Restitution of contractility in single ventricular myocytes of guinea pig heart

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Abstract

Objective: Our aim was to assess the extent to which changes in intracellular Ca2+ stores contribute to mechanical restitution in heart muscle. Methods: Single, isolated guinea pig ventricular cells were voltage clamped at -45 mV and stimulated continuously at 0.5 or 2 Hz with 200 ms depolarizing pulses (35°C). The recoveries of the peak of contraction force (Fp) and the calcium current (Ica) between beats were measured in contractions interpolated at various intervals (td) after a conditioning twitch. Recovery of SR Ca2+ load was inferred from the peak magnitude (Cp) of similarly interpolated contractures, induced by rapid application of 5 mM caffeine. Results: For a conditioning stimulus rate of 0.5 Hz, both Fp and Ica were very small for small td and recovered along similar time courses with a td of about 50 ms. Cp was maximal at as early a time after a previous contraction as could be measured, at which time Fp was 56% of maximal. Cp declined throughout the stimulus interval to about 50% of its maximal value. Similar results were obtained for a conditioning stimulus rate of 2 Hz, at which rate both Fp and Cp were increased by a factor of 2. Conclusions: The time course of mechanical restitution is coincident with the recovery of Ica from inactivation. Caffeine-releasable intracellular calcium stores are fully recovered soon after a contraction and well before mechanical restitution is complete.

Keywords: Contractile function; Caffeine; Guinea pig, ventricular cells; Calcium, intracellular concentration; Calcium channel, L-type

1. Introduction

The action potential duration and the force of contraction of cardiac muscle vary with the preceding stimulus interval. In regularly-stimulated guinea pig heart, an extra stimulus interpolated soon after a steady-state contraction elicits an action potential that is shorter in duration than the control, and a twitch of markedly less force than the control. The gradual restoration of the action potential duration and contractile force during diastole are called electrical and mechanical restitution, respectively [1-4]. Although these phenomena have been described in various preparations from different mammalian species, the underlying mechanisms remain uncertain. Electrical restitution is thought to be dependent on the recovery of membrane currents from inactivation and/or interval-dependent changes in the ionic composition of the intracellular solution [3,5]. Mechanical restitution has been explained in terms of (1) release of Ca2+ from the sarcoplasmic reticulum (SR) in proportion to the Ca2+ current (Ica) [6], (2) slow replenishment of the releasable Ca2+ store of SR [7,8], (3) time-dependent recovery of the SR release channel from inactivation [9], and (4) a slow reduction in activity of the SR calcium pump during diastole [10]. In fact, the restitution process could be any combination of these factors, whose contribution might vary with the inotropic state of the muscle.

In most previous studies mechanical restitution has been measured from multicellular preparations, and reflects a mixture of the effects of changes in the duration of the action potential and the effects of whichever other factors may be important in controlling contraction strength [7,11]. In our experiments we measured electrical and mechanical restitution (of force) in enzymatically isolated cells of

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guinea pig ventricle under voltage clamp conditions, where the duration of activation can be kept constant and the main trigger of Ca\(^{2+}\) release \((I_{\text{Ca}})\) can be accurately measured. Thus we can quantitatively estimate the importance of the recovery of \(I_{\text{Ca}}\) in mechanical restitution. Furthermore, with this preparation, we can estimate the SR Ca\(^{2+}\)-content from the current and force induced by fast application of caffeine to the cell, irrespective of the state of the SR calcium-release channel \([9,12]\). We find that electrical and mechanical restitution have similar time courses, while the recovery of caffeine-releasable calcium stores is much more rapid than either.

2. Methods

2.1. Enzymatic isolation of ventricular myocytes

Guinea-pigs weighing between 250 and 500 g were deeply anaesthetized with 65 mg/kg sodium pentobarbital. The heart was rapidly excised and the aorta cannulated for coronary perfusion with oxygenated solutions (35-36°C). The heart was first perfused for 5 min with a nominally Ca\(^{2+}\)-free, low sodium solution (LN) containing (mM): 100 NaCl, 10 KCl, 1.2 KH\(_2\)PO\(_4\), 50 taurine, 4 MgSO\(_4\), 20 glucose, 10 HEPES at pH 6.9. The perfusate was then switched to a solution of the same composition with the addition of 1 mg/ml collagenase (Sigma Type I), 1 mg/ml fatty-acid-free serum albumin (Sigma A-6003) and 100 μM of CaCl\(_2\). CaCl\(_2\) was added in 4 aliquots during the enzyme perfusion to bring the total added calcium to 200 μM. The heart was then minced and further incubated, with continuous stirring, in 5–10 ml of the same enzyme solution with the addition of 0.07 mg/ml protease (Sigma Type XIV). At 5–10 min intervals the supernatant, containing dissociated cells, was drawn off and replaced by more enzyme solution. The cell suspensions were diluted into LN, to which had been added 1 mM CaCl\(_2\) and 1 ml/100 ml penicillin/streptomycin (Sigma P-0781), and stored at 23°C. Also, some cell suspensions were allowed to settle for several minutes in enzyme solution (approx. 4°C). The supernatant was then decanted and the cells were covered with a depolarizing nutrient solution for storage at 4°C [13]. This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.2. Force measurement and voltage clamp

For experimentation, the cells were placed in a Lucite chamber (3.0 ml) and continuously superfused with a tip-warmed (35°C), modified Tyrode solution (NT) composed of (mM): 150 NaCl, 5.4 KCl, 1.2 MgCl\(_2\), 5 Hepes, 1.8 CaCl\(_2\), 5.5 glucose, pH adjusted to 7.4 with NaOH. Details of the force measurement from single cells has been described in previous papers [14,15]. In short, the ends of an isolated cell were attached to the tips of two glass rods (diameter = 76 μm) using poly-L-lysine (MW 30000–70000, Sigma) as a glue. The length, width and thickness of the cell were measured by rotating the rod pair by 90°, so that cross-sectional area and cell volume could be estimated. The edge movement of the rods (<3% of free cell length, i.e. 1–1.5 μm) was detected by two phototransistors located in the focal plane of the microscope objective (40×, Nikon). The force of contraction of a myocyte was calculated from the known compliance of the rods (0.76 m/N) and normalized to the cross-sectional area.

After a cell was successfully fixed on the rods, a patch pipet was attached near the center of the cell and the whole-cell clamp condition established. Patch pipets were about 2 μm in tip diameter with a resistance between 1.6 and 3 MΩ when filled with a solution containing (mM): 130 KCl, 10 HEPES, 5 K-oxaloacetate, 5 K-succinate, 1 MgCl\(_2\), 0.02 EGTA, 5 mM Na\(_2\)ATP, pH 7.4.

After obtaining the whole-cell voltage-clamp condition, a cell was stimulated initially at a regular rate of 0.5 Hz with 55 mV depolarizing pulses from a holding potential of −45 mV. The time course of mechanical restitution and the SR calcium content were then determined for various pulse protocols, as described below. In most cases, when the results of multiple protocols were to be compared, the protocols were applied in a completely bracketed format. For example, if the results from protocols a, b and c are to be compared, the protocols are applied to the cell in the pattern abca and the results from duplicate protocols are averaged before a comparison is made among the results. We report here only the results of complete experiments—i.e., those in which each protocol was applied in one cell.

2.3. Solution changes

Caffeine contractures were induced by brief exposure to otherwise normal Tyrode solutions containing 5 mM caffeine. The caffeine-containing solution was applied by means of a double jet system described previously [15]. The position of the jets was monitored by a LED/phototransistor pair, the output of which was recorded simultaneously with the electrical and mechanical signals from a cell. In most experiments the flow velocity was approximately 50 mm/s, permitting a fast and well-controlled release of intracellular calcium by caffeine. In other cases, noted in the text or figure legends, the flow rate was reduced to 13 mm/s to allow a larger percentage of complete experiments. Some experiments were made with both flow rates (in different cells) with no qualitative difference in the results.

2.4. Recording and analysis

Current and force signals were digitized (Instrutech, VR-100, 9 kHz/channel, 4 channels) and stored on video.
tape, with concurrent strip chart recording (Gould 2400). Off-line analysis was made by recreating the analog signal, redigitizing each channel at 2.5 kHz and analyzing the records with programs written in Asyst (Keithly-Asyst). The inward Ca\(^{2+}\) current (\(I_{\text{Ca}}\)) was estimated as the difference between the peak inward current and the steady-state current at the end of the pulse (visual estimate [16]). The peak force of the twitch in response to an electrical pulse (\(F_p\)) was measured, after filtering the signal at 100 Hz, as the difference between the force level just prior to the pulse and the maximum force during the pulse. For contractions interpolated at diastolic intervals of less than 200 ms, a control record was subtracted from the record of interest to remove the relaxation phase of the previous conditioning contraction. In practice, this affected \(F_p\) only for contractions interpolated at intervals less than 40 ms. The peak contracture force (\(C_p\)) is the difference between the force level before the solution-supply jets were moved and the maximal force. A subtraction similar to that described for electrically driven contractions was performed for contractures interpolated within 200 ms of a previous conditioning contraction.

2.5. Measurement of intracellular calcium store

To demonstrate the recovery of intracellular calcium stores between contractions, the cells were stimulated at 0.5 or 2.0 Hz, and caffeine contractures were elicited every 5–10 beats by switching the position of the perfusion jets at various times after a contraction. Ideally, in order to compare mechanical restitution with the recovery of releasable intracellular calcium, we would like to be able to release the intracellular stores by caffeine application as quickly as possible, preferably as quickly as the release initiated by depolarization, via calcium-induced calcium release [17,18]. With our technique, caffeine contractures had rising phases comparable to those of the electrically driven twitch, beginning after a latency of about 90 ms (including the 35 ms needed for the caffeine to reach the cell from the supply jet, Fig. 1). The time between the initial motion of the jets (lowermost trace in Fig. 1A) to the peak of contracture force for a 100 ms pulse of caffeine was 166 ± 13 ms (\(n = 5\)). Thus, the time between the onset of contracture force and the peak of force was about 76 ms, similar to the time to peak (TTP) of electrically driven contractions in these cells [14].

In addition to being very fast, the time courses of both the contracture and the caffeine-induced inward current were very reproducible (Fig. 1B). The 3 contractures for 100, 200 and 300 ms applications of caffeine were obtained sequentially, separated by 6 conditioning contractions. The rising phases of force and current are superimposable for all 3 contractures and the relaxation of the contractures in response to 200 and 300 ms caffeine applications were also essentially identical. The waveforms of the inward currents induced by caffeine were likewise very similar (lower traces, Fig. 1B). These currents reflect the extrusion of calcium by the Na/Ca exchange, following the release of calcium from the SR [19]. The similarity of the currents for all applications of caffeine of 50 ms duration or longer suggests that the release of calcium occurs very rapidly.

Relaxation of a contracture was slower for a 200 ms caffeine application than for a 100 ms application (Fig. 1B). The slow relaxation could simply reflect the continued presence of caffeine and its potentiating effect on the myofilaments [20]. Alternatively, some resequestration of calcium by the SR might occur during relaxation after the shorter caffeine application due to the rapid washout of caffeine. However, assuming that calcium release by caffeine is monotonically related to the SR content, neither incomplete release nor resequestration will qualitatively affect our principal results or conclusions. We conclude that the calcium release induced by the rapid application of caffeine for 100 ms is sufficiently fast and reproducible to reflect putative changes in SR calcium content during the period of mechanical restitution.
3. Results

3.1. Characteristics of the electrically driven contraction

Under our standard conditions, the electrically stimulated cell responded with a two-component contraction. The phasic component, which relaxed during depolarization, was the component which exhibited restitution (Fig. 2B). The more slowly developing tonic component relaxed only upon repolarization, and showed no restitution between beats (Fig. 2B). The tonic component remained steady for several hundred milliseconds if depolarization were prolonged, and relaxed immediately upon repolarization (not shown). It is not to be confused with the oscillatory contractions associated with calcium overload which appear after repolarization and which are associated with an oscillatory current. The phasic component is assumed to reflect the release of intracellular calcium stores and the entry of calcium from the extracellular space mainly via \( I_{Ca} \), while the tonic component presumably reflects a balance between calcium sources and sinks, and depends to a large extent on \( \frac{Na^+}{Ca^{2+}} \) exchange [21].

3.2. Restitution of \( I_{Ca} \) and twitch force

Mechanical restitution and recovery of \( I_{Ca} \) from inactivation was determined by interpolating extra beats at diastolic intervals (\( t_d \)) between 20 and 1000 ms, with 5–10 conditioning contractions separating successive test pulses (Fig. 2A). In Fig. 2C, \( F_p \) of the interpolated beat (or rate of rise of force, \( dF/dt_{max} \), or \( I_{Ca} \)) is plotted against \( t_d \) (i.e., the time between the end of the control clamp pulse and the beginning of the test clamp pulse).

\( I_{Ca} \), \( F_p \) and \( dF/dt_{max} \) recover very quickly after a previous depolarization, along similar trajectories (Fig. 2C and inset). The recovery of \( F_p \) is slightly slower than that of \( dF/dt_{max} \), possibly as a result of a small increase in the time to peak force (TTP) through the initial period of diastole (see below). The recovery of \( I_{Ca} \) appears to be biphasic, approaching its control value rather slowly after an initial fast phase of recovery (Fig. 2C, open circles).

For quantitative comparisons among \( I_{Ca} \), \( F_p \) and \( dF/dt_{max} \), we fitted a single exponential function to the averaged results from 6 cells (Fig. 2C, see Section 4). The recovery of \( I_{Ca} \) from inactivation had a time constant of 92...
ms, while the restitution of $F_p$ and $\frac{dF}{dt_{\text{max}}}$ had time constants of 103 and 71 ms, respectively. We also fitted single exponentials to data from individual cells and have plotted in Fig. 2D, for each cell, the time constant for the recovery of $I_{\text{Ca}}$ from inactivation ($K_i$) against the time constant for mechanical restitution ($K_r$). Though the rates of recovery of both $I_{\text{Ca}}$ and $F_p$ varied widely among cells in this study, the recovery of current and force always had similar time courses in a given cell, and so $K_i$ and $K_r$ were well correlated over the range of our results (Fig. 2D, $r = 0.92$). The standard errors of the values of the rate coefficients were too small to show in the figure, being, on average, about 0.007 of $K_i$ and 0.002 of $K_r$.

At the end of the period of mechanical restitution the ratio of $F_p$ of the interpolated contraction to that of the control was slightly greater than one, and similarly for $\frac{dF}{dt_{\text{max}}}$ (Fig. 2C). This ‘overshoot’ was consistently seen and means that $F_p$ and $\frac{dF}{dt_{\text{max}}}$ must decline during the latter part of the stimulus interval. This decline may be related to the decline of caffeine-releasable intracellular calcium stores that occurs throughout the stimulus interval (Fig. 3).

The TTP for interpolated contractions was smaller than for the conditioning contractions. At $t_d = 260$ ms the TTP was $80\% \pm 10\%$ (± s.d., $n = 6$) of the TTP of conditioning contractions, recovering slowly thereafter. A precise time course of the change in TTP was difficult to determine due to scatter in the data, which was, in turn, due to small amounts of mechanical noise. Nevertheless, for $t_d$ between 260 and 720 ms, the difference between the TTP of the interpolated beat and that of the immediately preceding conditioning contraction was statistically significant ($P < 0.05$, paired $t$-test).

3.3. Interpolated caffeine contractures

Caffeine is known to release $\text{Ca}^{2+}$ from the SR regardless of the state of the SR release channel [9,12]. Therefore, putative inactivation of the channel following a previous depolarization and release [12] should not prevent the

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Fig. 3. Diastolic decay of $C_p$ of interpolated contractures. (A) Caffeine contractures initiated between 5 and 1560 ms after a conditioning contraction. Duration of caffeine application = 100 ms. (B) Superimposed caffeine contractures and associated transient inward currents from panel A on an expanded scale; $t_d = 5$ ms (1), 705 ms (2) and 1560 ms (3). (C) Averaged values of $C_p$ and the $I_{\text{in}}$ from 5 experiments such as that in panel A. Each force measurement was normalized before averaging to the $F_p$ of the previous conditioning contraction, then divided by the value of $C_p$ for $t_d = 705$ ms before plotting. The integrated currents were averaged and divided by the value for $t_d = 705$ ms before plotting.
subsequent release of SR calcium by caffeine. Thus, if the SR calcium level were to change during the diastolic interval, the change should be reflected in the magnitude of the caffeine contracture and in the magnitude of the current accompanying the contracture.

In experiments analogous to those used to study mechanical restitution, caffeine was applied for 100 ms at intervals ranging between 5 ms and 1005 ms after a previous contraction, with one additional application at an interval of 1560 ms (Fig. 3). In contrast to the post-stimulation recovery of $F_p$, which is illustrated in Fig. 2, the strength of the interpolated caffeine contractures declined almost monotonically throughout the diastolic interval to 47% of its maximal value (Fig. 3). A small depression of $C_p$ at the beginning of the interval was probably due to a slight overlap of the caffeine application and the relaxation of the tonic phase of the previous contraction. In other experiments, we noted that when such overlap occurred, the waveform of the caffeine-induced current began much earlier and reached a smaller peak though the integral of the caffeine-induced current was not decreased (not shown). Consistent with this interpretation, $I_{a eff}$ declined smoothly throughout the interval to 58% of its maximal value. The waveform of both the contracture and the current remained relatively constant throughout that period (Fig. 3B). These results suggest that the SR calcium declines markedly between contractions under our experimental conditions.

However, the plots in Fig. 2C and Fig. 3C are not directly comparable. In Fig. 3C, $C_p$ and $I_{a eff}$ are plotted against $t_d$, as defined in Fig. 1, which means that the onset and the TTP's of the contractures are delayed by about 90 ms relative to depolarization-induced contractions plotted on the same time scale (as they are in Fig. 2). Also, the contracture results in Fig. 3C and the contraction results in Fig. 2C were obtained from completely separate groups of cells. Although the results in these two figures suggest that the recovery of contractility after a contraction occurs without a substantial change in the level of SR calcium, a more rigorous comparison was devised to demonstrate this point.

![Fig. 4. Comparison of caffeine contractures and electrically-driven contractions in the same cell. Panel A: Superimposed records, from one cell, of caffeine-induced contractures (A1) and electrically-driven contractions (A2) that were interpolated at diastolic intervals so arranged that the TTP of each contracture was nearly identical with the TTP of one of the contractions. (A1) Force (upper), current (middle) and position of the solution supply jets (lower), for three 100 ms caffeine applications. Conditioning pulses at 0.5 Hz, $t_d = -23, 127$ and 277 ms. $[\text{Ca}]_o = 1.8 \text{ mM}; \text{Temp.} = 35^\circ \text{C}$. (A2) Force (upper) and current (lower) for electrically-driven contractions from the same cell as in A1. $t_d = 70, 220$ and 370 ms. Panel B: Contraction at 370 ms and contracture at 277 ms from panel A are shown on an expanded time scale. The vertical scale of the contracture was adjusted to facilitate comparison of the time courses. Both records begin 350 ms after the end of the control pulse. Panel C: Comparison of $C_p$ and $F_p$ for 5 experiments of the kind shown in panel A. Ordinate: $F_p$ and $C_p$ were normalized to $F_p$ of the preceding conditioning pulse before averaging among the cells. The resulting averages are scaled so that both $C_p$ and $F_p$ are equal to 1 for the largest interval of interpolation. Abscissa: The mean TTP, from the end of the previous depolarizing pulse, of either contractions or contractures. Note that this means of plotting is different from that in Figs. 2 and 3, where the abscissa was $t_d$.](image-url)
3.4. SR content and mechanical restitution measured in the same cell

As pointed out earlier and shown again in Fig. 4B, the waveforms of contractions and contractures are very similar, suggesting that the processes leading from calcium release to contraction are similar in the two cases. Therefore, in order to compare depolarization-induced calcium release and caffeine-induced calcium release at the same points in time after a previous contraction, we have compared the peak force development among contractures and contractions having the same time to peak relative to the end of the previous depolarizing pulse (Fig. 4A). In 5 experiments, cells were driven at 0.5 Hz and extra pulses were interpolated at intervals of 70, 220 and 370 ms after the end of the previous depolarization (Fig. 4A2). Then, in the same cell, caffeine contractures were elicited by re-positioning the jets at intervals of -23, 126 and 277 ms after the end of the previous depolarization (Fig. 4A1). This protocol resulted in 3 contractures which had nearly the same times to peak as the 3 contractions interpolated in the first part of the protocol (Fig. 4C). It is clear from the experimental records in Fig. 4A and from the cumulative results in Fig. 4C that \( C_p \) of contractures elicited early in the diastolic period actually declines (slightly) at a time when \( F_p \) is still increasing markedly. The normalized values of \( C_p \) and \( F_p \) were significantly different (\( P < 0.001 \) for \( t_{ATTP} = 130 \) ms and \( P < 0.01 \) for \( t_{A,TTP} = 285 \) ms, paired \( t \)-test).

3.5. Voltage- and frequency-dependent potentiation

A possible problem in interpreting the foregoing results with respect to the underlying cause of mechanical restitution is that activation of the conditioning contractions under the protocol given in Fig. 2 may not depend much on intracellularly stored calcium. This is suggested by the fact that \( F_p \) of the contractions in response to stimulation at 0.5 Hz was reduced only to 64 ± 5% of control (mean ± s.d., \( n = 5 \)) by a prior application of 5 mM caffeine for 200 ms during the previous diastole (from experiments in Fig. 1). If it were assumed that caffeine released 100% of intracellular calcium (and that there was no re-uptake by the SR), so that the first post-caffeine contraction depended for activation entirely on extracellular calcium, the relative contributions of extracellular calcium and intracellular calcium to activation in conditioning contractions would be about 2:1 (64:36). This is a worst case since there may be re-uptake of calcium by the SR after the caffeine is removed. Furthermore, the calcium content of the SR is apparently much larger just after a depolarization than it is just before a depolarization (Fig. 3) and \( I_{Ca} \) is quite small immediately following a previous contraction. Therefore, contractions interpolated early in the stimulus interval may depend much more on intracellular calcium for activation than do the conditioning contractions. Nevertheless, it was of interest to study restitution under conditions in which conditioning contractions appear to depend to a greater extent on intracellular calcium stores for activation.

We first show that caffeine-releaseable SR calcium stores can be loaded both by increasing the pulse potential (to +30 mV) and by increasing the frequency of stimulation. The increase of pulse potential was useful to facilitate frequency-dependent potentiation, which is known to be voltage-dependent [22]. Cells were stimulated with 3 separate protocols in a bracketed format (Fig. 5, see Section 2.2): A, 0.5 Hz with the pulse potential = +10 mV; B, 0.5 Hz with pulses to +30 mV; C, 2.0 Hz with pulses to +30 mV. In each case caffeine contractures were interpolated by switching the position of the jets 20 ms prior to the end of the depolarizing pulse. Simply changing the pulse potential while stimulating at 0.5 Hz increased contracture strength slightly (\( C_p = 2.10 ± 1.42 \) and 2.73 ± 1.81 mN/mm² for +10 and +30 mV, respectively; \( n = 4, P < 0.05, \) paired \( t \)-test; Fig. 5A,B) but did not increase the strength of the conditioning contraction (\( F_p = 1.80 ± 0.98 \) and 1.73 ± 0.84 mN/mm² for +10 and +30 mV, respectively). Increasing the stimulus frequency to 2 Hz markedly increased both \( F_p \) and \( C_p \) (\( C_p = 3.82 ± 1.89 \) mN/mm² and \( F_p = 4.02 ± 1.40 \) mN/mm², Fig. 5C). The probability that a difference of more than 1 mN/mm² between \( C_p \) at 0.5 Hz and 2.0 Hz (+30 mV) was due to chance was very small (\( P < 0.005, \) paired \( t \)-test).

Fig. 5. Potentiation of contractions and contractures by increasing pulse voltage and/or frequency of stimulation. (A–C) Digitized records of force (upper) and current (lower) for 3 pulse protocols. (1) conditioning contractions, (2) contractures elicited by 200 ms applications of caffeine at \( t_p = -20 \) ms, and (3) the first contractions after the caffeine contracture. Holding potential = -45 mV; \([Ca]_o = 1.8 \) mM; flow velocity = 13 mm/s. (A) Pulse potential = +10 mV; basic rate = 0.5 Hz. (B) Pulse potential = +30 mV; basic rate = 0.5 Hz. (C) Pulse potential = +30 mV, basic rate = 2 Hz. In protocol C the depolarization immediately following the caffeine application was delayed (by omitting one beat) to permit washout of the caffeine.
Importantly, a caffeine application between contractions at 2 Hz reduced \( F_P \) of the first post-caffeine contraction to 0.30 ± 0.02 of control (Fig. 5C), while at 0.5 Hz, \( F_P \) of the first post-caffeine contractions were reduced to 0.66 ± 0.10 and 0.65 ± 0.04 of control for a pulse potential of +10 and +30 mV, respectively. The strong suppression of contraction by a prior application of caffeine suggests that the contraction at a stimulation rate of 2 Hz depends much more on intracellular calcium than does the contraction at 0.5 Hz. This result is consistent with the increased caffeine-releasable calcium content and the reduction of \( I_{Ca} \) characteristic of 2 Hz stimulation (Fig. 5).

3.6. Mechanical restitution in the potentiated state

In the potentiated state, it was not possible to study both the time course of mechanical restitution and the restoration of SR calcium stores in the same cells. Therefore, we compared mechanical restitution and recovery of \( I_{Ca} \) in one group of cells, and measured the strength of interpolated caffeine contractures in another group (Figs. 6 and 7).

In these experiments, restitution was first measured at a basic rate of 0.5 Hz, then at 2 Hz and then once more at 0.5 Hz. In each case extra stimuli were interpolated at \( t_d = 20, 60, 100 \) and 300 ms. \( F_P \) of the interpolated contractions was normalized to \( F_P \) of the contractions at \( t_d = 300 \) ms, which for the 2 Hz case was the conditioning contraction just preceding each interpolated contraction. \( F_P \) of the conditioning contractions at 2 Hz was 189 ± 31% (n = 3) of that at 0.5 Hz. Despite the increased dependence of contractility on intracellular calcium at the higher rate of stimulation, mechanical restitution and recovery of \( I_{Ca} \) from inactivation again had similar time courses (Fig. 6C,D). In these experiments, the time constants of recovery of \( I_{Ca} \) and \( F_P \) were 64 and 76 ms, respectively, for 0.5 Hz stimulation, and 79 and 70 ms for 2.0 Hz stimulation.

It should be noted that the TTP of contractions at 2 Hz was smaller than that at 0.5 Hz (Fig. 6B). The TTP's of interpolated beats at a basic rate of 2 Hz were even smaller than those of the conditioning contractions (e.g., 90 ± 3% for \( t_d = 260 \) ms, n = 3), similar to the results obtained at a basic rate of 0.5 Hz.

3.7. SR calcium content between beats in the potentiated state

In this series of experiments the cells were driven at 2 Hz and caffeine contractures were interpolated at \( t_d = -20 \) and 80 ms (Fig. 7A). \( C_p \) of the earlier contracture in each experiment was divided by that of the later contracture and the normalized \( C_p \) plotted against the TTP (Fig. 7). The data from the restitution experiments shown in Fig. 6D were replotted on the same time scale against their TTP's.

![Fig. 6. Restitution of contractility and recovery of \( I_{Ca} \) during diastole, with 2 Hz stimulation.](image-url)
Fig. 7. Comparison of caffeine contractures and electrically-driven contractions interpolated at equivalent times between potentiated contractions. (A) Superimposed records of conditioning contractions and contractures induced by 100 ms applications of caffeine for \( t_d = -20 \) and \( +80 \) ms, showing force (upper), current (middle) and position of solution-changing jets (lower). Conditioning stimuli at 2 Hz; holding potential = -45 mV; pulse potential = +30 mV; temp. = 35°C; [Cal] = 1.8 mM; flow velocity = 50 mm/s. (B) Comparison of Cr from 3 experiments such as in panel A and \( F_p \) for 3 experiments, replotted from Fig. 6. Ordinate: \( F_p \) and \( C_r \) were normalized to \( F_p \) of the preceding conditioning pulse before averaging among the cells. The resulting averages are scaled so that both \( C_r \) and \( F_p \) are equal to 1 for the largest interval of interpolation. Abscissa: The mean TTP, from the end of the previous depolarizing pulse, of contractions or contractures, as in Fig. 4. Note that this means of plotting is different from that in Figs. 2, 3 and 6, where the abscissa was \( t_d \).

The results of these experiments are quite similar to the results obtained at the lower rate of stimulation, though not as complete (compare Figs. 4 and 7). The difference between \( C_r \) and \( F_p \) at \( t_d \text{TTP} = 130 \) ms was highly significant (\( P < 0.001, t \) test). Likewise, the values of \( C_p \) (before normalization) at 130 and 230 ms were not significantly different. Thus, under conditions in which the caffeine releasable calcium has been almost doubled, mechanical restitution still occurs without a significant change in the SR calcium content, and its time course closely approximates that of the recovery of \( I_{Ca} \) from inactivation.

4. Discussion

We conclude that redistribution of intracellularly stored calcium is complete by at least the latter half of the period of mechanical restitution and that the rate of mechanical restitution is controlled mainly by the rate of recovery of \( I_{Ca} \) from inactivation. Support for this conclusion is provided by 3 observations: (1) caffeine-releasable stores actually decline slightly during the latter half of the period of mechanical restitution (Fig. 4), and so at least that portion of the restitution process cannot be attributed to increased availability of stored calcium; (2) the time courses of recovery of \( F_p \) and \( dF/dt_{\text{max}} \) are very similar to the time course of recovery of \( I_{Ca} \) under conditions in which the twitch strength should depend to a large extent on the level of caffeine-releasable intracellular stores (Figs. 2, 4, 6 and 7); (3) the rates of mechanical restitution and recovery of \( I_{Ca} \) correlate well in individual cells (Fig. 2D).

4.1. Intracellular calcium stores decline during diastole

One of the most often cited models of mechanical restitution is based on the hypothetical movement of calcium within the lumen of the SR in the period between contractions, such that the calcium taken up by the SR pumps during a contraction is not immediately available for subsequent release, but rather needs time to travel from the pumping sites to the release sites [23]. On the basis of this model it would be expected that the \( C_r \) of contractures initiated just after a previous contraction would increase with time after the contraction, as the SR stores became available (i.e., the time course of \( F_p \) and \( C_p \) just after a contraction should be similar). This is clearly not the case for the latter half of the period of mechanical restitution (Figs. 4 and 7). We interpret the nearly constant \( C_r \) of the caffeine contractures during the period of mechanical restitution to mean that the SR calcium stores are fully restored by this point in time and that the amount released by depolarization in this period depends directly on the magnitude of \( I_{Ca} \). This interpretation agrees with a previous similar conclusion based on the strength of rapid cooling contractures initiated just after a contraction [24]. An alternative explanation which we cannot exclude is that caffeine releases calcium from all parts of the SR, while depolarization releases calcium only from the junctional regions of the SR.

The decrease of caffeine-releasable calcium during diastole (Fig. 3) may be due to the spontaneous quantal release of calcium (‘sparks’) that has recently been demonstrated in both guinea-pig and rat cardiocytes [25,26]. The ‘spark’ rate has been shown to depend on the level of activation of the L-type calcium channel and thus to be voltage-dependent [25]. At the holding potential used in our experiments (-45 mV) the rate of loss of intracellular calcium could be in the mM/s range if the spark rate measured in rat heart applies under our experimental conditions [26]. On the other hand, twitch tension in guinea-pig heart declines between beats even at the normal resting potential (e.g., see [27]), at which potential the spark rate should be negligibly small.
4.2. The relation between $I_{Ca}$ and $F_p$

The time course of mechanical restitution is quite similar to that of the recovery of $I_{Ca}$ from inactivation, though not identical (Fig. 2). Identical time courses would not be anticipated since $F_p$ will depend on factors in addition to $I_{Ca}$—e.g., the amount of calcium available in the SR (Fig. 3) and the TTP at a given point in time during diastole. The calcium content of the SR appears to decline with time throughout the diastolic interval (Fig. 3), which would probably increase the apparent rate of mechanical restitution. The slow recovery of the TTP would, on the other hand, slow the recovery of $F_p$ but not the recovery of $dF/dt_{max}$. The data in Fig. 2 are consistent with these qualitative expectations.

The similarity of the rate of recovery of $F_p$ to that of $I_{Ca}$ is consistent with the known properties of the mechanism of e-c coupling in heart muscle. First, the sum of the calcium entering the extracellular space and that released from SR stores should be approximately proportional to $I_{Ca}$ since SR release is nearly proportional to $I_{Ca}$ [25] and since $I_{Ca}$ is also the main path of calcium entry at a pulse potential of +10 mV, with some contribution from Na$^+$/Ca$^{2+}$ exchange [28]. Second, $F_p$ has been shown to be proportional to the peak $[Ca]_i$ during a contraction [29]. Thus, $F_p$ and $dF/dt_{max}$ should be at least approximately proportional to $I_{Ca}$ if the SR calcium content is constant and the waveform of the $[Ca]_i$ transient does not vary greatly.

In a given cell, the rate of recovery of $F_p$ was well correlated with the rate of recovery of $I_{Ca}$ from inactivation though both rates varied widely among cells (Fig. 2D). This suggests that the similarity in time courses is not a coincidence, though it does not exclude the possibility that a third factor may control both rates.

The restitution of contractile force is faster in our preparation than in comparable preparations described in the literature. In voltage-clamped dog ventricular trabeculae at 30°C, Braveny et al. [30] found that $I_{Ca}$ recovered within 200 ms of the previous depolarization while force recovered with a half-time of 200–400 ms. The discrepancy here could well be due to the difference in experimental temperature. However, the mean time constant of mechanical restitution for guinea-pig ventricular myocytes in the present study (103 ms) is smaller than that measured in cat trabeculae at a similar experimental temperature (182 ms for a holding potential of -45 mV at 37°C) [6]. It is conceivable that the discrepancy here is related to the difference in extracellular calcium concentrations (3.6 mM in the earlier study) or the species difference, both of which might affect the rate of mechanical restitution [27].

The $I_{Ca}$ data are actually better fitted with the sum of two exponentials than with a single exponential, with time constants of 34 and 182 ms, comprising respectively 51 and 49% of the total current. The sums of squared deviations were $7.96 \times 10^{-3}$ and $1.69 \times 10^{-3}$ for the single and double exponential fits, respectively, and more detailed analysis indicated autocorrelation in the single exponential fit but not in the double exponential fit (Durbin-Watson statistic was 0.344 and 2.186, respectively). However, the $F_p$ data could not be reasonably fitted with two exponentials, probably due to the multiple influences on the time course of $F_p$ (e.g., changes in SR calcium content and changes in TTP). Thus, in order to compare the two sets of data, we fitted both with single exponentials.

The fast time constant for the recovery of $I_{Ca}$ from inactivation in our experiments (34 ms) is similar to the single exponential time constant measured in canine Purkinje cells (31 ms) at 37°C [31], and to the rate of action potential restitution in guinea-pig papillary muscles (38 ms) [32]. Biphasic recovery of $I_{Ca}$ from inactivation has been observed in Purkinje fibers of calf heart [33] and in the isolated myocytes of both rat and guinea-pig hearts [34], but no quantitative data are available under experimental conditions similar to ours.

4.3. Other models of restitution

It was recently proposed that both restitution and post-extrasystolic potentiation in heart muscle is the consequence of hyperactivity of the SR calcium pump immediately following a contraction-relaxation cycle [10]. One basis of the hypothesis was the observation that the TTP of early extrasystoles was smaller than later ones and that the reduction of the TTP could not be attributed to changes in the action potential or to changes in contraction strength per se. We likewise observed consistent shortening of the TTP during diastole. However, the diastolic changes of TTP were small in our experiments, while changes in the $dF/dt_{max}$ during restitution were large. Furthermore, if more rapid calcium pumping were to account for the small $F_p$ of a contraction which followed closely on another, a similar effect should be seen on $C_p$ and on the tonic phase of contraction, neither of which was the case. The consistent relationship between $I_{Ca}$ and $F_p$ suggests a causal relationship between these factors, and so we do not think that changes in SR pump activity can account for mechanical restitution, though it seems likely to play a role in frequency-dependent potentiation.

It is of interest that the TTP of contractions at 2 Hz was smaller than that at 0.5 Hz (Fig. 6B), a typical result of similar experiments with multicellular preparations of heart muscle [35]. This might be explained by a nonlinear relation between the intracellular calcium concentration and the rate of SR calcium pumping [36]. In any case, reduction of the TTP at high frequencies of stimulation is an important feature of cardiac muscle, and appears to be an intrinsic feature of e-c coupling in the cardiac cell.

To be complete, we should point out that the simplest explanation for our data is that: (1) activation of contraction does not depend on calcium-induced calcium-release (CICR) but rather depends entirely on extracellular cal-
cium entry via $I_{\text{Ca}}$; and (2) potentiation of contraction becomes more calcium-loaded. Although this interpretation goes against an immense body of data supporting the CICR hypothesis, a recent study with ferret myocytes has shown that CICR apparently fails when the calcium content falls to 60% of its maximal value [37]. Since we did not attempt to measure the relative calcium content of the SR in our experiments, we cannot exclude the possibility that we are working in a range in which CICR does not function.

4.4. Summary

Mechanical restitution and the recovery of $I_{\text{Ca}}$ from inactivation have very similar time courses in isolated, voltage-clamped guinea-pig cardiocytes, regardless of stimulation frequency. On the other hand, caffeine-releasable intracellular stores of calcium are replete before complete recovery of $I_{\text{Ca}}$. We conclude that mechanical restitution reflects recovery of the ability of membrane calcium channels to deliver activating calcium (to the myoplasm) and/or trigger calcium (to the SR release channels), and that the intracellular calcium stores are essentially constant during mechanical restitution.

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