Spontaneous Ca$^{2+}$ Release from the Sarcoplasmic Reticulum Limits Ca$^{2+}$-dependent Twitch Potentiation in Individual Cardiac Myocytes

A Mechanism for Maximum Inotropy in the Myocardium

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ABSTRACT We hypothesized that the occurrence of spontaneous Ca$^{2+}$ release from the sarcoplasmic reticulum (SR), in diastole, might be a mechanism for the saturation of twitch potentiation common to a variety of inotropic perturbations that increase the total cell Ca. We used a videomicroscopic technique in single cardiac myocytes to quantify the amplitude of electrically stimulated twitches and to monitor the occurrence of the mechanical manifestation of spontaneous SR Ca$^{2+}$ release, i.e., the spontaneous contractile wave. In rat myocytes exposed to increasing bathing [Ca$^{2+}$] ($[Ca_o]$) from 0.25 to 10 mM, the $[Ca_o]$ at which the peak twitch amplitude occurred in a given cell was not unique but varied with the rate of stimulation or the presence of drugs: in cells stimulated at 0.2 Hz in the absence of drugs, the maximum twitch amplitude occurred in 2 mM $[Ca_o]$; a brief exposure to 50 nM ryanodine before stimulation at 0.2 Hz shifted the $[Ca_o]$ of the maximum twitch amplitude to 7 mM. In cells stimulated at 1 Hz in the absence of drugs, the maximum twitch amplitude occurred in 4 mM $[Ca_o]$; 1 µM isoproterenol shifted the $[Ca_o]$ of the maximum twitch amplitude to 3 mM. Regardless of the drug or the stimulation frequency, the $[Ca_o]$ at which the twitch amplitude saturated varied linearly with the $[Ca_o]$ at which spontaneous Ca$^{2+}$ release first occurred, and this relationship conformed to a line of identity ($r = 0.90$, $p = <0.001$, $n = 25$). The average peak twitch amplitude did not differ among these groups of cells. In other experiments, (a) the extent of rest potentiation of the twitch amplitude in rat myocytes was also
limited by the occurrence of spontaneous Ca\textsuperscript{2+} release, and (b) in both rat and rabbit myocytes continuously stimulated in a given Ca\textsubscript{o}, the twitch amplitude after the addition of ouabain saturated when spontaneous contractile waves first appeared between stimulated twitches. A mathematical model that incorporates this interaction between action potential–mediated SR Ca\textsuperscript{2+} release and the occurrence of spontaneous Ca\textsuperscript{2+} release in individual cells predicted the shape of the Ca\textsubscript{o}-twitch relationship observed in other studies in intact muscle. Thus, the occurrence of spontaneous SR Ca\textsuperscript{2+} release is a plausible mechanism for the saturation of the inotropic response to Ca\textsuperscript{2+} in the intact myocardium.

**INTRODUCTION**

In cardiac muscle, the positive inotropic effect of increasing extracellular [Ca\textsuperscript{2+}] (Ca\textsubscript{o}) or adding cardiac glycosides or catecholamines to the bathing fluid results from a greater release of Ca\textsuperscript{2+} into the myoplasm after excitation (Allen and Kurihara, 1980; Morgan and Blinks, 1982; Wier and Hess, 1984; Endoh et al., 1986). However, once the maximal contractile response has been achieved, further increments in Ca\textsubscript{o}, glycosides, or catecholamines do not lead to stronger stimulated contractions but to a plateau and then a decrease in twitch strength (Dhalla and Braxton, 1968; Vassalle and Lin, 1979; Guarnieri et al., 1980; Lakatta and Lappe, 1981). In particular, rat cardiac preparations stimulated at low frequencies exhibit a saturation of their response to Ca\textsuperscript{2+} at a Ca\textsubscript{o} of 1.5–2.5 mM, a value much lower than that in other mammalian species studied under similar conditions (Capogrossi et al., 1986a). While the mechanisms that determine the maximum effect of inotropic interventions have not been precisely delineated (Allen et al., 1985; Kotake and Vassalle, 1986), it has been observed that the occurrence of spontaneous sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release in diastole can adversely affect the ensuing twitch (Suarez-Isla et al., 1984; Valdeolmillos and Eisner, 1985; Allen et al., 1985; Capogrossi et al., 1986c). Additional evidence indicates that the likelihood of spontaneous SR Ca\textsuperscript{2+} release occurring in the diastolic interval during regular electrical stimulation is increased by perturbations that increase the cell Ca load (Capogrossi and Lakatta, 1985; Capogrossi et al., 1986c).

Spontaneous SR Ca\textsuperscript{2+} release can occur in all mammalian cardiac tissues and has a marked species dependence (Kort and Lakatta, 1984). It has been determined from measurements of scattered light intensity fluctuations in papillary muscles (Kort et al., 1985a) and in the intact heart (Stern et al., 1985) and through direct observation of single cardiac cells (Capogrossi et al., 1986a) that unstimulated rat preparations can exhibit spontaneous SR Ca\textsuperscript{2+} release when Ca\textsubscript{o} is as low as 0.5 mM. Additionally, in "skinned" rat cardiac cells, spontaneous SR Ca\textsuperscript{2+} oscillations have been observed when the bathing Ca\textsubscript{o} is as low as 100 nM (Fabiato and Fabiato, 1975; Chiesi et al., 1981; Fabiato, 1983; Fabiato and Baumgarten, 1984), a value near that estimated for free myoplasmic [Ca\textsuperscript{2+}] in suspensions of intact rat myocytes (Powell et al., 1980; Sheu et al., 1984; duBell and Houser, 1986). In contrast to the rat, other species (e.g., unstimulated rabbit ventricular muscle or myocytes) do not show evidence of this phenomenon until Ca\textsubscript{o} is ≥10 mM (Kort and Lakatta, 1984; Capogrossi et al., 1986a). This
dependence of spontaneous SR Ca\(^{2+}\) release on Ca\(^{2+}\) and species has also been observed in comparing "skinned" rat and rabbit myocytes (Fabiato and Fabiato, 1978; Chiesi et al., 1981). Because spontaneous diastolic Ca\(^{2+}\) release is related to cell Ca loading, and because of its adverse effect on the ensuing twitch, we hypothesized that its occurrence could be a factor that limits the maximum twitch potentiation in response to a variety of perturbations that increase cell Ca loading. However, to establish such a role, the time when spontaneous SR Ca\(^{2+}\) release first occurs and its relationship to twitch saturation in a given cell need to be established.

In single cardiac cells, the mechanical manifestation of spontaneous SR Ca\(^{2+}\) release is readily visible as spontaneous sarcomere shortening, which begins locally and propagates along the cell as a contractile wave (Fabiato and Fabiato, 1972; Kort et al., 1985a; Capogrossi and Lakatta, 1985). Thus, it is possible to measure the extent of twitch amplitude in response to electrical stimulation and the occurrence of spontaneous contractile waves in a given myocyte. These were studied across a range of Cao during interventions that, in a given Cao, are known to either prevent the occurrence of spontaneous SR Ca\(^{2+}\) release between stimulated twitches or increase the likelihood of its occurrence. We used single myocytes from adult rats and rabbits because of the marked differences in conditions required for the spontaneous SR Ca\(^{2+}\) release to occur in these two mammalian species.

We observed that, without exception, under the experimental conditions employed, regardless of the method used to enhance contractility (e.g., an increase in Cao, addition of cardiac glycosides or isoproterenol to the bathing fluid, or permitting a period of rest following stimulation [rat myocytes]), saturation of the twitch amplitude in a given myocyte occurred simultaneously with the onset of spontaneous SR Ca\(^{2+}\) release. Additionally, as spontaneous diastolic SR Ca\(^{2+}\) release became more frequent, the twitch amplitude progressively decreased.

We have constructed a mathematical model to simulate in the intact myocardium this effect of spontaneous SR Ca\(^{2+}\) release as measured in single myocytes. This model, which assumes only that spontaneous Ca\(^{2+}\) release occurs heterogeneously throughout the tissue, predicts the pattern of saturation of twitch potentiation with an increase in Cao, or with rest following stimulation, which had been observed previously in intact tissue. We conclude that the occurrence of spontaneous heterogeneous diastolic SR Ca\(^{2+}\) release in individual myocytes is a mechanism that limits the maximum inotropic response of the heart to perturbations that act by enhancing cell Ca loading.

Some aspects of this work have been published in abstract form (Capogrossi et al., 1985, 1986b; Stern et al., 1987).

**METHODS**

Single myocytes from the left ventricle of 7-8-mo-old rats and adult rabbits (2-4 kg) were isolated as previously described (Capogrossi et al., 1986a). Briefly, we used a retrograde aortic perfusion with a low-Ca\(^{2+}\)-collagenase bicarbonate buffer at 37°C (pH 7.4), and then minced the left ventricle and mechanically dispersed the myocytes. Cells were placed
in a chamber on the stage of an inverted microscope (IM-35, Carl Zeiss, Inc., New York, NY) and continuously superfused at 37°C (pH 7.4) with HEPES buffer of the following composition (in millimolar): 137 NaCl, 5 KCl, 1.2 MgSO₄·7H₂O, 20 HEPES, and 15 dextrose; Ca₀ was varied between 0.5 and 10 mM. The picture of a single myocyte was projected onto a video monitor (WV-5200, Panasonic, Matsushita Communication Industrial Co., Ltd., Yokohama, Japan) through a TV camera (Panasonic) adapted to the microscope. Cells selected for study were rod-shaped, with a clear A-I pattern and sharp edges, and did not show blebs or granulations. All cells exhibited a synchronous contraction (twitch) in response to threshold stimuli (2–5 ms duration) delivered by a stimulator (SD9, Grass Instrument Co., Quincy, MA) through two platinum electrodes in the bathing fluid. In the absence of stimulation, these cells did not exhibit spontaneous twitches. To quantify cell movement along its long axis, either during a stimulated twitch or during a spontaneous contractile wave, the camera was rotated to align the sarcomere pattern perpendicularly to the video monitor raster lines, and a video dimension analyzer (305, Instrumentation for Physiology and Medicine, Inc., San Diego, CA) was used to measure cell length by video edge tracking. The signal was then transmitted to a chart recorder (Brush 220, Gould, Inc., Cleveland, OH) and to a computer (VAX 730, Digital Equipment Corp., Maynard, MA) for on-line analysis. The amplitude of a stimulated twitch was measured as the extent of cell shortening and expressed as percent of the resting cell length. The average twitch amplitude was determined by the mean of 12 consecutive twitches. All experiments were done at 37 ± 0.5°C. Ryanodine was purchased from Penick Corp., Lyndhurst, NJ. Isoproterenol and ouabain were purchased from Sigma Chemical Co., St. Louis, MO. All solutions were made fresh on the day of the experiment. Values are reported as means ± SEM.

Numerical computations and graphics for the mathematical model were done on a computer (PDP-10, Digital Equipment Corp.) using the modeling language MLAB (National Institutes of Health, Bethesda, MD).

RESULTS

Increasing Ca₀

Fig. 1 shows tracings of steady state twitch amplitude from a representative rat myocyte stimulated at three different rates in varying Ca₀. At 0.2 Hz (left-hand panels), the twitch amplitude increased as Ca₀ was increased between 0.5 and 2 mM. At 2 mM Ca₀, spontaneous diastolic contractile waves (arrows) occurred and the average twitch amplitude was maximum. The negative effect of wave occurrence on twitch amplitude was manifest not only on the first twitch after a spontaneous diastolic wave but also on subsequent twitches, as the twitch amplitude continued to increase until the next spontaneous diastolic wave occurred. Further increases in Ca₀ did not result in enhancement of the average twitch amplitude, but caused a greater number of spontaneous contractile waves during the diastolic interval, a marked variation of the amplitude of stimulated twitches, and a decline in the average twitch amplitude.

It has been shown previously that the localized spontaneous SR Ca²⁺ release that underlies the contractile waves, occurring between twitches during regular stimulation, can be suppressed by increasing the frequency of stimulation to a rate slightly faster than the delay interval between a twitch and a subsequent wave (Capogrossi et al., 1986c). Fig. 1 (right-hand panel) shows the effect of increasing the stimulation frequency to 1 Hz. In 0.5 mM Ca₀, the twitch
amplitude was reduced in comparison to the stimulation at 0.2 Hz in a similar Cao, as expected from previous studies, which describe a negative stimulation frequency-twitch strength relation in rat preparations (Orchard and Lakatta, 1985). However, unlike the case at 0.2 Hz, the average twitch amplitude continued to rise with increasing Cao up to 4 mM, and, at this Cao, occasional spontaneous contractile waves began to appear in the intertwitch interval. Increasing Cao to 6 mM made the spontaneous waves more frequent and was associated with marked variation in the amplitude of stimulated contractions and a decline in the average twitch amplitude. When the stimulation frequency was further increased to 3 Hz in 6 mM Cao (Fig. 1, bottom tracing), spontaneous SR Ca\(^{2+}\) release was suppressed, there was no contractile alternans, and the

![Figure 1](image_url)

**Figure 1.** Tracing of cell displacement of a rat myocyte stimulated at different frequencies in varying Cao. The left-hand panels show stimulation at 0.2 Hz. The appearance of spontaneous contractile waves is indicated by arrows only in the tracing at 2 mM Cao. The right-hand panels show stimulation at 1 Hz. The bottom panel shows stimulation at 3 Hz in 6 mM Cao.
average twitch amplitude increased to a value higher than in 6 mM Ca\textsubscript{o} during stimulation at either of the two slower frequencies.

Fig. 2 (filled symbols) more clearly illustrates the changes in the average twitch amplitude and its variation for the same cell depicted in the previous figure during stimulation at 0.2 Hz. Each point of the curve is the average of 12 consecutive twitches (see Methods). The variation in the amplitude of stimulated twitches that occurred after the appearance of spontaneous diastolic contractile waves is indicated by the shaded area. The numbers above the shaded area show the mean wave frequency per diastolic interval at each Ca\textsubscript{o}. Note that the average twitch amplitude saturated when diastolic waves first occurred and declined as diastolic waves became more frequent. This suggests that the saturation and subsequent decline in the average twitch amplitude in a single cardiac cell is related to the occurrence and the increase in the frequency of spontaneous SR Ca\textsuperscript{2+} oscillations. However, it does not indicate whether the time elapsed between a twitch and the beginning of the prior spontaneous contractile wave played a role in limiting the positive inotropic effect of increasing Ca\textsubscript{o}, as previously suggested (Capogrossi et al., 1986c). This time was calculated for the Ca\textsubscript{o} at which one or more spontaneous contractile waves appeared in each of the diastolic intervals after which the twitch amplitude was measured. During stimulation at 0.2 Hz, this was (in seconds): 1.8 ± 0.2 in 3 mM Ca\textsubscript{o}, 1.4 ± 0.2 in 4 mM Ca\textsubscript{o}, and 0.6 ± 0.1 in 6 mM Ca\textsubscript{o}. In the same myocyte during stimulation at 1 Hz, the peak average twitch amplitude shifted to 4 mM Ca\textsubscript{o}; at 6 mM Ca\textsubscript{o}, it declined from its peak (Fig. 1). An average of one wave occurred during each diastolic interval and twitch amplitude was 5.9% of the resting cell length (result not shown) vs. 8.2% when an average of one wave occurred in each diastole during stimulation at 0.2 Hz (Fig. 2). However, in the presence of an equivalent number of waves, i.e., one wave per diastolic interval, the time elapsed between the last spontaneous diastolic wave and the subsequent twitch during stimulation...
at 1 Hz was less than at 0.2 Hz and averaged, respectively, 0.25 ± 0.04 vs. 1.8 ± 0.2 s, which probably accounts for the greater depression in twitch amplitude at the higher frequency. This relation between the decrease in the interval between spontaneous and stimulated SR Ca²⁺ release and the decrease in the average twitch amplitude is compatible with time-dependent reloading of the releasable SR Ca²⁺ pool or time-dependent removal of inactivation of SR Ca²⁺ release mechanisms (Capogrossi et al., 1986c).

A response to Ca₀ similar to that depicted in Figs. 1 and 2 was observed in all cells studied and the average Ca₀-twitch amplitude curves for cells stimulated at 0.2 and 1 Hz are depicted in Fig. 3. The average Ca₀ at which waves first occurred was 1.9 ± 0.3 mM at 0.2 Hz and 4.6 ± 0.6 mM when the same cells were stimulated at 1 Hz. The average peak twitch amplitude was 10.9 ± 0.8 and 10.2 ± 0.8% of the resting cell length, respectively, at 0.2 and 1 Hz. The representation of the average data for n cells, as in Fig. 3, considers these cells as an ensemble, i.e., a tissue. Fig. 4 illustrates that the twitch amplitude of the ensemble can continue to increase over a range of Ca₀, even though some individual myocytes manifest SR Ca²⁺ oscillations. As Ca₀ continues to increase and a sufficient number of cells exhibit spontaneous Ca²⁺ release, the twitch amplitude plateaus, and when diastolic contractile waves are present in all myocytes, the average twitch amplitude declines from an optimum. Intact muscle shows the very same relationship between twitch amplitude and spontaneous

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**Figure 3.** Twitch amplitude at varying Ca₀ in rat myocytes continuously stimulated at 0.2 Hz (○) and at 1 Hz (●; n = 5), in the presence of isoproterenol at 1 Hz (○; n = 4), and after pretreatment with ryanodine at 0.2 Hz (○; n = 5). Cells stimulated at 0.2 and 1 Hz in the absence of isoproterenol or pretreatment with ryanodine are the same.

**Figure 4.** Average twitch amplitude in response to an increase in Ca₀ for myocytes stimulated at 1 Hz (from Fig. 3). The dotted line indicates how many of these cells exhibited spontaneous contractile waves in each Ca₀.
Ca\(^{2+}\) release measured as scattered light intensity fluctuations (cf. pp. 134–135): as \(C_{a_o}\) increases, the twitch plateaus and declines, while the magnitude of spontaneous Ca\(^{2+}\) release within the tissue increases monotonically with \(C_{a_o}\) (Lakatta and Lappe, 1981).

The relevant findings from Figs. 1–3 can be summarized as follows: (a) saturation of the twitch amplitude in response to \(C_{a_o}\) occurred when spontaneous contractile waves first appeared in the diastolic interval, regardless of the frequency of stimulation; (b) an increase in the stimulation frequency, a perturbation known to partially prevent the occurrence of spontaneous contractile waves (Capogrossi et al., 1986c), shifted the \(C_{a_o}\) at which the average peak twitch amplitude and spontaneous waves occurred; and (c) the maximum average twitch amplitude was similar at both stimulation frequencies, regardless of the \(C_{a_o}\) at which it was achieved. These findings are consistent with the hypothesis that spontaneous diastolic SR Ca\(^{2+}\) release limits \(C_{a_o}\) potentiation of twitch shortening.

**Ryanodine**

To further test the above hypothesis, we determined whether a partial inhibition of spontaneous SR Ca\(^{2+}\) release would shift to higher levels the \(C_{a_o}\) required for the peak twitch amplitude during stimulation at a given frequency. This was achieved by exposing additional cells to 50 nM ryanodine, a substance known to inhibit spontaneous SR Ca\(^{2+}\) oscillations in cardiac tissues (Stern et al., 1983; Capogrossi et al., 1984; Lakatta et al., 1985) for 10 min during stimulation at 0.2 Hz in 1.5 mM \(C_{a_o}\). This brief exposure to a low concentration of ryanodine caused an inhibition of spontaneous and triggered SR Ca\(^{2+}\) release, which could be overcome by increasing \(C_{a_o}\). Fig. 2 (open symbols) shows a representative cell studied in ryanodine in varying \(C_{a_o}\). As in the absence of ryanodine, the maximum twitch amplitude occurred when occasional diastolic contractile waves first appeared; however, the \(C_{a_o}\) at which the waves first occurred was markedly shifted to a higher level. After the appearance of one or more spontaneous contractile waves for each diastolic interval, a further increase in \(C_{a_o}\) resulted not only in a higher wave frequency but also in a decrease in the average interval between the beginning of the last diastolic wave and the ensuing twitch: 2.1 ± 0.3 s at 7 mM \(C_{a_o}\), 1.5 ± 0.4 s at 8 mM \(C_{a_o}\), and 0.6 ± 0.1 s at 10 mM \(C_{a_o}\). This strengthens the interpretation of the results of Fig. 2, i.e., the more marked negative inotropic effect of an increase in diastolic wave frequency is mediated, at least in part, by the higher likelihood for shorter intervals between the occurrence of the spontaneous wave and the ensuing stimulated twitch. Fig. 3 shows the average data for cells studied with ryanodine. The effect of pre-exposure to ryanodine was a shift to the right of: (a) the \(C_{a_o}\) at which the peak twitch amplitude was achieved, and (b) the initial appearance of spontaneous contractile waves, which occurred in all the cells in this group between 6 and 8 mM \(C_{a_o}\). The average \(C_{a_o}\) at which waves first appeared shifted from 1.9 ± 0.3 mM (n = 5) for the myocytes stimulated at 0.2 Hz and not pre-exposed to ryanodine (filled circles) to 7.0 ± 0.4 mM (n = 5) for the group stimulated at 0.2 Hz after exposure to ryanodine (open circles). The peak twitch amplitude in cells pre-exposed to ryanodine was 11.3 ± 1.8% vs. 10.9 ± 0.8% without ryanodine, not a significant difference.
**β-Adrenergic Stimulation**

If the hypothesis that is being tested is correct, perturbations that increase the likelihood of spontaneous diastolic SR Ca²⁺ release occurring should have an effect opposite to that of an increase in the frequency of stimulation or exposure to ryanodine, i.e., the peak twitch amplitude and spontaneous diastolic waves ought to occur in lower Ca₀. Fig. 3 (open diamonds) shows the Ca₀-twitch amplitude dose-response curve for myocytes stimulated at 1 Hz in 1 μM isoproterenol, a β-adrenergic agonist known to increase cell Ca loading and the likelihood of spontaneous SR Ca²⁺ release in the diastolic interval during stimulation (Capogrossi and Lakatta, 1985). The filled triangles depict cells stimulated at 1 Hz in the absence of the drug. Isoproterenol decreased the Ca₀ at which the maximal average twitch amplitude (which was 13.3 ± 1.7% of the resting cell length) occurred, and the average Ca₀ at which spontaneous contractile waves first appeared (2.1 ± 0.3 mM during exposure to the drug vs. 4.6 ± 0.6 mM in its absence).

Thus, under a variety of experimental conditions, the occurrence of spontaneous diastolic waves coincides with saturation of the steady state contractile response during electrical stimulation. This is clearly illustrated in Fig. 5, which shows the Ca₀ at which the maximal average twitch amplitude peaked and spontaneous contractile waves first occurred in a given cell, for all the protocols discussed thus far. Regardless of the different conditions under which the Ca₀ was varied, the Ca₀ at which the twitch amplitude peaked and that Ca₀ at which diastolic contractile waves first occurred are related by a single monotonic function, close to an identity.

**Cardiac Glycosides**

Cardiac glycosides are agents that produce a positive inotropic effect in cardiac muscle through an increase in Caₐ mediated by inhibition of the Na-K pump (Lee et al., 1980; Sheu and Fozzard, 1982); they have frequently been used to
experimentally induce states of high cell Ca loading (Li and Vassalle, 1984; Kotake and Vassalle, 1986). Under conditions of digitalis toxicity, spontaneous Ca\textsuperscript{2+} oscillations occur and have been related to some of the associated electrical and contractile abnormalities (Ferrier et al., 1973; Kass et al., 1978; Kass and Tsien, 1982; Valdeolmillos and Eisner, 1985; Allen et al., 1985). However, previous studies, using multicellular preparations, have failed to characterize the transition from a positive to a negative effect of an increase in cell Ca loading. If excess Ca loading in cardiac tissue is defined by a decrease in the inotropic state of the muscle and the appearance of "arrhythmias," it is possible that spontaneous diastolic Ca\textsuperscript{2+} release from the SR, which has been suggested as a cause for some

![Diagram](https://example.com/diagram.png)

**Figure 6.** Cell displacement of a representative rat myocyte in 0.5 mM Ca\textsubscript{o} regularly stimulated at 0.2 Hz and exposed to ouabain. Upper panel: after the addition of 300 μM ouabain, there is a progressive increase in the twitch amplitude until it saturates and a variation in the twitch amplitude appears. The lower panel shows tracings at higher chart speed, obtained at the times indicated by the letters in the upper panel. (a) Tracing obtained just before the addition of ouabain. Note that twitches are uniform and spontaneous diastolic contractile waves are absent. (b) Tracing obtained at the saturation of twitch amplitude. This individual twitch is also shown on an enlarged scale and it is apparent that the stimulated contraction occurs at the peak of a spontaneous contractile wave. (c) Tracing obtained during the plateau in twitch amplitude. Spontaneous contractile waves precede several twitches (arrows). Note that the average twitch amplitude at time c is less than at time b. The dotted line indicates a shift in diastolic cell length after the addition of ouabain (see text).

triggered and spontaneous arrhythmias in ventricular myocardium (Ferrier et al., 1973; Cranefield, 1977; Capogrossi et al., 1987), may also coincide with saturation of the positive inotropic effect of cardiac glycosides and may thus represent the point of transition into a state of excessive Ca loading. The question was addressed in the following experiments, which examined the influence of spontaneous diastolic waves on the twitch amplitude in single myocytes in a given Ca\textsubscript{o} during exposure to ouabain. Fig. 6 shows a representative example of a rat myocyte continuously stimulated at 0.2 Hz in 0.5 mM Ca\textsubscript{o}. The upper tracing, obtained at a slow chart speed, shows that after the addition of 300 μM ouabain to the bathing medium, there was a marked increase in the twitch amplitude, which then saturated. The three lower tracings were obtained at a higher chart speed at the time indicated by the letters. Spontaneous diastolic contractile waves
and variations in the twitch amplitude, which were absent in trace a, were present at trace c. Trace b shows the appearance of the first spontaneous contractile wave just before the fifth twitch.

We have previously shown that SR Ca\(^{2+}\) oscillations are not peculiar to rat preparations with intact sarcolemmal function but can occur in other mammalian species as well, and that, in the rabbit, they require a higher bathing Ca\(_{o}\) to become manifest (Kort and Lakatta, 1984; Capogrossi et al., 1986a). However, except for this difference in Ca\(_{o}\) dependence, they appear to have similar characteristics and functional sequelae in all species. In order to determine whether, in the rabbit, the occurrence of spontaneous diastolic waves limits the twitch response to cardiac glycosides, as it does in rat myocytes, we repeated the experiments depicted in Fig. 6 using rabbit myocytes. Fig. 7 shows tracings obtained from a representative rabbit cell regularly stimulated at 1 Hz in the presence of ouabain (see figure legend). As in rat myocytes, there was an increase
in contractility after the addition of the drug, with saturation of the twitch amplitude when spontaneous SR Ca\(^{2+}\) oscillations, manifested as diastolic contractile waves, first became evident, and in both cases the average twitch amplitude decreased as spontaneous SR Ca\(^{2+}\) release became more frequent.

It is apparent from both Figs. 6 and 7 that a shift in the diastolic cell length occurs after the addition of ouabain during the time when the twitch amplitude is increasing but before the appearance of the first spontaneous contractile wave (see the dotted line in the upper tracing of both figures). It is presently unknown whether such a change in cell length represents incomplete relaxation in the interval between potentiated twitches, related only to the passive viscoelastic properties of these unloaded myocytes, or whether it is indicative of residual Ca\(^{2+}\)-myofilament interaction. If the second possibility is correct, an increase in diastolic myoplasmic [Ca\(^{2+}\)] could herald the occurrence of spontaneous Ca\(^{2+}\) oscillations, as suggested previously (Fabiato and Baumgarten, 1984; Fabiato, 1985).

Rest Potentiation in the Presence of Spontaneous SR Ca\(^{2+}\) Release

The data presented so far suggest an important role for spontaneous SR Ca\(^{2+}\) release as a determinant of the maximum effect of perturbations that enhance the twitch amplitude through an increase in cell Ca loading. The results of prior studies have suggested that spontaneous diastolic Ca\(^{2+}\) release, manifested as a contractile wave, and action potential-mediated Ca\(^{2+}\) release during a twitch may occur from the same SR compartment (Allen et al., 1985; Capogrossi et al., 1986c). A time-dependent restitution of the mechanisms that govern Ca\(^{2+}\) release from this compartment could explain the limitation of the amplitude of the twitch that follows a spontaneous contractile wave. Additionally, a similar mechanism could underlie the potentiation of the increase in myoplasmic [Ca\(^{2+}\)] that causes the twitch and occurs with rest after a train of stimulation in rat muscle (Kurihara and Allen, 1982; Orchard and Lakatta, 1985). This hypothesis predicts that: (a) the time at which the twitch amplitude is maximal would be that just preceding a wave, i.e., the moment when SR Ca\(^{2+}\) loading is probably close to being the highest; (b) the time during which the twitch amplitude is minimal would be during the occurrence of a wave or immediately after an action potential–induced SR Ca\(^{2+}\) release, i.e., the moment that follows unloading of Ca\(^{2+}\) from the SR, regardless of whether this has occurred spontaneously or has been triggered electrically; (c) since an increase in Ca\(_o\) decreases the restitution time required for twitch recovery and decreases the delay interval for spontaneous diastolic Ca\(^{2+}\) release to occur after a twitch (Capogrossi and Lakatta, 1985; Capogrossi et al., 1986c), it should accelerate the restitution for twitch and wave without altering the peak twitch amplitude. These predictions were tested in the experiment illustrated in Fig. 8 for a representative rat myocyte studied in 2 and 4 mM Ca\(_o\). In this experimental protocol (Fig. 8A), a cell was stimulated at 2 Hz for 30 s; after cessation of stimulation, a test twitch was elicited after a rest period. The cycle was then repeated with a variation in the test interval. Fig. 8B shows that as the time from the prior stimulation increased, there was a progressive increase in the twitch amplitude in response to the test...
stimulus. However, the twitch amplitude did not reach a plateau but was interrupted by the occurrence of a spontaneous wave (arrow). An increase in \( \text{Ca}_0 \) from 2 to 4 mM accelerated the restitution of twitch amplitude and also decreased the time for the first wave to occur after the stimulation train. However, the peak twitch amplitude was the same in both \( \text{Ca}_0: \) 13.2 and 13.1\% of resting cell length, respectively, in 2 and 4 mM \( \text{Ca}_0 \). Fig. 8C shows the twitch amplitude in test excitations that were delivered after the onset of the first spontaneous twitch amplitude after a train of stimulation in a representative rat cardiac myocyte. The protocol employed is given in panel A. The cell was stimulated at 2 Hz for 30 s. The twitch amplitude in response to a test excitation at varying intervals after cessation of stimulation was then measured. After this measurement, the myocyte was stimulated again and the test interval was varied. At least 6 min was allowed to elapse between the stimulation trains to let any residual effect of stimulation on the cell dissipate. During these experiments, spontaneous contractile waves were present in the unstimulated state and did not occur during stimulation. In the example in panel A, \( \text{Ca}_0 \) was 2 mM and the test interval is indicated by a longitudinal arrow. Panel B shows the effect of \( \text{Ca}_0 \) on the restitution of twitch amplitude in 2 (O) and 4 (o) mM \( \text{Ca}_0 \). The abscissa indicates the time elapsed from the end of the stimulation train to the test stimulus, which in this panel always preceded the first contractile wave after stimulation. The arrow indicates the average time of occurrence of the first spontaneous contractile wave in each \( \text{Ca}_0 \). This time was 24.9 ± 0.3 (\( n = 4 \)) and 4.2 ± 0.9 s (\( n = 4 \)) in 2 and 4 mM \( \text{Ca}_0 \), respectively. Panel C shows the twitch amplitude of the test stimulus delivered either during the first or between the first and the second spontaneous contractile waves that occurred after stimulation in 2 and 4 mM \( \text{Ca}_0 \). Symbols are as in panel B. The abscissa indicates the time elapsed from the beginning of the first spontaneous contractile wave. The open and filled bars on the abscissa indicate the average duration of the spontaneous contractile waves in 2 and 4 mM \( \text{Ca}_0 \), respectively. The arrows indicate the average time of occurrence of the second contractile wave.
contractile wave after prior electrical stimulation. In both Ca, a similar pattern was observed when test excitations were delivered during the occurrence of the first or between the first and second contractile waves after cessation of prior stimulation. A decrease from the maximum twitch amplitude occurred when the test excitation and spontaneous SR Ca²⁺ oscillations occurred simultaneously, and a progressive increase in the twitch amplitude of the test excitation occurred with increasing time after the occurrence of the first wave until the second spontaneous Ca²⁺ oscillation occurred (arrows). Thus, like the peak twitch amplitude during regular stimulation in varying Ca, (Fig. 3), the extent of potentiation of the peak twitch amplitude between the first and second spontaneous contractile waves was similar regardless of Ca: 12.4 and 13.2%, respectively, in 2 and 4 mM Ca, and not different from the peak twitch amplitude obtained just before the first spontaneous contractile wave after stimulation. These data are not necessarily interpreted to indicate, however, that electrical stimulation just before the occurrence of a spontaneous SR Ca²⁺ release and a spontaneous SR Ca²⁺ oscillation induce a similar extent of unloading of Ca²⁺ from the SR.

**DISCUSSION**

In the present experiments, no exception to the case was found in which saturation of the positive inotropic response occurred in the absence of spontaneous SR Ca²⁺ release, manifested as a contractile wave. However, the hypothesis regarding the role of spontaneous SR Ca²⁺ release in the saturation of twitch amplitude in response to perturbations that enhance cell Ca loading is not intended to explain every aspect of beat-dependent inotropy in cardiac muscle. Other factors that vary with cell Ca loading in addition to the occurrence of SR Ca²⁺ release, including a reduction in intracellular pH or high-energy phosphates or an increase in inorganic phosphate, could lead to an inhibition of several mechanisms involved in excitation-contraction coupling (Vaughan-Jones et al., 1983; Fabiato, 1985; Kentish, 1986; Hoerter et al., 1986). Additionally, isoproterenol, via cAMP mediation, reduces the myofilament Ca²⁺ sensitivity (Ray and England, 1976). Changes in cell Mg²⁺ content can also profoundly affect excitation-contraction coupling mechanisms. Finally, inhibition of SR Ca²⁺ release by Ca²⁺ in the myoplasmic space needs to be considered (Fabiato, 1985).

If any of these factors changed to the required degree and this occurred simultaneously with the onset of spontaneous SR Ca²⁺ release, this factor, rather than or in addition to spontaneous SR Ca²⁺ release, could limit the twitch amplitude. However, the implications of some of these factors in lieu of spontaneous SR Ca²⁺ release as a cause for saturation of the twitch amplitude in the present study are more likely than others. Depletion of ATP can be excluded since SR Ca²⁺ oscillations require ATP for their occurrence (Chiesi et al., 1981). An increase in inorganic phosphate sufficient to limit the twitch amplitude would be expected to reduce the contractile wave amplitude (Kentish, 1986), an effect not observed in the present study. Additionally, an increase in inorganic phosphate is associated with a reduction of scattered light intensity fluctuations, the correlate of spontaneous waves in bulk muscle (Stern et al., 1986). A reduction in intracellular pH, in rat preparations at least, has recently been shown to
Spontaneous SR Ca\(^{2+}\) release and twitch potentiation exacerbate spontaneous SR Ca\(^{2+}\) release (Orchard et al., 1987). It is not known whether or in which direction cell Mg\(^{2+}\) changes with cell Ca loading. Inhibition of SR Ca\(^{2+}\) release (Fabiato, 1985) and of Ca\(^{2+}\) influx into the cell mediated by Ca\(^{2+}\) (Mitchell et al., 1983) is, however, a plausible mechanism for the saturation and decline in twitch amplitude with increasing and excessive cell Ca loading, respectively, since the localized elevations in myoplasmic [Ca\(^{2+}\)] that result from spontaneous SR Ca\(^{2+}\) release can exceed 1 \(\mu\)M in magnitude (Cobbold and Bourne, 1984; Fabiato, 1985). Thus, in addition to causing SR Ca\(^{2+}\) depletion, spontaneous SR Ca\(^{2+}\) release occurring sufficiently soon before a subsequent action potential can cause inhibition of both SR Ca\(^{2+}\) release and transsarcolemmal Ca\(^{2+}\) influx via Ca channels, and both factors could modulate the saturation of twitch amplitude during states of high cell Ca loading. Additionally, a steady increase in myoplasmic [Ca\(^{2+}\)] to a sufficiently high level could lead to inhibition of SR Ca\(^{2+}\) release and of the sarcolemmal Ca channel. However, the required myoplasmic [Ca\(^{2+}\)] in this regard is as high or higher than required for spontaneous SR Ca\(^{2+}\) release to occur (Chiesi et al., 1981; Fabiato and Baumgarten, 1984; Fabiato, 1985).

The present results show that the de novo appearance of spontaneous contractile waves during the diastolic interval in cardiac myocytes occurs with perturbations that potentiate the twitch amplitude by raising cell Ca loading. The major new findings of the present study are (a) that the maximum twitch potentiation that can be achieved by different perturbations in single cardiocytes is limited in all cases by an event common to each of them: the occurrence of spontaneous diastolic SR Ca\(^{2+}\) release; and (b) that the further increase in the frequency of the diastolic spontaneous SR Ca\(^{2+}\) oscillations by prolonged or more intense exposure to inotropic perturbations leads to a diminution in the average cell shortening during twitches.

While the data presented here for isolated cells show that when spontaneous SR Ca\(^{2+}\) release occurs between stimulated contractions, it can markedly influence the twitch amplitude in a given cell, similar effects are to be expected in intact muscle, in which the bulk properties reflect the average properties of its constituent cells. Indeed, in previous studies (Lakatta and Lappe, 1981), it was also observed that the positive portion of the force-frequency relation in cat muscle saturates when spontaneous Ca\(^{2+}\) release, monitored as scattered light intensity fluctuations, becomes excessive. More recent studies in rabbit cardiac muscle, which have shown similar results (Kort, A. A., and E. G. Lakatta, unpublished results), as well as experiments in rabbit myocytes in the present study, indicate that the role of spontaneous SR Ca\(^{2+}\)-release modulation of the peak twitch amplitude is not limited to rat preparations.

Mathematical Model of Effects of Spontaneous Ca\(^{2+}\) Release in Individual Myocytes on the Twitch in Intact Muscle

In order to explore the effects of spontaneous Ca\(^{2+}\) release on the contractility of intact muscle, we constructed a schematic mathematical model. The "contractility" of the "muscle" is represented by the average releasable Ca\(^{2+}\) of an ensemble of cells, subject to spontaneous Ca\(^{2+}\) release.

The model represents spontaneous Ca\(^{2+}\) release as a simple relaxation oscilla-
tor. When Ca from a store is released during the twitch, some is temporarily "lost" from that releasable store. The Ca\(^{2+}\) reloading of that store is depicted as a monoexponential process, tending toward an asymptotic level, \(y_0\), proportional to \(C_{ao}\), which, in our model, is defined as the "effective" \(C_{ao}\). The twitch amplitude in response to a test excitation at any time is proportional to the value of releasable stored Ca at that moment, \(y(t)\). If \(y(t)\) exceeds a threshold value, \(y_m\), the store is spontaneously released, resetting the system. It is important to note that this model is not intended as a complete model of the force-interval relationship. In its schematic form, it omits many phenomena. It ignores inhomogeneity of sarcomere lengths within cells and mechanical interaction among cells owing to spontaneous Ca\(^{2+}\) release (Kort and Lakatta, 1984; Kort et al., 1985a, b). Because only a single "compartment" of stored Ca is considered, the model has no memory for the effects of beats prior to the most recent one. In particular, the model does not consider explicitly the mechanism of spontaneous Ca\(^{2+}\) release, but makes use of one salient attribute, the fact that spontaneous release appears always to occur at a time when releasable stores have approximately the same level (see Fig. 8). The actual mechanism of spontaneous release is unknown. Fabiato (1985) has argued that it differs from "Ca-induced Ca release" and occurs only when the SR is "overloaded" with Ca. Model calculations (Stern et al., 1984) show that spontaneous release could occur as the result of regeneration of Ca-induced Ca release. Because the feedback gain for such regeneration depends on the SR Ca\(^{2+}\) concentration, the latter can show an approximately constant critical value at the onset of spontaneous release, even though sensing of Ca\(^{2+}\) does not occur within the SR. Incorporation of such a mechanistic model into the calculations presented here would be computationally prohibitive and would not add to our understanding. As seen below, the qualitative explanatory power of the simple schematic model is considerable, and shows that the phenomenon of spontaneous Ca\(^{2+}\) release can profoundly affect the behavior of whole muscle in ways that are not sensitive to the details of its mechanism.

In the schematic model, a single resting cell will execute periodic releases of Ca\(^{2+}\) with period

\[ t_m = \log \left( \frac{y_0}{y_0 - y_m} \right). \]

(1)

and between releases, the stored Ca will be given by

\[ y(t) = y_0(1 - e^{-t}), \]

(2)

where we have taken the time constant for reloading of the store as the unit of time.

We now consider an ensemble of cells, whose Ca stores are synchronously released by a stimulus at \(t = 0\). With time following this synchronous release, SR Ca\(^{2+}\) reloading occurs. If \(y_0\) is greater than threshold \(y_m\), each cell will execute independent oscillations until the next stimulus. During this interval, there will be progressive dephasing of cells owing to statistical variability in their oscillation frequencies (Stern et al., 1983). We represent this cell-to-cell variation (for
computational purposes) as being due to a statistical distribution of the release thresholds, $y_m$, which, for simplicity, are assumed to be normally distributed about a mean value (variability of uptake rates or of $y_0$ would give similar dephasing effects). The aggregate twitch amplitude for a stimulus at time $t$ is given by Eq. 2, evaluated for the time elapsed since the last spontaneous release, and ensemble-averaged over the distribution values of $y_m$. The time since the last release, for any given cell, will be that time since the last stimulus, less any whole multiples of the cell's oscillation period, $t_m$. We find, therefore, for the contractility at time $t$, in the presence of an effective $C_{ao}$, $y_0$, the expression

$$F(y_0, t) = y_0 \int_0^\infty \left(1 - \exp[t_m(y_m, y_0) \text{INT}[t/t_m(y_m, y_0)] - t]\right)P(y_m)dy_m,$$

where INT means "integer part of" and $P(y_m)$ is the probability distribution of the cellular thresholds.

Another quantity of importance is the frequency $R$ at which asynchronous spontaneous $Ca^{2+}$ releases (contractile waves) occur throughout the muscle. A given cell will undergo its $n$th spontaneous release when $t = nt_m$. The cells undergoing their $n$th release between $t$ and $t + dt$ are therefore those with values of $t_m$ between $t/n$ and $(t + dt)/n$, whose number is given, using Eq. 1, by

$$R_n dt = P[y_0(1 - e^{-t/n})]y_0/n e^{-t/n} dt.$$

Summing up over $n$, we find for the total number of release events per unit time

$$R(t) = \sum_{n=1}^\infty P[y_0(1 - e^{-t/n})]y_0/n e^{-t/n}.$$

Eqs. 3 and 5 are suitable for numerical evaluation, which was done using the modeling language MLAB.

We considered first the macroscopic twitch amplitude of a muscle stimulated regularly at frequency $f$, as a function of $C_{ao}$. This is given by Eq. 3, with $y_0$ as the independent variable and $t$ fixed at $1/f$. Fig. 9 shows the results for two stimulation rates. Initially, the twitch amplitude rises with increasing $C_{ao}$ to a degree inversely proportional to stimulation rate. Eventually, when the average spontaneous release frequency exceeds the stimulation rate, the twitch amplitude declines with further $Ca^{2+}$ increases. A strikingly similar biphasic $Ca^{2+}$-twitch relationship as the frequency of spontaneous oscillations increased with increases in $C_{ao}$ in rat muscle has been demonstrated previously (Lakatta and Lappe, 1981, Fig. 5). Because of the overdrive suppression of spontaneous $Ca^{2+}$ release by electrically stimulated $Ca^{2+}$ release (Capogrossi et al., 1986c), the $C_{ao}$ at which the peak twitch occurs is higher at higher stimulation rates, as shown in Figs. 1 and 3 of the present study for isolated cells, and as found in intact rat papillary muscles (Kort, A. A., and E. G. Lakatta, unpublished results) and in a recent study on the relationship between the rate of stimulation and the force developed by highly $Ca$-loaded canine Purkinje fibers (Kotake and Vassalle, 1986). A concept that emerges from the model and is depicted in Fig. 9 is that for the ensemble average of cells in which spontaneous $Ca^{2+}$ release occurs, maximum contractility never reaches the peak value of which one cell is capable because
some cells have spontaneously released before others reach the maximum average force. This raises the issue of what defines "excessive cell Ca loading" at the intact tissue level. As shown in Fig. 4, the average systolic shortening of an ensemble of cells can continue to increase, even though some cells begin to exhibit spontaneous Ca$^{2+}$ release. Thus, at the level of intact muscle, excessive Ca loading must be defined not by the presence of spontaneous Ca$^{2+}$ release in some cells but as that state when the magnitude of spontaneous Ca$^{2+}$ release is sufficient to limit the average systolic function among the cells, i.e., to limit twitch force.

Without adding any additional features to our model, we are at liberty to "slice" Eq. 3 the other way, keeping $C_{ao}$ fixed and varying $t$, the time elapsed since the last stimulus, i.e., as for the single myocyte in Fig. 8 of the present study. It then becomes a model of time-dependent restitution of contractility with Ca$^{2+}$ as a parameter (Fig. 10). In low $C_{ao}$ ($y_0$ in Eq. 3), restitution is a monotonic, roughly exponential process with the plateau contractility increasing in proportion to $C_{ao}$. If $y_0$ does not exceed $y_m$, no spontaneous release occurs and contractility is limited not by spontaneous Ca$^{2+}$ release, but by other factors that limit the level of $y_0$. When $C_{ao}$ exceeds the average threshold for the onset of spontaneous Ca$^{2+}$ release ($y_m$ in Eq. 1), the maximum contractility ceases to increase, and the pattern of restitution becomes oscillatory. A further increase
in Ca, shortens the period of the damped oscillations as well as lowering the plateau that occurs at long restitution times. The bulk rat cardiac muscle exhibits a Ca, dependence of restitution phenomena similar to that simulated by the model in Fig. 9 (Posner and Berman, 1969; Kort and Lakatta, 1985).

The frequency of asynchronous Ca$^{2+}$ release mentioned above can be observed indirectly by its contribution to diastolic tone, and more directly by laser scattering intensity fluctuations (Stern et al., 1983; Kort et al., 1985b). Each release event will make a tiny brief contribution to the tension of the muscle; the aggregate of these will give rise to an active diastolic tension proportional to the instantaneous rate of release events in the muscle (Stern et al., 1983). In Fig. 11 we have plotted restitution curves for twitch and resting force (taken to be proportional to the frequency of spontaneous Ca$^{2+}$ release) in high and low Ca,.

![Figure 11](https://example.com/figure11.png)

**Figure 11.** Restitution of average Ca$^{2+}$ release in response to an action potential (contractility), and active diastolic tension (proportional to mean frequency of spontaneous release events; Stern et al., 1983; Kort et al., 1985b) for two levels of "effective Ca," defined as in Fig. 9. The units of time and Ca$^{2+}$ are the same as in Figs. 9 and 10. The vertical scales are arbitrary, but are the same for panels A and B (see text for details).

from Eq. 5. As shown in the figure, the onset of oscillatory restitution of twitch force is accompanied by the development of damped periodic aftercontractions. The oscillations of twitch restitution lead the aftercontractions in phase by ~90 degrees, because the peak of contractility occurs when the largest number of cells are maximally Ca loaded and have not yet undergone spontaneous release. Oscillatory restitution and periodic aftercontractions have long been known to occur during high Ca loading, especially in the rat and the dog, species that are prone to spontaneous Ca$^{2+}$ release (Posner and Berman, 1969; Ferrier, 1976). The phase lag of the aftercontractions relative to contractility has also been observed (Ferrier, 1976; Lipsius and Gibbons, 1982). The present model interprets the aftercontraction as a "storm" of asynchronous spontaneous Ca$^{2+}$-release events, clustered in time because of the synchronizing influence of the previous stimulus. If this interpretation is correct, then scattered light intensity fluctuations, which are a manifestation of the microscopic motion owing to these release events, should vary in phase with the aftercontractions rather than with the
restitution of contractility. This has recently been confirmed (Kort, A. A., and E. G. Lakatta, unpublished results). Previous observations of the phase relationships between contractility and diastolic tension have been taken as evidence for oscillatory exchange of Ca$^{2+}$ among two or more anatomical intracellular compartments (Posner and Berman, 1969). Our model shows that these effects could be produced by the statistical effect of spontaneous Ca$^{2+}$ release, without assuming any compartmentation. It is clear, then, that the deduction of mechanisms of excitation-contraction coupling from the macroscopic behavior of whole muscle must be undertaken with great caution.

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