hancers (*15, 31*). Since expression of SV40 depends on host factors, it is not surprising that the factors important for the expression of the virus have a normal role in the control of cellular gene expression, as has been shown for SP1 (*32*).

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23. Total cellular RNA (25 μg) was analyzed by primer extension with 5′–end labeled synthetic oligonucleotide primers complementary to positions +15 to +34 of the CAT gene (*20*) and +58 to +81 of the TK gene (*24*). The extended products were approximately 100 and 130 bases long, indicating correct initiation from the hMT-II_A (*12*) and the SV40 early (*22*) promoters. The procedures used for primer extension analysis were as described by Walker *et al.* (*3*).
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Activation of Smooth Muscle Contraction: Relation Between Myosin Phosphorylation and Stiffness

KRISTINE E. KAMM AND JAMES T. STULL

Contraction and myosin light-chain phosphorylation were measured in electrically stimulated tracheal smooth muscle. Latencies for the onset of force, stiffness, and light-chain phosphorylation were 500 milliseconds. Myosin light chain was phosphorylated from 0.04 to 0.80 mole of phosphate per mole of light chain with a pseudo–first-order rate of 1.1 per second with no evidence of an ordered or negatively cooperative process. Following the period of latency, stiffness increased with phosphorylation and both increased more rapidly than isometric force. The linear relation between stiffness and phosphorylation during activation suggests independent attachment of each myosin head upon phosphorylation.

SMOOTH MUSCLE MYOSIN FILAMENTS are composed of myosin monomers that consist of two heavy chains [each with a molecular weight of 200,000 (200K)] and two each of two types of light chains (20K and 17K, respectively). Phosphorylation of the regulatory 20K light chains (P–light chain) of smooth muscle myosin by Ca^{2+} and calmodulin-activated myosin light-chain kinase results in an increase in the actin-activated magnesium-dependent adenosinetriphosphatase (Mg^{2+} ATPase) activity of myosin (*1, 2*). Conflicting biochemical data with smooth muscle myosin have been reported for two aspects of the activation process having important implications for regulation of contractile activity in the cell. (i) From kinetic studies, the mechanism of phosphorylation of the two heads of myosin in a filamentous form has been reported to conform to an ordered or negatively cooperative (*3, 4*), or an apparently random process (*5*). (ii) From studies on gizzard myosin, it has been concluded that both heads of myosin must be phosphorylated before the Mg^{2+} ATPase activity of either head can be activated by actin (*3, 4*). Data with myosin from bovine stomach and swine pulmonary artery, however, allow the possibility that the Mg^{2+} ATPase activity of each head is independently stimulated by phosphorylation of its P–light chain (*6*).

Contractile force in smooth muscle, as in skeletal muscle, is believed to result from the sliding of filaments due to the cyclic interaction of myosin with actin (*7*). In contrast to skeletal muscle, in which myosin P–light-chain phosphorylation modulates the contractility (*8*), P–light-chain phosphorylation in smooth muscle appears to be necessary for the contraction to occur (*2*). In addition, force development by a smooth muscle cell occurs only after a relatively long period of mechanical latency lasting some hundreds of milliseconds (*9*). Specific models relating mechanical activation to myosin P–light-chain phosphorylation in the smooth muscle cell cannot be tested in studies where cells in the tissue are slowly and asynchronously stimulated by agonist diffusing into the preparation or where values of phosphorylation vary only over a small range (*10*). We

Department of Pharmacology and Moss Heart Center, University of Texas Health Science Center, Dallas, TX 75235.

have described a preparation of bovine tracheal smooth muscle in which both myosin P–light-chain phosphorylation and force development are rapid and nearly maximal when strips are stimulated by neurotransmitter released during electric field stimulation of cholinergic nerves (*11*). That the rate of this contraction is independent of tissue cross-sectional area indicates that field stimulation avoids most of the diffusion problems delaying activation of cells in agonist-stimulated tissues. The studies reported here were undertaken to investigate (i) the kinetic properties of myosin P–light-chain phosphorylation in smooth muscle cells and (ii) the requirement for myosin P–light-chain phosphorylation for initiation of mechanical activity as assessed by force and stiffness. Strips of tracheal smooth muscle were prepared and mounted for measurement of isometric force (*11*). Platinum wire electrodes attached directly to the ends of the tissue allowed continuous field stimulation of the preparation between the time the organ bath was lowered and the tissue was frozen. Muscle strips were frozen with a rapid-release electronic freezing device (*12*). Stimulator, stimulus marker, and freeze hammer electronics were signaled by a switch triggered by the movement of the bath. Frozen muscle samples (7 to 10 mg) were analyzed for phosphate content of P–light chain by two-dimensional gel electrophoresis (*11, 13*).

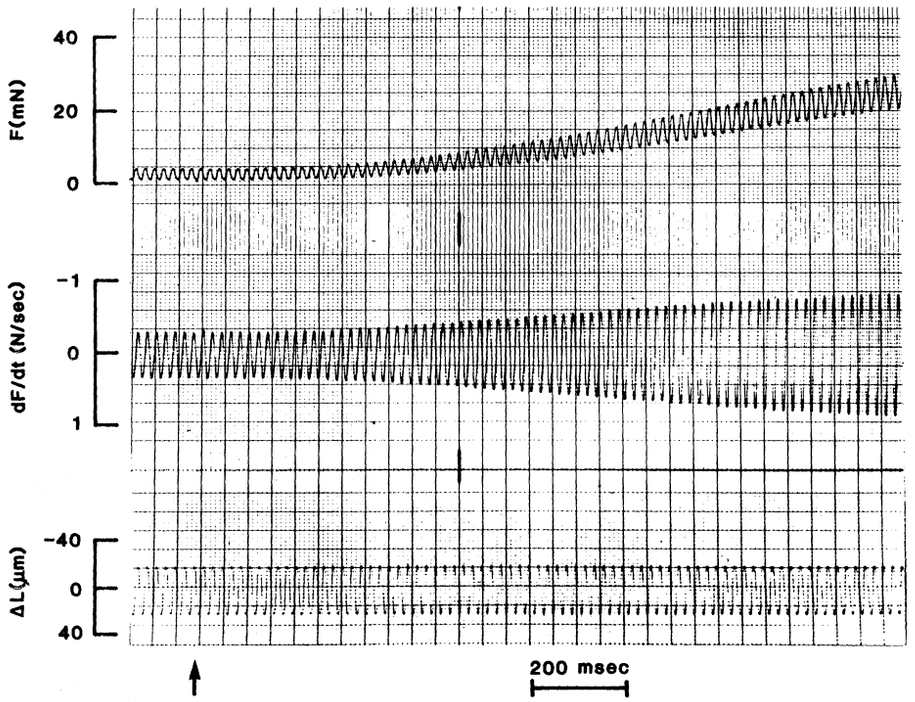

Fig. 1. Changes in force and stiffness in response to sinusoidal perturbations in muscle length during electric field stimulation of trachealis. Stimulus conditions were 0.2-msec, 16-V d-c pulses delivered at 50 Hz in the presence of 1 μ*M* propranolol and 0.1 μ*M* neostigmine, which resulted in an atropine-sensitive contraction of 0.8 maximal force developed in response to 1 μ*M* carbachol. Continuous stimulation was begun at the arrow. Muscle length was 7.9 mm and sinusoidal length oscillations (Δ*L*) of 40 μm were applied at a frequency of 50 Hz. The parameter *dF/dt* was used as an indicator of stiffness (*dF/dl*) since (i) $dF/dl = (dF/dt)/(dl/dt)$ and (ii) *dl/dt* is of constant amplitude and is in phase with *dF/dt*. Latency was estimated from the point of intersection of tangents to the force and *dF/dt* traces before stimulation and after the values started to increase.

Continuous measurements of stiffness were made during isometric contraction by measuring muscle force in response to sinusoidal length perturbations. Muscle strips were oriented horizontally between a servo motor (Cambridge Technology model 300H) and force transducer (Hewlett-Packard FTA 100). Tied ends of each strip were drawn through and cemented to the back of thin washers soldered to the tip of the lever arm and the arm of the force transducer. Strips, stretched to about 1.05 times the optimal length for force development (L_0), had an average length of 7.2 ± 0.2 mm ($n = 5$) (mean ± SEM used throughout the report). A function generator (Wavetek model 143) was used to drive the position control loop of the servo system, and position was monitored from the servo motor. Output signals were displayed on an oscillographic recorder (Hewlett-Packard 7754A). Strips were stretched and released with a 50-Hz sine wave of an amplitude that resulted in a total displacement equal to 0.5 percent of the muscle length (Fig. 1). These parameters were chosen so that measured stiffness depended only slightly on frequency of oscillation (*14*) and maximal force was not compromised by the magnitude of the oscillation. With frequencies from 0 to 150 Hz, neither the period of mechanical latency nor the initial rate of force development were altered. In a separate set of experiments, the time courses of stiffness and force measured at 30 Hz were the same as those reported here. Stiffness ($\Delta F/\Delta L$) was evaluated from the amplitude of the sinusoidal force response. Stiffness of the resting muscle was not diminished after calcium depletion by a 90-minute incubation in Ca^{2+}-free buffer containing 2 m*M* ethyleneglycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), indicating that stiffness of the resting muscle can be attributed primarily to mechanical properties independent of Ca^{2+}, presumably parallel elastic elements in the tissue. Also, it was assumed that possible contributions of weakly attached crossbridges to resting muscle stiffness would be essentially undetectable at physiological ionic strength and very low frequencies of oscillation used in this study. Active stiffness, the difference between total and passive stiffness, was expressed as a fraction of maximal active stiffness assessed on each strip after 60 seconds of field stimulation. At this time active stiffness [$5.8 \pm 0.5 \times 10^6$ N m^{-2} L_0^{-1} ($n = 5$)] measured by sinusoidal perturbation was not significantly different from that which we have calculated from the elastic recoil measured after isotonic quick releases to different afterloads [$6.1 \pm 0.6 \times 10^6$ N $m^{-2} \cdot L_0^{-1}$ ($n = 6$)] ($P > 0.1$). Active stress was $1.7 \pm 0.1 \times 10^5$ N m^{-2}.

Average values of force, stiffness, and P–light-chain phosphorylation during the first 5 seconds of contraction are shown in Fig. 2. The period of mechanical latency was estimated by measurements of both force and stiffness in each of six strips. Latency for force, 500 ± 64 msec, was not significantly different from latency for stiffness, 466 ± 68 msec (paired *t* test, $P > 0.1$). Myosin phosphorylation was very low (0.04 mole of phosphate per mole of P–light chain) in the resting muscle and was not significantly elevated after 500 msec of stimulation. No mechanical activity was detected before phosphorylation of myosin. These results indicate that the latency for force development could result from delays in phosphorylation of P–light chain. The sequence of events leading to activation of myosin–light-chain kinase (release, diffusion, and binding of neurotransmitter to receptors; receptor-mediated events causing release of calcium to the cytosol; diffusion and binding of calcium to calmodulin and Ca_4^{2+}·calmodulin activation of myosin–light-chain kinase) are probably the rate-limiting processes. There

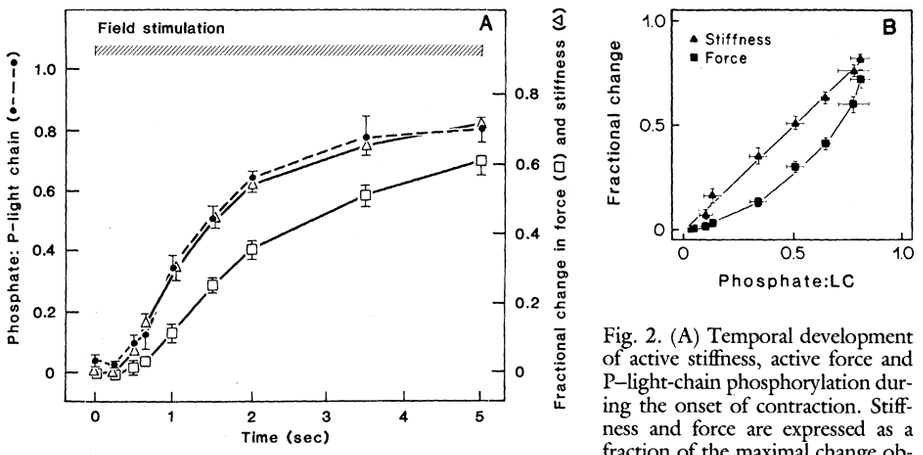

Fig. 2. (A) Temporal development of active stiffness, active force and P–light-chain phosphorylation during the onset of contraction. Stiffness and force are expressed as a fraction of the maximal change observed above values measured at rest (n = 5). P–light-chain phosphorylation (LC-P) are mean values from four to nine strips. (B) Mechanical data replotted as a function of LC-P. The method of unweighted least squares was used to relate the means of values of phosphorylation and stiffness since variances and sample sizes were consistent with the assumption of homogeneity of variance. A linear relation between phosphorylation and stiffness, $(\Delta F/\Delta L) = 1.01\ (\pm\ 0.03)$ LC-P $- 0.01\ (\pm\ 0.02)$, was obtained.

is no apparent delay in activation (assessed by stiffness) after phosphorylation of the myosin P–light chain.

Both stiffness and phosphorylation increased more rapidly than force after the 500-msec period of latency. The kinetics of myosin P–light-chain phosphorylation could be described with a pseudo-first-order rate of 1.1 sec^{-1}. These results on the intact tissue show no evidence of an ordered or negatively cooperative phosphorylation of the P–light chains of myosin.

Force developed by the cyclic interactions between myosin and actin is transmitted through elastic elements located in the tissue, the cells, and the contractile apparatus, whose compliance or stiffness has been evaluated. Morphological measurements as well as theoretical estimates (*14, 15*) have demonstrated that half or more of the tissue compliance is of intracellular origin. Further, the elastic responses measured on single amphibian smooth muscle cells exhibit behavior consistent with mechanical properties attributed to crossbridges in more highly structured skeletal muscle (*16*). In trachealis, as in taenia coli (*17*), active stiffness increased more rapidly than isometric force. Similar observations have been made for skeletal muscle fibers and bundles where stiffness is interpreted as an indication of the proportion of attached crossbridges (*18*). Furthermore, in tracheal smooth muscle, a linear relationship between stiffness and P–light-chain phosphorylation was observed during the first 5 seconds of contraction (Fig. 2). Although our experiments were not designed to dissect tracheal tissue compliance by determining the anatomical origin of various elastic elements, the linear relation between phosphorylation and stiffness indicates that a component of the elastic response may reside in the cellular contractile apparatus. These data raise the possibility that phosphorylation of myosin P–light-chain might allow the attachment of myosin to actin.

Independent versus cooperative stimulation of the actin-activated Mg^{2+} ATPase activity of myosin by phosphorylation can be interpreted from models predicting ways in which enzyme activity depends on the extent of P–light-chain phosphorylation (*4*). Interpretation of the manner in which force or stiffness depends on P–light-chain phosphorylation, however, is limited by our understanding of the transduction of actomyosin ATPase activity to mechanical output by the muscle strip. A linear relation allows models of stimulation in which either: (i) each head is independently stimulated upon phosphorylation regardless of the kinetics of phosphorylation, or (ii) myosin must be doubly phosphorylated for stimulation when the phosphorylation reaction is positively cooperative. An upwardly concave relation, in which the fractional activity equals the square of the P–light-chain phosphorylation, results from the model in which myosin must be doubly phosphorylated for stimulation and phosphate is incorporated randomly. There are to date no biochemical data to suggest positive cooperativity, and our data on tissue show no evidence of negative cooperativity for the process of myosin phosphorylation. Thus, the linear relation between stiffness and phosphorylation seems to reflect the model in which random phosphorylation of myosin heads leads to independent attachment. This model resembles results obtained on kinetics of skeletal muscle myosin phosphorylation and the relation between P–light-chain phosphorylation and isometric twitch potentiation in skeletal muscle (*8*). The possibility of cooperative effects of phosphorylation on activation of the tracheal smooth muscle is suggested by the relation between P–light-chain phosphorylation and force, which is consistent with a model in which force depends on the formation of doubly phosphorylated myosin (Fig. 2). However, it has also been proposed that the lag in force development after P–light-chain phosphorylation, which results in the observed nonlinear relation during the onset of contraction, could be attributed to delays in the expression of force through series elastic elements in the tissue (*19*). More definitive conclusions about the kinetic properties of myosin phosphorylation and the relation of phosphorylation to activation of contraction will require direct measures of levels of singly and doubly phosphorylated myosin in intact smooth muscle.

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