Rapid Ionic Modifications During the Aequorin-detected Calcium Transient in a Skinned Canine Cardiac Purkinje Cell

ALEXANDRE FABIATO

From the Department of Physiology, Medical College of Virginia, Richmond, Virginia 23298

ABSTRACT A microprocessor-controlled system of microinjections and microaspirations has been developed to change, within ~1 ms, the [free Ca^{2+}] at the outer surface of the sarcoplasmic reticulum (SR) wrapped around individual myofibrils (0.3–0.4 μm radius) of a skinned canine cardiac Purkinje cell (2.5–4.5 μm overall radius) at different phases of a Ca^{2+} transient. Simultaneously monitoring tension and aequorin bioluminescence provided two methods for estimating the peak myoplasmic [free Ca^{2+}] reached during the spontaneous cyclic Ca^{2+} release from the SR obtained in the continuous presence of a bulk solution [free Ca^{2+}] sufficiently high to overload the SR. These methods gave results in excellent agreement for the spontaneous Ca^{2+} release under a variety of conditions of pH and [free Mg^{2+}], and of enhancement of Ca^{2+} release by calmodulin. Disagreement was observed, however, when the Ca^{2+} transient was modified during its ascending phase. The experiments also permitted quantification of the aequorin binding within the myofibrils and determination of its operational apparent affinity constant for Ca^{2+} at various [free Mg^{2+}] levels. An increase of [free Ca^{2+}] at the outer surface of the SR during the ascending phase of the Ca^{2+} transient induced further release of Ca^{2+}. In contrast, an increase of [free Ca^{2+}] during the descending phase of the Ca^{2+} transient did not cause further Ca^{2+} release. Varying [free H^+], [free Mg^{2+}], or the [Na^+]/[K^+] ratio had no significant effect on the Ca^{2+} transient during which the modification was applied, but it altered the subsequent Ca^{2+} transient. Therefore, Ca^{2+} appears to be the major, if not the only, ion controlling Ca^{2+} release from the SR rapidly enough to alter a Ca^{2+} transient during its course.

INTRODUCTION

The skinned cardiac cell, which is obtained by microdissection of the sarcolemma from a segment of a single cell, offers an advantage over the skinned fiber from skeletal muscle (Natori, 1954) in that its small radius and the broad spacing of its myofibrils permit externally applied solutions to rapidly reach the outer surface of the sarcoplasmic reticulum (SR) that surrounds and packs individual myofibrils. In addition, changes in myoplasmic [free Ca^{2+}] are much slower in the cardiac intact cell than in the skeletal muscle intact cell (Fabiato, 1981*a*, 1982*a*). Accordingly, it is possible to change the myoplasmic [free Ca^{2+}] at the

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outer surface of the SR of a skinned cardiac cell more quickly than occurs during physiological activation: within ~1 ms as compared with the 4–5 ms of the time to peak of the fastest transsarcolemmal Ca^{2+} currents observed in isolated intact cardiac cells at 22°C (G. Isenberg, personal communication; see Isenberg and Klöckner, 1982, for data at 35°C).

This approach has been used in the present study to explore the factors controlling Ca^{2+} release and reaccumulation by the SR. The ionic composition of the medium bathing the skinned cardiac cell was changed during the course of the Ca^{2+} transient detected by simultaneously recording tension and aequorin bioluminescence. An increase of myoplasmic [free Ca^{2+}] produces an increase of the bioluminescence of the phosphoprotein aequorin (Blinks et al., 1976, 1982; Allen and Blinks, 1978, 1979; Wier, 1980; Wier and Isenberg, 1982; Wier and Hess, 1984; Hess and Wier, 1984). This change in aequorin bioluminescence considerably precedes the change in tension in the skinned cardiac cell (Fabiato, 1981*a*). Thus, a rapid change of the solution bathing the skinned cardiac cell can modify the aequorin bioluminescence transient at the beginning of the tension transient or even before its onset. Hence, excitation-contraction coupling can be studied by experimental manipulations between Ca^{2+} release from the SR and tension development.

Skinned cardiac cells from the Purkinje tissue of the adult dog were selected for these experiments because the intact cells from this tissue lack transverse tubules. This facilitates microdissection and eliminates the possibility of artifactual Ca^{2+} release caused by depolarization of sealed-over transverse tubules, which is a major problem in skinned skeletal muscle fibers (E. W. Stephenson, 1981). In addition, the release of Ca^{2+} from the SR results in a myoplasmic [free Ca^{2+}] significantly higher in this preparation than in skinned cells from other cardiac tissues (Fabiato, 1982*a*). This facilitates detection of aequorin bioluminescence. The relative amplitude of the tension transient resulting from the Ca^{2+} release is not, however, a larger percentage of the maximum Ca^{2+}-activated tension in the canine Purkinje cell than in other cardiac cells because the myofilaments are less sensitive to Ca^{2+} (Fabiato, 1982*a*). This preparation is very different from the skinned cardiac cells from ungulate Purkinje tissues, in which the SR is mostly superficial and releases little Ca^{2+}. In contrast, all the myofibrils of the skinned canine cardiac Purkinje cell are surrounded and packed by an abundant SR composed both of longitudinal tubules and of an exceptionally well-developed "extended junctional SR," which has the same structure as the terminal cisternae but makes no contact with the sarcolemma (Sommer et al., 1982). Finally, comparisons of the results obtained from skinned cells and intact cells are facilitated by the thorough description of the characteristics of the Ca^{2+} transient in intact multicellular preparations from the canine cardiac Purkinje tissue (Wier, 1980; Wier and Isenberg, 1982; Wier et al., 1983; Wier and Hess, 1984; Hess and Wier, 1984).

The purpose of these experiments was to investigate the effects of various ionic modifications at different times during the Ca^{2+} transient resulting from a spontaneous cyclic release of Ca^{2+} from the SR. The Ca^{2+} release occurred in the continuous presence of a bulk solution [free Ca^{2+}] sufficiently high for

overloading the SR (Fabiato, 1983). Because it was spontaneous, this Ca^{2+} release rendered methodological tests easier than they would have been for the Ca^{2+}-induced release of Ca^{2+}, which has to be triggered by a rapid increase of [free Ca^{2+}] at the outer surface of the SR of a previously quiescent skinned cell (Fabiato, 1983). After these tests had been accomplished, it was possible to do the more complex experiments reported in the two accompanying articles (Fabiato, 1985*a*, *b*), which study the mechanism and physiological relevance of the Ca^{2+}-induced release of Ca^{2+} from the SR.

The very rapid change of bulk solution [free Ca^{2+}] was made possible by the small volume (nanoliter [nl] range) of the bathing solution and by a microprocessor-controlled system that permitted extremely rapid microinjections and microaspirations because of the very small volume and incompressibility of the transmission fluid. The translation of these very rapid changes of bulk solution into rapid changes of [free Ca^{2+}] at the outer (i.e., cytoplasmic) surface of the SR of this very small preparation was facilitated by the broad spacing of the myofibrils. A major part of the Methods section consists of the description and testing of this microprocessor-controlled system of microinjections and microaspirations.

The methodological appraisal also required the study of some properties of aequorin, including: (*a*) its binding within the skinned cardiac cell, for which there was preliminary evidence (Fabiato, 1981*a*); (*b*) its affinity constant for Ca^{2+}, since a high concentration of aequorin (20–21 μM) was used to improve the signal-to-noise ratio; (*c*) the accuracy with which the peak myoplasmic [free Ca^{2+}] reached during Ca^{2+} release from the SR can be inferred from the amplitude of the light transient and a calibration of maximum light according to an adaptation of the method described by Allen and Blinks (1978, 1979).

Finally, the effects of calmodulin were also studied because this diffusible constituent, present in the intact cell, was reintroduced in the simulated intracellular milieu. Accordingly, a large portion of this article is methodological and serves as a basis for this and the accompanying studies (Fabiato, 1985*a*, *b*).

METHODS

General Description of the Experimental Setup

The experimental setup (Fig. 1) was an improved version of that previously described (Fabiato, 1981*a*). The high-power optics required for microdissection would have absorbed a tremendous amount of light, which would have limited the resolution of the bioluminescence recording. Accordingly, before recording bioluminescence, the microscope optics were removed by a microprocessor-controlled sliding mechanism that brought the cathode of the photomultiplier tube to 0.5 mm from the bottom of the chamber containing the preparation (chamber B in Fig. 1). The six micromanipulators and the microscope stage were attached to a vibration-free table (compressed air type) and had no physical contact with the microscope optics, which were attached to the sliding mechanism on a lower table (see Fig. 3 in Fabiato, 1981*a*). The microsyringes, their stepping motors, and the photographic camera with automatic film advance, which was used for recording the dimensions of the skinned cell, were attached to the wall of the lightproof Faraday cage enclosure of the setup without contact with the vibration-free table.

Skinned cardiac Purkinje cells were obtained from adult dogs used as controls for studies by other investigators. In all cases, the dogs had received no drug except for the anesthetics. A cardiac Purkinje cell was skinned and microdissected in chamber A (Fig. 1), in which *X*-axis and *Y*-axis movements were finely controlled. The skinned cell was 7–9 μm wide, 5–6 μm thick, and 20–40 μm long at the ~2.3-μm sarcomere length that was used for the experiments because it corresponded to maximum tension development. Preparations presenting obliquely branching myofibrils that were cut and not attached to the tension recording system were not used. Like skeletal muscle myofibrils (Street, 1983),

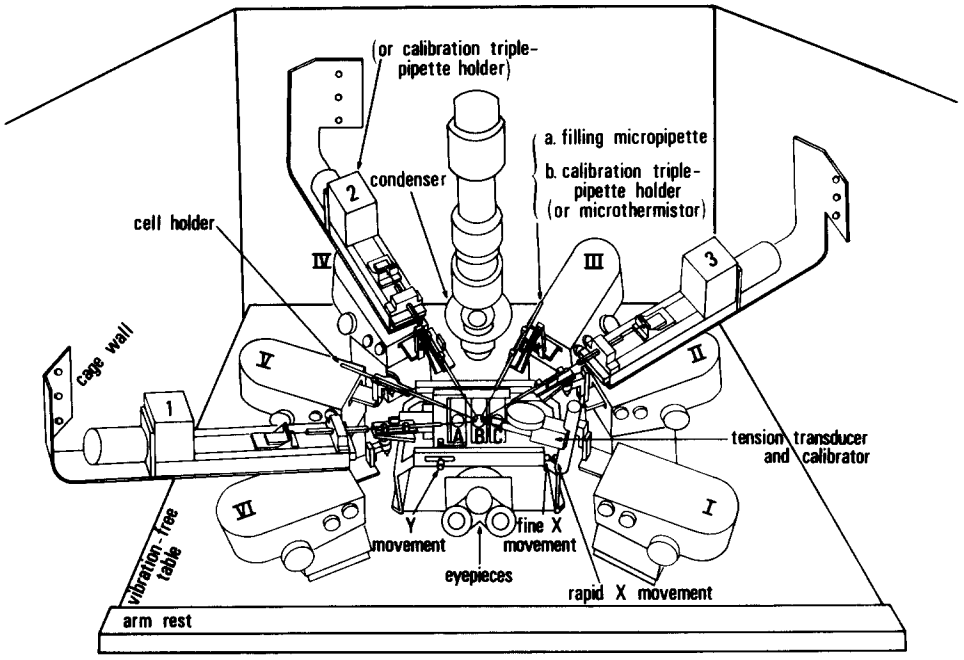

FIGURE 1. Location and function of the six micromanipulators. The captions in parentheses correspond to the setup for the present article, which was simpler than that for the two accompanying articles (Fabiato, 1985*a*, *b*).

cardiac myofibrils branch frequently. The branching is at a very small angle. Thus, when stretched by the tension recording system, all myofibrils ran nearly parallel, were at the same sarcomere length, and were connected to the transducer. Under these conditions, the aequorin bioluminescence transient always presented a single component of increase and decrease of light. In contrast, if a cut myofibril was not connected to the transducer, the aequorin bioluminescence transient presented two components, while the tension transient had only one. This was attributed to the asynchronous release of Ca^{2+} from the SR of the cut myofibril and from that of the rest of the preparation. Ca^{2+} release is strongly influenced by sarcomere length (Fabiato, 1980) and that of the unattached myofibril was much shorter than in the rest of the preparation. Accordingly, such preparations were not used.

After the microdissection had been completed, a rapid (<0.5 s), manually controlled movement of the stage along the *X* axis and a lifting and lowering of the preparation, which was attached to and protected by the cell holder, permitted the transfer of the

skinned cell into chamber B (Fig. 1), which contained water-saturated mineral oil (Aldrich Chemical Co., Milwaukee, WI). Then 1.5 nl of aequorin-containing solution was microinjected around the skinned cell, which resulted in the creation of an artificial chamber of very small volume. Chamber C was used for setting the microtools and filling the micropipettes with aequorin, as will be explained.

Micromanipulator I (Fig. 1) held the tension transducer and the tension calibrator, which was specially built from a microammeter and its needle (Fabiato, 1981*a*). Micromanipulator V, which was diametrically opposed to micromanipulator I, held the microtool used to microdissect and transfer the skinned cardiac cell; during tension recording, this microtool immobilized the end of the skinned cell. Micromanipulator III held the micropipettes used to fill the pipettes of the microinjection-aspiration system; during the experiment itself, it held a microthermistor (20-μm tip) monitoring the temperature in the microdrop surrounding the skinned cell in chamber B. The temperature was 22 ± 0.3°C in all of the experiments, except those studying the temperature dependence of the frequency of the cyclic contractions.

Micromanipulator IV held the small triple-micropipette micromanipulator shown in Fig. 2. These micropipettes were used to determine the maximum light by the injection of a solution at pCa 2.50 containing 0.068 mM total EGTA and the maximum tension by consecutive applications of solutions at pCa >9.00 and at pCa 4.25 in the presence of 10 mM total EGTA. The solution changes were effected by a vacuum-controlled microaspiration of the previous solution followed by a compressed air–controlled microinjection of the new solution. Air pressure and vacuum were triggered by solenoids commanded by duration-delay generators. The capillary force, which tends to aspirate solution into the micropipettes in the absence of air pressure, was compensated by a low air pressure precisely adjusted for each micropipette with a high-precision pressure regulator controlled with a highly sensitive gauge.

In the version used for the present study, the setup had only two hydraulically controlled micropipettes (numbers 1 and 3, held by micromanipulators II and VI, in Fig. 1) because I thought that more complexity would be beyond my ability.

Micropipettes and Microtools

Calcium decontamination of the micropipettes and microtools was essential in these experiments since they used a low [total EGTA]. Because heating displaces Ca^{2+} ions from glass, it was necessary to decontaminate the micropipettes and microtools of Ca^{2+} after their pulling and fire polishing, even though the pieces of glass tubing cut for making them had already been thoroughly Ca^{2+}-decontaminated. Tubing of 2 mm o.d. and 1 mm i.d. was used for all microtools and for the micropipettes employed for filling the hydraulically controlled micropipettes. Tubing of 0.79 mm o.d. and 0.40 mm i.d. was used for the glass tips of the hydraulically controlled microinjection-aspiration system. Both types of glass were cut into 9-cm segments that were bathed for 1 h in 70% perchloric acid at 80°C. This high temperature facilitated removal of the loosely bound Ca^{2+}. The tubing was then transferred to several successive baths of circulating, Ca^{2+}-free, bidistilled water and internally perfused with this water. Thereafter, the tubing was washed for 30 min in a solution containing 0.1 mM total EGTA buffered at pH 7.10 with 30 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES). It was then transferred to several successive baths of circulating, bidistilled, deionized water and very extensively perfused internally with bidistilled, deionized water. Finally, the tubing was dried in an oven and stored in an airproof, Ca^{2+}-free container.

The microtools for skinning, transferring, and holding the fixed end of the cell (long microtools) and for attaching the free end of the cell to the tension transducer (short

microtools) were made with a micropipette puller; their tips were occluded by heating. For accuracy of tension calibration, the short microtools required precise dimensions of overall length and location of the tapering, which were controlled under a high-power microscope (×60 objective, ×12 eyepieces). The microtools were submitted to all the steps of Ca^{2+} decontamination initially used for the pieces of glass tubing except for internal perfusion (since the tips of the microtools were occluded).

Two types of micropipettes were also made with a micropipette puller. Their tips were broken under a high-power microscope at exactly 7 μm i.d. The tip opening and the large

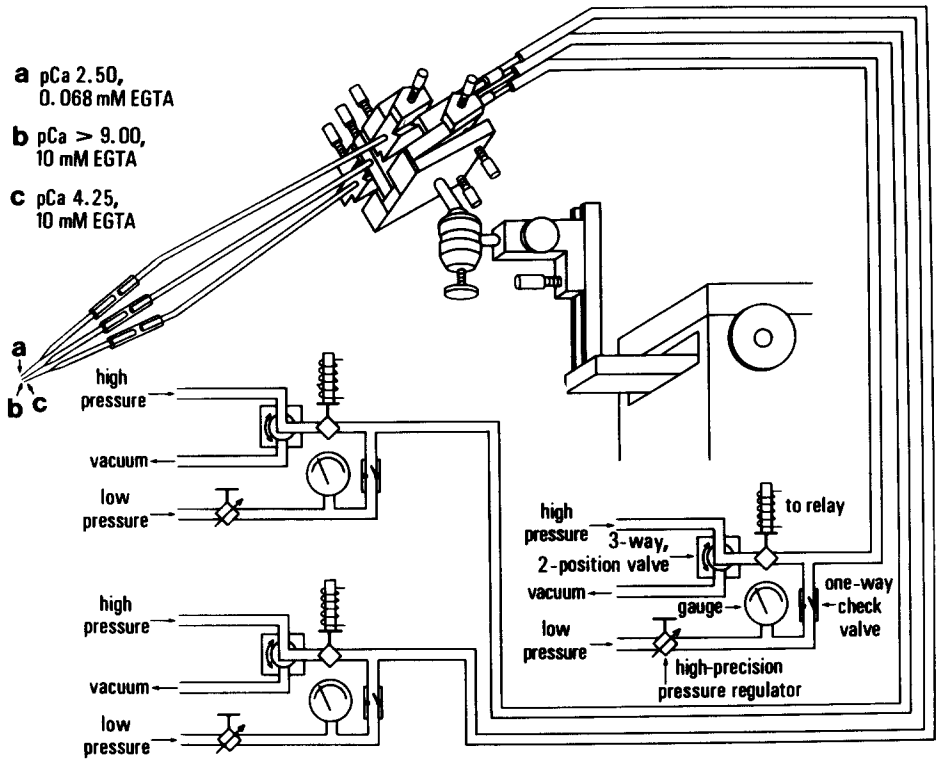

FIGURE 2. Triple-micropipette holder used for calibration of maximum aequorin bioluminescence (solution *a*) and of maximum tension (solutions *b* and *c*).

back opening were fire polished. The large micropipettes were cut at an overall length of 40 mm, whereas the small pipette tips for the hydraulically controlled microinjection-aspiration system had an overall length of 9 mm. The micropipettes were submitted to the same steps of Ca^{2+} decontamination used for the original pieces of glass tubing, including the extensive steps of internal perfusion, and dried in an oven.

The efficiency of this procedure of Ca^{2+} decontamination has been tested by the observation that 200 microinjections and microaspirations of a solution containing 1 mM EGTA in a Ca^{2+}-decontaminated micropipette resulted in an increase of the [total calcium] in the solution of <1 μM as measured by atomic absorption spectrophotometry. The experimental solutions contained less EGTA and hence were less likely to pull loose Ca^{2+} ions. In addition, they were not injected and reaspirated as many times. Thus, it was

estimated that the total calcium contributed by the glass micropipette was in the subnanomolar range. The possibility of a decrease of [free Ca^{2+}] by binding to the glass was checked by storing a solution at $-\log_{10}$[free Ca^{2+}] (pCa) 6.30 in the presence of 0.068 mM total EGTA, pMg 2.50, pMgATP 2.50, and pH 7.10 in a Ca^{2+}-decontaminated micropipette for 72 h. This resulted in no change of the [total calcium] in the solution injected from the micropipette, which was measured with a furnace atomic absorption spectrophotometer. Since the micropipettes were always used within 8 h after preparation, it was concluded that the binding of Ca^{2+} to the glass did not significantly alter the [free Ca^{2+}] in the experimental solutions.

Except when a high [total EGTA] was used, all micropipettes contained aequorin at the desired pMg, pMgATP, pH, and ionic strength. The aequorin was prepared by Drs. O. Shimomura and E. B. Ridgway. It was stored frozen in a solution containing 0.1 mM EGTA, twice the desired [KCl], and 30 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES). The pH had been adjusted to 7.10 at 22°C. On the day of the experiment, an aliquot of the aequorin-containing solution was thawed and 0.5 μl was removed with a microsyringe. This volume of aequorin-containing solution was mixed with an equal volume of a solution containing appropriate concentrations of ions, substrates, and buffers, so that the final concentrations would meet the specifications given by the computer program (Fabiato and Fabiato, 1979; Fabiato, 1981*a*). The tip of each filling micropipette was then filled with 2 nl of the aequorin-containing solution by vacuum aspiration. Mixing the two 0.5-μl solutions and filling the micropipettes was done within a few seconds in a water-saturated atmosphere. The filling micropipettes were stored at 2°C in a water-saturated atmosphere and used within a few hours. One set of micropipettes was filled in the morning and another set was filled in the afternoon.

All chemicals, including calmodulin, were obtained from Sigma Chemical Co. (St. Louis, MO). All the batches of EGTA used in the present experiments had a purity of 97.6% as indicated by the laboratory (not the catalog) of Sigma Chemical Co. As in all my previous experiments in skinned cardiac cells, the degree of purity of the EGTA measured by the supplier's laboratory was trusted. Using the method of Miller and Smith (1984), I found a degree of purity of 97.1 ± 1.1% (SD, $n = 7$) in one of the batches of EGTA.[1] The value given by the laboratory of Sigma Chemical Co. was within 1 SD of this result.

Description of the Microinjection-Aspiration System

To obtain very fast, accurate, and reproducible microinjections and microaspirations, it was necessary to use a nearly incompressible transmission fluid. Oil is compressible, and air bubbles render water compressible. Accordingly, bidistilled, deionized water that had been degassed by vacuum was used as a transmission fluid. It was also necessary that the microsyringes and tubing containing the transmission fluid would not expand or contract upon rapid injection or aspiration. These containers were made entirely of stainless steel or glass, except for a segment of noncompliant polyethylene tubing with an internal volume of 0.35 μl (*b* in Fig. 3). The interposition of this polyethylene tubing was necessary to prevent the transmission of vibrations from the stepping motor to the micropipette

[1] This SD, which is 10 times greater than that found by Miller and Smith (1984), is certainly related to my lack of experience in using this difficult method. The degree of purity of the Sigma EGTA given by Miller and Smith (1984) was not obtained with the batches that were used here. However, using their data would not influence the interpretation of the results. The range of pCa used for the calibrations of the aequorin light and tension transients was 5.40–5.90. With the degree of purity of EGTA found by Miller and Smith (1984), pCa 5.40 would in fact be pCa 5.32 and pCa 5.90 would be pCa 5.87.

and to permit the small change of angle of the transmission fluid system that occurred when the micropipette was lifted 1 mm.

The volumes of each component of the transmission fluid system are indicated in Fig. 3; the total was <9 μl. The microsyringe was moved by a stepping motor controlled in the half-step mode. The minimum unit of solution change, one half-step, was 52 pl.

The entire system (microsyringe, tubing, and glass tip) was filled with degassed water by microaspiration before the experiment. The microsyringe was then transferred to the stepping motor–controlled lead-screw system. This was a Unislide device (Velmex, East

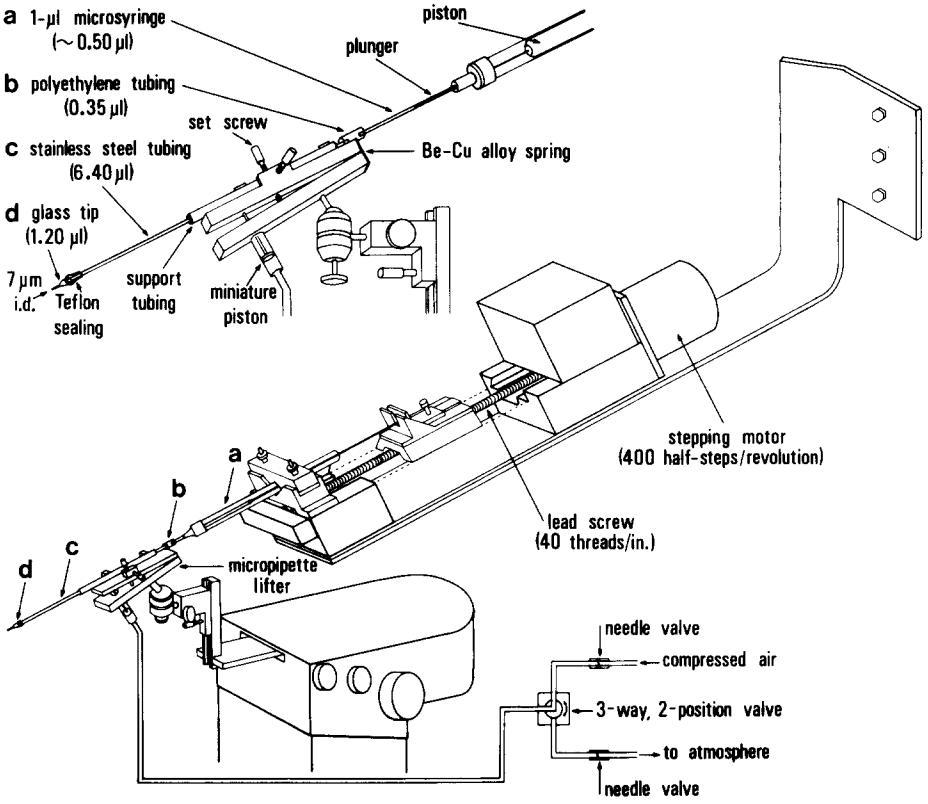

FIGURE 3. Hydraulically controlled microinjection-aspiration system. The volumes indicated in the upper part of the figure are those used in all the experiments reported in this article. The 1-μl microsyringe was about half filled with deionized and degassed water, which was contained in the needle and displaced by the plunger. The piston and barrel of the microsyringe served only to guide the movement of the fine plunger.

Bloomfield, NY) consisting of a set of sliding tracks moved by a fine lead screw (40 threads/in.). The lead screw was driven by an M062-FD09 Slo-Syn Stepping Motor (Superior Electric Co., Bristol, CT) that turned 400 half-steps per revolution.

The tip of each hydraulically controlled microinjection-aspiration pipette was placed in focus in chamber C, which contained oil (Fig. 1), and 0.1 μl of oil was aspirated using the remote-control push buttons, placed inside the lightproof Faraday cage (Fig. 4), of the microprocessor system.

One filling micropipette containing aequorin was placed on micromanipulator III (Fig. 1) and connected to a microinjection-aspiration system controlled by air and vacuum identical to that used for the calibration micropipettes shown in Fig. 2. Its tip was placed into the oil chamber, C, close to the tip of one of the hydraulically controlled micropipettes of the microprocessor-controlled microinjection-aspiration system. The filling pipette was emptied, forming a 2.0-nl drop in the oil. Exactly 1.8 nl of this drop was microaspirated into the tip of the hydraulically controlled micropipette. Thus, the ~9 μl of degassed water used as transmission fluid was separated by 0.1 μl of oil from the 0.0018 μl of aequorin-containing aqueous solution.

The procedures were repeated for the filling of the other hydraulically controlled microinjection-aspiration pipette(s) (only one was used in the present study; two were used in the studies reported in Fabiato, 1985*a*, *b*). The filling of the pipette with aequorin-containing solution was done in another area of the drop of oil. Chamber C was then replaced by a new chamber containing oil to eliminate the bioluminescence produced by the remaining amount of aequorin-containing solution. The two filled micropipette tips were precisely set in this newly filled chamber C, equally spaced from the center of the field.

After this microscopic setting, the micropipettes were lifted exactly 1.0 mm. This was done with a remote-controlled, specially built micropipette lifter (Fig. 3) by forcing with a micropiston the opening of an angle formed by two pieces of anodized aluminum backed by a beryllium-copper alloy spring. The maximum excursion of the micropiston was much more than 1 mm, but this distance was precisely reproducible. A set screw permitted the prior opening of the angle of the lifter so that the remaining excursion of the micropipette tip was limited to exactly 1.0 mm. The micropiston was remote-controlled from either inside or outside the lightproof cage (Fig. 4) by compressed air and a two-position valve (Fig. 3). It was necessary to slow down the lifting and lowering of the micropipette in order to prevent the movement from causing some extrusion of solution from the micropipette. This was done with needle valves, which slowed down the influx and efflux of air to and from the micropiston (Fig. 3).

The microthermistor was attached to an identical lifter and lowered into chamber B after the cell transfer. The hydraulically controlled micropipettes were lowered only when they had to be used to avoid a slow diffusion of ions between the microdrop and the solution contained in each micropipette. When a micropipette was lifted, its tip was only 1.5 mm from the 38-mm-diam cathode of the photomultiplier tube, which did not change significantly the amount of light detected by the tube (as predicted by the optical properties of photomultiplier tubes). Thus, the light produced by the two solutions containing aequorin at various [free Ca^{2+}] values was permanently monitored by the photomultiplier tube, whether the solution was still in a micropipette, injected into the chamber, or reaspirated into the micropipette in which it was originally contained. Finally, there was never mixing between the two solutions. This prevented the solution change from shifting the baseline (however, see Fig. 19) that resulted from the large resting glow of the micropipette tips and the very low dark current of the photomultiplier tube. This baseline, which was balanced by a counterbias voltage, was defined as the "zero" of the bioluminescence recording in the figures. Its amplitude was constant but several hundred times (depending upon the [free Ca^{2+}] of the aequorin-containing solutions in the micropipette tips) that of the transient light signals resulting from Ca^{2+} release from the SR.

The volume of solution surrounding the skinned cell was produced by 10 half-steps of the stepping motor (520 pl). Microinjection and microaspiration were effected in 10 ms (the period of each half-step was 1 ms). The velocity of flow ($\dot{v}$ in centimeters per second) through the opening of the pipette tips is given by:

$$\dot{v} = f/a,$$

where a is the area (in square centimeters), and f is the flow rate, which is equal to volume (in cubic centimeters) divided by time (in seconds). With a pipette tip of 7×10^{-4} cm i.d. (i.e., 3.5×10^{-4} cm radius) and a volume of 520×10^{-9} cm^3 microinjected or microaspirated in 1×10^{-2} s, the velocity of flow was:

$$v = (520 \times 10^{-9}/10^{-2})/[(3.5 \times 10^{-4})^2 \times \pi] = 135 \text{ cm/s}.$$

Note that only such a small volume of solution can be injected or aspirated around the skinned cell in a drop of oil in order to permit this rapid solution change. If a very small perfusion chamber of 1.31 μl had been built by boring into glass, then the same injection and aspiration of the content of the chamber in 10 ms would have caused a velocity of flow through the 7×10^{-4}-cm-diam opening of the pipette of 10 times the speed of sound (34×10^4 cm/s).

Microprocessor Control of the Microinjection-Aspiration System

The microprocessor system will be described following the flow chart shown in the right-hand part of Fig. 4 from bottom to top. The system was designed so that the control channel corresponding to each motor could be selected because the functions of the three channels differed. Only two channels, however, were used in the present study.

Each stepping motor was powered by an SP153B Preset Indexer (Superior Electric Co., Bristol, CT) composed of (*a*) an indexer specifying the number of steps, the movement direction, and the base speed, (*b*) a translator converting the number of steps into the number of half-steps, which could go up to 3,500/s, and (*c*) a driver translating the number of half-steps into impulses to the motor.

The indexer was controlled by a specially designed microprocessor device, hereafter termed the "controller," which used the same transistor-transistor logic input format as the indexer. The controller regulated the indexer functions (start, direction, and base speed) and monitored the outputs of the "done" line and "index" lines of the indexer. The "done" line gave a signal indicating when motion was complete. The two "index" lines monitored each half-step in the clockwise and counterclockwise directions, respectively. The controller also allowed selection between injection, aspiration, injection followed by aspiration after a preset delay, and aspiration followed by injection after a preset delay.

Arming (i.e., the function that permitted the indexer to respond to the start signal) could be done either manually with a push button or automatically by a time base. The arming and start signals were generated by adjustable delay generators for each channel. Each delay generator had the ability to function in a "paired-pulse" mode so that a given channel could be activated twice after a preset delay. The triggering of the delay generator was either by a push button (used for filling the micropipettes), or by a time base (used for the studies reported in Fabiato, 1985*a*, *b*), or by a signal generated by the microcomputer (used for the experiments reported in this article).

A microcomputer (model /09; Southwest Technical Products, San Antonio, TX) collected the data points of the aequorin bioluminescence signal every 5 ms and generated a 200-mV square wave, starting when the aequorin signal reached a preset threshold voltage and continuing until the voltage fell below this threshold. This computer-generated signal permitted the induction of aspiration-injection sequences at different phases of the Ca^{2+} transients caused by a spontaneous cyclic release of Ca^{2+} from the SR. The microcomputer signal was displayed on the fourth channel of the recorder. The microcomputer was used for many other purposes, as will be explained. The major features of this system (Fabiato and Wist, 1982) were that it was remote-controlled and it provided all information in analog form directly on one channel of the recorder. All sections of the

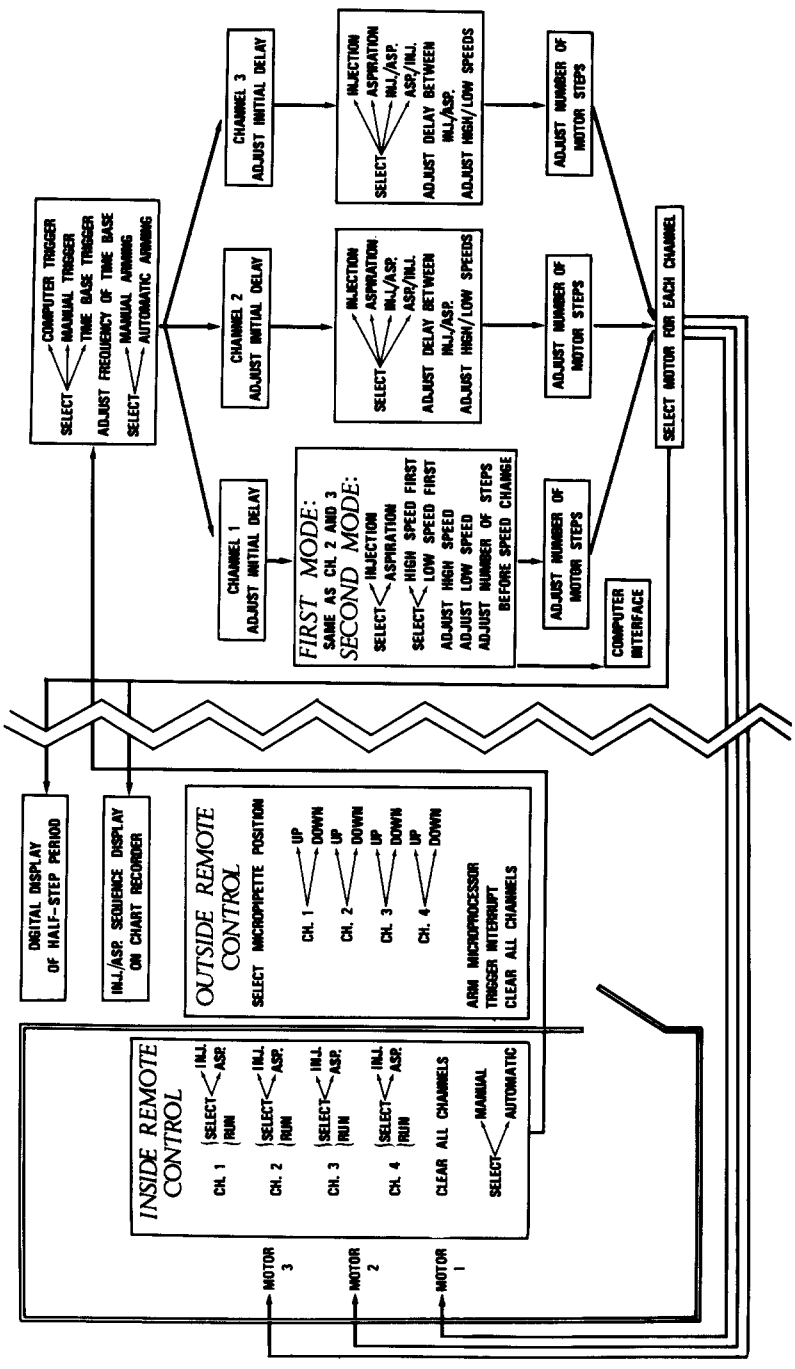

FIGURE 4. Flowchart of the functions of the microprocessor controlling the microinjection-aspiration system. The wavy double line indicates that a distance separated the microprocessor controls from the two remote controls. See Fig. 5*A* of this article and the Methods section in Fabiato (1985*b*) for the second mode of channel 1 (CH. 1) of the microprocessor and computer interface.

program were commanded not from a keyboard, but instead by sending interrupts to the computer with push buttons located close to the recorder and arranged to facilitate the experiments.

A single channel of the recorder (the third one) was used to display signals of the injections and aspirations generated by the controller. The signal was always upward for injection and downward for aspiration. The timing and duration of each signal corresponded to the exact timing and duration of each injection and aspiration. The amplitude of the signals was made different only to permit identification of each of the microprocessors. The amplifications of the signals corresponding to the three channels were adjusted in such a way that if two, or even all three, microprocessors had functioned simultaneously, in the same or in the opposite direction, no combination would have given the same voltage to the composite signal. This permitted identification of the operations that had occurred.

The microprocessor system required a number of adjustments before an experiment or a section of a given experiment. Although I could reach the control switches, potentiometers, and counters used for these adjustments when sitting in front of the recorder, the attention needed for the experiment made it necessary to have an outside remote-control box close to the recorder. A remote-control box inside the lightproof Faraday cage was needed for the filling of the micropipettes.

Both remote controls included a "clear all channels" button for use when an error in setting the number of steps of the indexer might have ruined the filling of the micropipettes or the experiment. A switch permitted the selection between injection and aspiration for each channel.

The outside remote-control box included a control of the position (up or down) of the lifter for each micropipette. This outside remote control was near the door of the lightproof Faraday cage so that these lifters could be used during work in the Faraday cage, which was done with the door open. During the actual recording, the door of the Faraday cage was closed and I was outside. The outside remote control permitted the microprocessor system to be armed when the recording traces were stable. It also contained a trigger interrupt in case an artifact that could ruin the tracing appeared on the recording after the arming.

The fourth channel was not commanded by the hydraulic microprocessor-controlled system but by an air- and vacuum-driven injection-aspiration system triggered by a relay. This system was used only for operating the filling micropipettes and was identical to that used for the calibration micropipettes shown in Fig. 2.

Tests of the Rapidity and Reproducibility of the Microinjection-Aspiration System

The rapidity of solution changes produced by the microinjection-aspiration system was tested on two previously developed setups. One included a 500-frames/s Photo-Sonics camera (Burbank, CA) (Fabiato and Fabiato, 1978*a*) and the other, a variable-wavelength microspectrophotometer (Fabiato, 1982*b*). For these tests, the micropipettes were oriented as in the experiments, at such an angle with respect to the skinned cell that the flow did not strike it directly.

On cinefilm, an injection of a pCa 7.90 solution appeared as an "inflating balloon." The injection resulted in a 127 ± 12% (SD, $n = 11$) increase of the width of the skinned cell. This change of width appeared and was completed between two frames, i.e., in <2 ms after the beginning of the injection. The injection produced no artifact on the tension recording, but the filtration with a cutoff frequency at 3 Hz could have eliminated a <2-ms artifact. A microaspiration appeared as a "deflating balloon" and was accompanied by a reduction of the width of the skinned cell.

This system permitted rapid mixing of solutions (Fig. 5*A*), a feature used for the aequorin calibration (see Figs. 8 and 9) and for producing a progressive change of [free Ca^{2+}] in the two accompanying studies (Fabiato, 1985*a*, *b*). The mixing of two solutions in the absence of a skinned cell was tested by first microinjecting water into oil to form a drop and then injecting with another micropipette a solution containing methylene blue

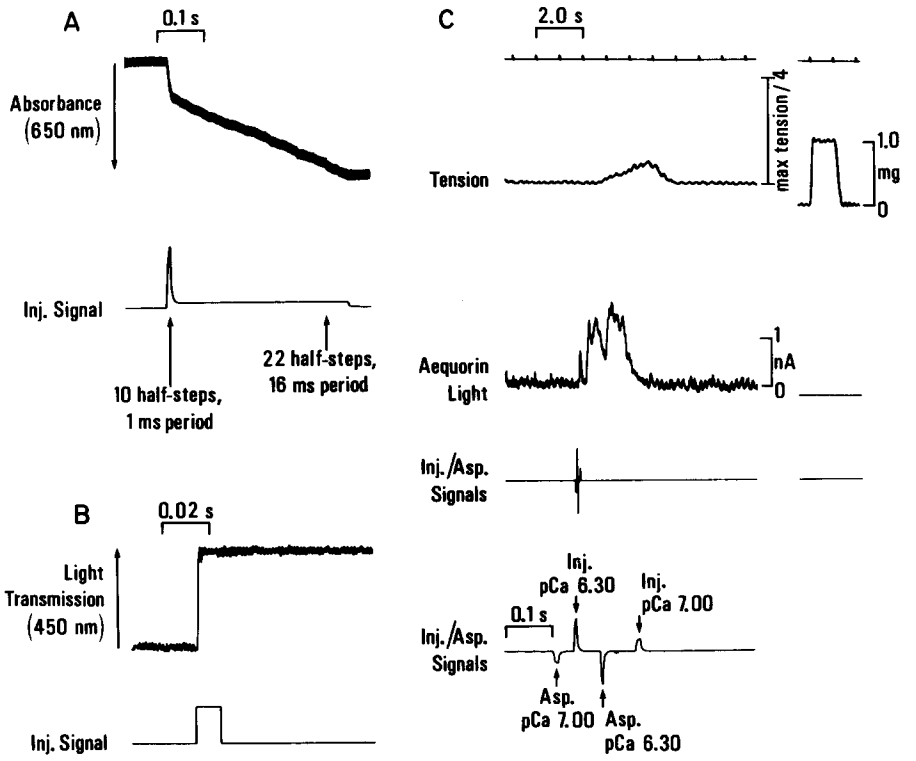

FIGURE 5. (*A*) Chart recording of light absorbance in a 10-μm-diam circular area, located at the same position as a skinned cardiac cell, during an injection of a solution containing methylene blue in a drop of water that had previously been injected into oil. Note that the time course of the injection signal was modified (compare with panel *B*) by the limited frequency response of the chart recorder. (*B*) Oscilloscope recording of light transmission in a 9-μm-wide, 6-μm-thick, 29-μm-long skinned canine cardiac Purkinje cell bathed in oil during the injection of 10 half-steps (1-ms period) of a solution of the same composition as used for the experiments but with pCa 7.90 (cutoff frequency of the filter = 300 Hz). (*C*) Attempt to produce a Ca^{2+}-induced release of Ca^{2+} by injection and aspiration in a 28-μm-wide, 19-μm-thick, 69-μm-long skinned canine cardiac Purkinje cell. Compare with Fig. 5 in Fabiato (1985*a*).

(Fig. 5*A*). During this injection, light absorbance was monitored at 650 nm in a circular area of diameter approximately equal to the width of a skinned cell, limited by a Czapski iris eyepiece, and precisely located in the position of the skinned cell. At a chart recording speed of 200 mm/s, no measurable delay was observed between the microinjection of the methylene blue solution and the increase of absorbance. The change in speed of the

stepping motor between two half-steps resulted, again with no measurable delay, in a change of slope of the absorbance tracing. This is consistent with the expected proportional change of concentration of methylene blue according to Bers's law.

In this study, solution changes were effected by a microaspiration of the previous solution followed by the microinjection of the new solution. The microaspiration removed all the solution except the fluid contained in the skinned cell. The microinjection consisted of forming a new drop of aqueous solution in oil and washing the fluid contained in the skinned cell. The delay between such a microinjection and the arrival of the flow of the solution into a skinned cell was measured by placing a skinned cell in its usual position, as for an experiment, and microinjecting a pCa 7.90 solution. A 10-ms microinjection, made with 10 half-steps that had a 1-ms period, caused an increase of light transmission after an average delay of 1.5 ms (n = 22, range = 1.2–1.8 ms) and with a rise time averaging 1.2 ms (n = 22, range = 1.0–1.4 ms) (Fig. 5*B*). This was attributed partly to the increase of light scattering that resulted from the increase of spacing of the myofibrils. In addition, the light absorption coefficient of water is lower than that of oil. Thus, the change of the solution in the skinned cell occurred long before the completion of the 10 half-steps of microinjection. In fact, a volume equal to that displaced by one half-step was sufficient to create a volume 25–50 times larger than that of the skinned cell. With a microinjection made with 10 half-steps that had a 0.5-ms period, as used in the study reported in Fabiato (1985*a*), the delay of increase of light transmission was reduced to an average of 1.1 ms (n = 17, range = 0.9–1.4 ms) and the rise time to an average of 0.8 ms (n = 17, range = 0.6–1.1 ms).

The myofibrils of skinned cells were widely spaced because of the lack of mechanical constraint related to the absence of sarcolemma. This effect became considerably more prominent when the preparation was made smaller because of the removal of intracellular structures that keep the myofibrils packed together in an intact cell. Under microscopic observation of preparations with the dimensions used in this study, the myofibrils were 0.7–0.8 μm wide, wider in the area of branching, and the spacing between them was about half of the width of a myofibril before the attachment of the skinned cell to the transducer. The spacing was reduced but still clearly visible, between 0.1 and 0.2 μm, after the skinned cell had been stretched and attached to the transducer.

Donnan osmotic forces related to the absence of sarcolemma are unlikely to play a major role in the spacing of the myofibrils. The myofilaments constituting the myofibrils, and perhaps the SR wrapped around them, bear negative charges. However, the electric field created by these aggregates of negative charges is unlikely to have an effect beyond a few angstroms in saline aqueous solution (McLaughlin, 1977). In contrast, the Donnan osmotic forces are responsible for the swelling of the myofilament lattice within individual myofibrils (Matsubara and Elliott, 1972). Polyvinylpyrrolidone and dextran at various molecular weights are large molecules that are excluded from the myofilament lattice. They produced a shrinkage of the individual myofibrils and, because of complex branching of the myofibrils, a reduction of the width of the skinned cell to a degree that increased with the concentration and the molecular weight of dextran or polyvinylpyrrolidone. However, a maximum reduction was obtained with 100 mg/ml of ~160,000-mol-wt polyvinylpyrrolidone, which decreased the width of the skinned cell by 24 ± 2.6% (SD, n = 9). Then the width of the individual myofibrils averaged 0.3–0.4 μm, an ~50% reduction. Thus, the reduction of the overall width of the preparation was not proportional to the reduction of the width of the individual myofibrils. After this maximum reduction with polyvinylpyrrolidone, the overall width of the preparation remained much larger than that of the skinned cell in oil: as previously indicated, the change from oil to aqueous solution increased the width of the preparation by an average of 127%. This demonstrates

that the spacing of the myofibrils is sufficient to permit the flow of a solution containing ~160,000-mol-wt polyvinylpyrrolidone between them.

From these observations, the following interpretation is proposed as a first approximation for the change of concentration of ions, especially Ca^{2+}, at the outer surface of the SR wrapped around individual myofibrils. This change of concentration is attributed mostly to hydraulic bulk flow rather than to diffusion of Ca^{2+} and other ions. The possibility of an unstirred layer at the outer surface of the SR cannot be discounted. Thus, diffusion of ions could still take place between the bulk solution and the outer surface of the SR, but this would be over a short distance relative to the overall minimum radius of the preparation. As previously calculated, the rate of flow through the micropipette opening is 135 cm/s, or even up to 270 cm/s in other studies (Fabiato, 1985*a*). The bulk flow strikes the oil-water interface and spreads in an approximately spherical manner, creating the aspect of an inflating balloon. The rebound flow is delayed by compression of the oil, accounting for most of the 1.5 ms between the microinjection and the decrease of light transmission recorded in the area of the skinned cell (Fig. 5*B*). The rebound is generated from all the internal surface area of the sphere, or rather the ellipsoid, formed by the drop of aqueous solution in oil. Hence, the bulk flow reaches the skinned cell in an approximately symmetrical manner in the three dimensions. This explains the absence of any large movement of the skinned cell during microinjection. The bulk flow is certainly reduced by turbulence in passing between the myofibrils, the few remaining mitochondria, and perhaps some intermyofibrillar cytoskeleton. However, the initial flow rate is so high with respect to the size of the skinned cell that the delay for penetrating between the myofibrils is very short.

This hydraulic bulk flow is fast compared with the slow rate of release of Ca^{2+} from the SR. Thus, the bulk solution free Ca^{2+} reached the vicinity of the outer surface of the SR that surrounds and packs all myofibrils (Sommer and Johnson, 1979) before any substantial Ca^{2+} release had time to take place from any part of the SR. Evidence will be presented in this and the next article (Fabiato, 1985*a*) that the hydraulic bulk flow is stopped by the SR, which binds and accumulates Ca^{2+} so that it prevents any direct activation of the myofilaments by externally applied solutions even in the presence of a very high [free Ca^{2+}] (see Fig. 7 in Fabiato, 1985*a*).

In a cylindrical preparation, the time for the average [free Ca^{2+}] to reach 90% of its equilibrium value following a step change in concentration at the outer surface is (Crank, 1975):

$$0.1\ r^2/D',$$

where r is the radius of the cylinder and D' is the apparent diffusion coefficient for Ca^{2+}. Using an apparent diffusion coefficient of 2×10^{-8} $cm^2 \cdot s^{-1}$ (Kushmerick and Podolsky, 1969) and a minimum radius of the preparation of 3×10^{-4} cm, the time required for the [free Ca^{2+}] to reach 90% of its equilibrium value would be:

$$0.1\ (3 \times 10^{-4})^2/(2 \times 10^{-8}) = 0.45 \text{ s}.$$

This 450-ms delay is at least one order of magnitude larger than the delay observed between a change of solution and a change of myoplasmic [free Ca^{2+}] resulting from Ca^{2+} release detected with aequorin (see photographic enlargements in Figs. 16–20), despite the time taken by the physiological processes modifying the Ca^{2+} release from the SR. In contrast, the diffusion of Ca^{2+} from the SR to the center of each myofibril with a 0.4-μm radius should take only 8 ms. This is an approximate figure since the apparent diffusion coefficient could be either lower than 2×10^{-8} $cm^2 \cdot s^{-1}$, especially at low [free Ca^{2+}], because of the fixed binding sites in the myofilament space, or higher than 2×10^{-8} $cm^2 \cdot s^{-1}$ because of the presence of the diffusible Ca^{2+} buffers calmodulin and EGTA.

The small size of the preparation was essential for the spacing of the myofibrils, which permitted the practically synchronous Ca^{2+} release from the SR surrounding all the myofibrils throughout the preparation. If larger skinned cells had been used, a rapid increase of [free Ca^{2+}] by injection and aspiration would have produced an aequorin light transient with multiple components. The experiment shown in Fig. 5*C* was done in a preparation with a cross-sectional area >10 times the average for the skinned cells used in the present study. The first component of the light signal may correspond to Ca^{2+} release from the outer surface of the SR packing the most superficial myofibrils in the preparation. The origin of the subsequent components was complex, and the following interpretation is based on properties of the Ca^{2+}-induced release of Ca^{2+} established in the next article (Fabiato, 1985*a*). The hydraulic bulk flow may have reached the SR surrounding some deeper myofibrils before Ca^{2+}-induced release of Ca^{2+} had been inhibited by the high [free Ca^{2+}] resulting from the release at the surface of the preparation (Fabiato, 1985*a*). This could explain the earliest components of the light signal following the initial one. After the end of the refractory period caused by the slow removal of the inactivation of the Ca^{2+} release process (Fabiato, 1985*a*), Ca^{2+} overload of the SR may have permitted a cyclic Ca^{2+} release, which would explain the latest components of the light transient. The tension transient resulting from this asynchronous Ca^{2+} release had an amplitude unrelated to that of the light transient. The peak of this tension transient was only 5% of the maximum tension (Fig. 5*C*). This contrasts with the induction of a tension transient of 30–35% of maximum tension by the same [free Ca^{2+}] trigger (from pCa 7.00 to 6.30) when applied to skinned cardiac cells that were only 7–9 μm wide and 5–6 μm thick (see Fig. 5 in Fabiato, 1985*a*). Even more dramatic differences were observed in skinned skeletal muscle fibers because their size can be made larger and the Ca^{2+} movements into and out of the SR are much faster (Fabiato, 1985*c*).

The major feature of the inward movement of Ca^{2+} in the small preparations used for the present study was that the SR constituted a barrier between the externally applied solutions and the myofilaments of each myofibril, which the SR surrounds and packs tightly. Inside this myofilament space, the inward movement of Ca^{2+} ions was slow because of diffusion with binding to fixed sites, particularly to troponin C. The following observations show that the slow diffusion also rendered the *net* outward movement of Ca^{2+} from the myofilament space to the bulk of the bathing solution negligible. Any loss of Ca^{2+} from the myofilament space to the bathing solution should be favored by increasing the volume of the solution or decreasing its [free Ca^{2+}]. A skinned canine cardiac Purkinje cell was bathed in 1.040 nl of aequorin-containing solution at pCa 6.30 in the presence of 0.068 mM total EGTA. It presented spontaneous contractions caused by a cyclic Ca^{2+} release from the SR. Then the solution was reaspirated, leaving only the fluid contained in the skinned cell surrounded by oil. This caused a more than 1,000-fold reduction of the volume of the bulk solution without changing its [free Ca^{2+}]. The frequency and amplitude of the cyclic contractions were not significantly modified during 7–12 min (range, $n = 12$), after which they decreased because of substrate exhaustion. Reinjection of 1.040 nl of the same solution, before the end of the 7–12-min period, again did not modify the frequency and amplitude of the cyclic contractions. Injection of 1.040 nl of a solution with the same [total EGTA] but a lower [free Ca^{2+}] (pCa 6.90), which should have favored the outward diffusional driving force for Ca^{2+}, did not reduce the amplitude of the next contraction but rather enhanced it before reducing the frequency and amplitude of the subsequent cyclic contractions.[2] These results indicate that the Ca^{2+}

[2] This finding, which provides a clue for the mechanism of the cyclic Ca^{2+} release of Ca^{2+} from the SR, is documented and discussed in another article (Fabiato, 1985*c*).

binding to troponin C (Johnson et al., 1979) and the Ca^{2+} reaccumulation into the SR are fast relative to the outward diffusion of Ca^{2+} from the myofilament space of individual myofibrils, with SR wrapped around them, to the bathing solution. The bulk solution [free Ca^{2+}] directly influenced the [free Ca^{2+}] in the myofilament space only when the [total EGTA] was increased from 0.068 to 10 mM (see Figs. 16 and 17).

In contrast, a change of [free Ca^{2+}] in the solution by microaspiration and injection very rapidly affected the portion of the outer surface of the SR that was accessible to the solutions by hydraulic bulk flow, as the results shown here will demonstrate. That microaspiration and injection were completed within the specified time was further demonstrated by the absence of artifact on the aequorin bioluminescence recording when a solution at a given pCa was aspirated in 10 ms and, after a delay of ~5 ms, a solution at another pCa was injected (e.g., see Fig. 18). If the previous solution had not been completely aspirated when the new solution was injected, a large artifact would have been produced by the exposure of the aequorin contained in the new solution to the different pCa of the previous solution, and vice versa.

The reproducibility of the volume produced by one half-step of the stepping motor was tested by microscopically measuring the diameter of the drop. It was also tested by measuring the area under the curve of the bioluminescence produced by the complete discharge with a saturating [free Ca^{2+}] of the aequorin contained in a drop produced by a half-step microinjection (see Fig. 7, *B* and *C*). Neither method detected any irreproducibility.

Solutions

The composition of the control solution was changed, with respect to that used previously (Fabiato, 1981*a*), in order to more closely simulate the Ca^{2+} buffering of the intracellular milieu. Calmodulin, a physiological intracellular Ca^{2+} buffer (Fabiato, 1983), was introduced at a concentration of 5.13 μM, which has been computed to correspond to its concentration in the intact canine cardiac Purkinje cell (Fabiato, 1985*b*). As previously (Fabiato, 1981*a*), two other physiological diffusible Ca^{2+} buffers were present in the solution: ATP at a concentration appropriate to obtain the desired pMg and pMgATP, and 12 mM phosphocreatine (with 15 U/ml creatine phosphokinase). EGTA was used to simulate the fixed Ca^{2+} buffers of the intact canine cardiac Purkinje cell: the Ca^{2+}-specific site of troponin C, and the binding sites at the outer face of the SR and at the inner face of the sarcolemma. The [total EGTA] necessary to simulate these fixed buffers varies with the pCa: 0.068 mM total EGTA was needed at pCa 5.45 under control conditions at pH 7.10 in the presence of 5.13 μM calmodulin (Fabiato, 1985*b*). A pCa of 5.45 was selected because it corresponds to the development of 40% of maximum tension, which is the average between 10 and 70%, the amplitude range of the phasic tension resulting from Ca^{2+} release from the SR (Fabiato, 1981*a*, 1982*a*). The [total EGTA] was increased to 0.092 mM when the pH was decreased to 6.70, and to 0.074 mM when calmodulin was deleted, in order to maintain the Ca^{2+} buffering constant at pCa 5.45.

The stability constants of the complexes between the four binding sites of calmodulin and, respectively, Ca^{2+}, Mg^{2+}, K^+, and Na^+ were those previously compiled from the literature (Klee and Vanaman, 1982; Fabiato, 1983). The stability constants for the complexes between the other ligands and the four cations were those used previously (Fabiato, 1981*a*).

The control solution was at pMg 2.50, pMgATP 2.50, ionic strength 0.170 M with K^+ and Cl^- as major ionic species, pH 7.10 buffered with 15 mM TES and 30 mM BES at 22°C. Whenever the solution departed from these specifications, it is indicated in the

figure captions.[3] BES is a more appropriate buffer than TES at pH 7.10 because its pK_a at 22°C is 7.14 vs. 7.44 for TES. Like TES, BES does not bind Ca^{2+} (Nakon and Krishnamoorthy, 1983) and is a derivative of taurine (Good et al., 1966), which is abundant in the intact cardiac cell. At concentrations up to 0.3 M, BES has no toxic effect on skinned cardiac cells (Fabiato, 1983). The solution included 15 mM TES because the aequorin had been prepared in the presence of TES. This high pH buffer concentration required an increase of the ionic strength from 0.160 (Fabiato, 1981*a*) to 0.170 M. Both values are likely to be near the physiological intracellular ionic strength, which is not known exactly.

The pH was measured at 22°C with a three-decimal-place pH meter (no. 701A; Orion Research Inc., Cambridge, MA), a Ross electrode (Orion no. 81-01), and a separate indifferent electrode (Orion no. 80-05) in order to avoid the error in pH measurements occasionally observed with some combination pH electrodes and with standard pH buffers at an ionic strength greatly different from that used for the experimental solution (Illingworth, 1981). The standard pH buffers recommended by Illingworth (1981) were used for calibration.

Except for the solutions used for calibration of maximum tension and maximum light, all solutions contained either 20 or 21 μM aequorin.[4] In the control solution, the pCa was generally 6.30. At this pCa, there was no significant discharge of the aequorin during the duration of an experiment (<1 h). This was demonstrated by comparing the area under the curve of maximum light produced by the discharge with an excess of Ca^{2+} of the same volume of aequorin-containing solution after various delays (see Fig. 7, *B* and *C*). The "zero" of the aequorin bioluminescence tracing did not drift significantly during the course of an experiment. This could have been predicted by the relationship between the rate constant of aequorin discharge at a given pCa (K_c) and the maximum rate constant in the presence of a saturating [free Ca^{2+}] ($K_{c\,max}$) (Blinks et al., 1982):

$$\log_{10}(K_c/K_{c\,max}) = \log_{10}(L/L_{max}),$$

where L is the bioluminescence at a given pCa, L_{max} is the maximum bioluminescence, produced by the instantaneous discharge of the aequorin, and $K_{c\,max}$ is 1.09 s^{-1}, the reciprocal of the "time constant" of aequorin light decay upon instantaneous discharge by an excess of free Ca^{2+} (0.92 s in the presence of pMg 2.50 at 22°C for the batch of aequorin used for the experiments, as explained later). With the batch of aequorin used at pCa 6.30 and pMg 2.50, $\log_{10}(L/L_{max})$ was −6.02, which gives a 267-h "time constant"

[3] The same is done in the accompanying articles (Fabiato, 1985*a*, *b*), in which the control conditions are identical. This facilitates cross-references between the articles. Several trials have shown that even nonspecialist readers can gather most of the information contained in these long articles by reading only the brief Discussion sections, which summarize the conclusions, and the extensive captions of the figures. The experiment illustrated in each figure is that for which the results were the closest to the average of the corresponding series, except for Fig. 19, which shows an atypical result.

[4] This information was obtained by comparison of the light produced by discharging with an excess of Ca^{2+} equal volumes of a solution containing the aequorin used (which was prepared by Drs. O. Shimomura and E. B. Ridgway) in the present study and of a solution containing a known concentration of aequorin, kindly provided by Dr. J. R. Blinks. The accuracy of this concentration is dependent upon the value of the extinction coefficient of aequorin, which is, perhaps surprisingly, three times that found for most other proteins (Prendergast and Mann, 1978). In addition, this estimate ignores the possibility of the presence of discharged aequorin in the batch used for the experiments. These uncertainties limit the general significance of the operational apparent stability constant of the Ca-aequorin complexes computed in one of the sections of the Results.

for the decay of aequorin light. The highest [free Ca^{2+}] used was pCa 5.50, for which $\log_{10}(L/L_{max})$ was −3.86 (see Fig. 10) in the presence of pMg 2.50. This gives a 111-min "time constant" for the decay of aequorin light.

Recording of Tension and Aequorin Bioluminescence

Tension was recorded with the same transducer and at the same cutoff frequency of 3 Hz as previously (Fabiato, 1981*a*). Aequorin bioluminescence was recorded with the same type of photomultiplier tube (D144A; EMI, Gencom Inc., Plainview, NY), but this one was obtained after extensive selection for dark current, which was only 0.04 nA at 920 V high voltage. The signal from the anode of the photomultiplier was filtered with a Butterworth double-pole, low-pass active filter (Lancaster, 1975). The cutoff frequency (f_c) was either 10 or 30 Hz.

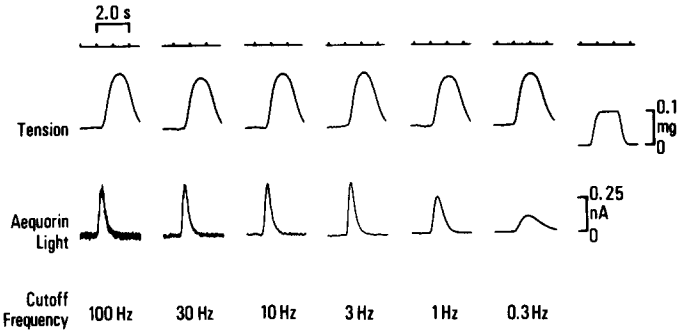

FIGURE 6. Effect of varying the cutoff frequency on the aequorin light signal recorded from an 8-μm-wide, 6-μm-thick, 29-μm-long skinned canine cardiac Purkinje cell. Tension was recorded simultaneously in order to give a time reference. The cutoff frequency of the filter for tension recording was constant at 3 Hz.

The attenuation of the highest frequency of signal (f) for a Butterworth filter is given by the following equation (Lancaster, 1975):

$$E_o/E_{in} = A_m/[1 + (f/f_c)^4]^{1/2},$$

where E_o is the output voltage, E_{in} is the input voltage, and A_m is the gain at midfrequency. It is apparent that if the highest frequency of the recorded signal does not exceed $0.3 \times f_c$, its attenuation will be <1%. The highest frequency of the aequorin bioluminescence signal was ≤1 Hz, which did not exceed $0.1 \times f_c$ even when f_c was 10 Hz. Thus, there was no attenuation of its amplitude. This is demonstrated in Fig. 6.

The phase shift (ϕ, given in degrees) for a Butterworth filter is given by the following formula:

$$\phi = \tan^{-1}[\sqrt{2} \times (f/f_c)]/[1 - (f/f_c)^2].$$

With $f/f_c = 0.1$, the phase shift was 8°. This produced so small a modification of the time to peak aequorin bioluminescence that it was not detectable at the recording speed used (Fig. 6).

The fastest component of the tension transient had a frequency of ~0.2 Hz. Thus, an f_c of 3 Hz more than sufficed to ensure that the fastest component was not attenuated. Similarly, the phase shift was <8°. However, the period was so long that an 8° phase shift would delay the peak of the tension transient by $5 \text{ s} \times (8/360) = 0.111$ s. Thus, the delay

between the peak of the aequorin bioluminescence and that of the tension transient was partly artifactual inasmuch as it was caused partly by the filtration.

Another cause of artifactual delay was the compliance of the tension transducer responsible for a shortening of ≤8% of the length of the preparation during maximum Ca^{2+} activation. However, the delay was not totally artifactual inasmuch as it could be

TABLE I

Dependence of the Period of the Cyclic Contractions on pCa, pMg, and Temperature

pCa	pMg 2.50	pMg 3.00	pMg 3.50	35°C, pMg 2.50, pH 6.90*
6.50	287.2±43.6‡	40.9±5.5	23.1±1.8	26.21±4.11
6.40	198.5±36.2	34.7±3.2	19.2±1.3	18.32±3.22
6.30	102.3±31.3	29.7±2.4	16.4±1.0	9.75±2.41
6.20	70.1±14.2	24.5±1.8	13.7±1.1	6.71±2.31
6.10	40.7±3.2	18.0±1.2	10.0±0.9	3.81±1.82
6.00	21.5±2.5	12.5±0.8	4.6±0.6	2.50±1.21
5.90	18.9±1.8	8.4±0.7	—	1.71±0.20
5.80	14.3±0.8	5.2±0.6	—	1.32±0.09
5.70	11.5±0.6	—	—	1.01±0.25
5.60	6.4±0.3	—	—	0.51±0.12
5.50	4.8±0.2	—	—	0.29±0.04
5.40	3.0±0.4	—	—	0.22±0.03

* The measurements at 35 ± 0.1°C, controlled with a heating and cooling stage (see Fig. 1 of Fabiato, 1985*a*), were done on high-speed cinefilm because the cutoff frequency of the transducer was too low to record the cyclic contractions. The skinned canine cardiac Purkinje cells were attached to the tension transducer, however, and stretched to ~2.3 μm sarcomere length, as in the other cases. The pH was set at 6.90 to take into account the decrease of the physiological intracellular pH that occurs when the temperature is increased (see Fabiato, 1985*a*, for discussion). In all other cases, the temperature was 22 ± 0.1°C, the pH was 7.10, and the period was that of the tension transients recorded with the transducer. In all cases, the pMgATP was 2.50, the ionic strength was 0.170 M with K^+ and Cl^- as the major ionic species, and the solutions contained 12 mM phosphocreatine, 15 U/ml creatine phosphokinase, and 5.13 μM calmodulin. The pH was buffered with 30 mM BES plus 15 mM TES.

‡ The value of the period is the mean ± SD in seconds (n = 5 in all cases). The differences between the values of the period of the cyclic contractions at two consecutive pCa values were significant in all cases, except for pCa 6.00 and 5.90 at pMg 2.50 and 22°C, and for the consecutive pairs of pCa values between pCa 6.30 and 5.90 at 35°C.

increased by lowering the temperature from 22 to 12°C (see Fig. 3 in Fabiato, 1985*a*) or decreased by >100 ms by raising the temperature from 22 to 37°C. This is consistent with the hypothesis (Fabiato, 1981*a*) that the delay is caused partly by the cross-bridge cycle, which includes slow reactions with temperature-dependent rate constants.

The results reported in the present and in the accompanying articles (Fabiato, 1985*a*, *b*) rely only on the amplitudes of the aequorin and tension transients, the accuracy of which was warranted by the recording system.

Statistical Analysis

All data are expressed in the form of mean ± standard deviation (SD). Student's t test was used for comparison of data, and differences were judged statistically significant for $P < 0.05$. The paired t test was used when appropriate. The number of experiments (n) is indicated for each experimental series. About twice as many experiments were rejected for the following reasons: (a) the presence of two components in the aequorin transient generally caused by branching myofibrils that were not attached to the tension transducer; (b) a variation in amplitude of either the aequorin or the tension transient under unchanged conditions; (c) large baseline shifts in light or tension recording.

RESULTS

The Ca^{2+} release from the SR, used to study the effects of ionic modifications during the Ca^{2+} transient, was spontaneous and cyclic. It was induced in the continuous presence of a sufficiently high steady state bulk solution [free Ca^{2+}] to overload the SR. As emphasized repeatedly (Fabiato and Fabiato, 1972; Fabiato, 1981*a*, 1983), this spontaneous cyclic release does not have the same mechanism as the Ca^{2+}-induced release of Ca^{2+}, which is triggered by a rapid increase of [free Ca^{2+}] at the outer surface of the SR of a previously quiescent skinned cell (Fabiato, 1985*a*). The overload of the SR with Ca^{2+} is a necessary condition for obtaining the spontaneous cyclic Ca^{2+} release, but may not be, by itself, the mechanism of this release, which is the subject of another article (Fabiato, 1985*c*). The frequency of the cyclic contractions resulting from the spontaneous cyclic release of Ca^{2+} from the SR is sensitive to bulk solution pCa, pMg, and temperature. It also decreases progressively with the prolongation of the time of the experiment, whereas the amplitude increases. Accordingly, the data shown in Table I were all obtained 15–45 min after the skinning of the cell. High-speed cinematography showed that the cyclic contractions were synchronous throughout the preparation for [free Ca^{2+}] up to pCa between 5.90 and 5.70 (range, $n = 16$), above which asynchronism was observed.

Estimate of the Free Ca^{2+} Concentration Reached at the Peaks of the Light and Tension Transients

At the end of each experiment, the aequorin-containing solution was microaspirated, leaving only the aequorin contained in the skinned cardiac cell. A solution at a saturating [free Ca^{2+}] of pCa 2.50 was then microinjected to discharge the aequorin contained in the skinned cell. The solution had the same constituents as the control experimental solution, including 0.068 mM total EGTA, pMg 2.50, pMgATP 2.50, 0.170 M ionic strength at pH 7.10 and 22°C. The same [total EGTA] as in the experimental solutions was used because EGTA might interfere with the Ca^{2+}-aequorin reaction (Ridgway and Snow, 1983). The recording (Fig. 7) was done with a current-to-voltage converter which was perfectly matched with that used for detecting the light transient caused by Ca^{2+} release from the SR but had a lower gain range. During the large light transient resulting from the discharge of the aequorin contained in the skinned cell, the microcomputer collected and stored data points every 5 ms. It calculated the area under the curve of aequorin bioluminescence and redisplayed it, after a

delay, as a rectangular wave recorded at a chart speed of 1 cm/s (Fig. 7*A*). The height of this rectangular wave was specified to be 0.8 cm, corresponding to 0.8 s, which was initially assumed to be the "time constant" of the light decay produced by the instantaneous mixing of a solution at saturating [free Ca^{2+}] with aequorin at 22°C, if one approximates the light decay as purely exponential (Allen and Blinks, 1979). The ordinate intercept of an exponential equals the area under the curve divided by the "time constant." Thus, the length of the

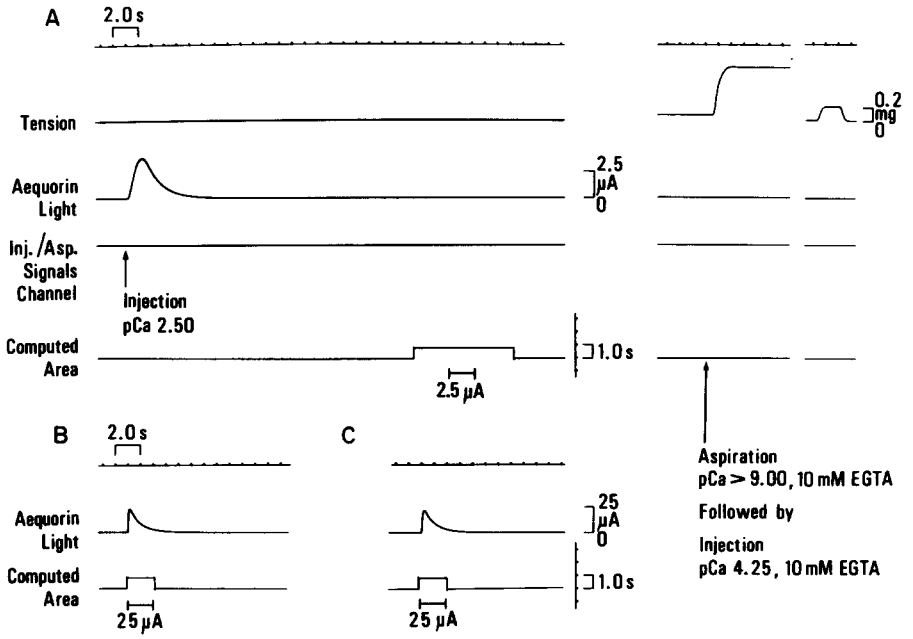

FIGURE 7. (*A*) Method of calibration of maximum light and maximum tension. Note that this calibration corresponds to that of the skinned cell used for the experiment illustrated in Fig. 18. (*B*) Discharge of the aequorin contained in a 52-pl drop of cell-free solution produced by one half-step of the motor. (*C*) Same as *B* but in a drop that had been left in the ion-free and water-saturated mineral oil for 30 min. Correction for the effect of free Mg^{2+} on the "time constant" of aequorin light decay requires multiplication of the height of the rectangular wave of computed area by 1.15, and division of its length by 1.15.

rectangular wave directly gave the amplitude of the maximum light that would be produced by mixing an excess of free Ca^{2+} with all the aequorin contained in the skinned cell. With this method of display, the time was shown vertically in order to avoid the risk that too high an intercept would exceed the amplitude of the recorder channel. In fact, the light decay caused by discharge of aequorin by a saturating [free Ca^{2+}] is not purely exponential. Thus, the 0.8 s should be referred to as the "ratio of total light to peak light," which still has a dimension in seconds (Blinks et al., 1982). This does not change the computation, and the length of the rectangular wave still gives directly the amplitude of the maximum light. For simplicity, this ratio will continue to be termed the "time constant."

The myoplasmic [free Ca^{2+}] reached at the peak of an aequorin light transient was computed from the ratio of the amplitude of the light transient signal to the maximum light and a calibration curve of aequorin. Initially, the calibration curve of the Mayo aequorin batch no. 1, kindly given to me by Dr. John R. Blinks, was used. However, each batch of aequorin may yield slightly different calibration curves under identical conditions because aequorin is a mixture of isoproteins (Blinks et al., 1982). In addition, batches of aequorin other than that used here had a toxic effect on the skinned cardiac cells. The toxic effect consisted of an increase in the frequency and a decrease in the amplitude of the cyclic contractions in the presence of a pCa 6.30 solution. Despite many experiments, no explanation for this toxic effect has yet been found. For these reasons, Dr. Blinks taught me his method of aequorin calibration so that I could apply it to the batch of aequorin used for the experiments reported in this and the next article (Fabiato, 1985*a*). Soon after, Dr. Blinks informed me of the finding of his student, Mr. E. D. W. Moore, that the "time constant" for the decay of light intensity is dependent upon the [free Mg^{2+}] in the solution in which the aequorin has been preincubated prior to its exposure to high [free Ca^{2+}] (Moore, 1984). Thus, the value of 0.8 s, used as the height of the rectangular wave in the calibration method, was inaccurate, which constituted one more reason for doing calibration curves with the same aequorin batch and under the same ionic conditions as used for the experiments. In addition, I had to modify the method of Allen and Blinks (1979) to take into account the problems related to the distribution of Ca^{2+} and other ions in skinned cardiac cells.

The calibrations were done with the microprocessor-controlled system of microinjection-aspirations used for the experiments (i.e., the recordings of tension and aequorin light transients in skinned cardiac cells with intact SR). The rapidity of this system exceeded that of the aequorin assay apparatus used by Allen and Blinks (1979). Identical results were obtained with the aequorin assay apparatus described by Blinks et al. (1978), which Dr. Blinks loaned to me for an extended period of time. The recording was done with the photomultiplier, the two current-to-voltage converters, and the amplifying system used for the experiments to cancel out any nonlinearity. The cutoff frequency was 300 Hz for the maximum light and 30 Hz for the light induced by [free Ca^{2+}] values lower than pCa 5.30.

Fig. 8 confirms the findings of Moore (1984), but with the aequorin batch and the ionic composition of the solutions used for the experiments. Complete discharge of aequorin was induced by a 5-ms microinjection of a large volume of a solution containing an excess of free Ca^{2+} with and without Mg^{2+} in both the low and high [free Ca^{2+}] solutions. During the display of the signal, the computer calculated the area under the curve of aequorin light and redisplayed it as a rectangular wave with a specified length equal to the amplitude of the peak light (as opposed to the specified height used for the calibration at the end of the experiment, as shown in Fig. 7). Hence, the height of the rectangular wave gave directly the "time constant." This "time constant" was 0.9201 ± 0.0014 s ($n = 62$) as compared with the average value of 0.82 s ($n = 4$) found in the absence of free Mg^{2+}, which is close to the 0.80 s used for the experiments. Accordingly, the length of the rectangular wave in Fig. 7 and the height of the

maximum light bars in Figs. 15 and 18–22 should be divided by 1.15 (which is equal to 0.92/0.80). The decrease of the rate of light production caused by free Mg^{2+} was more pronounced in the terminal part of the ascending phase. Thus, the comparison of the derivatives (dL/dt) gave a more accurate estimate of the

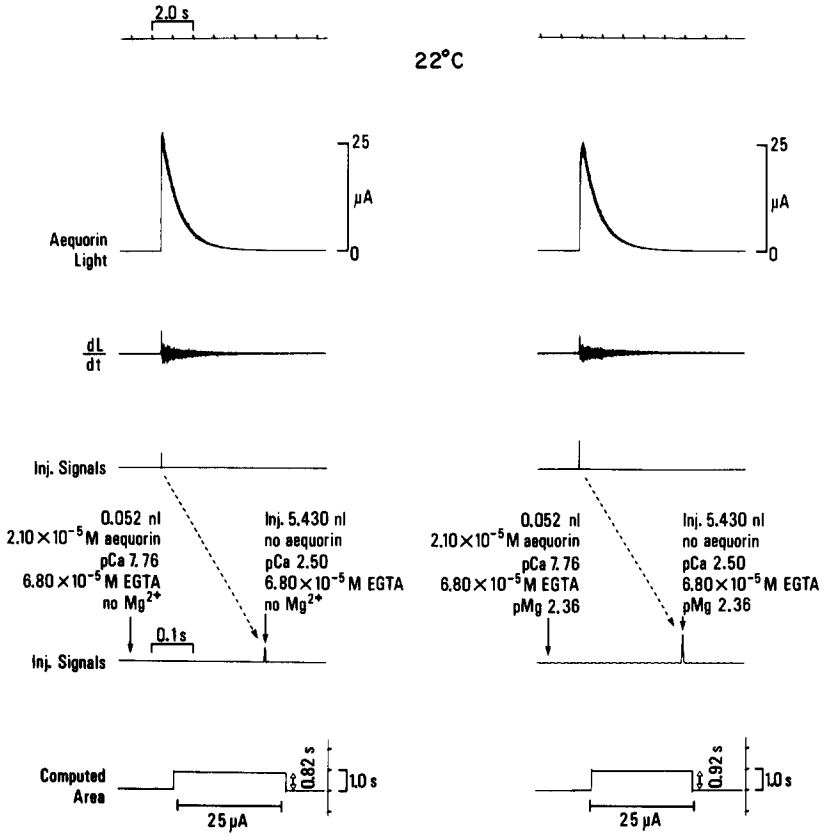

FIGURE 8. Determination of maximum light in the absence (left) and presence (right) of Mg^{2+}. See text for discussion of the values used for pMg and pCa. The pH was 7.03. Comparison of the computed area in the right-hand panel with that in Fig. 7 (*B* or *C*) shows that the same total light was obtained by discharging the aequorin contained in 52 pl of solution (the comparison requires multiplying the height, and dividing the length, of the rectangular wave in Fig. 7 by 1.15). This shows that the concentration of active aequorin had not changed significantly during the three years that separated these two measurements.

change of maximum rate of light production than a comparison of the time to peak light.

Two methods of aequorin calibration were used. The first was a solution calibration according to the protocol shown in the right-hand panel of Fig. 8, except that the pCa was varied (in values separated by 0.05 units). The $-\log_{10}$ of the ratio of the light obtained at each pCa value to the maximum light was plotted as a function of the pCa (Fig. 10, filled symbols with SD bars).

The second method consisted of discharging the aequorin contained in a skinned cell in which the ability of the SR to actively accumulate and release Ca^{2+} had been destroyed by 1 h of treatment with 0.5% (wt/vol) of the nonionic detergent polyoxyethylene 20 cetyl ether (Brij 58) (Eastwood et al., 1979). This was done to take into account the possibility of an influence of cellular constituents on the aequorin calibration curve. After 1 h of bathing in 1.040 nl (produced by 20 half-steps) of aequorin-containing solution at pCa 7.90 to permit the loading of the skinned cell with aequorin, this solution was reaspirated so that only the solution contained in the skinned cell remained. Then the amplitude of the light produced at a given pCa and the maximum light were determined in the same detergent-treated skinned cell. This was possible because of the extremely slow light decay in the [free Ca^{2+}] range used for the experiments. The current from the anode of the photomultiplier tube was transmitted to the two current-to-voltage converters used simultaneously in parallel. The injection of the low [free Ca^{2+}] solution (~30 times the volume of the skinned cell) caused a light transient that was recorded with the high-gain current-to-voltage converter but was not detectable with the low-gain one (Fig. 9). Although no photographic enlargement is provided, it can be measured that there was a <50-ms delay between the injection signal and the onset of the light and that a plateau of light was reached immediately. This is consistent with a diffusion of Ca^{2+} over only the ~0.4-μm radius of a single myofibril rather than over the ~3-μm minimum overall radius of a skinned cell. Even shorter delays (<20 ms) were obtained in skinned cells treated in the detergent for 24 h. After 2 s of recording to ensure that a plateau was reached, the high-gain current-to-voltage converter was disconnected by a mercury-contact switch that was remote-controlled, and after 100 ms an ~100-times-larger volume of solution at saturating [free Ca^{2+}] was injected. This resulted in the production of the total light. To obtain the theoretical maximum light, the area under the curve was redisplayed by the computer, this time with the specified "time constant" of 0.92 s according to the same principle used in Fig. 7. The $-\log_{10}$ of the ratio of light to maximum light was plotted as a function of the pCa (Fig. 10, open symbols without SD bars). Only a single determination was made for each pCa value because this required the preparation of one detergent-treated skinned cell each time.

It is of interest to compare the time course and amplitude of the maximum light obtained in this detergent-treated skinned cell and in the skinned cell with active SR used for Fig. 7. Even after correction for the inaccuracy of the "time constant," the peak light in Fig. 7 barely reached 40% of the theoretical maximum light, and its time to peak was ~500 ms. In contrast, the peak light in Fig. 9 was reached in <50 ms, and its amplitude was ~90% of the theoretical maximum light. These differences of percentage amplitude with respect to theoretical maximum light were attributed to the prevention by the SR of the direct activation of the myofilaments with the externally applied solution because the SR tightly surrounds the individual myofibrils. The Ca^{2+} accumulation and release by the SR therefore interfered with the activation of the myofilaments by externally applied solutions, even at a [free Ca^{2+}] as high as pCa 2.64 (see Fig. 7 in Fabiato, 1985*a*, for further elaboration on this point). The difference in the time to peak light may be caused partly by the cutoff frequency of the filter,

which was 30 Hz in Fig. 7 and 300 Hz in Fig. 9, but, as explained in Fig. 6, the filtration should little affect the amplitude of the peak light. This is further demonstrated by the almost identical peak-to-area ratio of the light signal recorded during rapid discharge of the aequorin contained in a 52-pl drop

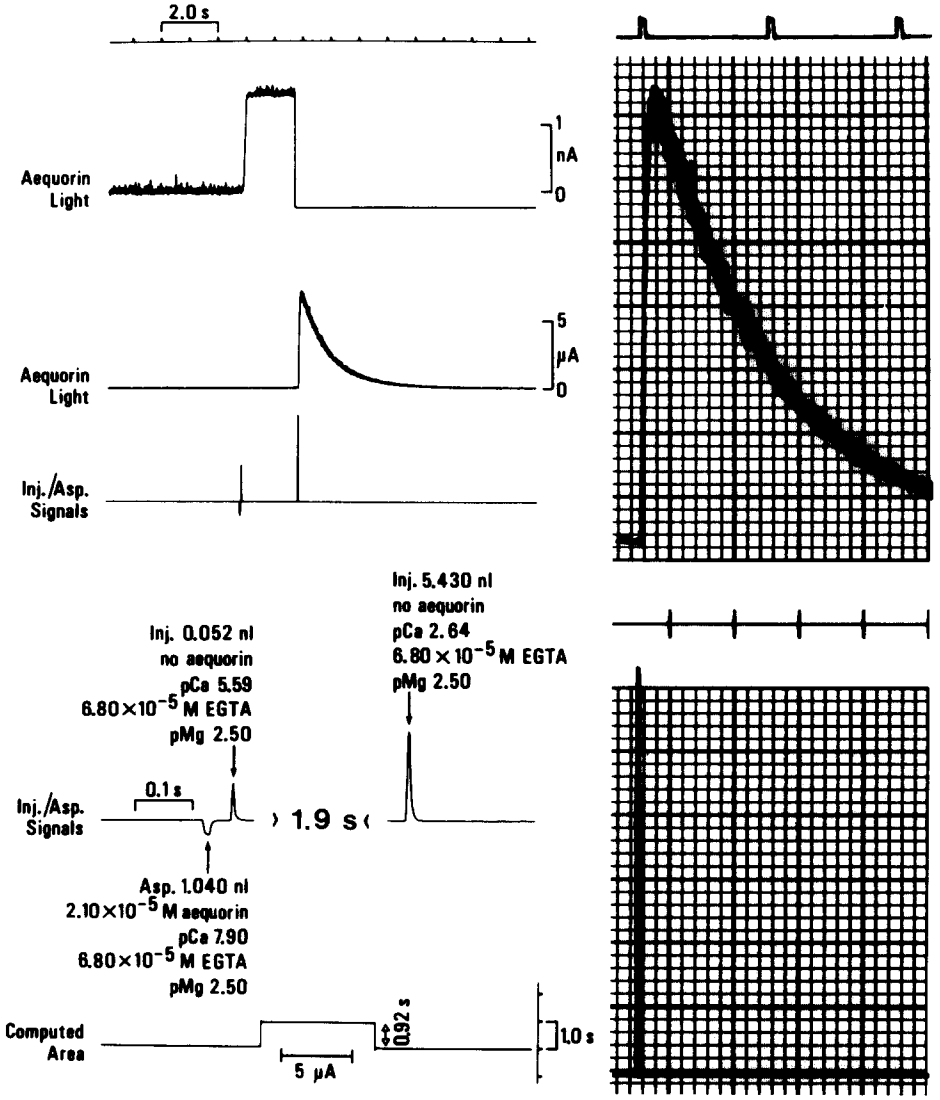

FIGURE 9. Calibration of maximum light in a detergent-treated skinned canine cardiac Purkinje cell that was 8 μm wide, 6 μm thick, and 32 μm long. The pH in the solution was 7.10. The rapid fall of the light signal shown in the upper trace was caused by the operation of the mercury-contact, remote-controlled switch. The photographic enlargement at the right of the figure shows the time relation between the injection of the solution at pCa 2.64 and the onset of the resulting light signal. The composition of the solutions that were injected or aspirated is indicated on the redisplay of the injection-aspiration signals at a 20-times-higher speed.

recorded at a cutoff frequency of, respectively, 30 Hz in Fig. 7, *B* and *C*, and 300 Hz in Fig. 8. The purpose of the protocol used in Fig. 9 was not to demonstrate this role of the SR but primarily to calibrate aequorin. Complete removal of the SR would have required a longer exposure to Brij 58 (Eastwood et al., 1979). In seven experiments similar to that of Fig. 9 but using skinned cells treated for 24 h with 1% (wt/vol) Brij 58, the peak light always exceeded 90% of theoretical maximum light and in one case was indistinguishable from it.

The composition of the solutions was different for the calibration in cell-free solutions (Fig. 8) and in a detergent-treated skinned cell (Fig. 9) to take into account the Donnan osmotic forces caused by the absence of the sarcolemma and the negative charges on the myofilaments (Elliott, 1973; D. G. Stephenson et al., 1981; Godt, 1981; Godt and Baumgarten, 1984). The ionic composition in the myofilament space was deemed to be the relevant one because of evidence, presented in the following section, that aequorin is concentrated in this space. It was not possible to record potential differences between skinned cells of the size used here and the solution because the microelectrode tip lodged itself between the widely spaced myofibrils.[5] The negative charges on the myofilaments should not vary with the size of the preparation. Hence, measurements were made between a microelectrode in 25–30-μm-wide, 16–20-μm-thick skinned canine cardiac Purkinje cells and an indifferent microelectrode in the solution. The potential was -4.11 ± 0.06 mV ($n = 28$). This value is slightly lower than that found in skinned fibers from skeletal muscle (Godt and Baumgarten, 1984) and in the same range as, but more precisely measured than, the value reported in cardiac fibers with disrupted sarcolemma from the rat ventricle (Fabiato and Fabiato, 1972). The Nernst equation for Ca^{2+} at 22°C is:

$$E_{Ca} = -28.6 \log_{10}[\text{free } Ca_i^{2+}]/[\text{free } Ca_o^{2+}],$$

where E_{Ca} is the equilibrium potential for Ca^{2+}, [free Ca_i^{2+}] is the average [free Ca^{2+}] within the skinned cell, and [free Ca_o^{2+}] is the average [free Ca^{2+}] in the solution. In a Donnan system, the equilibrium potential must be equal to the voltage. Thus, substituting -4.11 mV for E_{Ca} in the preceding equation and solving it for [free Ca_i^{2+}] gives:

$$[\text{free } Ca_i^{2+}] = [\text{free } Ca_o^{2+}] \times 10^{(4.11/28.6)} = [\text{free } Ca_o^{2+}] \times 10^{0.14}.$$

Hence, the average pCa in the myofilament space should be 0.14 units lower than that in the solution. This is an average pCa because within a few angstroms (two Debye zones; McLaughlin, 1977) around each myofilament, the [free Ca^{2+}] could be much higher both in skinned and in intact cardiac cells.

The correction factor for the pCa is independent of the [total EGTA] because the same change of concentration applies proportionally to the ionic charge of the different species of CaEGTA and the [free EGTA]. The [free Ca^{2+}] depends only on the ratio between these ionic species. The concentration of all anionic species should be lower in the myofilament space than in the solution.

Similarly, the [free Mg^{2+}] in the myofilament space should be 0.14 units lower

[5] The 0.3–0.4-μm tip of the microelectrode impaled a single myofibril but had a tendency to go through it and to be open to the space between this myofibril and a subjacent one.

than that in the solution, i.e., pMg 2.36 when the pMg in the solution was 2.50. The monovalent cation concentrations should be higher in the myofilament space than in the solution by 0.07 $-\log_{10}$ concentration units. This correction also applies to the pH even though pH = $-\log_{10}$ H^+ activity instead of $-\log_{10}$ [free H^+], because at constant ionic strength the activity is proportional to the molar concentration. Thus, a pH in the solution of 7.10 should yield a pH of 7.03 in the myofilament space, which is in the likely physiological range (Fabiato and Fabiato, 1979).

For the aequorin calibration in cell-free solution, the pMg was therefore 2.36 and the pH was 7.03 for the experiments done at pMg 2.50 and pH 7.10 in the bulk solution. The myoplasmic pCa values reached at the peak of the Ca^{2+} transient inferred from these solution calibrations were representative of the true average pCa values in the myofilament space. For the calibration in detergent-treated skinned cells, the situation was the same as in the experiments and, accordingly, pMg 2.50 and pH 7.10 were used, but the pCa values were 0.14 units higher than those plotted on the curve. Thus, the determination at pCa 5.59 obtained in Fig. 9 was plotted as 5.45 in Fig. 10. The appropriateness of this correction is supported by the good fit of the open circles in Fig. 10 by the curve drawn through the filled circles. Displacing these points, shown as open circles, by 0.14 pCa units would result in a considerably different curve.

The curve at pMg 2.50 had a slightly decreasing slope from 2.73 to 2.50 when the [free Ca^{2+}] was increased from pCa 5.90 to 5.40. If the aequorin-Ca^{2+} binding has a constant stoichiometry of 2.50 (Blinks et al., 1982), this change of the slope was in a direction opposite to that which would occur if the EGTA purity had been overestimated (Miller and Smith, 1984) and could be explained by a difference between aequorin batches or by a nonlinearity of the recording system. The latter would little affect the results because the same amplifying system was used for the experiments and calibration curves. On the other hand, the small change of slope may not have been apparent in the curves of Allen and Blinks (1979) and Blinks et al. (1982) because these investigators collected far fewer data than used here in the pCa range shown in Fig. 10.

The deletion of calmodulin (5.13 μM) did not modify the curve at pMg 2.50 (Fig. 10, filled triangles vs. filled circles). Preincubation with pMg 3.00 yielded the same "time constant" of 0.92 s as with pMg 2.50. The two calibration curves, in cell-free solution (Fig. 10, filled squares) and in detergent-treated skinned cells (Fig. 10, open squares), yielded consistent data when using pCa and pMg values 0.14 units higher and a pH 0.07 units higher for the skinned cell vs. the cell-free solution calibration. No calibration curve was done for pH 6.70 because it seemed well established that pH does not change the sensitivity of aequorin to Ca^{2+} in the range used here (Shimomura et al., 1962; Allen and Blinks, 1979; Blinks et al., 1982); this has been verified with the batch of aequorin used here both at 22°C (Fabiato, 1985*d*) and at 12°C (see Fig. 4 in Fabiato, 1985*a*).

The numbered horizontal lines in Fig. 10 and the corresponding vertical lines show the inference of the myoplasmic [free Ca^{2+}] (i.e., the average [free Ca^{2+}] in the myofilament space) reached at the peak of the Ca^{2+} transient from the aequorin calibration for each of the experiments illustrated in a figure.

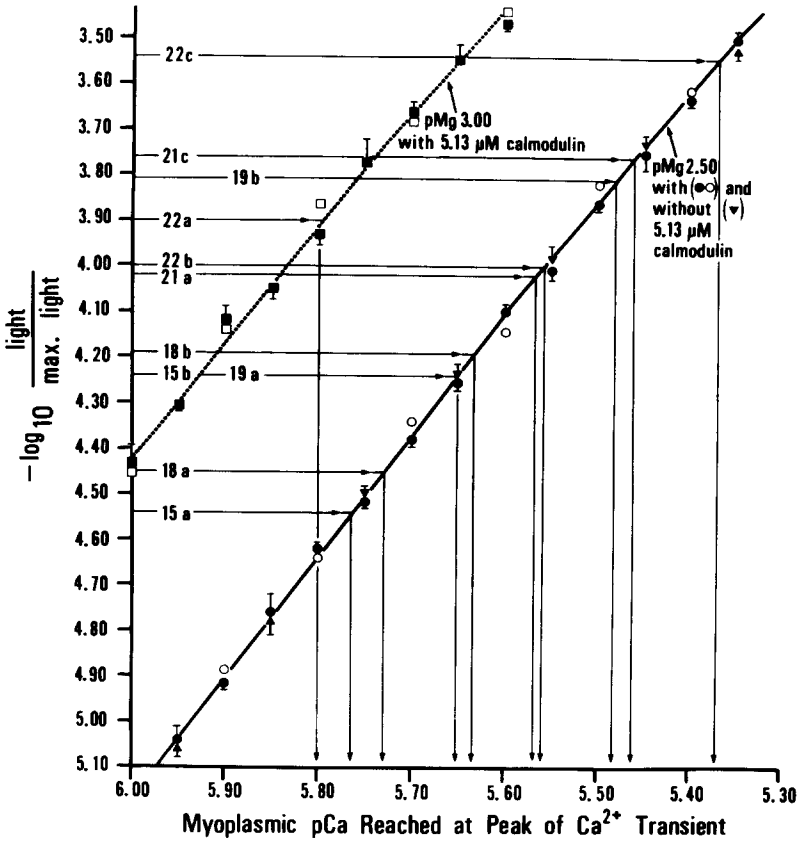

FIGURE 10. Inference of the peak myoplasmic [free Ca^{2+}] from the amplitude of the aequorin light transient. The calibration curves were obtained with the batch of aequorin used for the experiments, under the various conditions used for the experiments. Each filled symbol is the mean of six determinations of cell-free solution calibration with the SD shown in one direction only for clarity. Open symbols without SD bars correspond to single determinations in detergent-treated skinned canine cardiac Purkinje cells. The numbers on the horizontal lines correspond to figure numbers. The letters refer to the light transients in the figures, with *a* being the first one.

After the aequorin calibration, the maximum tension was determined by first applying a solution that contained 10 mM total EGTA and pCa >9.00 and otherwise had the same constituents as in the experimental solutions. This permitted relaxation of the skinned cell. Then a solution that contained 10 mM total EGTA and pCa 4.25 and was otherwise the same as the experimental solutions was used to induce a maximum tension. The [total EGTA] in the solution used for inducing maximum tension differed from that for maximum light because the application of pCa 2.50 in the presence of 0.068 mM total EGTA resulted in the development of a tension that had a very low rate of rise

and never reached the maximum tension developed in the presence of 10 mM total EGTA. This was caused by the competition for Ca^{2+} by a number of intracellular binding sites inside the skinned cardiac cell, including the mitochondria, which have a low affinity but a very high capacity for Ca^{2+} (Fabiato, 1981*a*, 1983). The ratio of the amplitude of a given tension transient to the maximum tension was used to infer the [free Ca^{2+}] reached during the Ca^{2+} transient from the tension-pCa curve.

Fig. 11 shows the tension-pCa curve under the different conditions used. The tension-pCa curve at pMg 2.50 and pH 7.10, in the presence of calmodulin, had a Hill coefficient of 2.09 ± 0.14 (Hill, 1913). The deletion of calmodulin (5.13 μM) had no significant effect on either the maximum tension developed by the myofilaments in the presence of a saturating [free Ca^{2+}] or the sensitivity of the myofilaments to Ca^{2+} as measured by the tension-pCa curve, for which none of the points were significantly different from those in the presence of calmodulin. Changes in pMg resulted in a parallel shift of the curve with no change of maximum tension, as previously found in mechanically skinned single cardiac cells (Fabiato and Fabiato, 1975), but in disagreement with the results reported in "chemically skinned" multicellular preparations (Donaldson et al., 1978). A change in pH resulted in a nonparallel shift of the curve, more pronounced at lower pCa values for a decrease of pH as previously described (Fabiato and Fabiato, 1978*b*). The curve at pH 6.70 did not fit Hill's equation for a single class of binding sites.

Because the tension-pCa relationship varies with the protocol (Brandt et al., 1980, 1982; D. G. Stephenson and Williams, 1981; Ridgway et al., 1983), how the tension-pCa curves were obtained is important. Skinned cardiac cells with similar dimensions were used. The solution changes were not done as described in the Methods section but as in a previous study (Fabiato, 1981*a*) with an air- and vacuum-driven system and seven microinjections and aspirations. One of the seven pipettes contained a solution at pCa >9.00 in the presence of 5.13 μM calmodulin, pMg 2.50, and pH 7.10. This was the relaxing solution to which a return was made between the application of each contracting solution. The second pipette contained a solution at pCa 4.25 in the presence of 5.13 μM calmodulin, pMg 2.50, and pH 7.10. This induced a maximum tension that was the reference for all the experiments. Thus, the application of this solution ended an experimental series. The five remaining pipettes contained solutions at various pCa values. These five pipettes were used in four series of increasing and decreasing pCa values. An experiment was discarded when the tension at a given pCa differed from that obtained in a previous series by >5%.

Only eight skinned cells were used for each curve: four at a given pH and pMg with or without calmodulin, and four with mixing values of pH and pMg. The resulting curves had standard deviations only about one-third of those previously reported for skinned single cardiac cells (Fabiato, 1981*a*, 1982*a*). The small solid circles correspond to the results obtained for a single determination of tension in a single skinned cell. The slope of the curve does not differ from that obtained with averaged data. Previous reports of a higher slope of the mammalian skeletal muscle tension-pCa curve when it was obtained from a single skinned fiber rather

than from pooled results (Brandt et al., 1980, 1982; D. G. Stephenson and Williams, 1981) are probably related to differences between more or less predominantly fast or slow fibers. Other experiments (Fabiato, 1985*d*; see also Fig. 6 in Fabiato and Fabiato, 1975) have demonstrated the absence of a hysteresis (Ridgway et al., 1983) in the tension-pCa relation of this preparation.

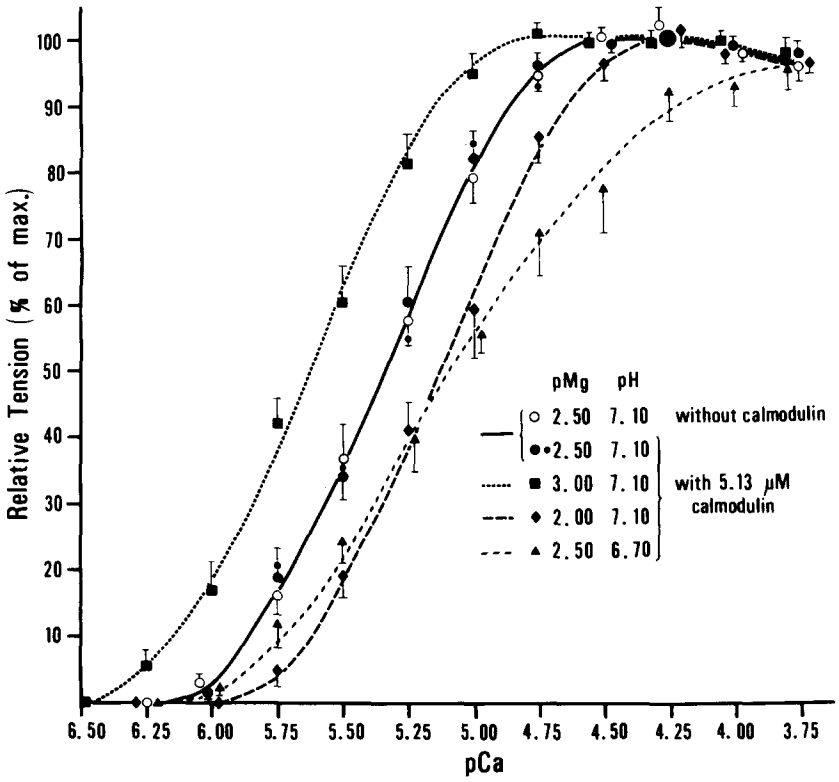

FIGURE 11. Tension-pCa curves under various conditions in skinned cardiac cells from the canine cardiac Purkinje tissue that were 7–8.5 μm wide, 5–6 μm thick, and 20–40 μm long. In all cases, the tension in a given skinned cell was expressed as a percentage of the maximum tension induced by pCa 4.25 with 5.13 μM calmodulin, pMg 2.50, and pH 7.10 in the same skinned cell. Each point is the mean of seven determinations, and each vertical bar is the SD shown in one direction only. The five small filled circles without SD bars correspond to the data obtained from a single skinned cell. In all cases, the [total EGTA] was 10 mM, the ionic strength was 0.170 M, and the temperature was 22 ± 0.3°C. The data points of the different curves were significantly different, except for the filled diamonds and filled triangles at pCa 5.25 and 5.50.

Fig. 12 shows the inference of the myoplasmic [free Ca^{2+}] reached at the peak of the tension transient for all the experiments illustrated in which the peak myoplasmic [free Ca^{2+}] was also inferred from an aequorin calibration. Fig. 13 shows the inference of the peak myoplasmic [free Ca^{2+}] for the experiments in which only a tension inference was made.

For this calibration of the myoplasmic [free Ca^{2+}] reached at the peak of the Ca^{2+} transient from the tension-pCa curve, the myofilaments, which were used as a sensor, were also responsible for the Donnan osmotic forces that complicated the measurements. Hence, the average pMg, pCa, and pH were lower in the myofilament space than in the bulk solution. Decreasing pMg and pH have effects opposite to those of decreasing pCa (Fabiato and Fabiato, 1975, 1978*b*).

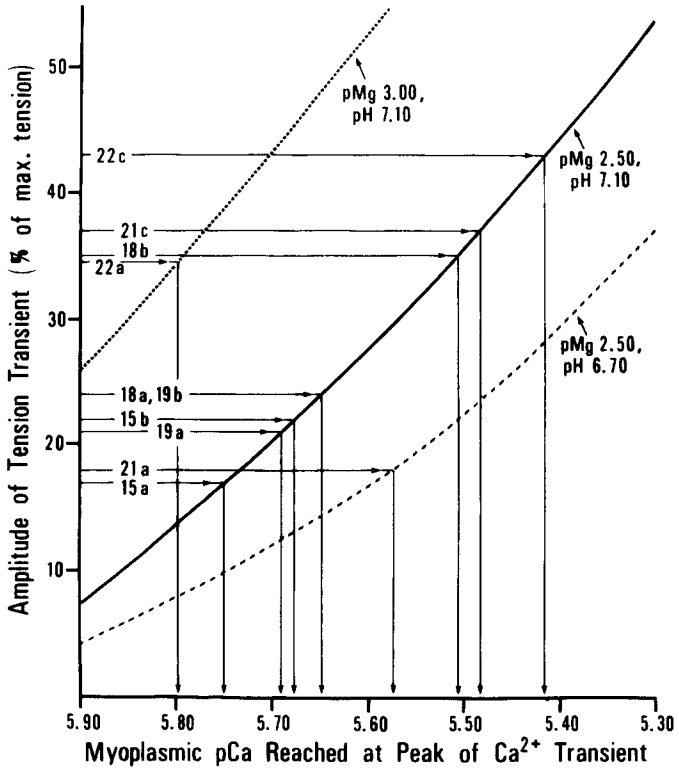

FIGURE 12. Inference of the peak myoplasmic [free Ca^{2+}] from the amplitude of the tension transient for the same experiments as in Fig. 10. The conventions for numbers and letters are the same as for Fig. 10. The data points and SD values for the tension-pCa curves at various pH and pMg values are shown in Fig. 11.

A tension-pCa curve was done at pMg 2.64 and pH 7.17 with each pCa value 0.14 units higher than the nominal values. This curve ($n = 6$ for each point; data not shown) was not significantly different from that labeled pMg 2.50 in Fig. 11. Thus, the values of myoplasmic pCa reached at the peak of the Ca^{2+} transient shown in Figs. 12 and 13 are accurate, within the limits of the statistical analysis, but the pMg and pH are not those in the myofilament space but those in the bulk solution.

Aequorin Binding in the Skinned Cardiac Cell

The determination of the maximum light was based on the assumption that microaspiration of the experimental solution removed all the aequorin except

that in the skinned cell. As stated, the volume displaced by one half-step of the motor was reproducible within the detection limits. However, the volume of a skinned cardiac cell was 30–50 times smaller than that of the solution displaced by one half-step. Thus, an uncertainty about whether exactly the volume of solution contained in the skinned cell was still present may be justified. However,

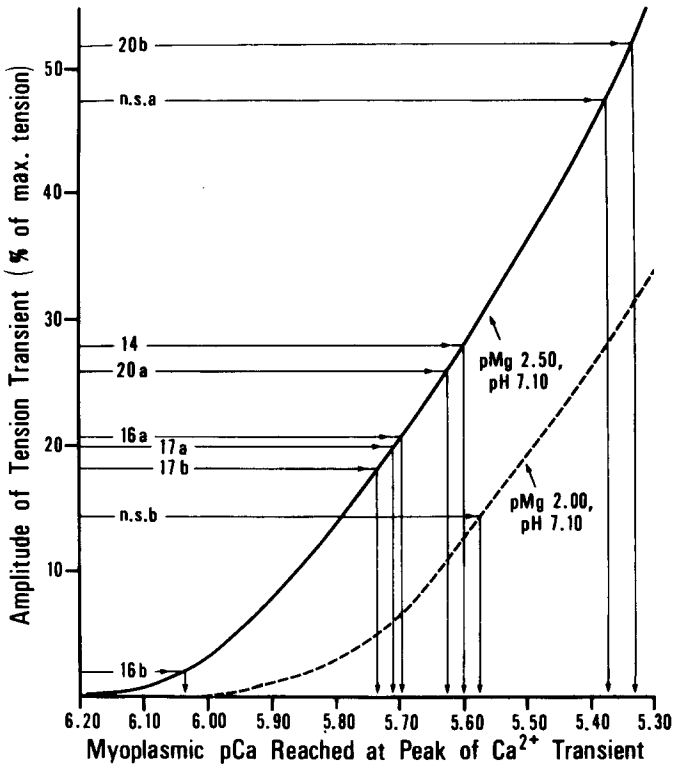

FIGURE 13. Inference of the peak myoplasmic [free Ca^{2+}] from the amplitude of the tension transient for the experiments in which an aequorin calibration was not done simultaneously. The conventions for numbers and letters are the same as in Figs. 10 and 12, but they refer to different experiments; *n.s.a* and *n.s.b* correspond to data not shown in a particular figure.

even if the microaspiration had removed some of the fluid contained in the skinned cell, this would not have significantly affected the calibration because aequorin binds and concentrates in the skinned cardiac cell, as will now be demonstrated.

After an experiment in a skinned cardiac cell with aequorin-containing solution, the skinned cell was removed and one half-step (52 pl) of the same aequorin-containing solution was injected into oil (Fig. 7). The complete discharge of the aequorin contained in this drop resulted in a maximum light 2.90 times that obtained from the skinned cardiac cell, which had a volume of 1.54 pl. The mitochondria occupy ~23% of the volume of the cardiac cell from the canine

Purkinje tissue (Fabiato, 1985*b*), leaving ~77% of the volume accessible to aequorin.[6] Therefore, aequorin within the skinned cell was concentrated by a factor of:

$$52/(1.54 \times 2.9 \times 0.77) = 15.1.$$

For 12 similar experiments, the concentration factor was 14.7 ± 2.1 after a 1-h exposure to aequorin.[7]

In five additional experiments, the skinned cell had been exposed to aequorin for 30 min only. The concentration factor was 15.3 ± 3.2, not significantly different from the value of 14.7 ± 2.1 obtained after a 1-h exposure to aequorin. This indicates that the loading and binding of aequorin were completed in 30 min in these cyclically contracting preparations.

The same protocol was applied to six skinned canine cardiac Purkinje cells that had been exposed to 1% (wt/vol) of the nonionic detergent Brij 58 for 12 h prior to the exposure to aequorin-containing solution for 1 h. At this concentration and exposure time, the detergent should not only prevent the SR from actively accumulating Ca^{2+} but should also physically destroy most of the SR and the mitochondria (Eastwood et al., 1979). The aequorin concentration factor was 17.1 ± 2.6, not significantly higher than without detergent treatment. That the concentration factor did not change when the SR and mitochondria were destroyed may suggest that aequorin binds to the myofilaments.

Four additional skinned cells were treated with detergent in the same way but were exposed to aequorin-containing solution for 30 min. The aequorin concentration factor was only 9.0 ± 4.8, significantly less than after 1 h. The difference observed in these skinned cells, which were quiescent because the SR was destroyed, contrasts with the lack of a significant difference when the same comparison was made in cyclically contracting skinned cells with intact SR. This suggests that the cyclic contractions may have some "squeezing" effect that would facilitate the access of aequorin to a restricted space in the myofilament lattice.

Another observation which suggests a loading of the skinned cardiac cell with aequorin is that of a progressive increase of the aequorin signal amplitude during

[6] The small volumes of the SR and of the molecules of the contractile proteins were neglected because they were estimated to be <5% of the cell volume, within the margin of uncertainty of the mitochondrial volume (Fabiato, 1985*b*). There is no reason to consider the large volume of the water content of the myofilaments excluded from the diffusion space of aequorin. On the contrary, the data suggest that aequorin concentrates in this myofilament space.

[7] This concentration factor is about twice that previously reported for skinned cardiac cells from the rat and rabbit ventricle (Fabiato, 1981*a*). The difference was caused by the lower precision of the method in the previous study, which had used a drop one-third of the size of a skinned cardiac cell. The volume of the drop had been inferred from its diameter as measured under the microscope. Since the drop was in contact with the glass, it was a flattened sessile drop (Davies and Rideal, 1963). It was assumed that the thickness of the drop was two-thirds of its width. This assumption was proven wrong by the more precise method used here. The sessile drop was, in fact, much more flattened. The calibration procedure with precise volume used here was recently applied to skinned cardiac cells from the rat ventricle. The results were in the same range as those obtained for the canine cardiac Purkinje cells, taking into account the fact that mitochondria occupy a larger volume (~36% of the cell) in the rat ventricle.

the 30-min equilibration period before the experiment (Fabiato, 1981*a*). Eleven experiments were done to study this phenomenon more precisely. The skinned cell was initially bathed in a solution at pCa 6.30 without aequorin (Fig. 14). Spontaneous cyclic contractions were observed. The solution was microaspirated,

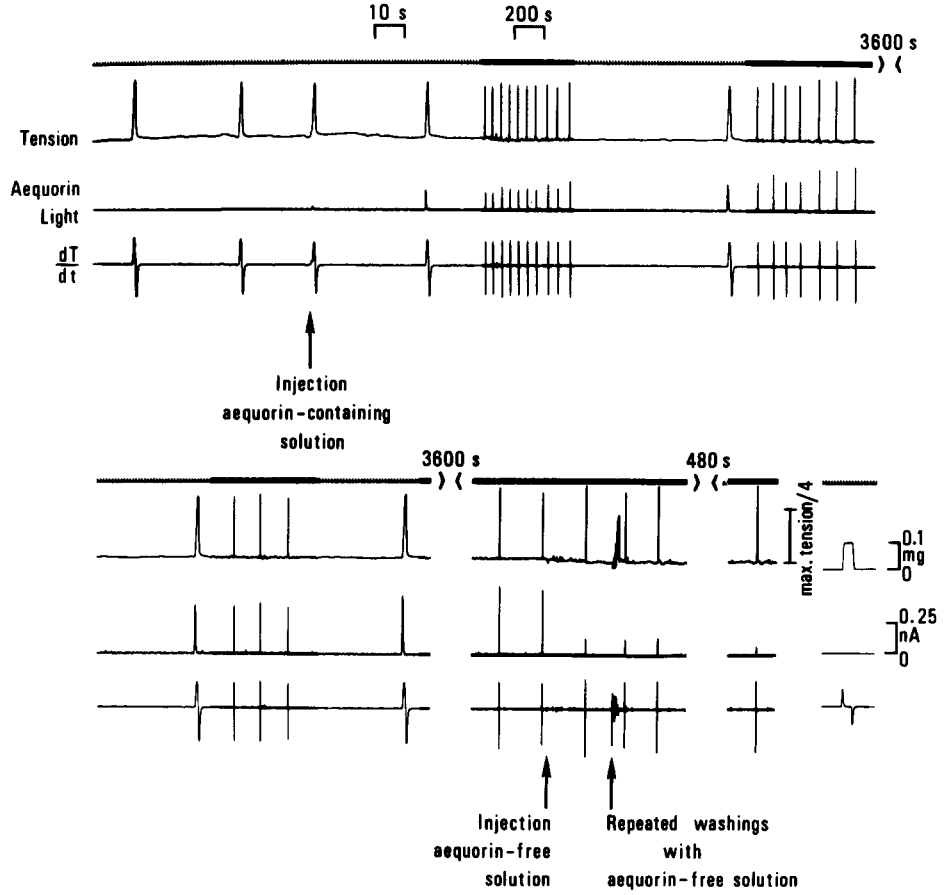

FIGURE 14. Aequorin loading in a skinned canine cardiac Purkinje cell that was 8.5 μm wide, 6 μm thick, and 26 μm long. Solution changes were effected by vacuum-induced aspiration and compressed air–induced injection (Fabiato, 1981*a*). The durations of the interruptions of the tracing are indicated. Note the small aequorin transient at the limit of visibility preceding the first tension transient, just after the injection of aequorin-containing solution. The concentration of aequorin was 21 μM. The repeated washings were accomplished by eight aspirations followed by eight reinjections of the aequorin-free solution.

and another at pCa 6.30, but with aequorin, was microinjected (Fig. 14, first arrow). The first Ca^{2+} transient following this microinjection gave a very small aequorin signal (at the limit of visibility in Fig. 14), while the amplitude and cycle of the tension transient were not modified by the addition of aequorin. The

second spontaneous Ca^{2+} transient presented an ~10-times-larger light signal. The amplitude of the aequorin light signal increased progressively by an additional factor of 3, while the amplitude of the tension transient was not modified during the next 30 min. It was at this time that the experiment was generally done, i.e., after a 30–60-min exposure to aequorin.

After further time had elapsed, the frequency of the cyclic Ca^{2+} release decreased and the amplitude of both the aequorin and the tension transients increased in a consistent manner, if the different stoichiometries of Ca^{2+} binding to aequorin and troponin C (Blinks et al., 1982) are taken into account. This cannot be taken as evidence for a further loading of the skinned cell with aequorin. In contrast, the first ~30 min corresponded to a very slow process during which aequorin progressively concentrated in the skinned cardiac cell.

When the aequorin-containing solution was removed and replaced by a solution at the same pCa but without aequorin (Fig. 14, second arrow), this resulted in the persistence of a light transient of amplitude equal to 30% of that in the presence of aequorin, while the tension was not modified. Washing the preparation eight times with aequorin-free solution by microaspirations and reinjections (Fig. 14, third arrow) did not further reduce the amplitude of the remaining light transient. This amplitude decreased with time, however, with a halftime of 8 ± 2 min ($n = 11$). The 70% decrease in amplitude of the light transient upon washing renders unlikely a tight binding to the myofilaments of most of the aequorin concentrated by a factor of ~15 in the skinned cell. The washing produced a rapid flow of solution through the preparation. This would explain the rapid decrease of the light transient amplitude, contrasting with its slow increase when the preparation was bathed in aequorin-containing solution with no flow of solution.

Because the process of aequorin concentration in the skinned cardiac cell was very slow and was accelerated by the contraction of the skinned cell, it was thought that aequorin could penetrate into a restricted space in the myofilament lattice, which is swollen in skinned cardiac cells. In seven experiments, similar to that shown in Fig. 14, the addition of 100 mg/ml dextran (~70,000 mol wt) to the solution shrank the skinned cardiac cell: the width decreased by $15 \pm 2\%$. These skinned cells were submitted to the same protocol used in Fig. 14. The frequency and amplitude of the cyclic contractions were not significantly different from those in the absence of dextran. Replacing this solution with one containing aequorin and the same concentration of dextran resulted in an aequorin transient about as large as the first transient recorded in Fig. 14. During a 1-h exposure to the aequorin-containing solution, the amplitude of this transient did not increase by more than a factor of 2 in any of the seven experiments. Irrespective of any possible effect of dextran on the aequorin sensitivity to Ca^{2+} (which has not been tested),[8] this result suggests that the slow concentration of aequorin in the skinned cardiac cell is facilitated by the swelling of the myofila-

[8] Similar experiments were done with various concentrations of polyvinylpyrrolidone. With 50 mg/ml polyvinylpyrrolidone, ~40,000 mol wt, the aequorin transient was completely abolished, even after a 1-h exposure to a solution containing 21 μM aequorin. The batch of polyvinylpyrrolidone used for this experimental series was sent to Dr. J. R. Blinks, who found that this concentration of polyvinylpyrrolidone decreased the light response of aequorin to Ca^{2+}.

ment lattice of individual myofibrils. The most likely hypothesis is that this concentration is caused by an accumulation of aequorin in the myofilament space without, however, a tight binding to the myofilaments. The mechanism of this concentration remains unclear. It is not related to an attraction of aequorin by the negative charges on the myofilaments since aequorin is also negatively charged.

Operational Apparent Stability Constant of the Ca-Aequorin Complexes

Introducing 21 μM aequorin did not change the amplitude or frequency of the cyclic contractions (Fig. 14). Most of the experiments were done at pMg 2.50, and the binding of Ca^{2+} to aequorin was believed to be negligible compared with that to 68 μM EGTA. However, the results with lower [free Mg^{2+}] (see Fig. 22) rendered a significant binding of Ca^{2+} to aequorin likely. Hence, the frequency of the cyclic contractions, which is highly sensitive to [free Ca^{2+}], was used to determine an operational apparent stability constant for the Ca-aequorin complexes at various pMg levels. According to generally accepted terminology (Fabiato and Fabiato, 1979), the term "apparent" was used because this stability constant was measured at pH 7.10 and corresponded to the global effect of all the Ca^{2+}-binding sites of aequorin. This was further qualified by the word "operational" because the determination was done in the presence of a specified pMg. The frequency of the cyclic contractions was determined at two pCa values for each pMg value: pCa 6.30, and the lowest pCa that could be used without significant discharge of aequorin during the experiment. The lowest pCa was higher at lower pMg (Table II). The frequency measurements were done 15–45 min after the transfer of the skinned cardiac cell.

Comparison of Tables I and II shows that adding 21 μM aequorin did not significantly modify the frequency of the cyclic contractions, either at pCa 6.30 or 5.50 in the presence of pMg 2.50. In contrast, at pMg 3.00 and 3.50, the presence of aequorin in the solution resulted in a significant decrease of the frequency of the cyclic contractions compared with that observed at the same pCa without Ca^{2+} binding to aequorin. In the presence of pMg 3.00, the period of the cyclic contractions at pCa 6.30 with aequorin was not significantly different from that at pCa 6.40 without aequorin, and the period of the cyclic contractions at pCa 5.80 with aequorin was slightly but was significantly longer than that at pCa 6.00 in the absence of aequorin. In the presence of pMg 3.50, the period of the cyclic contractions at pCa 6.30 with aequorin was not significantly different from that at pCa 6.50 without aequorin (but was significantly different from that at pCa 6.40), and the period at pCa 6.00 with aequorin did not differ significantly from that at pCa 6.30 without aequorin.

The changes of pCa, inferred from the frequency of the cyclic contractions, and the aequorin concentration[9] were entered in the computer program (Fabiato and Fabiato, 1979), which searched for the operational apparent stability constant

[9] As explained in the Methods section, the spontaneous cyclic Ca^{2+} release is dependent upon the [free Ca^{2+}] set in the bulk solution, which has a very large volume and reaches mostly by hydraulic bulk flow the outer surface of the SR packing individual myofibrils. Thus, the concentration of aequorin in the solution was entered in the computer program. The increase of concentration of aequorin by a factor of ~15 in the myofilament space was not relevant.

TABLE II

Dependence of the Period of the Cyclic Contractions on pMg in the Presence of 21 μM Aequorin

pCa	pMg 2.50	pMg 3.00	pMg 3.50*
6.30	91.2±28.4‡	35.9±2.8	21.2±1.3
6.00	—	—	15.8±1.2
5.80	—	14.2±0.6	—
5.50	4.6±0.3	—	—

* The temperature was 22 ± 0.1°C, the pH was 7.10, the pMgATP was 2.50, the ionic strength was 0.170 M, and the solution contained 12 mM phosphocreatine, 15 U/ml creatine phosphokinase, and 5.13 μM calmodulin. The pH was buffered with 30 mM BES and 15 mM TES.

‡ The period in seconds is the mean ± SD (n = 7 in all cases).

that best fitted the data. The results are shown in Table III. The concentration of aequorin at pMg 3.50 was entered as 20 μM instead of the 21 μM present initially, because some Ca^{2+}-induced discharge of aequorin presumably occurred at this low [free Mg^{2+}] during the time between the filling of the pipette and the measurements. The general significance of these operational apparent stability constants is limited by some degree of uncertainty about the concentration of aequorin, as previously explained.

An in vitro and accurate determination of the stability constants of all the Ca-aequorin complexes is not available because it would require knowledge of the number of Ca^{2+}-binding sites of aequorin and their affinity for Ca^{2+} before and after the luminescence reaction. The only value reported is 7×10^6 M^{-1} for one of the sites (Shimomura and Johnson, 1970), and it is not readily interpretable for reasons summarized by Blinks et al. (1982). Recently, Wier and Hess (1984) made a cuvette calibration of the aequorin bioluminescence as a function of [free Ca^{2+}]. They fitted the curve to an equation originally proposed by Allen et al. (1977) according to a "two-state" model of the Ca^{2+}-binding sites of aequorin. In the presence of 3.00 mM free Mg^{2+}, they found the stability constant of the "effective" state of the sites to be 2.60×10^6 M^{-1}, which is much higher than the value of $<1 \times 10^5$ M^{-1} that is proposed here for the operational apparent stability constant at about the same [free Mg^{2+}] (3.16 mM). This is not surprising, because the unbound Ca^{2+}-binding sites on the aequorin molecule are supposed to exist predominantly in an "ineffective" state with a low affinity for Ca^{2+} (Blinks et al., 1982).

TABLE III

Dependence of the Operational Apparent Stability Constant of the Ca-Aequorin Complexes upon pMg

pMg	Operational apparent stability constant
3.50	1.9×10^6 M^{-1}
3.00	5×10^5 M^{-1}
2.50	$<1 \times 10^5$ M^{-1}

Effects of Calmodulin on Ca^{2+} Release and Accumulation by the Sarcoplasmic Reticulum

The inclusion of 5.13 μM calmodulin[10] in the solution dramatically increased the amplitude of both the aequorin transient (by 92 ± 17%, n = 13) and the tension transient (by 29 ± 3%, n = 13) (Fig. 15), but did not modify the sensitivity of the myofilaments to Ca^{2+} (Fig. 11). The duration of the cycle of the spontaneous Ca^{2+} release significantly decreased from 72 ± 3 to 64 ± 2 s (n = 13). The less

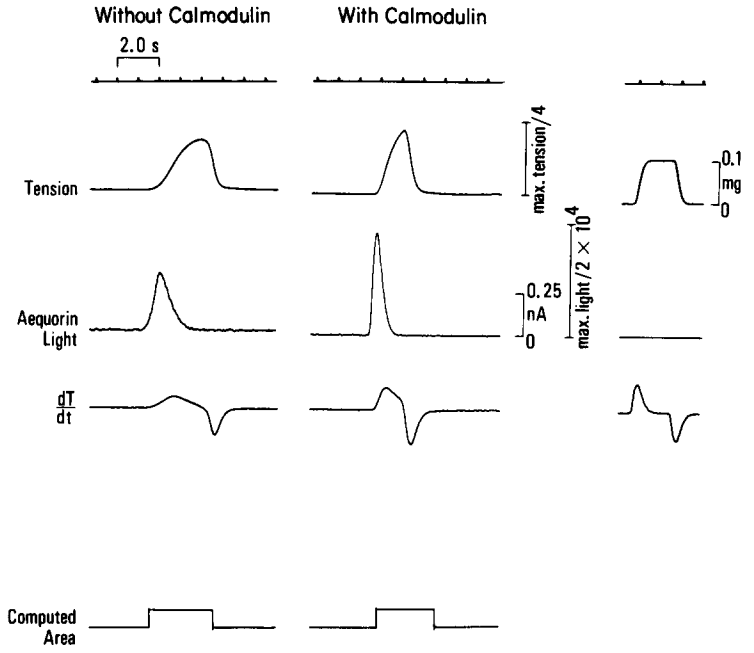

FIGURE 15. Effect of 5.13 μM calmodulin in a skinned canine cardiac Purkinje cell that was 7 μm wide, 5.5 μm thick, and 25 μm long. The [total EGTA] was 0.068 mM, pMg 2.50, pMgATP 2.50, pH 7.10, ionic strength 0.170 M, and temperature 22°C. In this figure and in Figs. 18–22, the calibration bar of maximum light had to be divided by 1.15 before computing the ratio of light to maximum light shown in Fig. 10.

pronounced relative effect on the tension than on the light transient amplitude is consistent with the different stoichiometries of the binding of Ca^{2+} to troponin C and aequorin (Blinks et al., 1982). There was excellent consistency for the peak myoplasmic [free Ca^{2+}] inferred from, respectively, light (Fig. 10) and tension calibrations (Fig. 12). For the light transient, adding calmodulin significantly increased the peak myoplasmic [free Ca^{2+}] from pCa 5.76 ± 0.03 (n = 13)

[10] This concentration is the result of computations (Fabiato, 1985*b*) that do not warrant an accuracy to the second decimal place. However, it is no more difficult to prepare a solution with this precision than with rounded-off numbers, and it was simpler to limit the assumptions and approximations to those precisely indicated (Fabiato, 1985*b*).

to pCa 5.65 ± 0.05 (n = 13). For the tension transient, the increase of myoplasmic [free Ca^{2+}] was from pCa 5.76 ± 0.04 (n = 13) to pCa 5.67 ± 0.03 (n = 13). The area under the curve of aequorin bioluminescence decreased by 11 ± 3%. This demonstrates that the amplitude of the peak of the tension transient is related not to the total but to the peak light.

Microinjection of a solution at pCa 6.30 with 5.13 μM calmodulin during a spontaneous Ca^{2+} release, initiated with pCa 6.30 but without calmodulin, had no effect on this light and tension transient but potentiated the subsequent one (n = 4). Microinjection of the same calmodulin-containing solution at any time during the interval between two spontaneous releases failed to induce a Ca^{2+} release but again potentiated the subsequent release (n = 8). The degree of potentiation increased as the microinjection of calmodulin-containing solution was applied earlier in the preceding cycle (n = 8). With a microinjection at or before the peak of the preceding light transient, the potentiation reached its maximum for the next Ca^{2+} transient. The peak of the Ca^{2+} transient corresponds approximately to the beginning of the phase of rapid Ca^{2+} reaccumulation into the SR (Fabiato, 1985*a*). These results suggest that the primary effect of calmodulin is an enhancement of the rate and extent of Ca^{2+} accumulation into the SR. The effect on the rate and amplitude of Ca^{2+} release appears to be a consequence of a higher Ca^{2+} content of the SR at the time of the release.

Preincubating a skinned canine cardiac Purkinje cell for 5 min with 5.13 μM calmodulin in the presence of a [free Ca^{2+}] varying between pCa 7.90 and 5.30 resulted in no significant variation of the amplitude and frequency of the spontaneous light and tension transients subsequently observed in the presence of pCa 6.30 and 5.13 μM calmodulin (n = 11). This could suggest that, if Ca^{2+} binding to calmodulin is essential for it to phosphorylate phospholamban (Bilezikjian et al., 1981) or other proteins (Plank et al., 1983), the effects observed in skinned canine cardiac Purkinje cells with 5.13 μM calmodulin are not through a phosphorylation. These effects could be through a direct stimulation of the SR Ca^{2+} pump protein as proposed by Lopaschuk et al. (1980). Alternatively, 5.13 μM calmodulin could, by mass action, cause the formation of enough Ca-calmodulin complexes to saturate the phosphorylation system even in the presence of pCa 7.90 (as kindly suggested to me by Drs. W. G. L. Kerrick and E. G. Kranias). This possibility cannot be excluded, because a dose-response curve of calmodulin was not done. The goal of these experiments was not to define the mechanism of action of calmodulin but to reintroduce it by including it in the solution bathing the skinned cardiac cell at its presumably physiological intracellular concentration (Fabiato, 1985*b*).

The effects of cyclic AMP on the SR (Fabiato, 1981*b*) were additive to, but independent of, those of calmodulin (n = 4). Cyclic AMP-dependent protein kinase was not routinely added because it seems to remain bound to the intracellular structures. Cyclic AMP-dependent protein kinase alone (0.1–1.0 μM) had no effect on either the sensitivity of the myofilaments to Ca^{2+} or the Ca^{2+} release and reaccumulation by the SR (Fabiato, 1981*b*). Adding cyclic AMP-dependent protein kinase (0.1–1.0 μM) did not modify the effect of calmodulin on the SR or its absence of effect on the Ca^{2+} sensitivity of the myofilaments.

Effects of Decreasing Free Ca^{2+} Concentration at Different Phases of the Ca^{2+} Transient

A decrease of the bulk solution [free Ca^{2+}] during the ascending phase of the aequorin light transient rapidly curtailed this transient (Fig. 16). The tension transient decreased to 10 ± 2% (n = 11) of its original amplitude. In all of the experiments, the amplitude of the remaining light transient was much less depressed than that of the tension transient, especially if the different stoichiometries of Ca^{2+} binding to troponin C and aequorin were taken into account. The myoplasmic [free Ca^{2+}] at the peak of the tension transient (Fig. 13) was decreased by 0.33 ± 0.05 pCa units (n = 11). No aequorin calibration was possible, because of the 10 mM total EGTA used to obtain a rapid decrease of [free Ca^{2+}] in the myofilament space.

A decrease of bulk solution [free Ca^{2+}] during the descending phase of the aequorin light transient resulted in a curtailment of the light transient with a significant (paired t test) but small decrease of the peak of the tension transient by 7.5 ± 4.1% (n = 11) (Fig. 17). This was observed in the 11 skinned cells at various timings of the decrease of bulk solution [free Ca^{2+}] in the descending phase of the light transient, even <25 ms after the peak (n = 3). The myoplasmic [free Ca^{2+}] reached at the peak of the tension transient (Fig. 13) was decreased by only 0.03 ± 0.02 pCa units (n = 11).

Application of 10 mM total EGTA always caused a decrease of the baseline of the aequorin signal, which went offscale in Figs. 16 and 17. This was attributed to the removal of Ca^{2+} from the aequorin concentrated in the myofilament space by the high [total EGTA] used in these two experimental series only. As explained in the Methods section, this was the only circumstance under which there was evidence of rapid net outward diffusion of Ca^{2+} from the myofilament space to the bathing solution. This result does not imply the effect of the [total EGTA] or [free $EGTA^{4-}$] on the sensitivity of aequorin light to Ca^{2+} that Ridgway and Snow (1983) have described. The present study provides no argument for or against this suggestion, but has taken the most cautious approach by always using the same [total EGTA] for both aequorin calibration and experiments, except in these two experimental series, for which a high [total EGTA] was required to rapidly decrease the [free Ca^{2+}] in the myofilament space despite the low on and off rates of Ca^{2+} binding to EGTA (Fabiato, 1985*a*). By recording at a lower sensitivity, it was verified that after its decrease the baseline light remained steady without any redevelopment of light: i.e., the transient aequorin signal was indeed curtailed rather than merely shifted offscale.

Effects of Increasing Free Ca^{2+} Concentration at Different Phases of the Ca^{2+} Transient

A rapid increase of [free Ca^{2+}] by microaspiration of the solution at pCa 6.30 and microinjection of the solution at a higher [free Ca^{2+}] should produce a Ca^{2+}-induced release of Ca^{2+} from the SR (Fabiato, 1985*a*). Accordingly, an explanation of the rationale for the experimental series consisting of increasing the bulk solution [free Ca^{2+}] during the spontaneous Ca^{2+} release requires reference to information that is discussed in detail in the next article (Fabiato, 1985*a*).

Before studying the effect of an increase of bulk solution [free Ca^{2+}] during the Ca^{2+} transient, the optimum [free Ca^{2+}] trigger to induce a Ca^{2+} release during the interval between spontaneous Ca^{2+} releases was sought. Aspiration of the solution at pCa 6.30, followed ~50 ms later by the reinjection of the same

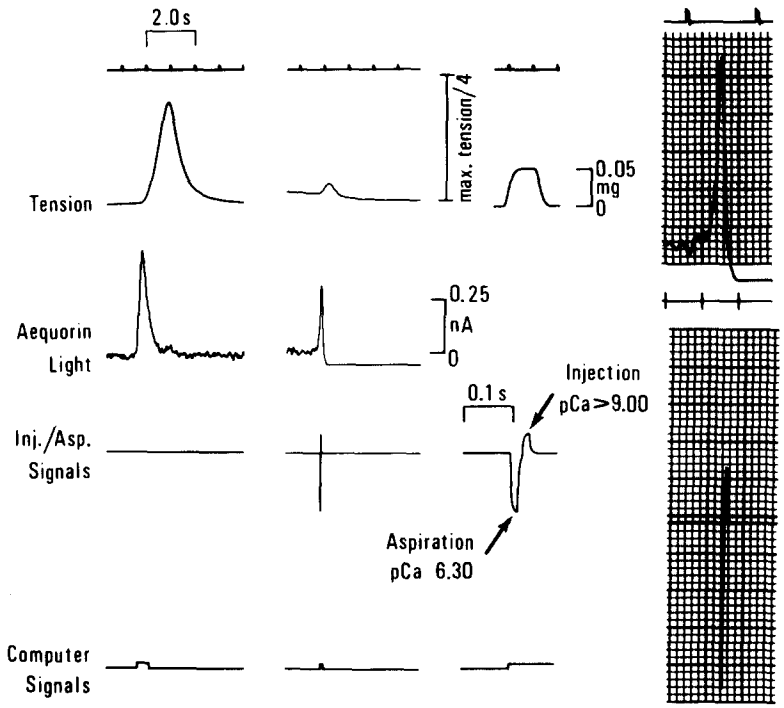

FIGURE 16. Effect of decreasing [free Ca^{2+}] during the ascending phase of the aequorin light transient in a skinned canine cardiac Purkinje cell that was 7 μm wide, 5.5 μm thick, and 26 μm long. The preparation was cyclically releasing Ca^{2+} (69-s cycle). The pMg was 2.50, pH 7.10, and the solution contained 5.13 μM calmodulin. The solution at pCa 6.30 contained 0.068 mM total EGTA; that at pCa >9.00 contained 10 mM total EGTA. The ascending phase of the computer signal was triggered by the aequorin light signal as soon as it reached a specified threshold voltage. The computer signal, in turn, permitted the triggering of the microprocessor-controlled aspiration and injection sequence after a specified delay. The computer and injection-aspiration signals were redisplayed at a 20-times-higher speed, as shown under the signal given by the tension calibrator. The right-hand panel of the figure is a photographic enlargement of a portion of the same tracing, but with the original grid lines, which shows the relationship between the injection signal and its effect on the aequorin bioluminescence recording. The enlarged time scale (1 s) is at the top (note that the vertical bars between the two traces are those printed on the tracing paper and do not correspond to a time scale). The same organization of the panels was used for all subsequent figures.

solution, had no effect at any time during the interval between spontaneous Ca^{2+} releases (n = 9). No artifact was observed on either the tension or the light recording, for reasons explained in the Methods section. This is not illustrated in a particular figure because Fig. 21 shows the absence of an artifact on the

tension and light tracings during the change of solution without a change of pCa. The minimum increase of [free Ca^{2+}] to induce a Ca^{2+} release was from pCa 6.30 to either pCa 6.00 (n = 5) or pCa 5.90 (n = 4), i.e., from 0.50 μM free Ca^{2+} to 1.00 or 1.26 μM free Ca^{2+}, a 0.50- or 0.76-μM increase. This is ~10 (8.33 or 12.67) times more than the minimum increase of [free Ca^{2+}] necessary to obtain a Ca^{2+}-induced release of Ca^{2+} when the skinned canine cardiac Purkinje cell was initially quiescent in the presence of pCa 7.00. Then an increase of bulk solution [free Ca^{2+}] from pCa 7.00 to pCa 6.80, i.e., an increase of [free Ca^{2+}] by 0.06 μM, was sufficient to trigger a Ca^{2+} release (see Fig. 9 in Fabiato, 1985*a*).

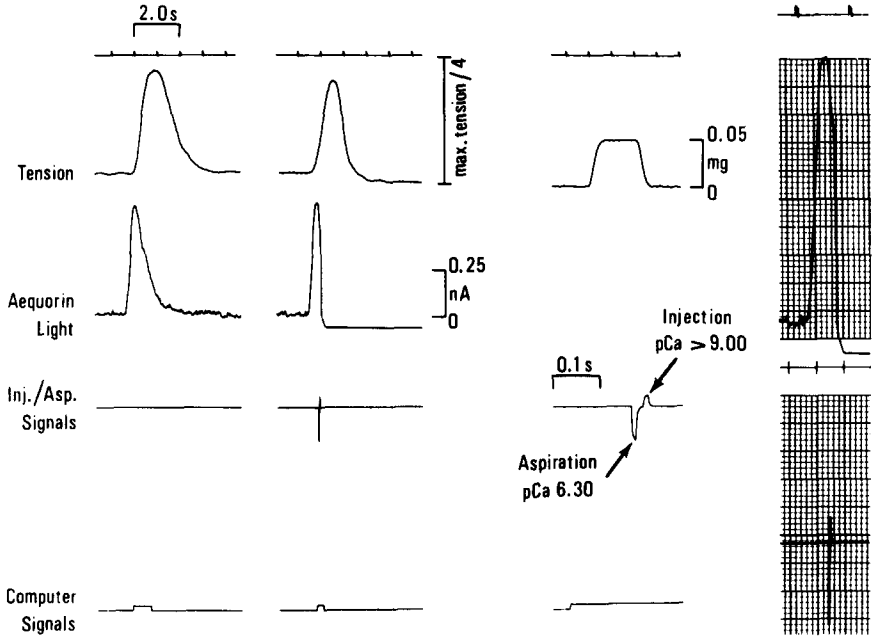

FIGURE 17. Effect of decreasing [free Ca^{2+}] during the descending phase of the aequorin light transient in a 7-μm-wide, 5.5-μm-thick, 28-μm-long skinned canine cardiac Purkinje cell that was cyclically releasing Ca^{2+} (62-s cycle). The pMg was 2.50, pH 7.10, and the solution contained 5.13 μM calmodulin. The solution at pCa 6.30 contained 0.068 mM total EGTA; that at pCa >9.00 contained 10 mM total EGTA.

Thus, the continuous presence of a high [free Ca^{2+}] at the outer surface of the SR partly inhibited Ca^{2+}-induced release of Ca^{2+} (see Fabiato, 1985*a*, for further discussion). The optimum increase of bulk solution [free Ca^{2+}] for triggering Ca^{2+}-induced release of Ca^{2+} was from pCa 6.30 to 5.50 (n = 9), the same final pCa that was optimum as a trigger starting from an initial [free Ca^{2+}] of pCa 7.00 (see Fig. 9 in Fabiato, 1985*a*). The myoplasmic [free Ca^{2+}] reached at the peak of the Ca^{2+} transient elicited by this optimum [free Ca^{2+}] trigger increased when the delay between the previous spontaneous release and the triggering of Ca^{2+}-induced release was increased. This suggests a reloading of the SR between the spontaneous Ca^{2+} releases. With this information, an increase of bulk solution

[free Ca^{2+}] from pCa 6.30 to pCa 5.50 was used to study the effect of increasing [free Ca^{2+}] at different times during the Ca^{2+} transient. A pCa of 5.50 always corresponded to a [free Ca^{2+}] higher than the myoplasmic [free Ca^{2+}] reached at the peak of the undisturbed spontaneous Ca^{2+} release (Fig. 10). As explained elsewhere (Fabiato, 1985*a*), it was not possible to establish the exact value of the [free Ca^{2+}] at the outer surface of the SR resulting from a bulk solution pCa of 5.50.

Increasing the bulk solution [free Ca^{2+}] during the ascending phase of the aequorin light transient resulted in an additional Ca^{2+} release detected with aequorin after a delay of ≤25 ms. The amplitude of the tension transient was increased (Fig. 18). Comparing the peak myoplasmic [free Ca^{2+}] inferred from aequorin light (Fig. 10) and tension (Fig. 12) showed that in 8 of the 10 skinned cells the peak myoplasmic [free Ca^{2+}] reached after the additional Ca^{2+} release inferred from the tension transient was larger than that inferred from the aequorin bioluminescence transient. The average increase of myoplasmic [free Ca^{2+}] resulting from the Ca^{2+} release was 0.10 ± 0.02 pCa units (n = 10) for the aequorin transient and 0.14 ± 0.04 pCa units (n = 10) for the tension transient; this difference is significant.

Identical experiments, but without aequorin light recording, on the high-speed cinematography setup (Fabiato and Fabiato, 1978*a*) showed that this contraction, which was enhanced by the Ca^{2+}-induced release of Ca^{2+} triggered with the application of pCa 5.50, was synchronous throughout the preparation.

Aequorin could not be used for microinjections with [free Ca^{2+}] higher than pCa 5.50 because the aequorin discharge in the pipette was too rapid. An increase of bulk solution [free Ca^{2+}] from pCa 6.30 to pCa 5.00, with a delay of 50–100 ms after the onset of the tension transient, did not significantly modify (even with a paired t test) this tension transient or its rate of tension development (n = 6). Although aequorin light was not recorded, this early increase of [free Ca^{2+}] certainly took place during the ascending phase of the Ca^{2+} transient. The negative result contrasts with the prominent depression of the Ca^{2+} transient caused by Ca^{2+}-induced release of Ca^{2+} when a pCa of 5.00 was applied during its ascending phase (see Fig. 8 in Fabiato, 1985*a*). This suggests that spontaneous cyclic Ca^{2+} release and Ca^{2+}-induced release of Ca^{2+} do not occur through the same mechanism. A moderate increase of bulk solution [free Ca^{2+}] in the ascending phase of the spontaneous Ca^{2+} transient triggered further Ca^{2+} release, whereas a supraoptimum increase of [free Ca^{2+}] merely inhibited this additional Ca^{2+} release caused by a Ca^{2+}-induced release of Ca^{2+} without perturbing the spontaneous Ca^{2+} release or activating the myofilaments directly (see Fabiato, 1985*a*, for further discussion).

An increase of [free Ca^{2+}] (to the same level, pCa 5.50, as used in the ascending phase) during the entire descending phase of the aequorin light transient resulted in no additional Ca^{2+} release (Fig. 19).[11] If the skinned cell was left in the high

[11] With this high [free Ca^{2+}] trigger, a small deflection of the aequorin light signal was detected at the time of solution change in only 2 of the 12 experiments (Fig. 19). The absence of mixing of the solutions during the change precluded discharge of aequorin contained in the large volume of solution bathing the skinned cell. However, it could have been expected that the aequorin concentrated in the myofilament space would always be discharged by the higher

[free Ca^{2+}] solution, a spontaneous Ca^{2+} release occurred after a delay of several hundred milliseconds, at the end of the aequorin transient. The amplitude of this delayed spontaneous release of Ca^{2+} was such that the myoplasmic [free Ca^{2+}] inferred from the aequorin bioluminescence (Fig. 10) was always much larger than that inferred from the tension transient (Fig. 12). Thus, comparing the peak myoplasmic [free Ca^{2+}] of this delayed spontaneous release with that of the control release showed an increase in myoplasmic [free Ca^{2+}] of 0.17 ± 0.04

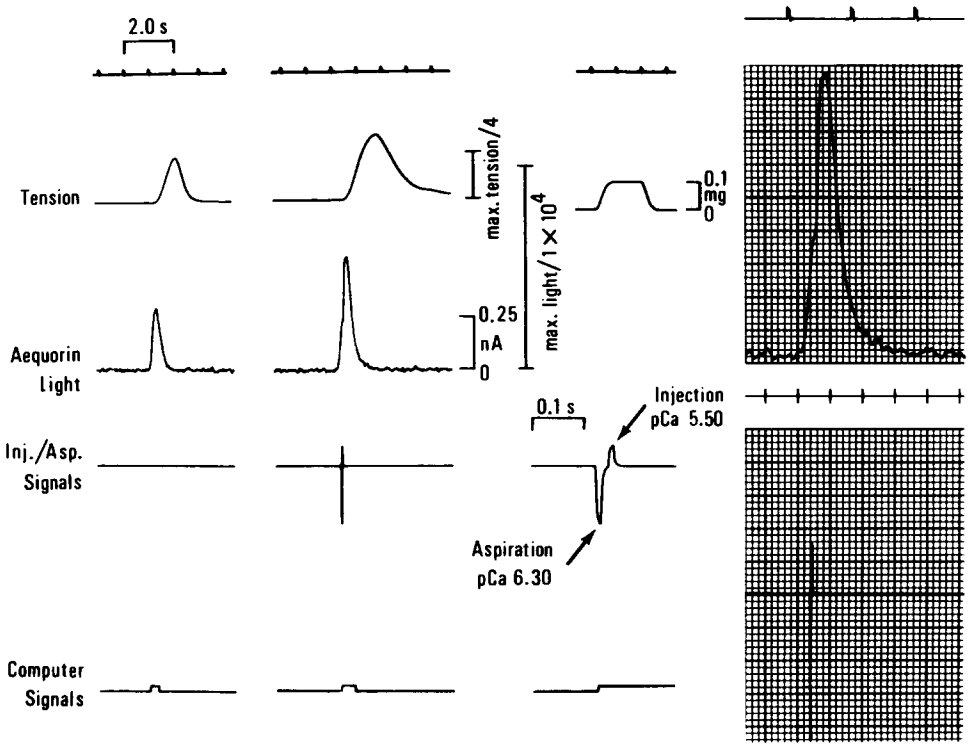

FIGURE 18. Effect of increasing [free Ca^{2+}] during the ascending phase of the aequorin light transient in an 8-μm-wide, 6-μm-thick, 32-μm-long skinned canine cardiac Purkinje cell that was cyclically releasing Ca^{2+} (69-s cycle) in the presence of 0.068 mM total EGTA, pMg 2.50, pH 7.10, and 5.13 μM calmodulin. Note that the calibrations of maximum tension and maximum light from this skinned cell are shown in Fig. 7*A*.

[free Ca^{2+}] solution. The observation that this did not occur in this experimental series, and the absence of a significant difference of the peak myoplasmic [free Ca^{2+}] transient inferred from aequorin light and tension calibrations during Ca^{2+}-induced release of Ca^{2+} (see Fig. 9 in Fabiato, 1985*a*), suggest that the externally applied solution did not reach the myofilaments directly but only induced Ca^{2+} release from, or Ca^{2+} accumulation into, the SR that superficially packs the myofibrils. A light signal was detected at the time of the replacement, by aspiration and injection, of a solution at pCa 7.00 by a solution at [free Ca^{2+}] as low as pCa 6.50 in skinned cells with SR destroyed by a 12-h exposure to 1% (wt/vol) Brij 58.

pCa units (n = 12) for the aequorin transient and 0.05 ± 0.02 pCa units (n = 12) for the tension transient. This discrepancy is explained by the previously mentioned asynchronism of the spontaneous Ca^{2+} release in the presence of a bulk solution pCa of 5.50. Localized areas of high [free Ca^{2+}] artifactually increased the aequorin signal. Meanwhile, the early contraction of some myofibrils pulled other, still relaxed myofibrils and thereby artifactually diminished the amplitude of the overall tension recorded from the ends of the preparation. Identical

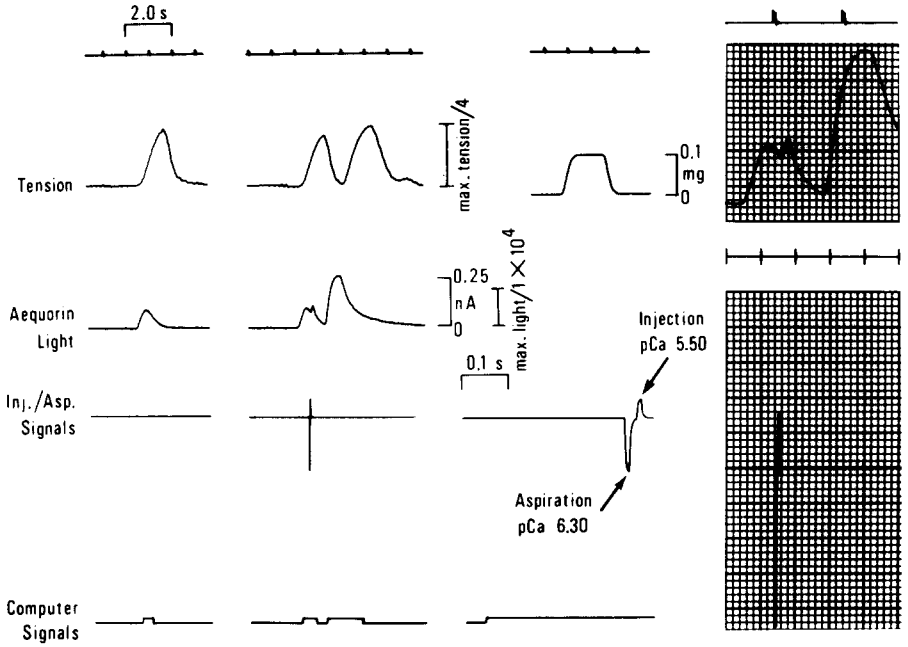

FIGURE 19. Effect of increasing [free Ca^{2+}] during the descending phase of the aequorin light transient in a 7-μm-wide, 5.5-μm-thick, 28-μm-long skinned canine cardiac Purkinje cell that was cyclically releasing Ca^{2+} (64-s cycle) in the presence of 0.068 mM total EGTA, pMg 2.50, pH 7.10, and 5.13 μM calmodulin. The small deflection of the aequorin light signal at the time of the injection of this [free Ca^{2+}] (pCa 5.50), which was the highest used, was observed only in this and one other experiment in a series of 12.

experiments, but without aequorin light recording, done on the high-speed cinematography setup confirmed the asynchronism, although it was generally not apparent on the tension recording because of the low-frequency response of the transducer. This asynchronism was, as previously noted, a property of the spontaneous Ca^{2+} release in the continuous presence of a high bulk solution [free Ca^{2+}]. It was not merely related to the early occurrence of the Ca^{2+} release after a previous release. High-speed cinematography demonstrated that the contraction was homogeneous throughout the preparation when the early release was induced at the same time as in Fig. 19 with respect to the previous contraction, but with either caffeine or via Ca^{2+}-induced release of Ca^{2+} triggered by a rapid and homogeneous increase of [free Ca^{2+}] at the outer surface of the SR.

The major finding of this experimental series was that an additional Ca^{2+} release could be triggered by externally applied Ca^{2+} during the ascending but *not* the descending phase of the spontaneous aequorin light transient. This could have been caused by either an inhibition of the Ca^{2+} release process or by the SR having been emptied by the just-completed Ca^{2+} release. The latter hypothesis is rendered unlikely by the observation that a solution containing the ionophore A23187 at 10 μM applied during the descending phase of the light transient resulted in a very large Ca^{2+} release after a short delay (Fig. 20). The peak myoplasmic [free Ca^{2+}] increased by 0.27 ± 0.04 pCa units (n = 8), as inferred from the tension calibration (Fig. 12). The aequorin calibration was not used because of the possibility of an effect of the ionophore on the Ca^{2+}-aequorin reaction, which has not yet been eliminated (Dr. J. R. Blinks, personal communication). Similar results were obtained with 20 (n = 3) or 60 mM (n = 3) caffeine, which induced a large Ca^{2+} release without a significant delay during the descending phase of the Ca^{2+} transient. The light transient induced by even 60 mM caffeine was, however, always smaller than that elicited by the ionophore A23187 at 10 μM. These results are not shown because Figs. 15 and 16 of the next article (Fabiato, 1985*a*) demonstrate that even 5 mM caffeine induced a Ca^{2+} release without a significant delay during the descending phase of the Ca^{2+} transient.[12]

Effects of Changing the Concentration of Ions Other Than Ca^{2+} During the Course of a Ca^{2+} Transient on This and the Subsequent Ca^{2+} Transients

An increase of pH from 6.70 to 7.10 resulted in no modification of the corresponding aequorin light or tension transient (Fig. 21). The subsequent spontaneous Ca^{2+} release was dramatically modified, however. The cycle was shortened to 37 ± 4% (n = 12) of its value at pH 6.70. The amplitude of the aequorin transient was increased to an average of 179% of the value at pH 6.70. The amplitude of the tension transient was increased to an average of 207% of the value at pH 6.70, more than the percentage of increase of the aequorin light transient. At a constant pH, the percentage change of the light is always greater than that of the tension signal because of the different stoichiometries of Ca^{2+} binding to troponin C and aequorin. However, increasing the pH from 6.70 to 7.10, the pH range used, does not significantly modify the Ca^{2+}-aequorin reaction (Shimomura et al., 1962; Allen and Blinks, 1979; Blinks et al., 1982; Fabiato, 1985*d*; and Fig. 4 of Fabiato, 1985*a*), while it increases the sensitivity of the myofilaments to Ca^{2+} (Fig. 12). The inference of myoplasmic [free Ca^{2+}] from the tension transient with the appropriate tension-pCa curves (Fig. 12) showed an increase of peak myoplasmic [free Ca^{2+}] of 0.10 ± 0.03 pCa units (n = 12). This value was not significantly different from that inferred from the aequorin calibration, 0.11 ± 0.02 pCa units (n = 12) (Fig. 10). The subsequent transients of cyclic Ca^{2+} release sometimes showed a further increase of peak myoplasmic [free Ca^{2+}] during two or three cycles, while the cycle duration remained constant

[12] These data, reported in the next article (Fabiato, 1985*a*), indicate that an appropriate [free Ca^{2+}] trigger releases the same amount of Ca^{2+} as 5 mM caffeine. The two types of Ca^{2+} release present an equal dependence upon the Ca^{2+} loading of the SR after the end of the refractory period of the Ca^{2+}-induced release of Ca^{2+} (Fabiato, 1985*a*).

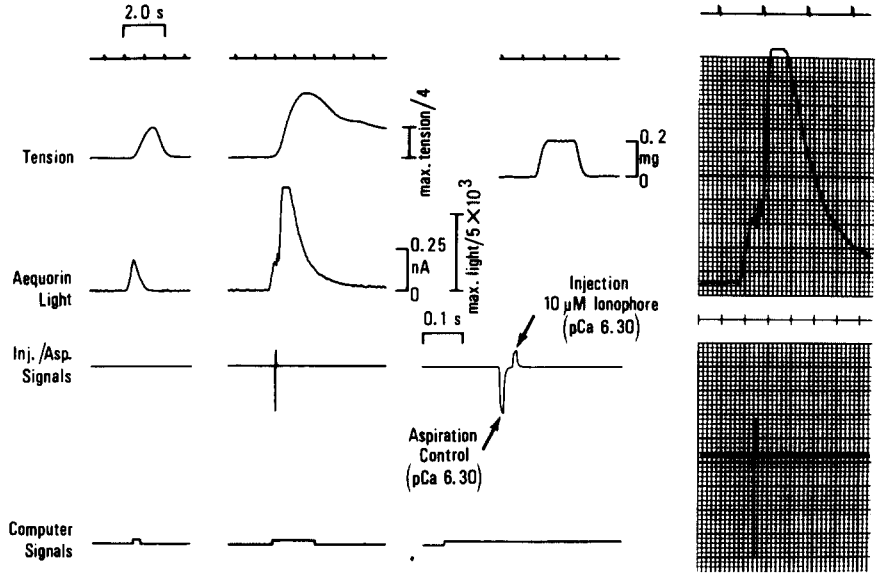

FIGURE 20. Effect of applying the Ca^{2+} ionophore A23187 early in the descending phase of the aequorin light transient in an 8-μm-wide, 6-μm-thick, 31-μm-long skinned canine cardiac Purkinje cell that was cyclically releasing Ca^{2+} (68-s cycle) in the presence of 0.068 mM total EGTA, pMg 2.50, pH 7.10, and 5.13 μM calmodulin.

at an average of 19 s. This progressive increase of peak myoplasmic [free Ca^{2+}] was not recorded in all of the 12 experiments; accordingly, it was not analyzed statistically.

Increasing the pH from 7.10 to 7.40 during the ascending phase (n = 5), and from 6.70 to 7.10 (n = 8) or 7.10 to 7.40 during the descending phase (n = 4), and decreasing the pH from 7.40 to 6.70 during the ascending (n = 5) or descending (n = 4) phase of the aequorin light transient did not modify the corresponding light and tension transients. Increasing the pH increased the

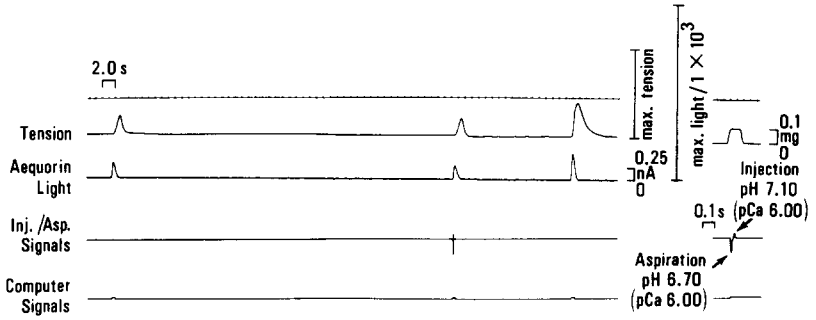

FIGURE 21. Effect of increasing pH from 6.70 to 7.10 in a 7-μm-wide, 5.5-μm-thick, 25-μm-long skinned canine cardiac Purkinje cell in the presence of pCa 6.00, 0.068 mM total EGTA, pMg 2.50, and 5.13 μM calmodulin.

frequency of the subsequent Ca^{2+} transients and their peak myoplasmic [free Ca^{2+}] with no significant differences between the values inferred from tension and light calibrations. Decreasing the pH had the opposite effects.

The experiments increasing the [free Mg^{2}] from pMg 3.00 to 2.50 during the ascending phase of the aequorin light transient were done before the determination of the operational apparent stability constant of the Ca-aequorin complexes. The problems related to using aequorin with a change of [free Mg^{2+}] will be explained in describing a typical experiment (Fig. 22). Without taking into account the Ca^{2+} binding to aequorin, an increase of [free Mg^{2+}] from pMg 3.00 to 2.50 during the ascending phase of the aequorin light transient decreased the amplitude and the slope of the ascending phase of this light transient (photographic enlargement at the right of Fig. 22). The aequorin calibration data (Fig.

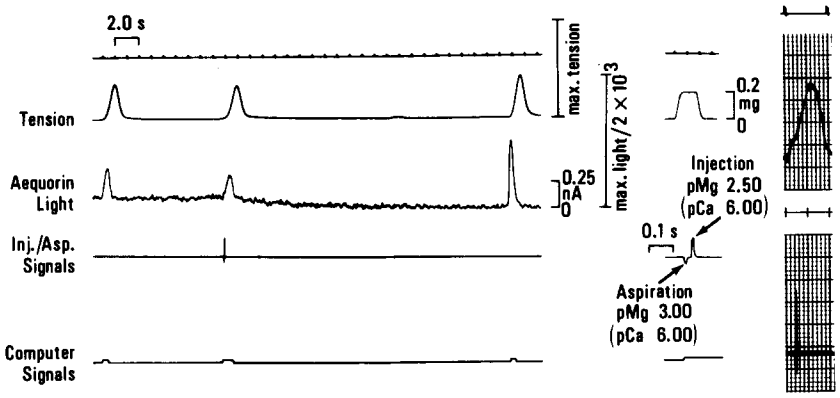

FIGURE 22. Effect of increasing [free Mg^{2+}] from pMg 3.00 to 2.50 in an 8-μm-wide, 6-μm-thick, 23-μm-long skinned canine cardiac Purkinje cell in the presence of pCa 6.00, 0.068 mM total EGTA, pH 7.10, and 5.13 μM calmodulin. The pCa has been computed without taking into account the binding of 21 μM aequorin to Ca^{2+}.

10) indicate that the decreases in amplitude and slope were caused by the decreased sensitivity of aequorin to Ca^{2+} at higher [free Mg^{2+}]. In fact, the peak myoplasmic [free Ca^{2+}] reached during the Ca^{2+} transient increased from pCa 5.80 (point 22*a* in Fig. 10) to 5.56 (point 22*b* in Fig. 10). However, this did not take into account the significant binding of aequorin to Ca^{2+} in the presence of pMg 3.00. Taking into account the operational apparent stability constant of 5×10^5 M^{-1} (Table III) showed that the myoplasmic pCa at the peak of the control Ca^{2+} transient at pMg 3.00 was pCa 5.54, which is not distinguishable from the pCa value of 5.56 obtained after application of pMg 2.50. It can be concluded that an increase of [free Mg^{2+}] during the ascending phase of the aequorin-detected Ca^{2+} transient had *no* effect on this transient.

The decreased resting light at high [free Mg^{2+}], apparent in Fig. 22, is consistent with the finding by Allen et al. (1977) that the Ca^{2+}-independent component of the aequorin light decreases at higher [free Mg^{2+}]. This decrease of resting light did not correspond to a decrease of myoplasmic [free Ca^{2+}]. On

the contrary, computations indicated that the myoplasmic [free Ca^{2+}] increased from pCa 6.15 to 6.00 because of the decreased operational apparent stability constant of the Ca-aequorin complexes from 5×10^5 M^{-1} to $<1 \times 10^5$ M^{-1} (Table III). The subsequent light transient was considerably increased in amplitude. Computations indicated that it reached a myoplasmic pCa of 5.37 (point 22*c* in Fig. 10), which is consistent with the pCa of 5.42 inferred from the tension transient (point 22*c* in Fig. 12). The amplitude of the light and tension transients did not further increase during the spontaneous Ca^{2+} releases following this first release subsequent to the solution change.

In nine similar experiments, it was confirmed that after correction for (*a*) the decreased aequorin sensitivity to Ca^{2+} caused by increased [free Mg^{2+}], and (*b*) the change in the operational apparent stability constant of the Ca-aequorin complexes, there was no significant modification of the amplitude of the Ca^{2+} transient during which [free Mg^{2+}] was increased. The amplitude of the subsequent Ca^{2+} transients was considerably increased, as inferred from the amplitudes of both the light and the tension transients. The frequency of the cyclic contractions was considerably decreased.

An increase of [free Mg^{2+}] from pMg 3.00 to 2.50 during the descending phase of the light transient did not significantly modify the tension transient. The aequorin transient was curtailed, which shortened its duration. Again, appropriate corrections indicated that there was no modification in the peak Ca^{2+} release. The amplitudes of the subsequent light and tension transients were increased by values that were not significantly different from those observed with increased [free Mg^{2+}] during the ascending phase.

A bulk solution pMg of 2.50 was optimum, or near optimum, for the amplitude of the Ca^{2+} release from the SR. A further increase of [free Mg^{2+}] from pMg 2.50 to 2.00 decreased the Ca^{2+} release during the spontaneous releases subsequent to that during which the change was applied. The peak myoplasmic [free Ca^{2+}] inferred from the tension-pCa curve (data not shown, labeled "*n.s.*" in Fig. 13) decreased by 0.21 ± 0.04 pCa units ($n = 8$). The aequorin recording was not interpreted because the light was so low in the presence of pMg 2.00 that the signal-to-noise ratio was extremely poor.

Total replacement of K^+ by Na^+ did not change the corresponding tension and aequorin transients except for some small notches on the aequorin transient at the time of solution change in some experiments. The frequency of the subsequent spontaneous Ca^{2+} releases was slightly decreased. The amplitude of the aequorin transient decreased by $8 \pm 3\%$ ($n = 8$) and that of the tension transient decreased by $3 \pm 2\%$ ($n = 8$). The interpretation of these small effects of substitution of Na^+ for K^+ on the aequorin transient and the tension transient is uncertain because this substitution decreases the aequorin response to Ca^{2+} as well as the sensitivity of the myofilaments to Ca^{2+} (Moisescu and Ashley, 1977; Blinks et al., 1982; Fabiato, 1985*e*).

DISCUSSION

Control of Ca^{2+} Release by Ca^{2+} Ion

The major finding from these experiments is that the same increase of [free Ca^{2+}] that induced further Ca^{2+} release during the ascending phase of the

aequorin light transient failed to do so during the descending phase. The present data do not exclude the possibility that the first few milliseconds of the descending phase of the Ca^{2+} transient might be caused partly by a decrease of myoplasmic [free Ca^{2+}] corresponding to Ca^{2+} binding to troponin C, as suggested by experiments in barnacle muscle; in this muscle, however, the Ca^{2+} control of the myofilament contraction may be different (Ridgway et al., 1983; Ridgway and Gordon, 1984). For the skinned canine cardiac Purkinje cell, evidence will be presented that most of the several hundred milliseconds of this descending phase correspond to Ca^{2+} reaccumulation into the SR (Fabiato, 1985*a*). Experiments using caffeine to estimate the rate of Ca^{2+} reaccumulation into the SR demonstrated that the descending phase of the Ca^{2+} transient has the same timing as the phase of rapid Ca^{2+} reaccumulation into the SR. Thereafter, Ca^{2+} accumulation continues for many seconds but with a clear-cut rate change (see Fig. 16 in Fabiato, 1985*a*).

Why is Ca^{2+} unable to induce further Ca^{2+} release during the phase of rapid Ca^{2+} reaccumulation into the SR? The Ca^{2+} ionophore and caffeine experiments suggest that this is not because the Ca^{2+} releasing pool of the SR is empty. Thus, the process permitting Ca^{2+} to induce further (net) Ca^{2+} release must be inhibited during the phase of rapid Ca^{2+} reaccumulation. This could be explained by two mechanisms that are discussed in the next article (Fabiato, 1985*a*): (*a*) competition between the Ca^{2+} pump and the molecule gating the Ca^{2+} release channel, or (*b*) a time-dependent inactivation of the Ca^{2+} channel. In addition, too large an increase of [free Ca^{2+}] at the outer surface of the SR inhibits the additional release of Ca^{2+} triggered by the Ca^{2+}-induced release process. Therefore, of the ions studied, Ca^{2+} appears to be the only one capable of rapidly activating and inactivating Ca^{2+} release.

The other ions studied (H^+, Mg^{2+}, Na^+, K^+) did not modify the Ca^{2+} release and accumulation by the SR during the course of a Ca^{2+} transient. Therefore, they are not candidates for the triggering and inactivation of Ca^{2+} release. After a delay, however, the steady state [free H^+] and [free Mg^{2+}] modify Ca^{2+} accumulation into the SR and Ca^{2+}-induced release of Ca^{2+} from the SR (Fabiato and Fabiato, 1975, 1978*b*). These other ions, especially H^+, might also be counterions helping to balance the change of charge distribution across the SR membrane that results from Ca^{2+} release (Somlyo et al., 1981).

For methodological reasons, these results have been obtained for the Ca^{2+} transient caused by spontaneous cyclic Ca^{2+} release from the SR. It seems plausible that once the Ca^{2+} release has been induced, its ionic control may be independent of the mechanism that had initiated the release. However, it was necessary to ascertain this by verifying that the conclusions of this article held true for ionic modifications during the course of the Ca^{2+}-induced release of Ca^{2+} elicited by a rapid increase of [free Ca^{2+}] at the outer surface of the SR of a previously quiescent skinned cardiac cell. This was done in the next article (Fabiato, 1985*a*) for the role of Ca^{2+} in controlling Ca^{2+} release at different times during the Ca^{2+} transient. Extensive experiments also confirmed the completely negative results with respect to the effects of substitution of Na^+ for K^+ and of changing pH, which did not affect the corresponding Ca^{2+} transient elicited by Ca^{2+}-induced release of Ca^{2+} from the SR. These negative results required the

most complex technique, including four microprocessor-controlled microinjection-aspirations because the low and high [free Ca^{2+}] solutions, which were needed to trigger Ca^{2+} release, had to be duplicated at different pH values or $[Na^+]/[K^+]$ ratios. The results are reported in two articles (Fabiato, 1985*d*, *e*) that may be considered appendices to the present series.

Measurement of the Peak Myoplasmic Free Ca^{2+} Concentration Reached During Ca^{2+} Release

Preliminary results in skinned cardiac cells from the rat and rabbit ventricle (Fabiato, 1981*a*) had suggested a good agreement between the peak myoplasmic [free Ca^{2+}] inferred from (*a*) the amplitude of the light transient and a cuvette calibration of the [free Ca^{2+}] dependence of aequorin bioluminescence, and (*b*) the amplitude of the tension transient relative to maximum tension and the tension-pCa curve. However, this agreement could have been fortuitous since it was observed under a single set of conditions. In addition, this consistency was unexpected. Because of the Ca^{2+} buffering within the cardiac cell with binding sites having generally unknown on and off rates (Fabiato, 1983), it seemed unlikely that the transient myoplasmic [free Ca^{2+}] could be inferred from the steady state tension obtained for the determination of a tension-pCa curve. Similarly, the likeliness of myoplasmic [free Ca^{2+}] gradients during the circulation of Ca^{2+} from the SR to the myofilaments, together with the high stoichiometry of the Ca^{2+}-aequorin reaction, rendered plausible a disproportionate contribution to the light signal by the increase of [free Ca^{2+}] in the localized area near the SR (Blinks et al., 1982).

The present results show that, under a variety of ionic conditions and levels of positive inotropism induced by changing the ionic concentrations or adding calmodulin, the values of peak myoplasmic [free Ca^{2+}] measured with the two methods during spontaneous cyclic Ca^{2+} releases from the SR were highly consistent. The next article (Fabiato, 1985*a*) demonstrates the same consistency for the Ca^{2+} release elicited by the process of Ca^{2+}-induced release of Ca^{2+} from the SR (with some discrepancies at low temperature, however). Because of the large amount of data accumulated, the consistency can no longer be considered fortuitous, at least in this preparation.[13] This does not necessarily mean, however, that the [free Ca^{2+}] is homogeneous in the myoplasm during Ca^{2+} release. Since aequorin appears to concentrate in the myofilament space, both troponin C and aequorin may sense the free Ca^{2+} when it reaches the myofilaments, underestimating the change of [free Ca^{2+}] at the outer surface of the SR. This effect may be minimized if Ca^{2+} circulates only over a very short distance because it is released from all segments of the SR, including the longitudinal SR that tightly packs the myofibrils (Fabiato, 1981*a*). The considerable phase shift between the peaks of light and tension transients is not an obstacle to this hypothesis. The

[13] Similar measurements done during Ca^{2+}-induced release of Ca^{2+} from the SR of small skinned fibers from the frog semitendinosus having the same dimensions (Fabiato, 1985*c*) showed that the [free Ca^{2+}] inferred from aequorin calibration was always much higher than that inferred from tension calibration. It must be noted that the rate of light increase during Ca^{2+} release was ~50 times higher in this preparation than in the skinned canine cardiac Purkinje cell.

almost complete absence of effect of a decrease of myoplasmic [free Ca^{2+}] at any time after the peak of the light transient on the amplitude of the corresponding tension transient indicates that part of the delay between the peaks of aequorin light and tension transients is caused by reactions subsequent to the presumably very rapid Ca^{2+} binding to troponin C.

These conclusions do not necessarily apply to the aequorin-detected Ca^{2+} transient in intact cells of multicellular cardiac preparations (Allen and Blinks, 1978, 1979; Wier, 1980; Allen and Kurihara, 1980, 1982; Morgan and Blinks, 1982; Allen and Orchard, 1983; Wier et al., 1983; Wier and Hess, 1984; Hess and Wier, 1984) for the following reasons.

First, it is not likely that aequorin concentrates in the myofilament space in these intact cells, because they do not undergo the swelling of the myofilament lattice, which appears to be important in the concentration of aequorin in the myofilament space of skinned cardiac cells.

Second, when length changes and ionic or pharmacological interventions increase the sensitivity of the myofilaments to Ca^{2+}, this can be caused by an increase of Ca^{2+} binding to the myofilaments, although the physiological and biochemical data are not always in agreement (e.g., see Discussion in Fabiato and Fabiato, 1978*b*). The [total calcium] bound to the myofilaments is always many times greater than the [free Ca^{2+}] that they sense (Solaro et al., 1974; Fabiato, 1983). Because the affinity of the Ca^{2+}-specific site of troponin C is in the range of [free Ca^{2+}] reached at the peak of the Ca^{2+} transient, this binding site represents the major intracellular Ca^{2+} buffer in this [free Ca^{2+}] range (Fabiato, 1983). Accordingly, increased Ca^{2+} binding to the myofilaments should decrease the [free Ca^{2+}] available for binding to aequorin because of the limited amount of total calcium entering through the sarcolemma of an intact cell. Therefore, whenever an intervention increases the Ca^{2+} binding to the myofilaments of an intact cell, a decreased amplitude of the aequorin light transient should not be interpreted as necessarily reflecting a decrease in the transsarcolemmal Ca^{2+} influx or Ca^{2+} release from the SR. Similarly, when the Ca^{2+} binding to the myofilaments is increased, the absence of modification of the Ca^{2+} transient could correspond to an increase in transsarcolemmal Ca^{2+} influx or in Ca^{2+} release from the SR. The opposite rationale should be used for qualitatively interpreting the aequorin signal under conditions decreasing the Ca^{2+} binding to the myofilaments. This problem is greatly minimized with the skinned cardiac cell because it is surrounded by a very large Ca^{2+} sink, the bathing solution, which has an ~1,000-times-larger volume than the skinned cell.

Third, the Ca^{2+} transient reaches its peak much more rapidly in intact cardiac muscle at 35°C than in skinned cardiac cells at 22°C. The inconsistency between the peak myoplasmic [free Ca^{2+}] inferred from tension and aequorin light with rapid ionic modifications during the ascending phase of the light transient in a skinned cardiac cell suggests that the consistency during undisturbed Ca^{2+} transients may occur because enough time is given for the myofilaments to "catch up" with the SR in terms of [free Ca^{2+}]. Although aequorin and tension measure the same myoplasmic [free Ca^{2+}] at the peak of the Ca^{2+} transient, there is a discrepancy during the ascending phase of the Ca^{2+} transient. During this phase, the [free Ca^{2+}] probably reaches a high level at the outer surface of the SR,

which would be underestimated by aequorin because (*a*) it is less concentrated at the outer surface of the SR than on the myofilaments, and (*b*) the "time constant" of its light response is ~20 ms at 22°C in the presence of pMg 2.50 with the aequorin batch used for these experiments. Only at the peak of the Ca^{2+} transient, when the Ca^{2+} wave has reached the myofilaments, would the two methods give the same measurement. The same coincidence may not occur in intact cardiac muscle. This is not a definitive conclusion. The way in which the data have been presented, fully documenting typical experiments, should permit readers to make their own interpretations with respect to this problem.

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