Myosin Phosphorylation and the Cross-Bridge Cycle in Arterial Smooth Muscle

Abstract. *Phosphorylation of the 20,000-dalton light chain of myosin is closely correlated with cross-bridge cycling in arterial smooth muscle. Evidence is presented that dephosphorylation can produce an attached, noncycling cross-bridge (latch-bridge) which is responsible for the high economy of force maintenance in this tissue.*

The concentration of calcium [Ca^{2+}] in myoplasm is believed to be the link between excitation events at muscle cell membranes and initiation of the actin-myosin interaction. The actin-activated adenosinetriphosphatase activity of myosin isolated from vertebrate smooth muscle is dependent on phosphorylation of the 20,000-dalton myosin light chain (LC 20) by an endogenous Ca^{2+}-dependent myosin light chain kinase (MLCK) (*1-3*). This evidence suggested that increases in myoplasmic [Ca^{2+}] may initiate contraction in smooth muscle by activating MLCK. The hypothesis is supported by observations showing an increase in phosphorylated LC 20 in isometrically contracting smooth muscles (*4-5*). The results of the study described here show that light chain phosphorylation is closely correlated with the rate of the actin-myosin interactions and suggest that developed force can be maintained by the presence of attached, noncycling cross-bridges produced by dephosphorylation.

Our experiments were performed on smooth muscle strips obtained from the media of swine carotid arteries, adjusted to their optimum length for active force generation (L_0) (*6-7*). Mechanical measurements on this preparation reflect cellular contractile function (*8*). Unstimulated tissues exhibited a low level of tone, and the isometric contraction induced by substitution of 109 m*M* K^+ for Na^+ in isosmotic physiological salt solution can be maintained for at least 2 hours with little change. Maximum isometric force (F_0) was a function of the [Ca^{2+}] in the high K^+ solution. The F_0 was $2.9 \pm 0.4 \times 10^5$ (± standard deviation) newtons per square meter in the presence of 1.6 m*M* Ca^{2+} in the experiments reported here (Fig. 1a), rising to a maximum value of $3.9 \pm 0.2 \times 10^5$ N/m^2 with 25 m*M* Ca^{2+}. Steady-state active stress (total stress − passive stress at L_0) should provide an estimate of the number of cross-bridges developing force additively (*9, 10*). A reproducible method to estimate the total number of attached cross-bridges (*11-15*) is to determine the total stress less the passive elastic stress at which the activated muscle yields when subjected to a quick-stretch of sufficient rate (> 0.05 L_0/sec) and magnitude (> 0.018 L_0) (*7, 12*). This measurement, here termed the load-bearing capacity, is shown in Fig. 1a.

Relations between force and velocity were determined, and V_0, the shortening velocity at zero external load, was obtained as an estimate of the mean cross-bridge cycling rate (*9, 16*). Velocities were obtained from steady-state shorten-

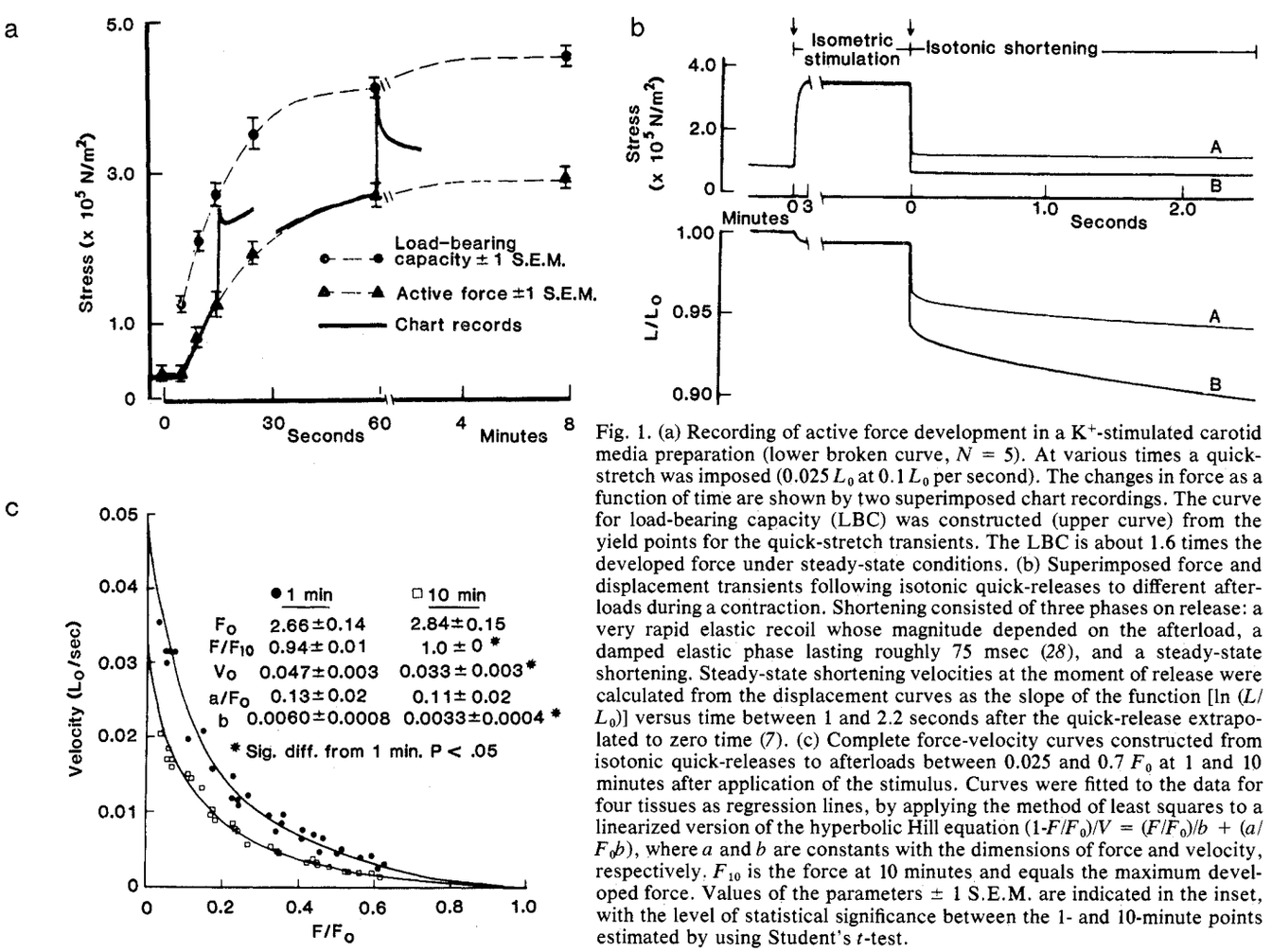

Fig. 1. (a) Recording of active force development in a K^+-stimulated carotid media preparation (lower broken curve, $N = 5$). At various times a quick-stretch was imposed (0.025 L_0 at 0.1 L_0 per second). The changes in force as a function of time are shown by two superimposed chart recordings. The curve for load-bearing capacity (LBC) was constructed (upper curve) from the yield points for the quick-stretch transients. The LBC is about 1.6 times the developed force under steady-state conditions. (b) Superimposed force and displacement transients following isotonic quick-releases to different afterloads during a contraction. Shortening consisted of three phases on release: a very rapid elastic recoil whose magnitude depended on the afterload, a damped elastic phase lasting roughly 75 msec (*28*), and a steady-state shortening. Steady-state shortening velocities at the moment of release were calculated from the displacement curves as the slope of the function [ln (L/L_0)] versus time between 1 and 2.2 seconds after the quick-release extrapolated to zero time (*7*). (c) Complete force-velocity curves constructed from isotonic quick-releases to afterloads between 0.025 and 0.7 F_0 at 1 and 10 minutes after application of the stimulus. Curves were fitted to the data for four tissues as regression lines, by applying the method of least squares to a linearized version of the hyperbolic Hill equation $(1\text{-}F/F_0)/V = (F/F_0)/b + (a/F_0b)$, where a and b are constants with the dimensions of force and velocity, respectively. F_{10} is the force at 10 minutes and equals the maximum developed force. Values of the parameters ± 1 S.E.M. are indicated in the inset, with the level of statistical significance between the 1- and 10-minute points estimated by using Student's t-test.

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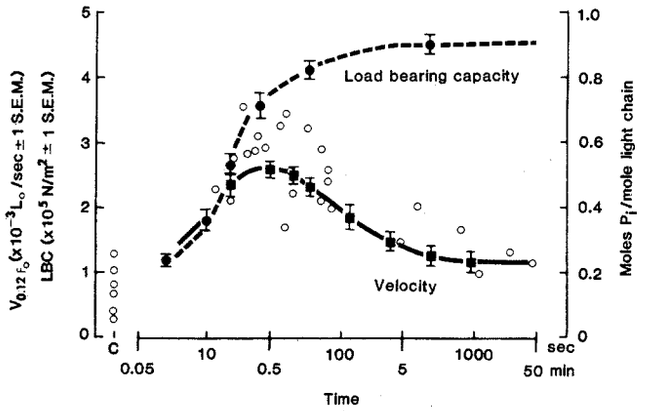

Fig. 2. Load-bearing capacity (●), shortening velocity at an afterload of 0.12 F_0 (■), and fractional phosphorylation of tissue LC 20 (○) as a function of time on a log scale after K^+ stimulation of the carotid media preparation. The mechanical data points are averages for a series of five preparations (± 1 S.E.M.), with extensions of the curves based on time course studies in other tissues. Each phosphorylation determination represents a single tissue, frozen at the indicated times (N = 30), and C designates unstimulated tissues.

ing rates after a quick-release to a given afterload (Fig. 1b), by means of an electromagnetic lever system (*7*). The force-velocity curves varied when quick-releases were performed at different times after the contraction was initiated. The two curves illustrated in Fig. 1c show that at times when the tissue was generating near steady-state force there were marked decreases in shortening velocity with time, as indicated by the values of V_0 and b. The data indicate that cross-bridge cycling rates change with time when F_0 and the load-bearing capacity remain relatively constant.

The parameter a/F_0, which is a measure of the concavity of the force-velocity curve, exhibited no significant changes with time. Therefore, shortening velocity obtained at any given afterload was proportional to the true value of V_0. Accordingly, shortening velocity ($V_{0.12}$) at a load of 0.12 F_0 was used to assess changes in cycling rates as a function of time during a contraction. $V_{0.12}$ was 0.44 V_0 ± 0.02 S.E.M. (standard error of the mean; N = 11) for force-velocity curves at 1, 3, and 10 minutes, and this ratio was not significantly different in paired comparisons at any time (Student's t-test). Shortening velocity increased rapidly after the high K^+ solution was applied, peaked by 30 seconds, and subsequently declined to a stable value after approximately 20 minutes of stimulation (Fig. 2). True shortening velocities in the early part of the response were underestimated because the fixed afterload of 0.12 F_0 would represent an appreciably greater effective load while force was still increasing. Similar phasic velocity changes were observed when tissues were activated with electrical field stimulation, norepinephrine, and histamine, although quantitative differences were found in the time course of the response and the magnitude of the peak velocity and developed force (*7*).

Amounts of phosphorylated LC 20 during K^+-induced isometric contractions interrupted at various times by quick-freezing of the tissues are also illustrated in Fig. 2. Phosphorylation was determined by the shift in the isoelectric point of the phosphorylated LC 20 when the polypeptides were resolved by a two-dimensional gel electrophoresis system (*17*). Unstimulated tissues exhibit low, but significant, amounts of phosphorylated LC 20 (Fig. 2). This may be attributed to the presence of tone and to a small (< 15 percent) systematic overestimate arising from the introduction of a negative charge on the polypeptide by carbamylation or other reactions during tissue processing (*17*). Phosphorylation preceded force development and was proportional to the increase in load-bearing capacity, as would be predicted if LC 20 phosphorylation is the Ca^{2+}-dependent regulatory mechanism for initiating the actin-myosin interaction. Peak levels of phosphorylation were about 0.65 mole of P per mole of LC 20 with the high K^+ solution containing 1.6 mM Ca^{2+}, which is submaximal for force development. However, phosphorylation started to decline before force reached maximum values, reaching a steady intermediate level after approximately 20 minutes. There was a striking correlation between light chain phosphorylation and shortening velocities (Fig. 2). A similar correlation was evident when histamine was the stimulus.

Our experimental approach, which assumes that load-bearing capacity is an estimate of the number of attached cross-bridges and that V_0 or V (at a constant afterload) provides an estimate of cross-bridge cycling rates, is consistent with data from structural, biochemical, and mechanical studies on striated muscle. However, our results were obtained during imposed mechanical transients which, in themselves, may alter the level of activation (*18*). But since the mechanical perturbations were always the same, changes in the estimated parameters with time should reflect real changes in the contractile system. Furthermore, the rate of isometric force redevelopment after a step shortening to 0.95 L_0 showed the same time-dependent changes as the isotonic shortening velocities (*7*). Our extension of this methodological approach to a smooth muscle tissue involves the additional reasonable assumption that the general cross-bridge sliding filament mechanism operates in this tissue (*19*).

The curve for load-bearing capacity (Fig. 2) is a type of conventional active-state curve, and presumably reflects the increase in myoplasmic [Ca^{2+}] to a steady-state value because (i) developed force in glycerol-extracted tissue from the carotid media is a function of the [Ca^{2+}] (*20*), and (ii) active stress developed by KCl-depolarized smooth muscles is dependent on extracellular Ca^{2+} and is a function of the [Ca^{2+}] in the bathing solution (*7, 21*). The increase in phosphorylation on stimulation (Fig. 2) is also an expected consequence of increasing [Ca^{2+}] with activation of the MLCK (*1-5*). Thus, the initial response to stimulation is consistent with the hypothesis that activation of the contractile apparatus involves an obligatory Ca^{2+}-stimulated phosphorylation of the LC 20. However, the carotid media exhibits behavior not seen in striated twitch fibers: shortening velocity decreases with time although the numbers of cross-bridges generating force remain constant (Figs. 1a and 2). This behavior has also been observed in rabbit urinary bladder (*22*) and amphibian slow skeletal muscle (*23*).

Mechanical data must be cautiously interpreted, but the following hypothesis is plausible. We suggest that cross-bridge cycling depends on LC 20 phosphorylation. This proposal is consistent with biochemical data for a close correlation of LC 20 phosphorylation with both actin-activated adenosinetriphosphatase activity (*1-3, 17*) and shortening velocity (Fig. 2). The second part of the hypothesis is concerned with the evidence that cross-bridges can remain attached and generating a force when the phosphorylated fraction capable of cycling decreases to about 25 percent. It seems reasonable that the MLCK-myosin light chain phosphatase system can act on attached as well as on free cross-bridges, and we suggest that dephosphorylation of an attached cross-bridge arrests the cycle. Such an at-

tached, noncycling cross-bridge might be termed a "latch-bridge," capable of maintaining force but acting as an internal load on the remaining cross-bridges to produce the decrease in velocity (*10*). Attached, noncycling cross-bridges have been postulated in resting smooth muscle (*24*), and their existence is fairly well established for invertebrate smooth muscles of the catch type (*25*).

The basis for the decrease in phosphorylated LC 20 with time cannot be inferred from our data, but must reflect a decrease in the relative activity of kinase to phosphatase. This could come about by enzymatic alteration [see (*26*) for one hypothesis] or different reaction rates for these enzymes when acting on free or attached cross-bridges.

Our results have important functional implications for vascular smooth muscle. On stimulation, there is a fairly rapid initiation of the actin-myosin interaction which has been shown to require phosphorylation of the LC 20. The subsequent decline in phosphorylation and in cycling rates, with maintained numbers of cross-bridges, would produce a high holding economy already demonstrated in this tissue (*27*). This is consistent with the physiological role of arterial smooth muscle, which normally functions tonically, resisting increases in arterial diameter due to blood pressure.

PATRICK F. DILLON
MARK O. AKSOY
STEVEN P. DRISKA
RICHARD A. MURPHY

Department of Physiology, University of Virginia School of Medicine, Charlottesville 22908

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Chemical Defense Mechanisms on the Great Barrier Reef, Australia

Abstract. *Seventy-three percent of all exposed common coral reef invertebrates, from four phyla (42 species) tested, are toxic to fish. This represents the first evidence of the high incidence of toxicity in the marine tropics among several phyletic groups comprising dominant species. Most of the remaining exposed species have structural defense mechanisms against predation by fish. Of cryptic invertebrates, 25 percent are toxic to fish. The relation between toxicity, fish feeding behavior, community structure, and evolutionary theory is discussed.*

On a visit to the Lizard Island Research Station, north Great Barrier Reef, Queensland, Australia, between 18 May and 5 June 1979, I extended previous research (*1-4*) by conducting studies on common species from several phyla. The results of the laboratory toxicity tests are presented in Table 1 (*5*).

Sponges showed patterns similar to those reported (*4, 6*), that is, there is a relatively high percentage of toxic exposed species although some cryptic species are also toxic. Of the 10 exposed species, four were nontoxic. Of the nontoxic species, *Carteriospongia foliascens* and *Carteriospongia*? sp. are packed with sand grains and are very tough. *Pericharax heteroraphis* has large spicules that are very densely packed, and ? *Hemimycale* sp. is difficult to tear. These characteristics probably serve as physical deterrents to predators; certain of these species may contain mildly noxious compounds not detected by current methods (*4*).

The most toxic sponge was *Haliclona* sp., a cryptic species; toxicity is characteristic of the family Haliclonidae (*4, 6*). The other toxic cryptic sponge (*Chondrilla* sp.) was mildly toxic. Both *Haliclona* sp. and *Chondrilla* sp. may occur as either exposed (subject to fish predation) or cryptic forms since certain species of *Haliclona* show both distributional modes, as does the mildly toxic Caribbean sponge *Chondrilla nucula* Schmidt (*4*).

All but a single species of soft coral studied were toxic. Vermeij (*7*) proposed that those zoanthids and octocorals (*Xenia, Clavularia*) that lack stinging nematocysts may have chemical defenses against grazers since they appear to be eaten by very few fish. The toxicity of *Xenia* sp. in my study supports this contention. Both gorgonian species tested

Table 1. Bioassays of the toxicity to fish of common coral reef animals from Lizard Island, Great Barrier Reef, Australia. A total of 42 frozen species were tested in Sydney; 73 percent of all exposed organisms tested were toxic to goldfish. By exposed is meant that part of the body is exposed to fish or that the animal is exposed to fish during part of the day.

Taxonomic group	Number of species				
	Tested	Exposed		Cryptic	
		Toxic	Non-toxic	Toxic	Non-toxic
Sponges	16	6 (60)*	4	2	4
Soft corals	8	7 (88)	1		
Gorgonians	2	2 (100)			
Asteroids	1		1		
Crinoids	1	1 (100)			
Holothurians	12	6 (100)			6
Ascidians	2		2		

*Numbers in parentheses represent the percent of the total exposed.

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