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19. We thank T. Tse for help in extracting the chitin, E. Schallenberger for trapping the animals, L. E. Ullberg for photographs, and B. E. Weitbrecht for art.

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## Active Shortening Retards the Decline of the Intracellular Calcium Transient in Mammalian Heart Muscle

Abstract. *When active shortening of the cat papillary muscle was allowed at any time during a contraction, the intracellular concentration of free calcium ions, detected with the calcium-sensitive bioluminescent protein aequorin, was higher than at comparable times in isometric twitches. The difference was not attributable to the differences of length involved or to motion artifacts, and must have been related to the act of shortening or the difference in force development in the two types of contractions. This observation and the phenomenon of shortening deactivation are both consistent with the hypothesis that attachment of cross bridges increases the affinity of the myofilaments for calcium ions.*

It is well known that in many kinds of striated muscle the total duration of contraction is less when the muscle is allowed to shorten during a twitch than when it is constrained to contract isometrically throughout (*1*, *2*). The mechanism of this so-called "shortening deactivation" has not been elucidated, but an obvious possibility is that the intracellular $Ca^{2+}$ transient responsible for excitation-contraction coupling might somehow be abbreviated when shortening is permitted to occur. To test this possibility we used the calcium-sensitive bioluminescent protein aequorin (*3*–*5*) to monitor intracellular calcium transients in the cat papillary muscle—a muscle in which shortening deactivation is prominent under normal conditions (*2*). The results effectively rule out the mechanism just considered and suggest an alternative that is consistent with other evidence.

Aequorin was prepared and microinjected as described previously (*4*). Multiple superficial cells of papillary muscles from the right ventricles of adult cats were injected with the photoprotein; it was usually necessary to inject 30 to 100 cells to obtain satisfactory light signals. After injection, the muscles were mounted vertically between a small clamp (ventricular end) and a 9-0 Tevdek thread (tendinous end) attached to a servo-controlled electromagnetic lever (*6*). A narrow extension of the base of the glass muscle bath (*7*) extended a short distance axially into an ellipsoidal reflector, allowing the muscle to be positioned near one focal point of the reflector; a photomultiplier (EMI 9635A) was mounted so that its photocathode was at the other focal point. This arrangement, which is similar in principle to one illustrated elsewhere (*5*), was designed to optimize light gathering and to minimize any artifacts in the light signal that might result from twisting or rotation of the muscle during shortening. Light (recorded as photomultiplier anode current with time constant 1 msec), force, velocity, and length signals were recorded in analog form on magnetic tape for later analysis. It was usually necessary to average 8 to 64 light signals obtained under identical conditions to obtain a satisfactory signal-to-noise ratio. So that all records would be made under comparable conditions with respect to loading history (*8*, *9*), each test contraction was preceded by seven free-loaded isotonic contractions. Light signals during such contractions did not change detectably during the time required to acquire the signals that were averaged and compared in a given experimental protocol. Except when noted otherwise, experiments were conducted with the initial muscle length set at $L_{max}$ (that is, the muscle length at which tension development was maximal); temperature was 30°C; the stimulus interval was 4 seconds.

Figure 1A shows records made during isometric (a) and free-loaded isotonic (b) contractions starting from the same muscle length. The aequorin signals rise to the same peak level in the two types of

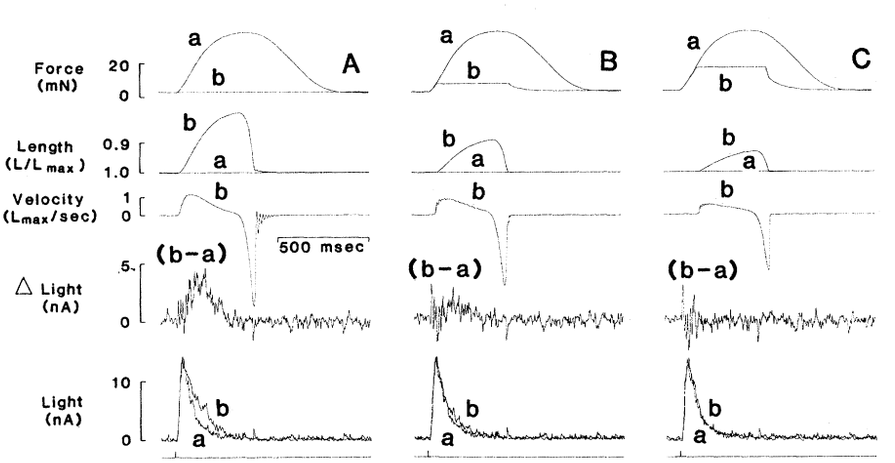


Fig. 1. Aequorin signals of a cat papillary muscle contracting against various loads. Tracings from top downward in all panels: 1, force; 2, shortening; 3, velocity of shortening; 4, light of shortening; 5, aequorin signal; and 6, stimulus. Tracings 1, 2, and 5 show two superimposed twitch contractions—one isometric (a) and one in which shortening occurred (b). The light of shortening is the difference between the two averaged aequorin signals (b − a) obtained by digital electronic subtraction (note that the fourth tracing is displayed with twice the gain of the fifth). Twenty-four signals were averaged. (A) Tracings from an isometric (a) and a free-loaded isotonic contraction (b). The aequorin signals reach the same peak, but the decline of the aequorin signal ($Ca^{2+}$ transient) is initially faster during force development (a) than during shortening (b). The total duration of the light signal is essentially the same in the two contractions. The light of shortening (the difference between the two light signals) becomes apparent some 35 msec after the onset of shortening. (B) The muscle now lifts a light load during the isotonic contraction. The light of shortening is less pronounced. (C) The muscle lifts a heavier load. The light of shortening is practically absent. All tracings in this figure are from the same muscle. Characteristics: length at $L_{max}$, 5.25 mm; mean cross-sectional area, 0.33 $mm^2$; ratio of resting to total tension (resting + active) at $L_{max}$, 0.079.

contraction, but contrary to our initial hypothesis, the $Ca^{2+}$ transient recorded during isotonic shortening (b) declines later than that accompanying the rise of force in isometric contractions (a). The difference between the two light records (b − a) is shown as Δ light in Fig. 1 and will be referred to henceforth as the "extra light" associated with shortening (*10*) or "light of shortening." The extra light is first distinguishable some 35 msec after the onset of shortening, and follows a time course rather similar to that of the velocity of shortening, although with a delay of 35 to 40 msec. In after-loaded isotonic contractions, in which a short period of isometric force development precedes active shortening (Fig. 1B), the onset of the extra light is delayed until after shortening begins. The delay is greater and the extra light is less the larger the after-load against which the muscle contracts (compare Fig. 1, B and C). Results similar to those shown in Fig. 1 have been obtained in each of 11 experiments. Six other experiments gave unclear results because the light signals were too noisy to allow small differences to be resolved. We have never observed clear-cut changes of a type qualitatively different from those shown in Fig. 1. In a few exceptionally bright preparations, the differences between the aequorin signals under isometric and isotonic conditions were clear in individual contractions (that is, without signal averaging).

Motion per se was shown to have a negligible effect on the light signal in experiments with muscles onto which particles from a luminous watch dial had been rubbed to give continuous light emission from a region comparable to that normally injected with aequorin. Differences in the muscle length at which light emission occurred were found not to be important: aequorin signals (not shown) recorded under isometric conditions at the extremes of length involved were not perceptibly different (*11*). Experiments of the type illustrated in Fig. 2 show that shortening is associated with extra light production whether it occurs early or late in the time course of the $Ca^{2+}$ transient and that the extra light can be "turned off" if shortening is stopped before the end of the aequorin signal.

The light of shortening almost certainly reflects an elevation of intracellular calcium ion concentration [$Ca^{2+}$] and therefore must result from an increase in the rate of delivery of calcium to the cytoplasmic space, a decrease in the rate of its removal, or a combination of the two. The sarcoplasmic reticulum and the surface membrane play important roles in these processes, and the possibility must be kept in mind that either or both might be involved in generating the light of shortening. However, we are not aware of specific mechanisms by which the functions of these structures could be altered to produce the changes observed (*12*). The process responsible for the light of shortening must be capable of sensing changes in force or length and of acting very rapidly to change intracellular [$Ca^{2+}$]. This requirement points to the contractile apparatus itself and to the most attractive explanation of the light of shortening that has come to our attention so far. The affinity of troponin C for calcium is increased by attachment of rigor links between the thick and thin filaments of the myofibrils (*13*). It has been suggested that the attachment of force-generating cross-bridges may have a similar effect (*14*). If that is true, allowing the muscle to shorten will decrease the affinity of troponin C for calcium, so that the myofilaments will have less calcium bound to them at the end of a period of shortening than at a comparable time under isometric conditions. Since by that time the $Ca^{2+}$ transient is over (see Figs. 1 and 2), calcium cannot be regained by troponin C, and the terminal phase of relaxation must take place with a reduced degree of activation. Though other factors may be involved, the mechanism just proposed could account for shortening deactivation as well as the light of shortening and thus resolve the apparent paradox presented by the association of shortening deactivation with prolongation of the aequorin signal. The same molecular mechanism has been invoked to account for changes in the aequorin signals of barnacle muscle fibers (*15*) and cat papillary muscles (*16*) after quick releases and to explain changes in the time course of the aequorin signal of mammalian heart muscle resulting from changes in initial fiber length (*16*).

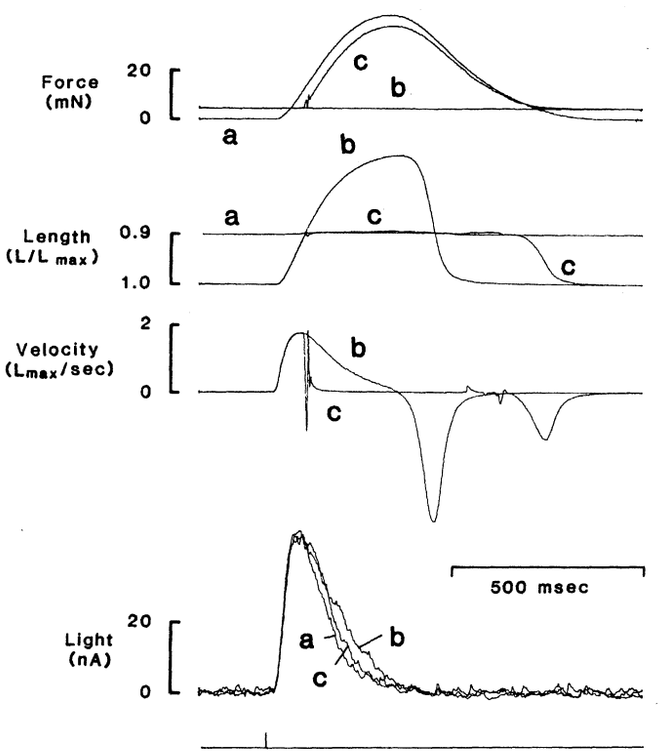


Fig. 2. Influence of abruptly stopping shortening on the aequorin signals of a cat papillary muscle. Tracings as in Fig. 1, except that the averaged ($N$ = 48) records from three different kinds of contraction are superimposed, and no differential light signal is displayed. Contraction (a) is an isometric twitch at 90 percent of $L_{max}$; contractions (b) and (c) start as free-loaded isotonic contractions from $L_{max}$, but in contraction (c) shortening is stopped abruptly at 90 percent of $L_{max}$. The light signal of the stopped contraction (c) deviates from that of the wholly isotonic contraction (b) about 45 msec after shortening is stopped, and drops to join the isometric signal (a). (In some experiments, the aequorin signal of the stopped contraction (c) rejoined that of the isometric contraction (a) earlier than shown here.) Muscle characteristics: length at $L_{max}$, 7.0 mm; mean cross-sectional area, 0.85 $mm^2$; ratio of resting to total tension at $L_{max}$, 0.077.


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7. The bath contained 50 ml of a physiological salt solution with the following composition (m*M*): NaCl 94, $NaHCO_3$ 24, KCl 5, $MgSO_4$ 1, $Na_2HPO_4$ 1, $CaCl_2$ 2.25, sodium acetate 20, and glucose 10. The solution was equilibrated at 30°C with a gas mixture of 95 percent $O_2$ and 5 percent $CO_2$; the *p*H was 7.4.
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10. The difference signal could equally well be plotted downward (a − b) and thought of as "missing light" associated with tension development. Although, as will be apparent from the discussion, the latter approach may prove to have a sounder mechanistic basis, we have adopted the former for the purpose of this presentation because we believe that most readers will be accustomed to thinking of the isometric as the fundamental mode of contraction, and of shortening as a complicating event.
11. Our observation that the time course of the aequorin signal was essentially the same in isometric contractions at two fiber lengths would appear at first glance to be in conflict with a recent report by Allen and Kurihara (*16*), who found that decreases in fiber length tended to prolong the aequorin signal. There is, in fact, no real conflict, for the length changes we have studied were comparatively small, and comparable changes in their experiments produced no more than a 6 to 7 percent change in the duration of the aequorin signal. This change is less than the standard deviation of their measurements, and in the absence of information from greater length changes, would not have been considered significant. We too see a change in the time course of the aequorin signal with greater changes in length. The important point is that changes in the aequorin signal associated with shortening are very much greater than those produced by alterations in length per se.
12. The duration of the plateau of the action potential is known to be longer in isotonic than in isometric contractions (*9*), but since in a given contraction the light of shortening occurs earlier than the change of the action potential, it seems unlikely that the prolongation can be the cause of the change in the $Ca^{2+}$ transient.
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## *Dipetalonema viteae* (Nematoda: Filarioidea): Culture of Third-Stage Larvae to Young Adults in vitro

Abstract. *Infective third-stage larvae of* Dipetalonema viteae *(Nematoda: Filarioidea) were cultured to young adults in a cell-free culture system. Third-stage larvae from the tick vector grew, developed, and molted twice in a medium containing NCTC 135 and Iscove's modified Dulbecco's medium supplemented with fetal bovine serum under a gas phase of 95 percent nitrogen and 5 percent carbon dioxide. The availability of such a culture system for filariids should facilitate studies of their immunology, biochemistry, and sensitivity to drugs.*

Filarial nematodes are important parasites of man and other animals throughout the world; diseases caused by these organisms, such as African river blindness, are especially targeted for study by the World Health Organization (*1*). The culture of filariids in vitro, which thus far has had only limited success (*2*), would be valuable for studies of the immunology and biochemistry of the parasites as well as for the screening of drugs. We now report the culture of a filarial nematode from the third larval stage through two molts to the young adult in a cell-free culture medium. Prior exposure of the larvae to the mammalian host was not required.

We used *Dipetalonema viteae* (Onchocercidae) as a model in developing a culture system for the vertebrate phase of a filariid life cycle, and started with third-stage larvae obtained directly from the arthropod vector. This nematode in jirds (*Meriones unguiculatus*) or hamsters (*Mesocricetus auratus*) has been used in a number of studies on filariasis, including immunological (*3*) and biochemical (*4*) investigations. It has also been used for the screening of potential filaricides in vivo (*5*).

*Dipetalonema viteae* has a typical filariid life cycle. The arthropod vector, an argasid tick (*Ornithodorus tartakovskyi*), releases third-stage larvae in the vertebrate host while taking a blood meal. These larvae grow and develop in the subcutaneous tissues of the vertebrate and molt on days 7 to 8 (third molt) to the fourth stage and again on days 20 to 22 (fourth molt) to the adult stage.

Cultures were prepared and medium changes were made under aseptic conditions in a laminar flow hood. They were initiated with 12 to 17 third-stage larvae obtained from infected ticks (*6*). The larvae were inoculated into 16 by 125 mm glass culture tubes containing 2 ml of a 1:1 (by volume) mixture of NCTC 135 (*7*) and Iscove's modified Dulbecco's medium (IMDM) (*8*) supplemented with 5, 10, 20, or 30 percent nonheat-inactivated fetal bovine serum (FBS-NHI; M.A. Bioproducts) (*9*). The medium in the culture tubes was gassed with 95 percent $N_2$ and 5 percent $CO_2$ for 60 seconds at a flow rate of 65 ml/sec, before and after inoculation with larvae. Culture tubes were closed with nontoxic rubber stoppers and kept stationary and vertical at 36.5° ± 0.5°C. The medium was changed every 4 days (*10*).

The molt to the fourth larval stage was usually first observed between days 11 and 15 after initiation of culture. However, molting also occurred as early as day 8 and as late as day 20. Molting from the fourth stage to the adult occurred after 42 days of culture. Several hundred male and female fourth-stage larvae were obtained over the course of these studies, and a small number of them developed to adults. The growth and development of worms was influenced by the lot and concentration of the FBS supplement. Of the two lots of FBS-NHI tested, lot 948 resulted in the best development of larvae to the young adult. In media supplemented with 10 and 20 percent FBS-NHI from this lot, 50 percent (12 worms) and 40 percent (10 worms), respectively, of the worms were young adults at the termination of cultures on day 71. Only 4 percent of the worms were young adults in media supplemented with 5 percent FBS-NHI (lot 948). In media supplemented with 10 and 20 percent FBS of lot 873, 4 to 15 percent of the worms completed the fourth molt. Incomplete fourth molts, with worms tangled in partially shed cuticles or debris on the bottom of culture tubes, were seen in 4 to 25 percent of the worms in cultures supplemented with 10 or 20 percent of both lots of FBS. Most of the other worms in cultures were fourth-stage larvae, except in media supplemented with 5 percent FBS-NHI in which most worms had not developed beyond the advanced third larval stage. Survival of larvae in media supplemented with 30 percent FBS-NHI was decreased in comparison with media supplemented with 10 and 20 percent FBS. Larvae survived for 47 to 49 days,