of caged Ca\textsuperscript{2+} or caged ATP (see Fig. 1 for description of excitation-contraction delays). Note the significantly greater delays after caged release of Ca\textsuperscript{2+} in the absence (delays 1 + 2 + 3 + 4; A) or presence of 40 μM CaM (delays 2 + 3 + 4; B). In the presence of elevated Ca\textsuperscript{2+} and exogenous CaM, release of caged ATP (delays 3 + 4; C) substantially shortened the delay before force was generated. Thio phosphorylation of MLC\textsubscript{20} with ATP\textsubscript{S} resulted in the shortest delay (delay 4; D) in force generation.

Fig. 8. Summary of the temporal delays in force generation of porcine ASM strips after flash photolysis of caged Ca\textsuperscript{2+} or caged ATP (see Fig. 1 for description of excitation-contraction delays). Flash photolysis of caged Ca\textsuperscript{2+} in the absence of exogenous CaM (delays 1 + 2 + 3 + 4) resulted in significantly greater delay compared with that in the presence of 40 μM CaM (delays 2 + 3 + 4). In the presence of elevated Ca\textsuperscript{2+} and exogenous CaM, flash photolysis of caged ATP (delays 3 + 4) further shortened the delay. Thio phosphorylation of MLC\textsubscript{20} with ATP\textsubscript{S} resulted in the shortest lag (delay 4) in force development. *, †, and ‡, Statistically significant (P < 0.05) differences with sequential pairwise comparisons.

Fig. 9. Time required to reach maximum steady-state force after photolytic release of caged Ca\textsuperscript{2+} or caged ATP. Note that the longest temporal delays were associated with delays 1 and 2 (see Fig. 1 for description of excitation-contraction delays).
with previous evidence of such spatial and temporal heterogeneities of \([\text{Ca}^{2+}]_i\) regulation.

The physiological significance of \([\text{Ca}^{2+}]_i\) oscillations remains a subject of investigation. In porcine ASM cells, ACh-induced \([\text{Ca}^{2+}]_i\) oscillations display an agonist concentration- and time-dependent modulation of amplitude, frequency, and propagation velocity (27, 28). However, the peak or maximum \([\text{Ca}^{2+}]_i\) level reached (relative to 0 nM) remains relatively constant across time and ACh concentration. These previous data can be interpreted as a limited SR \([\text{Ca}^{2+}]_i\) store being the source of these oscillations. Accordingly, it can be hypothesized that the major physiological significance of local propagating \([\text{Ca}^{2+}]_i\) oscillations lies in their providing an effective mode of regulating the global cellular \([\text{Ca}^{2+}]_i\) level using only a limited pool of SR \([\text{Ca}^{2+}]_i\). In contrast to the local response, the time-dependent modulation of \([\text{Ca}^{2+}]_i\) oscillations at a single ACh concentration is reflected by global \([\text{Ca}^{2+}]_i\), as a biphasic response, with no observed oscillations (28). Accordingly, the significance of propagating \([\text{Ca}^{2+}]_i\) oscillations may lie in the establishment of global force level by spatial and temporal integration of \([\text{Ca}^{2+}]_i\); by slower \([\text{Ca}^{2+}]_i\)-dependent physiological processes that are involved in force production (28). The results of the present study using both single cells and multicellular strips are consistent with this hypothesis.

As shown in Fig. 1, the overall scheme of excitation-contraction coupling in smooth muscle involves several processes that follow a rise in \([\text{Ca}^{2+}]_i\). In the first set of experiments, the sum delay between all of these processes that follow the rise in \([\text{Ca}^{2+}]_i\) was measured, including four molecules of \([\text{Ca}^{2+}]_i\) binding to CaM, diffusion and binding of Ca-CaM to MLCK, activation of the Ca-CaM-MLCK complex, MLC20 phosphorylation, and activation of cross-bridge cycling. Among these processes, uncaging of \([\text{Ca}^{2+}]_i\) by flash photolysis and the actual binding of \([\text{Ca}^{2+}]_i\) to CaM have been shown to be extremely fast (19) and are unlikely to contribute to the delay in force generation. Furthermore, the kinetics of Ca-CaM binding to MLCK has also shown to be rapid (33). In an elegant study in rabbit vascular smooth muscle, Zimmermann et al. (34) estimated that the contribution of this reaction in the presence of 40 μM CaM was insignificant to the hundreds of milliseconds delay in force generation. Because the present study was conducted under similar conditions, Ca-CaM-MLCK complex formation was unlikely to have contributed to the delay in porcine ASM.

The present study found that addition of 40 μM CaM significantly decreased the delay between \([\text{Ca}^{2+}]_i\) and force. This is consistent with findings in vascular smooth muscle and suggests that, in a permeabilized multicellular preparation, dissociation and diffusion of CaM from some internal binding site is relatively slow compared with \([\text{Ca}^{2+}]_i\) binding to CaM and subsequent Ca-CaM activation of MLCK. In the study by Zimmermann et al. (34), it was demonstrated that even though significantly smaller concentrations of CaM, such as 0.1 μM, can result in increased \([\text{Ca}^{2+}]_i\) sensitivity and steady-state levels of force generation, the delay in force generation is affected only by much higher CaM concentrations. On the basis of this finding, these authors concluded that, although rapidly activating CaM is available for steady-state force generation, CaM recruitment from intracellular stores is a limiting factor in the rate of force activation. However, in the present study, the delay to the onset of force generation after release of caged \([\text{Ca}^{2+}]_i\) in the presence of 40 μM CaM was comparable to that observed in the intact ASM cell. These results suggest that there may be sufficient cytosolic CaM within an intracellular region of an ASM cell and that CaM recruitment may not rate limiting. In vascular smooth muscle, Zimmermann et al. (34) also argued that, because the time delay with addition of extremely high levels of CaM was still significantly high compared with that with flash photolysis of ATP, diffusion of CaM itself may contribute minimally. Instead, it has been suggested that isomerization of Ca-CaM-MLCK (33) accounts for a nondiffusional component of the time delay.

Flash photolysis of caged ATP in the presence of elevated \([\text{Ca}^{2+}]_i\) and exogenous CaM drastically decreased the delay in force generation, suggesting that MLC20 phosphorylation is extremely fast, compared with recruitment of CaM. Indeed, previous studies have demonstrated rates of MLC20 phosphorylation from 1–3 s⁻¹ (14, 34) that would yield time delays similar to those found in the present study. In this regard, activity of MLCK itself is thought to be non-limiting (34). With phosphorylated MLC20, the delay in force generation due to cross-bridge recruitment is minimal compared with more proximal processes. These data are consistent with previous studies using flash photolysis of caged ATP under rigor conditions in smooth (34) and striated muscles (10). It should be noted that although the delay to the onset of force generation was only ~500–800 ms, the time required to reach maximum steady-state force was much longer (~15–80 s; Figs. 7–9).

The extent of MLC20 phosphorylation depends on the balance between MLCK and myosin light chain phosphatase (MLCP) activity. Agonist stimulation may...
shift the balance toward MLCK-mediated MLC$_{20}$ phosphorylation by promoting Ca$_{2+}$-dependent MLCK activity and reducing MLCP activity. Certainly removal of [Ca$^{2+}]$, (e.g., after release of caged diazo-2) would shift the balance toward MLC$_{20}$ dephosphorylation and thereby promote force relaxation. The results of the present study clearly indicate that this shift toward MLC$_{20}$ dephosphorylation and force relaxation is a much slower process. After photolytic release of diazo-2 and subsequent reduction of [Ca$^{2+}]$, the onset of force relaxation was observed only after ~1.2 s, compared with a temporal delay of ~0.5–0.8 s for force generation after release of caged Ca$^{2+}$. In addition, the t$_{1/2}$ for force relaxation was ~57 s, which was much slower than the time required for force to reach half maximum (~16 s, without exogenous CaM). The slower kinetics for force relaxation imply a slow inactivation of Ca-CaM-MLC complex and/or a slow rate of MLCP-mediated MLC$_{20}$ dephosphorylation after abrupt Ca$^{2+}$ chelation induced by photolysis of diazo-2.

The present study also found that delays in force generation were significantly less in single myocytes than in multicellular strips. This difference may be partially attributable to noncontractile elements in strip preparations that dampen the force response. Furthermore, different cells within a strip may differ in their kinetics of excitation-contraction coupling processes sufficiently to cause heterogeneity in the force response, thus further delaying overall contraction.

In conclusion, the present study demonstrates spatial and temporal delays in excitation-contraction coupling in porcine ASM cells. The results indicate that the greatest contribution to the delay in force generation occurs from recruitment of intracellular CaM, whereas phosphorylation of MLC$_{20}$ and cross-bridge recruitment are much faster in activation. These temporal delays are important in the excitation-contraction coupling during ACh-induced [Ca$^{2+}]$ oscillations, in which spatial delays in [Ca$^{2+}$] elevation due to propagation may further delay cellular contraction.

We thank Dr. Anna Boukatina for advice on technical issues. This work is supported by National Institute of General Medical Sciences Grants GM-56686 (to G. C. Sieck) and GM-57816 (to Y. S. Prakash) and by the Mayo Foundation, Rochester, MN.

REFERENCES
26. Pabelick CM, Prakash YS, Kannan MS, and Sieck GC. Spatial and temporal aspects of calcium sparks in porcine trae...


