# Simulated Calcium Current Can Both Cause Calcium Loading in and Trigger Calcium Release from the Sarcoplasmic Reticulum of a Skinned Canine Cardiac Purkinje Cell

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ABSTRACT Skinned canine cardiac Purkinje cells were stimulated by regularly repeated microinjection-aspiration sequences that were programmed to simulate the fast initial component of the transsarcolemmal $Ca^{2+}$ current and the subsequent slow component corresponding to noninactivating $Ca^{2+}$ channels. The simulated fast component triggered a tension transient through $Ca^{2+}$-induced release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR). The simulated slow component did not affect the tension transient during which it was first introduced but it potentiated the subsequent transients. The potentiation was not observed when the SR function had been destroyed by detergent. The potentiation decreased progressively when the slow component was separated by an increasing time interval from the fast component. The potentiation was progressive over several beats under conditions that decreased the rate of $Ca^{2+}$ accumulation into the SR (deletion of calmodulin from the solutions; a decrease of the temperature from 22 to 12°C). In the presence of a slow component, an increase of frequency caused a positive staircase, and the introduction of an extrasystole caused a postextrasystolic potentiation. There was a negative staircase and no postextrasystolic potentiation in the absence of a slow component. These results can be explained by a time- and $Ca^{2+}$-dependent functional separation of the release and accumulation processes of the SR, rather than by $Ca^{2+}$ circulation between anatomically distinct loading and release compartments. The fast initial component of transsarcolemmal $Ca^{2+}$ current would trigger $Ca^{2+}$ release, whereas the slow component would load the SR with an amount of $Ca^{2+}$ available for release during the subsequent tension transients.

## INTRODUCTION

Calcium loading of the sarcoplasmic reticulum (SR) by the transsarcolemmal $Ca^{2+}$ influx is strongly suggested by different types of experiments in intact adult mammalian cardiac muscle. The analysis of the force-frequency relationships (Koch-Weser and Blinks, 1963; Kruta and Braveny, 1968; Wood et al., 1969; Bass, 1976; Edman and Jóhannson, 1976; Wohlfart, 1982; Wohlfart and Noble, 1982; Pučelik et al., 1983) and voltage-clamp studies of the relation between $Ca^{2+}$ current and contraction (Beeler and Reuter, 1970; Morad and Goldman,

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1973; Winegrad, 1979) suggest that $Ca^{2+}$ influx during a given beat is used at least partly to load the SR with $Ca^{2+}$ that can be released during the subsequent beats. Inasmuch as there is strong evidence that the transsarcolemmal $Ca^{2+}$ influx can load the SR, it may seem paradoxical that one of the hypotheses for the mechanism of intracellular $Ca^{2+}$ release is that the transsarcolemmal influx of $Ca^{2+}$ would be the trigger for the $Ca^{2+}$ release from the SR. How can the transsarcolemmal $Ca^{2+}$ influx induce both $Ca^{2+}$ release from the SR and $Ca^{2+}$ loading in the SR? This is the question that the present article addresses.

The rationale for these experiments was derived from the observations that (*a*) increasing the [free $Ca^{2+}$] at the outer surface of the SR causes $Ca^{2+}$ release

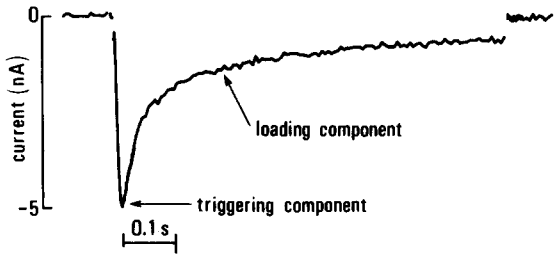


FIGURE 1. Fast and slow components of the $Ca^{2+}$ current recorded during a voltage-clamp experiment in an isolated intact ventricular cell from the adult guinea pig. Unpublished experiment by B. P. Bean, K. S. Lee, and R. W. Tsien, using the suction pipette technique for voltage clamp with internal perfusion (Lee and Tsien, 1982, 1983). The extracellular Tyrode solution contained $Na^+$ as the major cation, 5 mM $CaCl_2$, and 0.010 mM tetrodotoxin to block the fast $Na^+$ current. The intracellular medium in the suction pipette contained 75 mM $K_2HPO_4$, 75 mM K-aspartate, and 5 mM EGTA, at pH 7.00 buffered with 10 mM HEPES. The temperature was 22°C. The $Ca^{2+}$ current was induced by a depolarization from −40 to +20 mV over 735 ms. The protocol included a leak subtraction to eliminate the inward current insensitive to $Ca^{2+}$ blocking agents. Under these conditions, the $K^+$ conductance was very low despite the use of $K^+$ rather than $Cs^+$ in the intracellular perfusion medium (Marban and Tsien, 1982). Thus, the recording is practically the $Ca^{2+}$ current.

when the rate of increase is high but leads to $Ca^{2+}$ accumulation when the rate is low; and (*b*) because of the time- and $Ca^{2+}$-dependent inactivation of the $Ca^{2+}$-induced release of $Ca^{2+}$, increasing the [free $Ca^{2+}$] cannot induce $Ca^{2+}$ release from the SR during the refractory period following a previous $Ca^{2+}$ release (Fabiato, 1985*b*).

A microprocessor-controlled microinjection-aspiration system was programmed to first produce a high rate of increase of [free $Ca^{2+}$] in the solution bathing a skinned cell from the canine cardiac Purkinje tissue. This should trigger $Ca^{2+}$ release from the SR because of the high rate. Then an approximately seven-times-slower increase of [free $Ca^{2+}$] was produced, which should cause $Ca^{2+}$ loading because of its low rate and its occurrence during the refractory period of the $Ca^{2+}$-induced release process.

Recent voltage-clamp data have shown that the $Ca^{2+}$ current has these char-

acteristics: a fast initial component followed by a slow component corresponding to noninactivating $Ca^{2+}$ channels (Marban and Tsien, 1982; Lee and Tsien, 1982, 1983; Tsien, 1983). The $Ca^{2+}$ current shown in Fig. 1 was not simulated exactly, because this figure was kindly provided by Dr. R. W. Tsien after the completion of the experiments reported here. In addition, it must be noted that the amplitude of the slow component relative to that of the peak current is highly variable in intact cardiac muscle (McDonald, 1982; Tsien, 1983; Fabiato and Baumgarten, 1984). As will be explained and emphasized, the conclusions of this study are not dependent upon specific values of the fast and slow components of the transsarcolemmal $Ca^{2+}$ current or, rather, of the transsarcolemmal total calcium influx (perhaps including pathways other than the $Ca^{2+}$ current) during the action potential.

## METHODS

### *Preparation and Solutions*

Skinned cardiac cells of a volume ~2% of the intact cell were obtained from the adult canine cardiac Purkinje tissue by microdissection (Fabiato, 1985*a*). The skinned cells were 7–9 μm wide, 5–6 μm thick, and 20–40 μm long. An isolated intact cardiac cell obtained by enzymatic treatment from the canine Purkinje tissue was 33 ± 4 μm (SD, $n = 20$) wide, 21 ± 6 μm thick, and 102 ± 17 μm long when stretched to a sarcomere length of 2.3 μm.[1]

All solutions were at $-\log_{10}$[free $Mg^{2+}$] (pMg) 2.50, pMgATP 2.50, pH 7.10 buffered with 45 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), ionic strength 0.170 M with $K^+$ and $Cl^-$ as major ionic species. The solutions contained 12 mM phosphocreatine and 15 U/ml creatine phosphokinase. Generally, the solutions contained 55 μM EGTA and 5.13 μM calmodulin. The temperature was maintained constant, generally at 22 ± 0.1°C, with a heating and cooling stage (Fabiato, 1985*b*). In order to depress the rate of $Ca^{2+}$ accumulation into the SR, some experiments were done either with deletion of calmodulin or with a decrease of the temperature from 22 to 12°C (Fabiato, 1985*b*). The stability constants between multiple cations and ligands were those previously listed (Fabiato, 1981, 1983, 1985*b*).

### *Microinjection-Aspiration System*

The three hydraulically controlled microinjection-aspiration pipettes used were placed on micromanipulators II, IV, and VI (as indicated in Fig. 1 of Fabiato, 1985*a*). The tension transducer and tension calibrator were on micromanipulator I; the microtool used for microdissecting, holding, and transferring the skinned cell was on micromanipulator V; and the calibration triple-pipette holder (see Fig. 2 of Fabiato, 1985*a*) was on micromanipulator III.

One pipette contained a solution at pCa 7.00 (except for the experimental series illustrated by Fig. 11*A*) and the second contained a solution at pCa 5.00. The third was used for aspirating the mixed solutions. To change the solutions rapidly without an excessive flow rate through the pipette, the volumes had to be very small. This was

[1] The method of isolation of a $Ca^{2+}$-tolerant canine cardiac Purkinje cell was similar to that used for mammalian ventricular cells (Fabiato, 1981), except that a step of microdissection was included between an initial exposure to enzymes for digestion of the connective tissue and a final enzymatic treatment for separation of the bundles into individual cells. This technique will be described in detail elsewhere (Fabiato, A., manuscript in preparation).

achieved by microinjection in 10 ms of 0.4–9.4 nl of the aqueous experimental solution around the skinned cell, which was in a chamber containing ~2 × $10^5$ nl of ion-free, water-saturated mineral oil (Fabiato, 1985*a*). Additional, larger volumes were injected at a seven-times-lower speed; the total volume of aqueous solution bathing the skinned cell was 1.6–11.9 nl (1,100–8,200 times the volume of the skinned cell), depending upon the experimental conditions. An increase of [free $Ca^{2+}$] outside the SR simulating that which results from the transsarcolemmal $Ca^{2+}$ current was obtained by first injecting a volume of solution at pCa 7.00 specified by a number of half-steps (generally 30) of one stepping motor, and then, without aspiration of this solution, injecting a variable volume of solution at pCa 5.00, with a variable number of half-steps of another stepping motor. Tests of the rapidity and reproducibility of the mixing of the two solutions have been reported (see Fig. 5*A* in Fabiato, 1985*a*). Despite the low on and off rates of $Ca^{2+}$ binding to EGTA, this method did not result in an overshoot of the [free $Ca^{2+}$] above the level specified at the end of the fast increase of [free $Ca^{2+}$]. The overshoot was prevented by the rapid buffering of the [free $Ca^{2+}$] mostly by calmodulin (Fabiato, 1985*b*), especially since the ratio of calmodulin to EGTA was higher in this study (5.13 μM/55 μM) than in the preceding one (5.13 μM/68 μM; see Figs. 12 and 13*D* in Fabiato, 1985*b*).

The microinjection of pCa 5.00 was done with channel 1 of the microprocessor system used in the second mode (see Fig. 4 in Fabiato, 1985*a*). In this mode, the controller permitted the base speed of the stepping motor to be changed between two consecutive half-steps. Binary-coded decimal rotary switches permitted specification of the number of half-steps at the high speed. A counter system detected each half-step from the "index" line of the controller. When the half-steps reached the number selected, the counter system triggered the speed change by commanding a set-reset circuit to change the resistor controlling the oscillator of the base speed circuit. The motor continued to run at the second specified (low) speed until the overall number of half-steps specified on the indexer was reached. At this time, the "done" line of the indexer gave a signal indicating that the overall motion was completed. This signal reset the counter to zero and changed the base speed back to its original setting. An adjustable time base commanded the series of microinjections and the aspiration (there was only a single aspiration in the series). The arming (i.e., the function that permitted the indexer to respond to a start signal given by the microprocessor) was done automatically by the time base.

A special display mode was used to record the signal of fast and slow injections. This caused the amplification of the upward signal displayed on the recorder to decrease abruptly to a preset value during the transition from rapid to slow injection. The composite signal was displayed on one channel, generally channel 3, of the recorder, together with the upward signal of the injection of the solution at pCa 7.00 and the downward signal of the aspiration of the mixed solutions (see Fabiato, 1985*a*, for details).

### *Inference of the Myoplasmic Free $Ca^{2+}$ Concentration Reached at the Peak of the $Ca^{2+}$ Transient*

Because the rapid and slow increases of [free $Ca^{2+}$] were obtained by mixing, at a variable rate, two solutions at pCa 7.00 and 5.00, aequorin could not be used to measure the change of myoplasmic [free $Ca^{2+}$] caused by the $Ca^{2+}$ release from the SR. A large total amount of aequorin contained in the solution at pCa 7.00 would have been discharged upon exposure to a higher [free $Ca^{2+}$], causing a large light artifact during mixing. Accordingly, the [free $Ca^{2+}$] reached at the peak of the $Ca^{2+}$ transient was inferred only from the amplitude of the tension transient. Thus, at the end of the experiment, the only calibration was that of maximum tension. This was done with the triple-pipette holder but with only two pipettes containing solutions at pCa >9.00 and 4.25 in the presence of

10 mM total EGTA. After the induction of complete relaxation by injecting pCa >9.00, maximum tension was elicited by full activation with pCa 4.25. The vertical bar at the end of each tracing, preceding the signal given by the tension calibrator, indicates the value of half of the maximum tension in all cases (Figs. 3–12). The myoplasmic [free $Ca^{2+}$] (i.e., the [free $Ca^{2+}$] in the myofilament space) at the peak of the tension transient was obtained from the ratio of the amplitude of the tension transient to the maximum tension and the appropriate tension-pCa curve: the curves in the presence and absence of calmodulin were identical (Fabiato, 1985*a*), whereas a decrease of the temperature increased the $Ca^{2+}$ sensitivity of the myofilaments (Fabiato, 1985*b*).

### *Computation of the Instantaneous Change in Bulk Solution Free $Ca^{2+}$ Concentration and of the Simulated Transsarcolemmal Total Calcium Influx*

A microcomputer (model /09; Southwest Technical Products, San Antonio, TX) was used to compute (*a*) the instantaneous change of [free $Ca^{2+}$] in the bulk of the solution bathing the skinned cardiac cell, which resulted from the mixing of the low- and high-[free $Ca^{2+}$] solutions, and (*b*) a first approximation of the total calcium influx across the sarcolemma that the $Ca^{2+}$ current should produce in an intact cell to obtain the same instantaneous change of [free $Ca^{2+}$] that occurred in the solution bathing the skinned cell.

The $Ca^{2+}$ buffering at various [free $Ca^{2+}$] levels in the EGTA-containing solution was different from that in an intact cardiac cell. Accordingly, data banks were set up to give the [total calcium] necessary to obtain specified pCa values listed in narrow steps of 0.01 unit from pCa 8.00 to 5.00. This was done for the solutions buffered with EGTA under various conditions and for the intracellular physiological buffering at the appropriate temperature. The data banks were stored on the same disk as the computer program. The calculation of the physiological buffering took into account the steady state affinities of all known $Ca^{2+}$ buffers present in the intact canine cardiac Purkinje cell, as will be explained.

At the beginning of the experiment, the keyboard of the microcomputer was used to select the appropriate pair of data banks for physiological and bulk solution $Ca^{2+}$ bufferings. It was also used to enter the values of the variables that did not change during a given experiment: the number of half-steps of solution at low [free $Ca^{2+}$], and the pCa values of the low-[free $Ca^{2+}$] and high-[free $Ca^{2+}$] solutions. All the other data were collected directly from the microprocessor, and all other commands for data collection and display were done by push buttons that were near the recorder in order to facilitate the experiment.

At the end of the experiment, a push button permitted a rerun of the microinjection-aspiration sequence, during which the computer collected the following data from channel 1 of the microprocessor system: (*a*) pulses for each half-step of the stepping motor given by the "index" line of the microprocessor (Fabiato, 1985*a*), and (*b*) rectangular waves indicating the total and fast running times of the stepping motor. Because the pulses given by the "index" line were separated by intervals as short as 0.5 ms, the collection of the data had to be at a high speed permitted only by Assembly language. To simplify the software, the information collected in Assembly was transferred into Pascal, and the rest of the program was continued in this language.

For each half-step of the motor, a simple dilution calculation gave the [total calcium] that resulted from the mixing of the two solutions. Then the microcomputer searched in the bulk solution data bank for the two closest pCa values. These pCa values were transformed into [free $Ca^{2+}$] values. A linear interpolation in 100 parts was made between these two values, permitting a very precise definition of the [free $Ca^{2+}$], which gave the value of one point for the curve of bulk solution [free $Ca^{2+}$] (Figs. 3–5 and 7–12). This

curve is perfectly smooth because of the linear interpolation in 100 parts between values separated by already as little as 1/100 of a pCa unit. From the identified value of bulk solution [free $Ca^{2+}$], the program went into the data bank for physiological $Ca^{2+}$ buffering in which the same linear interpolation was made. The preceding value of physiological [total calcium] was subtracted from the value just found and the difference was divided by the time interval. This gave one data point for the curve of simulated total calcium influx (Figs. 3, 4, and 7–12). The data points for each curve were stored in "buffers," made by setting aside certain areas of the random access memory of the computer.

When the computation was completed, a push button simultaneously triggered the display of the two resulting curves and a redisplay of the injection-aspiration signals of the microprocessor, which were recorded at a high speed. The descending phase of the curve of bulk solution [free $Ca^{2+}$] was triggered by the microprocessor activating the motor for the reinjection of the solution at pCa 7.00. The portion of the curve between the beginning of the aspiration of the mixed solutions and about two-thirds of the reinjection of the solution at pCa 7.00 was dotted by retouching the figure prints (Figs. 3, 5, and 7–12) to indicate that the instantaneous [free $Ca^{2+}$] at the outer surface of the SR was not actually known during this time. A printout of the digital data (bulk solution [free $Ca^{2+}$] and $\Delta$[total calcium]/$\Delta t$) was also provided. This was useful in ensuring the accuracy of the analog recording, especially since it was done at the limit of the response speed of the pen.

### *Computation of the Physiological Steady State $Ca^{2+}$ Buffering of an Intact Canine Cardiac Purkinje Cell*

The concentrations of $Ca^{2+}$ ligands (in moles per liter of cell volume accessible to $Ca^{2+}$) in an intact canine cardiac Purkinje cell (Table I) were computed according to the rationale used for mammalian ventricular cells (Fabiato, 1983). There are no data in the literature on the concentrations of these $Ca^{2+}$ buffers for the canine cardiac Purkinje cell, but data are available for ventricular cells (Fabiato, 1983). Hence, the concentrations were inferred from those reported for the canine ventricular cell with the following corrections. It was assumed that the concentration of troponin C was proportional to the "concentration of myofilaments." The latter was inferred from the ratio of maximum tension per cross-sectional area induced by full $Ca^{2+}$ activation with pCa 4.25 after chemical skinning with 10 mM EGTA and 0.5% (wt/vol) of the nonionic detergent polyoxyethylene 20 cetyl ether (Brij 58). The maximum tension was 16.83 g/mm$^2$ for the intact canine cardiac Purkinje cell and 19.63 g/mm$^2$ for the intact canine ventricular cell; thus, the ratio was 0.857. The same correction was applied in calculating the concentration of $Ca^{2+}$-binding sites at the outer face of the SR, assuming that the SR volume is proportional to that of the myofilaments. For the binding sites at the inner face of the sarcolemma, the smaller surface-to-volume ratio of the canine cardiac Purkinje cell ($0.137 \times 10^7$ cm$^2$/liter according to Eisenberg and Cohen, 1983, a result kindly communicated long before the publication of their article) as compared with the ventricular cell ($0.50 \times 10^7$ cm$^2$/liter; see Fabiato and Baumgarten, 1984, for references) was taken into account.

For computing the concentrations of cellular $Ca^{2+}$ buffers in moles per liter of cell water accessible to $Ca^{2+}$ from the concentrations in moles per kilogram wet weight of tissue (Table I), the volume excluded by the mitochondria was assumed to be 23.2% of the cell volume, which is an average between the values measured in the mammalian ventricular cell (Page, 1978) and in the ungulate cardiac Purkinje cell (Mobley and Page, 1972). The mitochondria were considered excluded volume for $Ca^{2+}$ movements because their $Ca^{2+}$ affinity is too low to buffer the resting [free $Ca^{2+}$] (Fabiato, 1985*c*; however, see Fabiato, 1984) and their $Ca^{2+}$ transport is much too slow to influence the amplitude and relaxation rate of a physiological contraction (Fabiato, 1983, 1984, 1985*c*).

The stability constants for these $Ca^{2+}$ buffers were those used for adult mammalian ventricular cells (Fabiato, 1983), except for the $Ca^{2+}$ binding to troponin C, which was computed to have a stability constant of $3.000 \times 10^5$ $M^{-1}$ from a comparison of the shift of the pCa inducing 50% activation of, respectively, canine ventricular and canine cardiac Purkinje skinned cells (Fabiato, 1982).

The concentrations and stability constants of intracellular $Ca^{2+}$ buffers were used to establish the data bank for physiological intracellular $Ca^{2+}$ buffering that the microcomputer program used to calculate the instantaneous simulated total calcium influx. If direct information becomes available for the $Ca^{2+}$ buffering in the intact canine cardiac Purkinje cell, it will be simple to rerun these data with corrected data banks for physiological $Ca^{2+}$ buffering.

TABLE I

*Total Concentrations of Ligands Participating in Physiological $Ca^{2+}$ Buffering in the Intact Canine Cardiac Purkinje Cell*

| Ligand | Concentration (mol/liter cell water accessible to $Ca^{2+}$) |
|---|---|
| Phosphocreatine | $1.2 \times 10^{-2}$ |
| Calmodulin (1) | $5.13 \times 10^{-6}$ |
| Calmodulin (2) | $5.13 \times 10^{-6}$ |
| Calmodulin (3) | $5.13 \times 10^{-6}$ |
| Calmodulin (4) | $5.13 \times 10^{-6}$ |
| External binding site on SR | $3.49 \times 10^{-5}$ |
| $Ca^{2+}$-specific site of troponin C | $5.17 \times 10^{-5}$ |
| High-affinity internal binding site on sarcolemma | $2.65 \times 10^{-4}$ |

The numbers 1–4 correspond to the four sequential $Ca^{2+}$-binding sites of calmodulin (Klee and Vanaman, 1982). The concentration of the additional ligand, ATP, was inferred from the pMgATP and the pMg, which were both 2.50. The physiological pH is thought to be 7.10 at 22°C, and the ionic strength is thought to be ~0.170 M with the [total potassium] and [total sodium] computed as previously described (Fabiato and Fabiato, 1979; Fabiato, 1981).

The diffusible $Ca^{2+}$ buffers calmodulin, ATP, and phosphocreatine were included in the solutions at the appropriate concentrations. The fixed $Ca^{2+}$ buffers on the SR, myofilaments, and sarcolemma were simulated by the appropriate [total EGTA]. This permitted the formation of the data banks that the microcomputer program used to calculate the instantaneous bulk solution [free $Ca^{2+}$]. The [total EGTA] needed to simulate the fixed $Ca^{2+}$ buffers varied with the pCa, as indicated in Table II. The pCa reached at the peak of the $Ca^{2+}$ transient varied widely in this study, making the selection of an appropriate [total EGTA] in the solution difficult. In fact, this was not necessary inasmuch as it was demonstrated in the preceding article that the [total EGTA] little influences the amplitude of the tension transient because $Ca^{2+}$ binds much more rapidly to troponin C than to EGTA (see Fig. 10 in Fabiato, 1985*b*). Thus, the [total EGTA] was 0.055 mM in all the experiments, except for those in the absence of calmodulin, where it was 0.060 mM. Note that decreasing the temperature to 12°C did not change the intracellular $Ca^{2+}$ buffering significantly, because of the various changes in different directions of the stability constants for the various cation-ligand complexes.

Fig. 2 indicates the transsarcolemmal total calcium influx that would be required to induce twitches of various levels of relative amplitude with two, perhaps physiologically

TABLE II

*Total EGTA Concentration Necessary to Simulate the Fixed $Ca^{2+}$ Buffers of the Intact Canine Cardiac Purkinje Cell as a Function of the pCa*

| pCa | [total EGTA] | pCa | [total EGTA] |
|---|---|---|---|
| | *mol/liter* | | *mol/liter* |
| 7.00 | $1.9 \times 10^{-5}$ | 5.90 | $4.5 \times 10^{-5}$ |
| 6.80 | $2.2 \times 10^{-5}$ | 5.80 | $5.0 \times 10^{-5}$ |
| 6.70 | $2.3 \times 10^{-5}$ | 5.70 | $5.5 \times 10^{-5}$ |
| 6.60 | $2.5 \times 10^{-5}$ | 5.60 | $6.0 \times 10^{-5}$ |
| 6.50 | $2.6 \times 10^{-5}$ | 5.50 | $6.5 \times 10^{-5}$ |
| 6.40 | $2.8 \times 10^{-5}$ | 5.45 | $6.8 \times 10^{-5}$ |
| 6.30 | $3.0 \times 10^{-5}$ | 5.40 | $7.0 \times 10^{-5}$ |
| 6.20 | $3.4 \times 10^{-5}$ | 5.30 | $7.7 \times 10^{-5}$ |
| 6.10 | $3.7 \times 10^{-5}$ | 5.20 | $8.3 \times 10^{-5}$ |
| 6.00 | $4.0 \times 10^{-5}$ | 5.10 | $9.0 \times 10^{-5}$ |

The fixed $Ca^{2+}$ buffers are on the inner face of the sarcolemma, the outer face of the SR, and the myofilaments (Fabiato, 1983).

relevant (Fig. 1), values of time to peak total calcium influx in an intact cardiac cell. The computations were done with data reported in the preceding article (see Figs. 12 and 14 in Fabiato, 1985*b*; to facilitate comparison, the same symbol is used for each pCa trigger value). The rationale and equation for converting the transsarcolemmal total calcium influx into $Ca^{2+}$ current in microamperes per square centimeter (or microamperes per

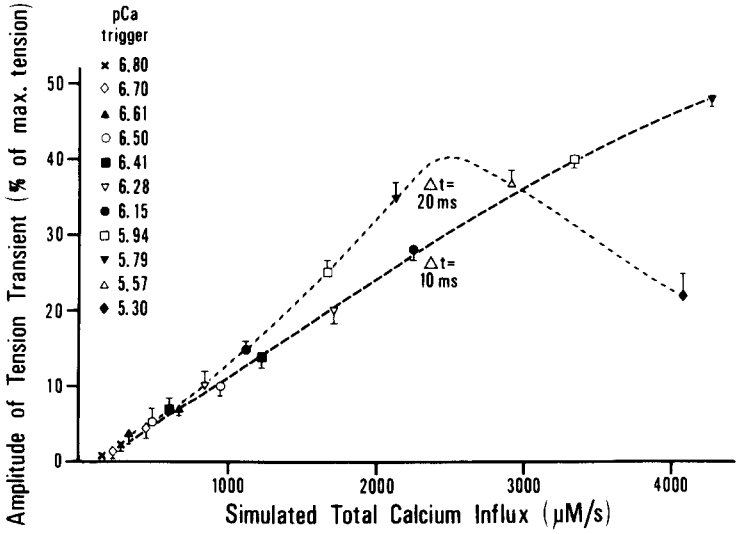


FIGURE 2. Relative amplitude of the tension transient as a function of the peak of the simulated transsarcolemmal total calcium influx. The transsarcolemmal total calcium influx has been computed from the change of [total calcium] inferred from the data bank giving [total calcium] as a function of pCa under the steady state intracellular $Ca^{2+}$ buffering conditions of an intact canine cardiac Purkinje cell. Each point is the mean of eight determinations, and each vertical bar is the SD shown in one direction only for clarity. The tension data are those used in Figs. 12 and 14 in Fabiato (1985*b*).

microfarad, since the specific capacitance of the sarcolemma is ~1 $\mu F/cm^2$) is given elsewhere (Fabiato and Baumgarten, 1984), together with a review of the literature on the amplitude and rate of rise of the fast initial component of the $Ca^{2+}$ current. The proportionality between the simulated total calcium influx and tension shown in Fig. 2 is consistent with that observed between $Ca^{2+}$ current and tension in intact cardiac muscle (New and Trautwein, 1972; Gibbons and Fozzard, 1975; McDonald et al., 1975).

Despite these similarities, the simulated total calcium influx in skinned cardiac cells cannot readily be used to infer an expected range of values for the $Ca^{2+}$ current in an intact canine cardiac Purkinje cell. $Ca^{2+}$ does not flow uniformly across the sarcolemma but flows through discrete channels (Fabiato, 1983). However, the simulation of the $Ca^{2+}$ current by a homogeneous $Ca^{2+}$ flow is valid if the distance between the sarcolemma and the SR is equal to at least half the distance between two channels. It should be noted that the previous calculation of the distance between two $Ca^{2+}$ channels (Fabiato, 1983) was a considerable overestimate. It was based on the only available data (Reuter et al., 1982), which were obtained with an intracellular perfusion with 96 mM $BaCl_2$. This has now been demonstrated to increase considerably the current through a single channel (Cavalié et al., 1983).

When comparing the data reported here with the $Ca^{2+}$ current in intact cardiac cells, one cannot discount the possibility that $Na^+$-$Ca^{2+}$ exchange may participate in the transsarcolemmal $Ca^{2+}$ influx (Mullins, 1981). However, from available data on intracellular $Ca^{2+}$ and $Na^+$ activities and on the coupling ratio of the $Na^+$-$Ca^{2+}$ exchange, this process would contribute to the transsarcolemmal $Ca^{2+}$ influx only if it was fast enough to transport a significant amount of $Ca^{2+}$ during the interval between the depolarization of the action potential and the time at which the increase of myoplasmic [free $Ca^{2+}$] resulting from $Ca^{2+}$ release from the SR causes $Na^+$-$Ca^{2+}$ exchange to function in the $Ca^{2+}$ influx mode (Fabiato and Baumgarten, 1984). Finally, the major difference between a skinned cell and an intact cell is that in the former the $Ca^{2+}$ sink is practically unlimited, whereas in the latter it is limited by the transsarcolemmal $Ca^{2+}$ influx (Fabiato, 1985*a*, *b*).

### *Statistical Analysis*

Data are expressed in the form of mean ± standard deviation (SD). Student's *t* test was used for comparing data, and differences were judged statistically significant for $P < 0.05$. The paired *t* test was used when appropriate.

## RESULTS

### *Potentiation of the Subsequent Tension Transient by the Slow Component of the Simulated Total Calcium Influx*

Fig. 3 shows an example of the effect of introducing a slow component of the simulated transsarcolemmal total calcium influx. The skinned canine cardiac Purkinje cell was stimulated by a sequence of injections and one aspiration repeated at regular intervals of 26 s. Initially, the skinned cardiac cell was bathed in a solution at pCa 7.00 (30 half-steps). Then seven half-steps of solution at pCa 5.00 were injected in 10 ms and mixed with the solution at pCa 7.00. This increased the bulk solution [free $Ca^{2+}$] from 0.10 to 0.26 $\mu$M (pCa 6.59), which triggered a tension transient of 11% of maximum tension (the first transient in Fig. 3), corresponding to a peak myoplasmic [free $Ca^{2+}$] of pCa 5.84 (see Fig. 12 or 13 in Fabiato, 1985*a*). After several hundred milliseconds, the mixed solutions were aspirated. Finally, the 30 half-steps of solution at pCa 7.00 were reinjected.

For the induction of the second tension transient, seven half-steps of solution at pCa 5.00 were again injected in 10 ms and mixed with the volume of solution at pCa 7.00 produced by 30 half-steps. This resulted in the same peak [free $Ca^{2+}$] trigger of 0.26 $\mu$M. However, the injection of solution at pCa 5.00 was continued for 51 additional half-steps at one-seventh of the original base speed (with an interval between two half-steps of 10 ms instead of 1.43 ms). This resulted in a

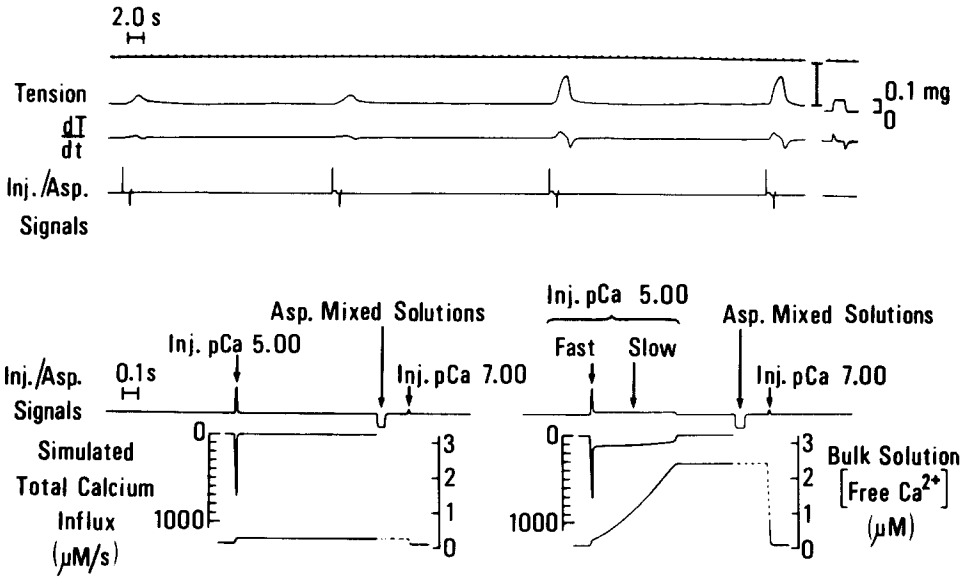


FIGURE 3. Demonstration of the potentiation of the following tension transient by a slow increase of [free $Ca^{2+}$] during a tension transient. The left-hand high-speed recording of the injection-aspiration signals and of the synchronous computer display corresponds to the first tension transient; the right-hand high-speed recording corresponds to the last three tension transients. The experiment was done in an 8.5-$\mu$m-wide, 6-$\mu$m-thick, 30-$\mu$m-long skinned canine cardiac Purkinje cell. In this and all the subsequent figures, the vertical bar preceding the signal given by the tension calibrator corresponds to one-half of the maximum tension. The ratio of the amplitude of the tension transient to the maximum tension and the tension-pCa curve (see Fig. 12 or 13 in Fabiato, 1985*a*) permitted inference of the peak myoplasmic [free $Ca^{2+}$].

final bulk solution [free $Ca^{2+}$] of 2.45 $\mu$M (pCa 5.61). The corresponding tension transient (the second transient in Fig. 3) was not modified. The stimulation of the skinned cell was terminated, as previously, by the aspiration of the mixed solutions and the reinjection of 30 half-steps of solution at pCa 7.00. The following tension transient, induced with the same pattern of increase of bulk solution [free $Ca^{2+}$], was considerably potentiated, reaching 32% of maximum tension, which corresponded to a peak myoplasmic [free $Ca^{2+}$] of pCa 5.55 (see Fig. 12 or 13 in Fabiato, 1985*a*). The rates of tension development and relaxation were also considerably increased, as shown on the tension derivative trace

($dT/dt$).[2] The same pattern of stimulation with a slow component was continued for the subsequent tension transients, and the potentiation remained stable: the subsequent contraction amplitudes did not differ significantly from the amplitude observed for the second tension transient with a slow component.

The same result was obtained in 33 similar experiments, including the one shown in Fig. 3. During the stimulation without a slow component and during the first $Ca^{2+}$ transient induced with a slow component of increase of bulk solution [free $Ca^{2+}$], the amplitude of the tension transient averaged 7.5% of maximum tension, corresponding to a myoplasmic pCa of 5.90 ± 0.05 at the peak of the $Ca^{2+}$ transient. The tension transient following that for which the slow component of increase of bulk solution [free $Ca^{2+}$] was first introduced and all subsequent transients induced with such a stimulation averaged an amplitude of 34% of maximum tension, corresponding to a peak myoplasmic [free $Ca^{2+}$] of pCa 5.53 ± 0.02. Thus, the potentiation was highly significant.

After the slow component had been deleted, the potentiation persisted on the subsequent tension transients, which were elicited with only a fast component of increase of bulk solution [free $Ca^{2+}$], from 0.10 to 0.26 $\mu$M in 10 ms. The potentiation decreased progressively over seven or eight tension transients elicited at regular intervals of 25 or 26 s. Thus, it took 175–200 s for the tension transient to return to its original amplitude before potentiation. These data are not shown, because a similar decay of potentiation in the absence of calmodulin is illustrated in Fig. 6. If the preparation was not stimulated, it remained in the potentiated state for a longer time: 620 ± 35 s ($n$ = 33) was required for the tension transient to return to its nonpotentiated amplitude. This delay may be related to that necessary to obtain a rested state contraction in intact cardiac muscle (Allen et al., 1976; Beresewicz and Reuter, 1977; Lewartowski et al., 1978; Reiter et al., 1978; Pučelik et al., 1983). Some investigators interpret their results as suggesting that the rested state contraction depends mostly (Beresewicz and Reuter, 1977) or totally (Lewartowski et al., 1978) upon the transsarcolemmal $Ca^{2+}$ current, and that the subsequent contractions depend on $Ca^{2+}$ release. Others (Reiter et al., 1978) propose that the rested state contraction depends on $Ca^{2+}$ release from the SR. The present results in skinned cardiac cells suggest that both the rested state contraction and the subsequent contractions are through $Ca^{2+}$-induced release of $Ca^{2+}$ from the SR. The rested state contraction, however, would be more dependent upon the transsarcolemmal $Ca^{2+}$ influx trigger for $Ca^{2+}$ release because the SR has been unloaded by $Ca^{2+}$ leak during the rest period. Again, it must be emphasized that the transsarcolemmal $Ca^{2+}$ influx is not necessarily limited to the $Ca^{2+}$ current blocked by $Ca^{2+}$ antagonists (Reiter et al., 1984) but may be, for instance, via $Na^{+}$-$Ca^{2+}$ exchange.

As indicated, the fast and slow injections of solution at pCa 5.00 in a preset volume of solution at pCa 7.00 were a simulation of the fast and slow components of the transsarcolemmal $Ca^{2+}$ current. The aspiration of the mixed solutions and

[2] The same consistency between an increase of tension amplitude and of rates of tension development and relaxation was observed whenever a potentiation occurred. This will not be repeated for the other figures showing a change in $dT/dt$.

the reinjection of the solution at pCa 7.00 were a simulation of the processes of $Ca^{2+}$ extrusion across the sarcolemma: $Na^{+}$-$Ca^{2+}$ exchange and sarcolemmal $Ca^{2+}$ pump (see Fabiato, 1983, for a discussion). Deleting these latter two steps (aspiration of mixed solutions and reinjection of solution at pCa 7.00) resulted in a cyclic repetition of the contraction (Fig. 4). This already suggests (but does

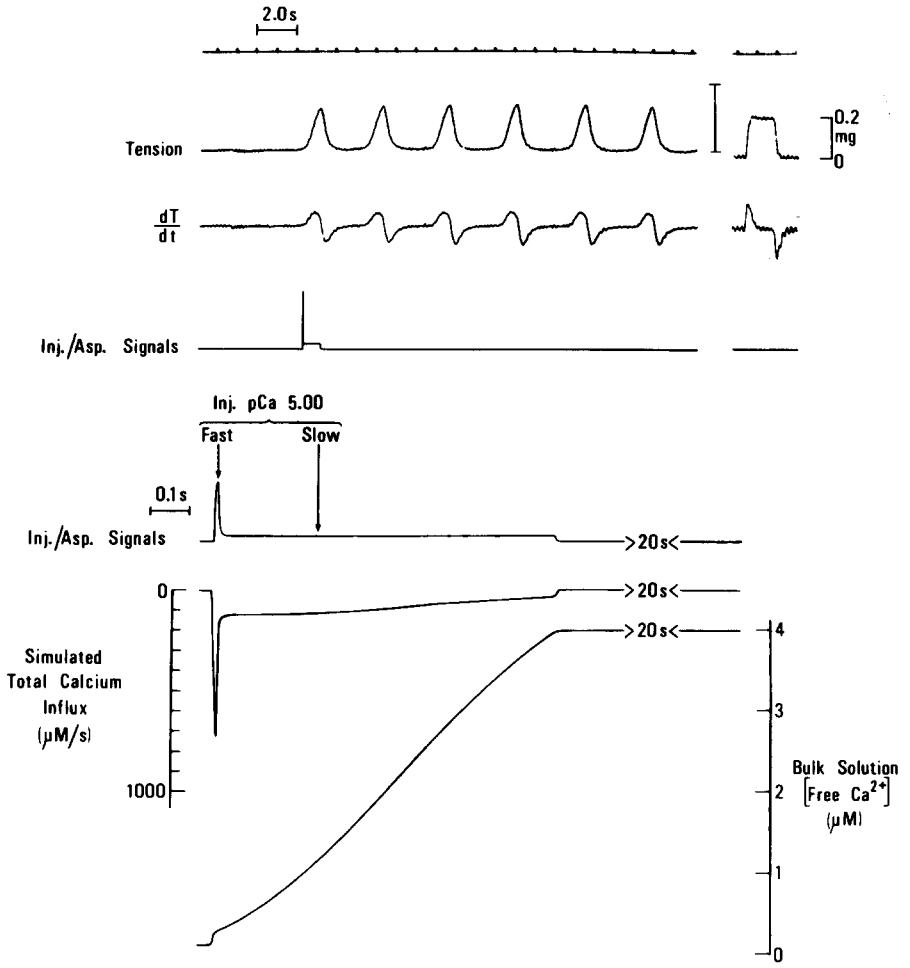


FIGURE 4. Effect of eliminating from the programmed injection-aspiration series the steps corresponding to the simulation of the transsarcolemmal $Ca^{2+}$ efflux mechanisms. The experiment was done in an 8-μm-wide, 6-μm-thick, 28-μm-long skinned canine cardiac Purkinje cell. The first tension transient had been potentiated by three previous stimulations at 25-s intervals. Each included a slow component of increase of [free $Ca^{2+}$], as well as an aspiration of the mixed solutions and a reinjection of the solution at pCa 7.00. The elimination of the last two steps permitted the occurrence of cyclic contractions corresponding to a spontaneous release of $Ca^{2+}$ from an overloaded SR (Fabiato, 1985*a*).

not yet demonstrate) a spontaneous $Ca^{2+}$ release permitted by a $Ca^{2+}$ overload of the SR (see Fig. 7 in Fabiato, 1985*b*). The first tension transient shown in Fig. 4 occurred after three previous tension transients induced by the complete microinjection-aspiration series at regular intervals of 25 s. The cyclic repetition of the contraction resembled that observed under conditions inhibiting the processes of $Ca^{2+}$ extrusion across the sarcolemma of intact cardiac muscle. This occurs, for instance, during digitalis intoxication (Kass and Tsien, 1982).

*Potentiation Is Related to the Sarcoplasmic Reticulum Function*

The same type of activation by a fast and then a slow increase of [free $Ca^{2+}$] from mixing solutions at pCa 5.00 and 7.00 was applied to nine skinned canine cardiac Purkinje cells that had been treated for 2 h with 0.5% (wt/vol) of the nonionic detergent Brij 58 to destroy the ability of the SR to actively accumulate $Ca^{2+}$ (Fabiato, 1985*a*). The first contraction shown in Fig. 5*A* is the one for which the stimulation with a slow component of increase of bulk solution [free $Ca^{2+}$] was applied for the first time. The following contraction was not potentiated, nor were the subsequent ones (not shown). For all contractions, the increase of [free $Ca^{2+}$] resulted in tension development, and the return to pCa 7.00 resulted in rapid relaxation. When the aspiration of the mixed solutions and the reinjection of the solution at pCa 7.00 were omitted (Fig. 5*B*), a sustained tension was obtained. It was approximately equal to that predicted from the bulk solution [free $Ca^{2+}$] reached at the end of the slow increase of [free $Ca^{2+}$] and the tension-pCa curve (see Fig. 11 in Fabiato, 1985*a*). When the slow component of increase of bulk solution [free $Ca^{2+}$] was deleted (Fig. 5*C*), no tension was elicited because the [free $Ca^{2+}$] reached at the peak of the fast component was below the threshold of activation of the myofilaments (see Fig. 11 or 13 in Fabiato, 1985*a*).[3]

In summary, a detergent-treated skinned cardiac cell responded by a given level of tension to a given level of bulk solution [free $Ca^{2+}$] independently of the rate at which the [free $Ca^{2+}$] had been changed. Similar results were obtained in all nine experiments of this series. Therefore, the previously described potentiation was related to the SR function.

*Potentiation Is Related to the $Ca^{2+}$ Loading of the Sarcoplasmic Reticulum by the Slow Component of Increase of Free $Ca^{2+}$ Concentration*

The protocol illustrated in Fig. 3 was repeated under conditions that depress the rate of $Ca^{2+}$ accumulation into the SR: a decrease of the temperature to 12°C or deletion of calmodulin from the solution (Fabiato, 1985*b*). These modifications did not eliminate the potentiation but slowed its development over several contractions. For both experimental interventions, the skinned canine cardiac

[3] Other experiments in detergent-treated skinned canine cardiac Purkinje cells demonstrated that a transient suprathreshold increase of bulk solution [free $Ca^{2+}$] of the same rate and duration used here for the fast component of increase of bulk solution [free $Ca^{2+}$], still in the presence of 0.055 mM total EGTA, elicited about the same tension as the same increase of [free $Ca^{2+}$] applied continuously and in the presence of 10 mM total EGTA. This contrasts sharply with the results observed in the presence of a functional SR (Fabiato and Baumgarten, 1984; Fabiato, 1981, 1985*a*).

Purkinje cells were stimulated every 25 or 26 s, as under the control conditions. This time interval was insufficient to permit complete reloading of the SR, which would have required ~42 s in the absence of calmodulin and ~72 s at 12°C (Fabiato, 1985*b*).

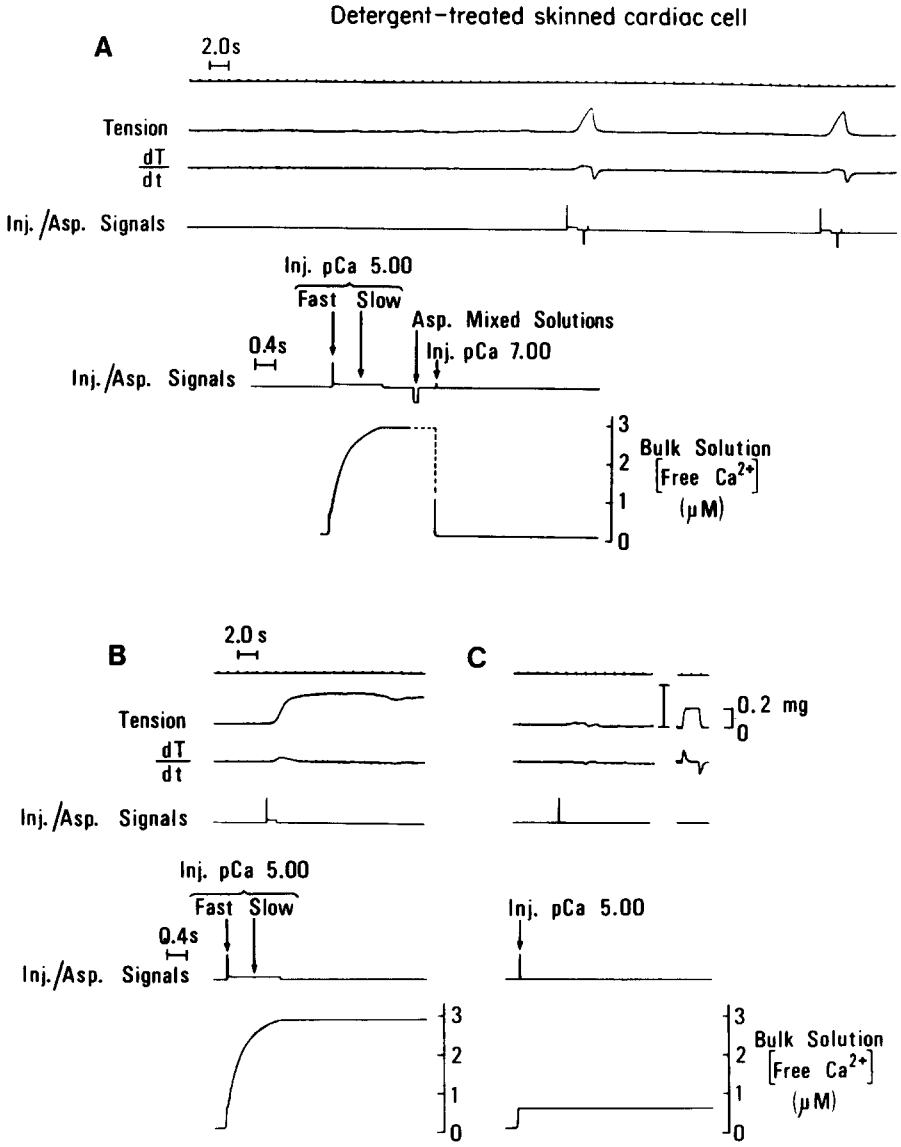


FIGURE 5. Effect of the stimulation of a detergent-treated skinned canine cardiac Purkinje cell by the same type of injection-aspiration series used in skinned cells with intact SR for the experiments illustrated in Figs. 3 and 4. The skinned cell was 9 μm wide, 6 μm thick, and 32 μm long and had been exposed to 0.5% (wt/vol) Brij 58 for 2 h.

In the experiment shown in Fig. 6, the skinned canine cardiac Purkinje cell was stimulated at regular intervals of 25 s in the absence of calmodulin. Initially, 30 half-steps of solution at pCa 7.00 were injected, and the increase of bulk solution [free $Ca^{2+}$] was produced by injecting 14 half-steps of solution at pCa 5.00 in 10 ms (with a 0.71-ms interval between half-steps). Because of the absence of calmodulin, there was an overshoot of the bulk solution [free $Ca^{2+}$] during the rapid mixing of the solutions (see Fig. 13*E* in Fabiato, 1985*b*), and the computation of simulated total calcium influx was meaningless. The amplitude

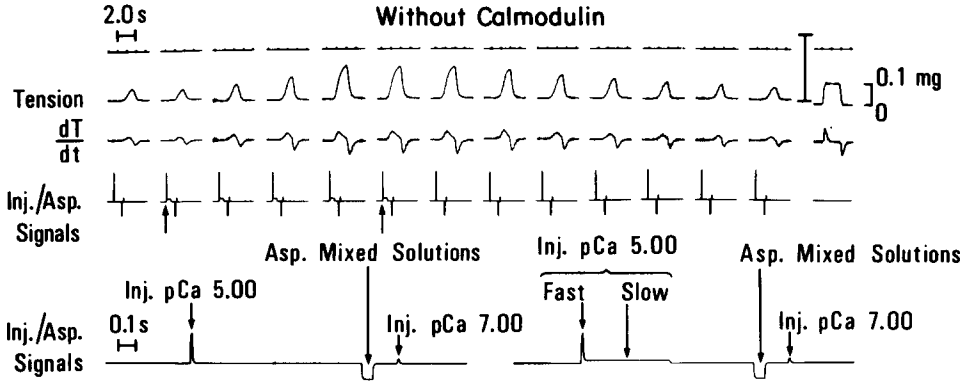


FIGURE 6. Effect of deleting calmodulin on the potentiation caused by the introduction of a slow component of [free $Ca^{2+}$] increase. The preparation was stimulated at regular intervals of 25 s. The arrows indicate the first and last tension transients that were triggered with a slow component of increase of [free $Ca^{2+}$]. The high-speed recording of injection-aspiration signals on the right corresponds to the tension transients for which the increase of [free $Ca^{2+}$] included a slow component; the high-speed recording on the left corresponds to all the other tension transients. The experiment was done in a 7.5-$\mu$m-wide, 5.5-$\mu$m-thick, 22-$\mu$m-long skinned canine cardiac Purkinje cell.

of the resulting tension transient was 9% of maximum tension, corresponding to a peak myoplasmic [free $Ca^{2+}$] of pCa 5.88. The series ended, as under control conditions, by the aspiration of the mixed solutions and the reinjection of the solution at pCa 7.00. Starting with the second tension transient (the first arrow in Fig. 6), a slow component of increase of bulk solution [free $Ca^{2+}$] was introduced that consisted of 45 half-steps (with a 10-ms interval between half-steps) of continued injection of the pCa 5.00 solution. The corresponding tension transient was not modified but the subsequent one was potentiated. The potentiation increased over the next two tension transients and then remained stable, with an amplitude of the tension transient reaching 27% of maximum tension, which corresponded to a peak myoplasmic [free $Ca^{2+}$] of pCa 5.61. Elimination of the slow component (after the second arrow in Fig. 6) did not modify the potentiation of the next tension transient, but the potentiation decayed over the four tension transients following it.

This protocol was used in seven experiments. The tension transient triggered

without a slow component averaged 8% of maximum tension, corresponding to a peak myoplasmic [free $Ca^{2+}$] of pCa 5.89 ± 0.04. The potentiation was completed in three to four tension transients, with an amplitude of the tension transient reaching an average of 28% of maximum tension, which corresponded to a peak myoplasmic [free $Ca^{2+}$] of pCa 5.60 ± 0.02. The potentiation decayed over three to five tension transients, i.e., 75–125 s. If the preparation was not stimulated, 388 ± 29 s ($n$ = 7) was required to obtain a tension transient of amplitude equal to that of the nonpotentiated one. This delay is significantly shorter than in the presence of calmodulin. Therefore, the depression of the rate and extent of $Ca^{2+}$ accumulation into the SR by deletion of calmodulin caused a more progressive development but a faster decay of the potentiation.

For the study of the effect of introducing a slow component of [free $Ca^{2+}$] increase at 12°C (Fig. 7), the instantaneous bulk solution [free $Ca^{2+}$] and simulated total calcium influx were computed because five experiments similar to that shown in Fig. 13*D* of Fabiato (1985*b*) but done at 12°C showed no overshoot of [free $Ca^{2+}$] during the rapid mixing of the solutions at pCa 7.00 and 5.00. Initially, 30 half-steps of solution at pCa 7.00 were injected, and the increase of the bulk solution [free $Ca^{2+}$] was produced by injecting 19 half-steps of solution at pCa 5.00 in 10 ms (with a 0.53-ms interval between half-steps). This resulted in a [free $Ca^{2+}$] trigger of 0.65 μM (pCa 6.19), which induced a tension transient of 26% of maximum tension. Taking into account the effect of decreasing the temperature on the tension-pCa curve (see Fig. 4 in Fabiato, 1985*b*), the peak myoplasmic [free $Ca^{2+}$] was pCa 5.77. The introduction of a slow component of [free $Ca^{2+}$] increase, consisting of 180 additional half-steps (with a 10-ms interval between half-steps) of solution at pCa 5.00, gave a bulk solution [free $Ca^{2+}$] of 6.61 μM (pCa 5.18). This had no effect on the corresponding tension transient (the second transient in Fig. 7). A potentiation was apparent in the following tension transient, increased over the two subsequent ones, and remained stable thereafter, with an amplitude of the tension transient of 70% of maximum tension, corresponding to a peak myoplasmic [free $Ca^{2+}$] of pCa 5.35.

The simulated total calcium influx was that which would have been necessary to produce in an intact cell at 22°C the increase of [free $Ca^{2+}$] obtained in the bulk solution. Thus, the temperature change was considered an intervention depressing the rate of $Ca^{2+}$ accumulation into the SR rather than a simulation of a decrease of temperature in an intact cell, which should have been accompanied by an increase of intracellular pH (Fabiato, 1985*b*).

This protocol was used in nine experiments, and the contraction transient induced in the absence of a slow component of increase of [free $Ca^{2+}$] averaged 22% of maximum tension, corresponding to a peak myoplasmic [free $Ca^{2+}$] of pCa 5.81 ± 0.04. The potentiation built up over three to four tension transients and reached a peak that averaged 64% of maximum tension, corresponding to a peak myoplasmic [free $Ca^{2+}$] of pCa 5.39 ± 0.04. After elimination of the slow component, the potentiation decayed over three to five tension transients. If the preparation was not stimulated, 692 ± 29 s ($n$ = 9) was required for the tension transient to return to the nonpotentiated amplitude. This delay is significantly longer than under control conditions. Thus, a decrease of the temperature

diminished both the rate of establishment and the rate of decay of potentiation. The difference between the effects of deleting calmodulin and decreasing the temperature on the rate of decay of potentiation may be related to the observation that, although both interventions depress the rate of $Ca^{2+}$ accumulation into the SR, deleting calmodulin depresses the steady state level of $Ca^{2+}$ accumulation into the SR to a much larger extent than does decreasing the temperature from

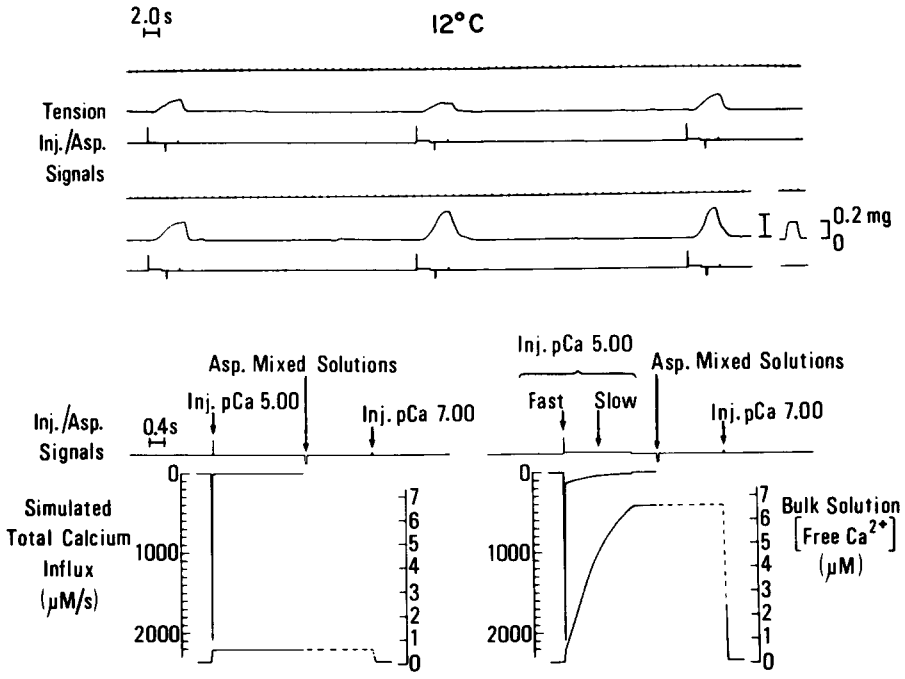


FIGURE 7. Progressive potentiation at 12°C. The first tension transient was elicited without a slow component of increase of [free $Ca^{2+}$], and the corresponding high-speed recording of injection-aspiration signals and of the synchronous computer display is shown on the left. All the other tension transients were elicited with a slow component of [free $Ca^{2+}$] increase, and the corresponding high-speed recording is shown on the right. The experiment was done in a 7-μm-wide, 5-μm-thick, 20-μm-long skinned canine cardiac Purkinje cell.

22 to 12°C (Fabiato, 1985*b*). In addition, a decrease of temperature appears to decrease the rate of passive leak of $Ca^{2+}$ from the SR.

These two series of experiments demonstrate that the potentiation is related to a $Ca^{2+}$ accumulation into the SR induced by the slow component of the increase of [free $Ca^{2+}$] at the outer surface of the SR. The slow component does not induce further $Ca^{2+}$ release from the SR during the corresponding tension transient but causes accumulation into the SR of an amount of $Ca^{2+}$ available for release during subsequent tension transients. The buildup of the potentiation is, accordingly, slower when the rate of $Ca^{2+}$ accumulation is lower.

*Dependence of the $Ca^{2+}$ Loading upon the Timing and Rate of the Slow Component of Simulated Calcium Influx*

The experiments shown in Fig. 8 were done in the same skinned canine cardiac Purkinje cell. This figure compares a control (Fig. 8*B*) similar to that shown in Fig. 3, with an experiment in which the slow component of the increase of bulk solution [free $Ca^{2+}$] was identical in amplitude but delayed by 300 ms (Fig. 8*A*).[4] During the tension transient elicited by an increase of bulk solution [free $Ca^{2+}$] without a slow component and during the first $Ca^{2+}$ transient elicited with a slow component, the tension transient was ~7% of maximum tension, corresponding to a peak myoplasmic [free $Ca^{2+}$] of pCa 5.92. In the control, the potentiation was maximum at the first tension transient following that for which the slow component of increase of bulk solution [free $Ca^{2+}$] was introduced and reached 34% of maximum tension, corresponding to a peak myoplasmic [free $Ca^{2+}$] of pCa 5.53 (Fig. 8*B*). When the slow component of increase of bulk solution [free $Ca^{2+}$] was delayed by 300 ms (Fig. 8*A*), the potentiation was incomplete during the first tension transient following that for which the slow component had been introduced. The potentiation was complete at the second tension transient but reached only 26% of maximum tension, corresponding to a peak myoplasmic [free $Ca^{2+}$] of pCa 5.62.

The same protocol was applied to 12 skinned canine cardiac Purkinje cells with various intervals between the fast and slow components of increase of bulk solution [free $Ca^{2+}$]. In all cases, the degree of potentiation decreased when the interval was increased. This result indicates that the potentiation is maximum when the slow component is applied during the phase of rapid $Ca^{2+}$ reaccumulation into the SR immediately following the $Ca^{2+}$ release. As the delay following $Ca^{2+}$ release increases, the rate of $Ca^{2+}$ reaccumulation into the SR decreases (Fabiato, 1985*b*). This accounts for the decrease in the potentiation caused by the slow component when its onset is delayed.

The degree of potentiation is also dependent upon the rate of increase of bulk solution [free $Ca^{2+}$] during the slow component. If an early additional fast increase of [free $Ca^{2+}$] is applied instead of an early slow increase, the potentiation of the subsequent contraction transient is very weak (see Fig. 8 in Fabiato, 1985*b*). This occurs even if the bulk solution [free $Ca^{2+}$] at the end of the additional fast increase is higher than that which would have been reached at the end of the slow component (Fig. 3 of this article vs. Fig. 8 in Fabiato, 1985*b*). This still occurs if the decrease of bulk solution [free $Ca^{2+}$] by reinjection of pCa 7.00 is delayed by a time equal to the total duration of a slow component of increase of bulk solution [free $Ca^{2+}$]. Thus, it appears that when the increase of bulk solution [free $Ca^{2+}$] is too fast, the (net) $Ca^{2+}$ accumulation into the SR is unable to take full advantage of it.

[4] In this particular experimental series, the fast component of increase of bulk solution [free $Ca^{2+}$] from 0.10 to 0.26 $\mu$M was applied in 15 ms instead of the 10 ms used in all the other experiments (the interval between half-steps was 2.14 ms instead of 1.43 ms). The interval between half-steps for the slow component of increase of bulk solution [free $Ca^{2+}$] was 10 ms, as in the other experimental series.

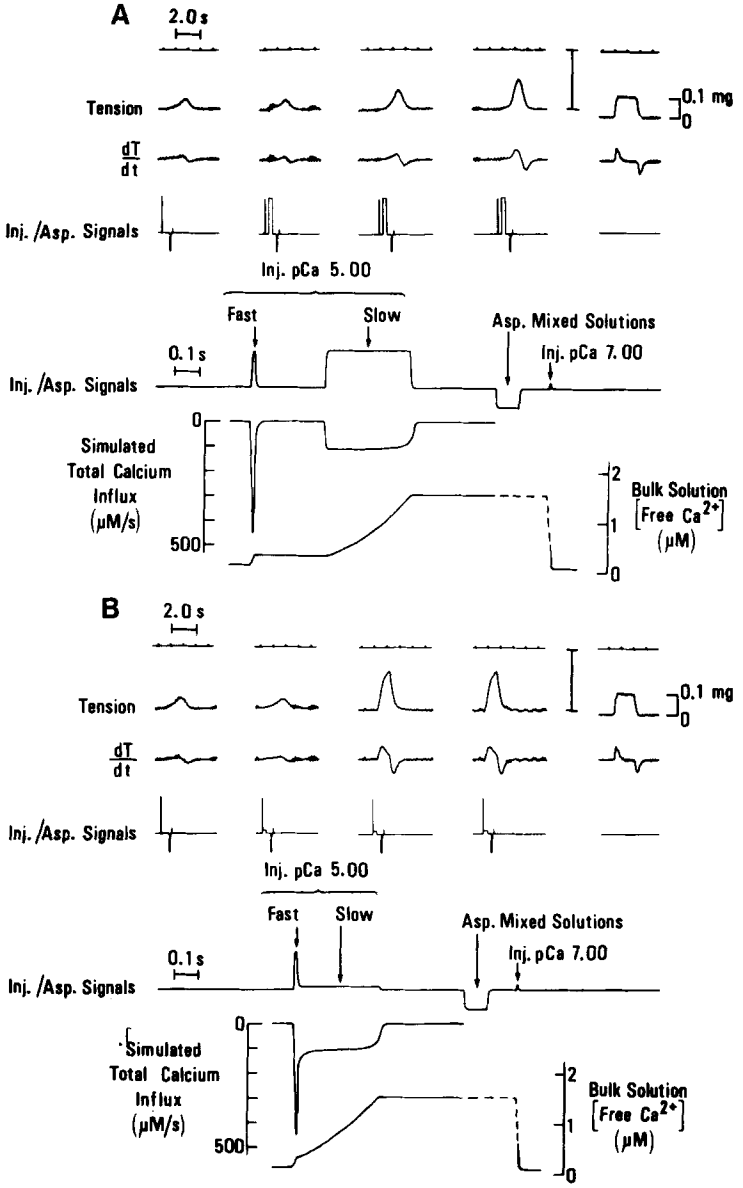


FIGURE 8. Effect of delaying the slow component of the increase of [free $Ca^{2+}$] on the potentiation of the subsequent $Ca^{2+}$ transient. The experiments shown in the two panels were done in the same skinned canine cardiac Purkinje cell, which was 7 μm wide, 5 μm thick, and 21 μm long. In both panels, the first tension transient was elicited without a slow component of increase of [free $Ca^{2+}$]. No high-speed recording of the injection-aspiration signals and computer display is shown for this first transient. The high-speed recordings correspond to the subsequent tension transients for both panels *A* and *B*. The preparation was stimulated at regular intervals of 25 s.

In summary, the slow component of the $Ca^{2+}$ current in the intact cardiac cell (Fig. 1) presents the optimum conditions for $Ca^{2+}$ loading of the SR because (*a*) it occurs as early as possible during the phase of rapid $Ca^{2+}$ reaccumulation into the SR, and (*b*) its rate of increase of [free $Ca^{2+}$] is low. This does not imply, however, that the $Ca^{2+}$ current is necessarily the only source of transsarcolemmal total calcium influx during the action potential of an intact cardiac cell.

### *Force-Frequency Relationships of Potentiated and Nonpotentiated Tension Transients*

The experiments shown in Figs. 9 and 10 were done in the same skinned canine cardiac Purkinje cell, but with a change of the amplification for the tension recording. In the absence of a slow component of the increase of bulk solution [free $Ca^{2+}$], an increase of frequency of the stimulation by the microinjection-aspiration series resulted in a negative staircase: three tension transients after an approximately fivefold increase of frequency, the amplitude of the tension transient had decreased from 7 to 5% of maximum tension, corresponding to a decrease of the peak myoplasmic [free $Ca^{2+}$] from pCa 5.92 to 5.96 (Fig. 9). In seven similar experiments, the negative staircase was completed two to five tension transients after the increase in frequency (Figs. 9 and 11*B*).

The same skinned canine cardiac Purkinje cell was stimulated in the presence of a slow component of increase of bulk solution [free $Ca^{2+}$] (Fig. 10). An approximately fivefold increase of frequency resulted in a positive staircase. The control tension transient at 26-s intervals was 35% of maximum tension, corresponding to a peak myoplasmic [free $Ca^{2+}$] of pCa 5.51. The increase of frequency first decreased the amplitude of the tension transient to 18% of maximum tension, corresponding to a peak myoplasmic [free $Ca^{2+}$] of pCa 5.73. The tension amplitude increased over the second and third tension transients to reach 23% of maximum tension, corresponding to a myoplasmic pCa of 5.67. In seven similar experiments, the positive staircase was completed in three to four tension transients after the frequency change (Figs. 10 and 12*F*).

Thus, in the same skinned canine cardiac Purkinje cell either a negative staircase or a positive staircase could be obtained, depending upon the absence or presence of a slow component of increase of [free $Ca^{2+}$]. The absence of a slow component of the simulated transsarcolemmal total calcium influx resulted in force-frequency relationships that were similar to those of the intact adult rat ventricular cell (Meijler et al., 1962; Henderson et al., 1969; Fabiato, 1981), in which the action potential plateau is short and the $Ca^{2+}$ current is deprived of a slow component because all, or almost all, $Ca^{2+}$ channels inactivate within 10–20 ms (Mitchell et al., 1983; Josephson et al., 1984). In contrast, the presence of a slow component of increase of [free $Ca^{2+}$] resulted in force-frequency relationships somewhat similar to those of the intact ventricular cell of adult mammals other than the rat (Kruta and Bravený, 1960; Koch-Weser and Blinks, 1963; Fabiato, 1981), in which the action potential plateau and the slow component of the $Ca^{2+}$ current are well developed (Marban and Tsien, 1982; Lee and Tsien, 1982, 1983; McDonald, 1982; Tsien, 1983). It should be noted, however, that the intact ventricular cell from mammals other than the rat reaches greater steady state tension and aequorin light transients (at the end of the positive

staircase) with a 5-s as compared with a 25-s interval at 22°C (Koch-Weser and Blinks, 1963; Allen and Kurihara, 1980; Morgan and Blinks, 1982). This is not the case for the intact canine cardiac Purkinje tissue, in which the steady state tension and aequorin light transients increase when the duration of the interval is increased up to at least 100 s (Wier, 1980).

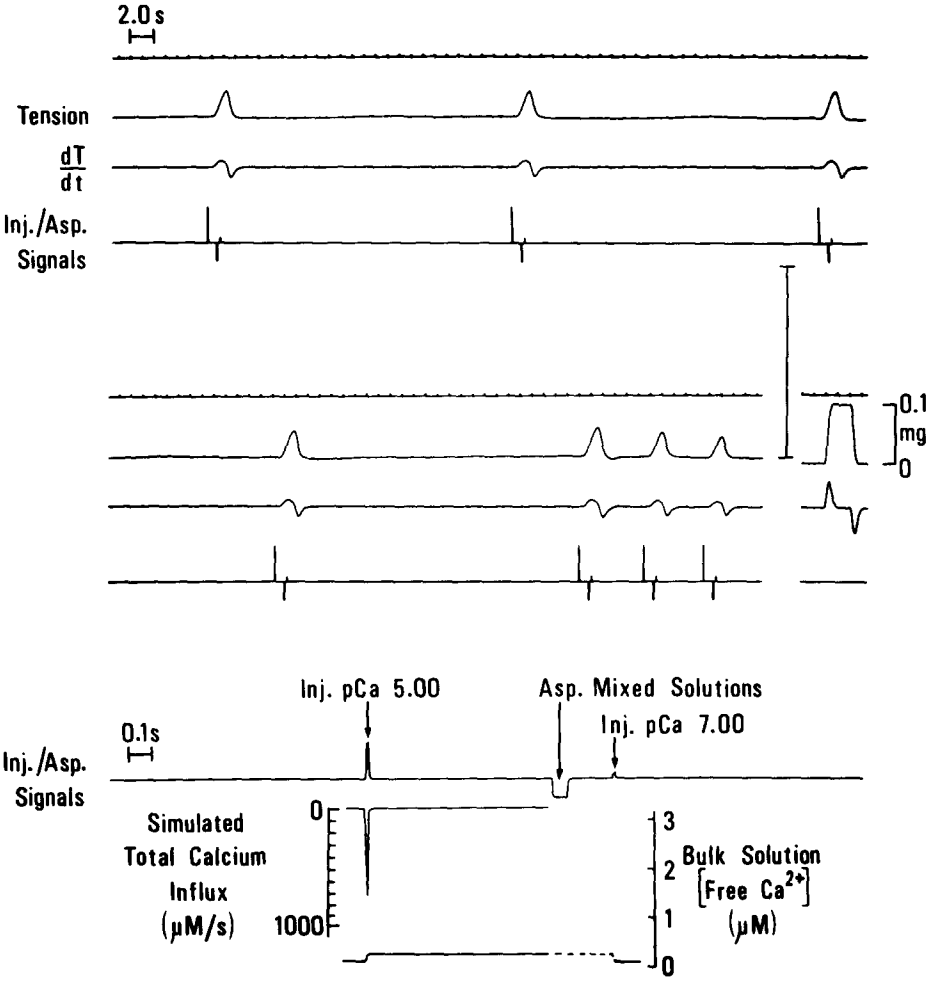


FIGURE 9. Negative staircase caused by an increase of frequency by a factor of ~5 in a 7.5-μm-wide, 5.5-μm-thick, 24-μm-long skinned canine cardiac Purkinje cell stimulated by an injection-aspiration series without a slow component of increase of [free $Ca^{2+}$]. This experiment was done in the same skinned cell used for Fig. 10, but with a different tension calibration scale.

In skinned canine cardiac Purkinje cells, the negative staircase did not occur when the resting [free $Ca^{2+}$] in the solution was too low to permit effective filling of the SR, even if a high [free $Ca^{2+}$] was used as a trigger. Fig. 11*A* shows the absence of a negative staircase when the resting pCa was 7.75 in the same skinned

cell in which a negative staircase was observed (note that the Δ[free $Ca^{2+}$] trigger was much lower) when the resting pCa in the solution was 7.00 (Fig. 11*B*). This suggests that, at least in a skinned cardiac cell, the negative staircase is caused by a progressive reduction in the degree of refilling of the SR during the interval

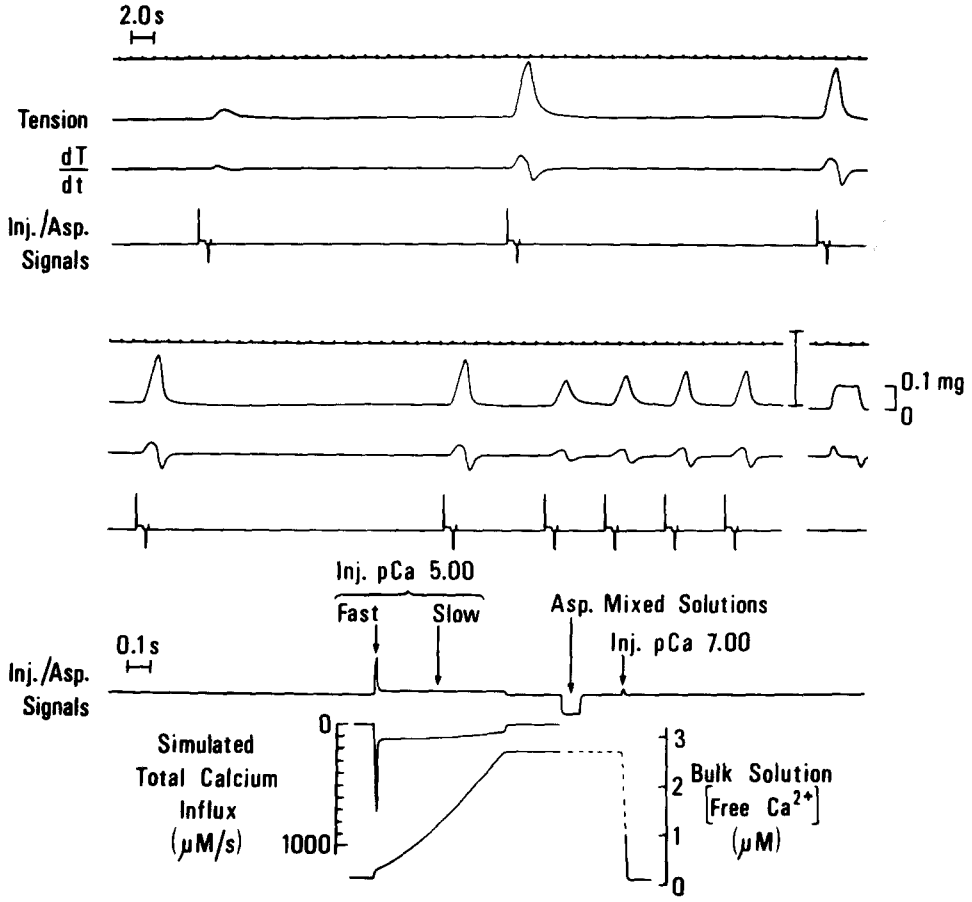


FIGURE 10. Positive staircase (in the same skinned cell used for Fig. 9) caused by an increase of frequency by a factor of ~5 as the skinned cell was stimulated by an injection-aspiration series with a slow component of increase of [free $Ca^{2+}$]. The initial tension transient in this figure is the first one during which a slow component of [free $Ca^{2+}$] increase was applied. This did not result in any modification of this tension transient, which was of amplitude about equal to that shown in Fig. 9 but was recorded at a 2.5-times-lower amplification. The subsequent tension transient was strongly potentiated.

between tension transients resulting from the increase of frequency. This is consistent with the continuation of a slow loading of the SR during ~16 s in the presence of pCa 7.00 at 22°C (see Fig. 16 in Fabiato, 1985*b*).

When a skinned canine cardiac Purkinje cell was stimulated by a microinjection-aspiration series in the absence of a slow component of bulk solution [free

$Ca^{2+}$] increase, the interpolation of a premature stimulation (i.e., a simulated extrasystole) never produced a postextrasystolic potentiation. The following tension transient was always decreased in amplitude.

When the preparation was stimulated with a microinjection-aspiration series that included a slow component of increase of bulk solution [free $Ca^{2+}$] (Fig. 12*A*), a simulated extrasystole produced a postextrasystolic potentiation that

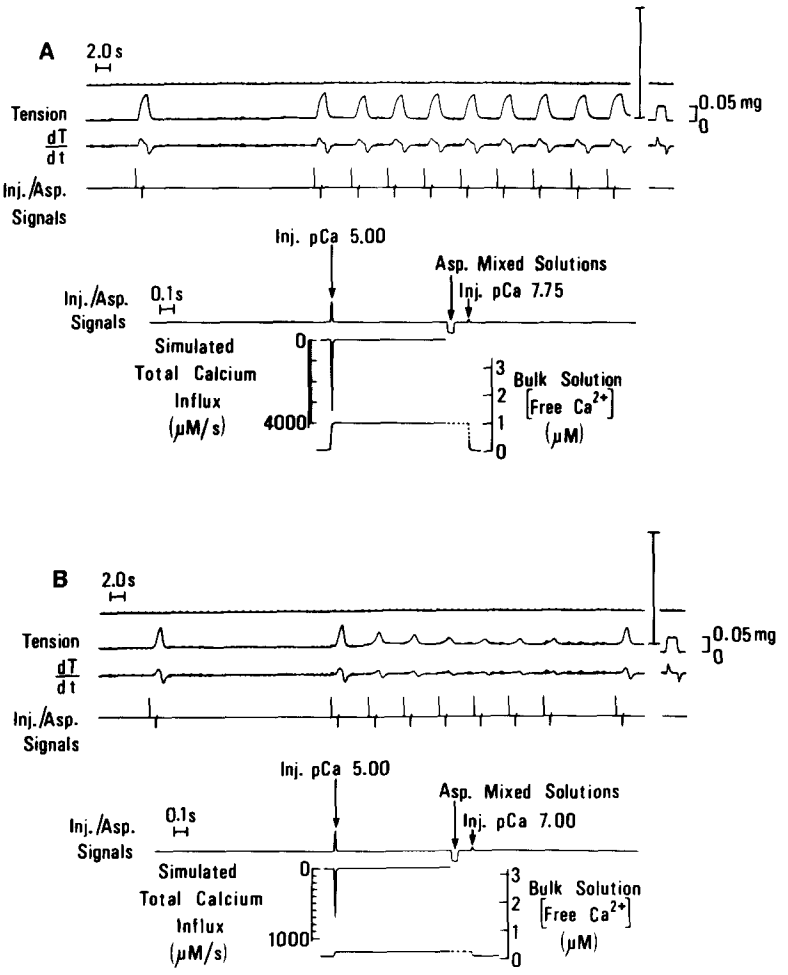


FIGURE 11. Absence of a negative staircase when the resting [free $Ca^{2+}$] in the solution bathing the skinned cell was low, which greatly diminished the $Ca^{2+}$ loading of the SR during the interval between tension transients (panel *A*), and presence of a negative staircase when the resting [free $Ca^{2+}$] was high enough to permit a large loading of the SR with $Ca^{2+}$ during the interval between tension transients (panel *B*). In both panels *A* and *B*, the increase of frequency was by a factor of ~5, and the injection-aspiration series was without a slow component of increase of [free $Ca^{2+}$]. The experiment was done in an 8.5-μm-wide, 6-μm-thick, 29-μm-long skinned canine cardiac Purkinje cell.

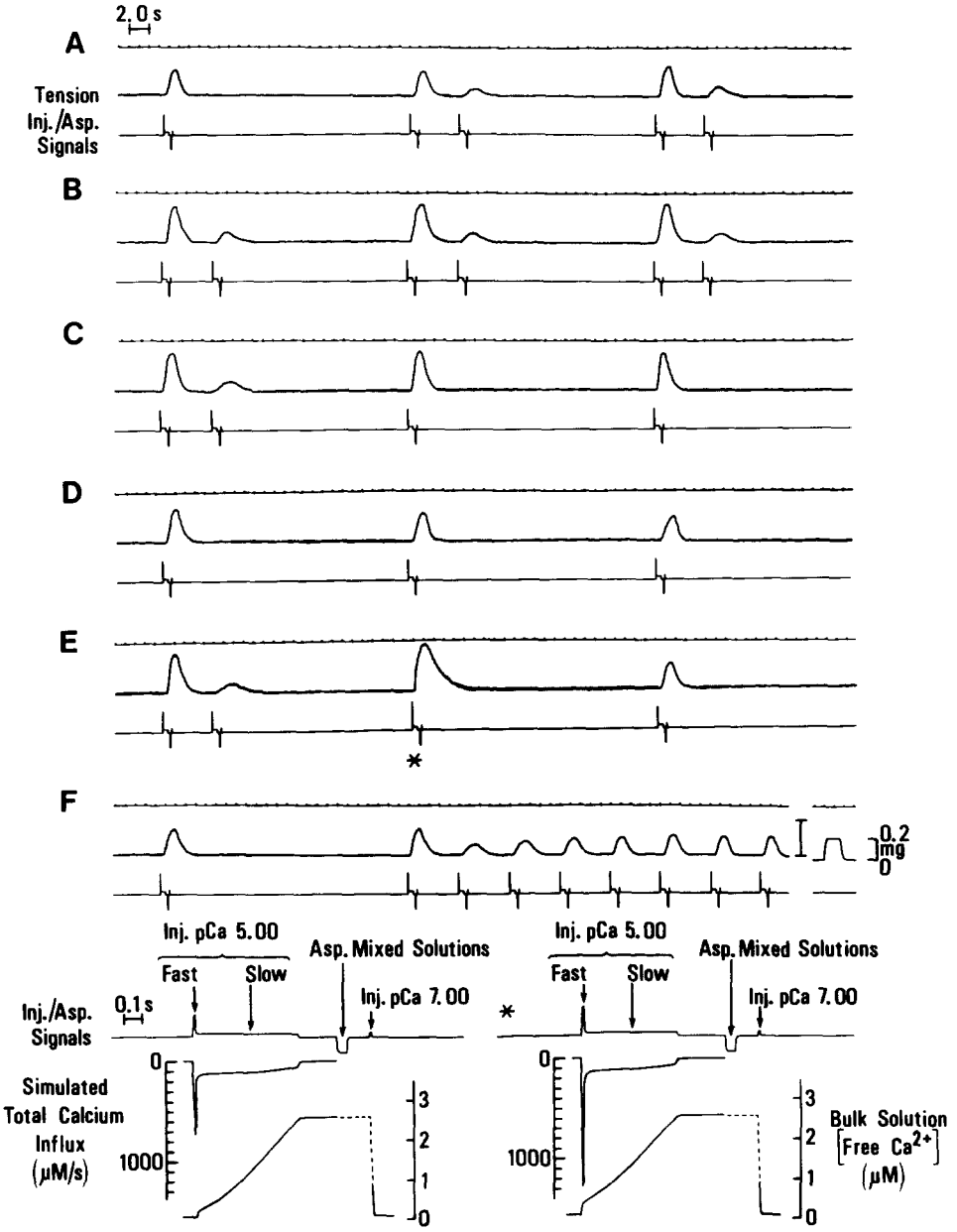


FIGURE 12. Simulation of postextrasystolic potentiation and of the decay of this potentiation in a 7.5-μm-wide, 5.5-μm-thick, 22-μm-long skinned canine cardiac Purkinje cell. The high-speed recording on the left corresponds to all the stimulations except that indicated by an asterisk, for which the high-speed recording is shown on the right.

built up over two to three tension transients (Fig. 12*B* and 12 similar experiments). Upon interruption of the simulated "paired-pulse stimulation" (i.e., stimulation with interpolated extrasystoles; see Cranefield and Hoffman, 1968), the potentiation decreased progressively over four to five tension transients (Fig. 12, *C* and *D*, and 12 similar experiments).

Although this progressive buildup and decay of the potentiation occurred over a much smaller number of contractions than in the intact heart (Cranefield and Hoffman, 1968), it could suggest that $Ca^{2+}$ has to circulate from an accumulation compartment to a release compartment in the SR, as proposed by investigators working in intact cardiac muscle (Kruta and Bravený, 1968; Wood et al., 1969; Bass, 1976; Edman and Jóhannsson, 1976; Wohlfart, 1982; Wohlfart and Noble, 1982; Pučelik et al., 1983). In fact, the experimental intervention shown in Fig. 12*E* demonstrates that the extra amount of $Ca^{2+}$, which is used over several tension transients during the decay of the simulated postextrasystolic potentiation, is already entirely available for release at the first tension transient following the end of the simulated paired-pulse stimulation. The first tension transient in Fig. 12*E* was preceded by seven transients elicited with a paired-pulse stimulation. Thus, the postextrasystolic potentiation had reached its plateau. The second tension transient was elicited by a larger increase of bulk solution [free $Ca^{2+}$], and the paired-pulse stimulation was interrupted. This resulted in a large tension transient, and the following tension transient immediately reached the control amplitude. Therefore, all the extra $Ca^{2+}$ available for potentiation decay can be released in a single tension transient.

This experiment was done 12 times by using various levels of [free $Ca^{2+}$] trigger to produce the large tension transient unloading the $Ca^{2+}$ accumulated during potentiation. When the [free $Ca^{2+}$] trigger was too large, the tension transient following the large one was smaller than control, but the subsequent one returned to the control value. When the [free $Ca^{2+}$] trigger was too small, the subsequent tension transient was still slightly potentiated. By trial and error, it was found that an increase of bulk solution [free $Ca^{2+}$] from pCa 7.00 to 6.41 in 10 ms (instead of the control increase of [free $Ca^{2+}$] from pCa 7.00 to 6.60 in 10 ms) was appropriate to release all the extra $Ca^{2+}$ contained in the SR (Fig. 12*E*). Then the amplitude of the tension transient following the large one was indistinguishable from that of control in four of five experiments; it was slightly less in the fifth one.

This result strongly argues against the hypothesis of anatomical compartmentation for $Ca^{2+}$ loading and $Ca^{2+}$ release by the SR. The compartmentation is only functional: it is dependent upon the timing and rate of change of [free $Ca^{2+}$] at the outer surface of the SR and the interactions of the time- and $Ca^{2+}$-dependent processes of $Ca^{2+}$ accumulation and release.

## DISCUSSION

The major implication of these studies is that the transsarcolemmal $Ca^{2+}$ influx can either trigger the $Ca^{2+}$ release from the SR when it is fast or load the SR with $Ca^{2+}$ when it is slow and occurs during the refractory period of the process of $Ca^{2+}$-induced release of $Ca^{2+}$. Hence, the slow component of the $Ca^{2+}$ current

corresponding to noninactivating $Ca^{2+}$ channels (Marban and Tsien, 1982; Lee and Tsien, 1982, 1983; Fig. 1 of this article) may be at least one source of $Ca^{2+}$ loading of the SR.

The hypothesis that part of the $Ca^{2+}$ current of the mammalian cardiac action potential can be a source of $Ca^{2+}$ loading of the SR has been suggested from voltage-clamp experiments (Morad and Trautwein, 1968; Beeler and Reuter, 1970; Gibbons and Fozzard, 1975). The hypotheses implied, however, either that $Ca^{2+}$ would not be the trigger for the $Ca^{2+}$ release or that the release and loading compartments of the SR would be anatomically distinct. For instance, the terminal cisternae of the SR could have been the release site and the longitudinal SR could have been the accumulation site, as suggested by autoradiography in skeletal muscle (Winegrad, 1968). This hypothesis is strongly supported for skeletal muscle excitation-contraction coupling (Winegrad, 1982), although recent electron-probe microanalyses have not confirmed the long delay implied in this $Ca^{2+}$ recirculation (Somlyo et al., 1981). In contrast, there is no evidence for different sites for $Ca^{2+}$ release and accumulation in cardiac muscle (Winegrad, 1979, 1982).

The data with aequorin in skinned cardiac cells (Fabiato, 1981, 1983) already suggested that the longitudinal SR is capable of both accumulating and releasing $Ca^{2+}$. Thus, the observation of a well-developed $Ca^{2+}$-induced release of $Ca^{2+}$ in skinned cardiac cells from tissues deprived of transverse tubules (such as the Purkinje tissue of the dog and the ventricular tissue of the pigeon), and in which the skinning had removed all superficial couplings, proved that the release was not from the terminal cisternae. However, it has not yet been possible to demonstrate completely that the release was from the free longitudinal SR, even in skinned cells from the canine cardiac Purkinje tissue, because this tissue presents an "extended junctional SR," which is morphologically similar to the SR of the terminal cisternae but makes no contact with transverse tubules or the surface membrane (Sommer et al., 1982). A survey of tissues of a variety of species in the animal kingdom failed to uncover a tissue with a well-developed $Ca^{2+}$-induced release of $Ca^{2+}$ but without a prominent "extended junctional SR." This obviously did not prove that the "extended junctional SR" is the only release site but merely failed to support in a compelling manner the hypothesis that the release also takes place from the free longitudinal SR (Fabiato, 1981, 1983). The data reported here suggest a mechanism through which $Ca^{2+}$ release and accumulation could take place with only a functional compartmentation caused by the time and $Ca^{2+}$ dependence of the activation and inactivation of $Ca^{2+}$ release from the SR and of the $Ca^{2+}$ accumulation into the SR.

The data suggest that $Ca^{2+}$ loading of the SR participates in the force-frequency relationships of the intact mammalian heart. However, they do not entirely explain these relationships. For instance, movements of $Ca^{2+}$ across the sarcolemma through $Na^{+}$-$Ca^{2+}$ exchange and "$Na^{+}$ pump lag" (Lado et al., 1982; Langer, 1983) participate in the long-term changes of tension amplitude of the positive staircase, which takes place over more contractions in intact than in skinned cardiac cells. Frequency-dependent changes in the duration of the action potential plateau (Boyett and Jewell, 1980) might also play a role in the positive

staircase in the frog ventricle (Chesnais et al., 1978), where the SR seems to play no role in beat-to-beat $Ca^{2+}$ release and accumulation (Fabiato, 1983; but see Winegrad, 1979). There is redundancy in the control of cardiac excitation-contraction coupling. The same phenomenon, such as the positive staircase, may be explained by the handling of $Ca^{2+}$ by either the sarcolemma, the SR, or both.[5]

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[5] The appraisal of the potential physiological role of $Ca^{2+}$-induced release of $Ca^{2+}$ from the SR is left to the readers. They may be concerned about the low rates of $Ca^{2+}$ release and accumulation. These unphysiological rates might be related partly to the absence of diffusible physiological constituents other than those included in the solutions, although more additions than reported have been tried without success. Another factor is the broad spacing of the myofibrils and of the elements of the SR wrapped around them, which could eliminate some positive feedback. For instance, in an intact cell the beginning of the $Ca^{2+}$ release from one area of the SR may accelerate the release from neighboring areas. Correcting the unphysiological spacing of the myofibrils would preclude the rapid changes of [free $Ca^{2+}$] at the outer surface of the SR that are necessary for the experiments.

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