Biphasic Contractions Induced by Milrinone at Low Temperature in Ferret Ventricular Muscle: Role of the Sarcoplasmic Reticulum and Transmembrane Calcium Influx

CLAIRE O. MALECOT, DONALD M. BERS, AND BERTRAM G. KATZUNG

The effects of milrinone were studied in ferret papillary muscle stimulated at various rates and temperatures from 23° to 36°C. In voltage-clamp experiments, 50 μg/ml (0.237 mM) milrinone induced a 2.1-fold increase in calcium current at 28° or 36°C. At 50 μg/ml, milrinone transiently increased contractility in all muscles at 28°C, but its steady-state effect was either increased (+50%) or decreased (−24.7%) steady-state twitch amplitude. A negative inotropic effect always occurred below 27°C. Milrinone decreased the total twitch duration and split the twitch into two components (P1 and P2) in the absence of any evidence of aberrant conduction. Increasing milrinone concentration from 50 to 300 μg/ml decreased P1 and increased P2. Ryanodine (100 mM) or caffeine (10 mM) suppressed P1. Contractions elicited after 30 seconds of rest were also biphasic in the presence of milrinone, but not in its absence. P2 of post-rest contraction was increased by caffeine or calcium (10 mM) and decreased by cobalt (2 mM) when drugs were applied at the beginning of the rest. Ryanodine and caffeine also suppressed P1 of post-rest contraction. The evidence suggests that P1 may be caused by Ca release from the sarcoplasmic reticulum and P2 by increased Ca influx during the action potential via the calcium channel. It is also suggested that P2 may be present under control conditions, but to a lesser extent, and masked by a large P1. (Circulation Research 1986; 59:151-162)

In a recent paper, we reported that amrinone, a bipyridine inotropic drug, caused a positive or negative inotropic effect, depending on the duration of the depolarizing pulse used to evoke the contraction.1 Because milrinone, a congener of amrinone, has been reported to have greater potency and lower toxicity, we initiated a study of this agent. In order to increase the inotropic instability of our papillary muscle preparations, we began the work using a temperature of 28°C. We found that a previously unreported effect of milrinone — biphasic twitches — was unmasked at this lower temperature. We therefore extended this project to study the basic mechanism of the inotropic actions of milrinone and the nature of the biphasic twitches induced by the drug.

It is now recognized that both amrinone and milrinone inhibit phosphodiesterase in cardiac tissue.2 Thus, it might be predicted that milrinone, like amrinone, could increase transmembrane calcium flux. This prediction is readily testable. It is not clear what mechanisms would lead to the development of a biphasic twitch pattern. However, biphasic contractions have been reported under a variety of conditions that prolong the depolarized state of the membrane. These conditions include artificial lengthening of the action potential duration or long voltage-clamp pulses3-5 or pharmacological interventions such as caffeine.6 In most of these interventions, the second component of contraction is characterized by a maintained tension or contracture whose duration is correlated with the duration of the depolarization (for review, see Coraboeuf).7 This tonic component of contraction has been attributed to a shift of the sodium-calcium exchange equilibrium favoring Ca uptake with membrane depolarization.8-9

There are other conditions under which the second component of contraction does not appear as maintained tension, but rather as a phasic twitch, with a very delayed time to peak compared to the "normal" twitch ("first component"). This is the case for rested-state contractions elicited after 15- to 30-minute rest periods.10,11 Beresewicz and Reuter suggested that the rested-state contraction may be primarily regulated by the calcium ions entering the cell (via the calcium channel) during the action potential, whereas the steady-state contraction may be more dependent on the calcium released from the sarcoplasmic reticulum (SR).11 These results suggest that Ca can contribute by different routes to the development of several tension components and that these tension components are separable under certain physiologic or pharmacologic conditions. In the present study, we found that under appropriate conditions milrinone induces two-component contractions in which the first component is attributable to SR calcium release and the second may be more directly caused by calcium influx. A preliminary report of these results has been presented.12
Materials and Methods

Tissue Preparation

Young ferrets (0.7–1 kg) of either sex were anesthetized with sodium pentobarbital (30 mg/kg IP). The heart was quickly removed and placed in normal physiologic solution, maintained at room temperature or 36°C. Papillary muscles or thin trabeculae (0.1–0.6 mm in diameter) were dissected under a microscope, and a small loop of 10–0 surgical silk suture was tied on each end. Muscles were mounted horizontally in a 0.1-ml experimental chamber, with one end fixed and the other fastened to a piezoresistive transducer (Aksjeselskapet Mikroelektronik, Norway) or to a Grass force-displacement transducer (FT.03). Muscles were stretched at the start of the experiment to a steady length that yielded 90% of maximum force. Preparations were allowed to recover for at least 45 minutes before collection of experimental data was begun. Muscles were paced at 0.5 Hz, using either field stimulation or punctate stimulation applied through a bipolar electrode positioned on the surface of the muscle at the fixed end. We did not find any differences in the responses of the preparation to milrinone attributable to mode of stimulation.

For voltage-clamp experiments, muscles were mounted in a single sucrose gap system composed of three compartments. The anterior (test) compartment was perfused with normal or drug-containing physiologic solution, the middle with isotonic sucrose solution, and the posterior (current injection chamber) with isotonic potassium solution. In these experiments, tension development induced by the tip of the papillary muscle (less than 0.3 mm in length) was recorded with a Grass force-displacement transducer (FT.03).

Data Recording and Analysis

We recorded membrane potential with conventional glass microelectrodes (10–30 MΩ) filled with 3 M KCl, connected to an operational amplifier (Analog Devices 545) or an electrometer amplifier (W.P. Instruments Inc., model KS 700). Electrical and mechanical activities were displayed on an oscilloscope and on a rectilinear chart recorder.

The slow inward current, \( i_M \), was elicited with steps from a holding potential set at −40 mV to completely inactivate the sodium current. Because the outward current was negligible at the end of the 200-msec test pulse applied from the holding potential, we measured the slow inward current as the difference between the current flowing at the end of the 200-msec pulse and the maximum peak inward or minimum peak outward current (method of New and Trautwein). Temperature coefficients “R10” were calculated by linear regression analysis of the equation:

\[ \log_{10} (Y) = \{\log_{10} (R_{10})/10\} \cdot T \]

where Y is the variable studied [time to peak or time to + (dT/dt)_{max}]. and T is the temperature expressed in degrees Celsius. As noted by Bennett, the thermal ratio \( R_{10} \) is analogous to the \( Q_{10} \) but is preferred over \( Q_{10} \) since the latter was defined as a ratio of rates. Monophasic twitch tension was measured at the peak of the twitch. For the biphasic twitches observed in the presence of milrinone, we measured the tension maxima and times-to-maxima of the earlier (P1) and later (P2) components of the twitch. In some cases the two components were not sufficiently separated and one peak was not well defined. In such cases, measurements for that component were made at the time the rising or relaxation phase of the twitch showed a marked change in its time course (e.g., a shoulder). However, because of the overlap of P1 and P2, measurements of these components can only be viewed as an approximation of the actual contributions of the two components to the observed twitch.

Data are expressed as the mean ± SD except where noted. Drug effects were evaluated by statistical analysis of paired data, using Student’s t test, analysis of variance, or \( \chi^2 \) test. \( p < 0.05 \) was regarded as significant.

Perfusion, Solutions, and Drugs

Preparations were superfused at a constant flow rate with prewarmed oxygenated solutions. Complete renewal of the solution in the experimental chamber was achieved in less than 3 seconds. For most experiments, temperature was maintained constant at 28 ± 0.2°C, using a Peltier thermoelectric device (Bailey Instruments Inc., Saddle Brook, N.J.). For determinations of the thermal ratio \( R_{10} \) of the different parameters of contraction, temperature was varied between 18 and 37°C. Normal physiologic solution consisted of (millimolar): NaCl, 140; KCl, 6; CaCl 2, 2; MgCl 2, 1; HEPES, 5 (N-2-hydroxyethylpiperazine-N’-2 ethanesulfonic acid); glucose, 11. In some experiments, KCl concentration was lowered to 4 mM. The isotonic potassium solution used for sucrose gap studies contained (millimolar): KCl, 140; CaCl 2, 1.8; MgCl 2, 1; HEPES, 5; glucose, 11. The pH of the perfusate was adjusted to 7.40 ± 0.002 with 1 N NaOH. The isotonic sucrose solution consisted of (millimolar): sucrose, 300; CaCl 2, 0.040; and glucose, 11. All solutions were continuously bubbled with 100% O 2.

Milrinone (50–300 μg/ml; MW = 211.1), kindly provided by Sterling-Winthrop Research Institute (Rensselaer, N.Y.), was made up in a concentrated stock solution (5 mg/ml, 23.7 mM) of the powder dissolved in HCl solution (23.7 mM acid in water). Aliquots were then added to the perfusing medium to give the final concentration. The pH of the milrinone-containing solution was always readjusted to 7.40 ± 0.002 with 1 N NaOH before perfusion. Ryanodine was obtained from Penick Corporation (Lyndhurst, N.J.).

All the observed drug-induced effects except those caused by ryanodine were easily reversible.

Results

General Effects Of Milrinone

Slow Inward Current. Because we previously found that amrinone increased the slow inward current of ferret papillary muscles,1 we tested the effect of milrin-
on this current. Figure 1A shows the effect of 50 µg/ml (0.237 mM) milrinone (filled circle) on the time course of the slow inward current elicited in a representative ferret papillary muscle by applying a 200-msec depolarizing pulse from the holding potential (−40 mV) to −10 mV at 0.5 Hz and 28°C. The average effect of this concentration in four muscles was a 110% increase in the maximum slow inward current that could be elicited by a test pulse to around −10 mV. The time to peak current was slightly but not significantly shortened by the drug. Figure 1B indicates that milrinone (filled circles) increased the slow inward current at all activating potentials between −40 and +20 mV, without any change in the potential at which the current reached its maximum (around −10 mV). Milrinone did not modify the threshold of the current and did not change its apparent reversal potential. These effects suggest that milrinone increased the slow inward current via an increase of calcium conductance. Effects of milrinone on the slow inward current at all activating potentials were also tested in preparations stimulated at 1 Hz at 36°C for more direct comparison with our prior observations with amrinone. We found that the increase in the inward current (measured at the voltage step that elicited the maximum current) produced by 0.237 mM (50 µg/ml) milrinone in three preparations averaged 100.7 ± 9.2%. Amrinone (0.5 mM) under the same conditions increased the slow inward current by 79 ± 15%.

Effects of Milrinone on Action Potential Parameters at 0.5 Hz and 28°C

<table>
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<tr>
<th>Amplitude (mV)</th>
<th>E_R (mV)</th>
<th>OS (mV)</th>
<th>APD_{0} (msec)</th>
<th>APD_{90} (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>95.6</td>
<td>−75.4</td>
<td>20.3</td>
<td>165.9</td>
</tr>
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<td>±SE</td>
<td>1.6</td>
<td>2.5</td>
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<td>8.6</td>
</tr>
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<td>Milrinone</td>
<td>99.4</td>
<td>−77.7</td>
<td>21.6</td>
<td>189.6</td>
</tr>
<tr>
<td>±SE</td>
<td>2.1</td>
<td>1.2</td>
<td>1.7</td>
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<tr>
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<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
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</tr>
</tbody>
</table>

Milrinone 50 µg/ml, 0.5 Hz. Data are means ± SEM. E_R = resting potential; OS = overshoot; APD_{0} = action potential duration at 0 mV; APD_{90} = action potential duration at −60 mV. Milrinone values were compared to control values using a paired t-test.

Effects of Milrinone on Twitch Tension. Figure 2 shows the effect of 50 µg/ml milrinone on the tension of twitches elicited by action potentials in two representative ferret papillary muscles stimulated at 0.5 Hz at 28°C. In all the preparations studied, milrinone exerted a transient positive inotropic effect, followed by a decrease in contractility to a new steady-state level as drug exposure was continued. In some muscles, the
new inotropic steady state was above the control level (steady state, +50.0 ± 25.3%, n = 24; transient maximum, +80.0 ± 27.2%). In the remainder, the new steady-state level was below the control amplitude (steady state, −24.7 ± 13.4%, n = 16; transient maximum, +28.6 ± 12.7%). Possible reasons for this difference in steady-state response are discussed below (see “Discussion”). In most muscles, a second component of tension (P2) appeared within 10 minutes of drug exposure. The first peak (P1) diminished as the second component (P2) developed (Figure 2).

Milrinone's positive inotropic effect might be augmented by an increased calcium release from the sarcoplasmic reticulum in addition to the measured increase in slow inward current. We therefore tested the effects of milrinone in the presence of ryanodine, a compound that has been shown to inhibit calcium release from the sarcoplasmic reticulum. At a concentration of 100 nM, ryanodine inhibited the twitch tension by 81.5 ± 8.8% (n = 4). Addition of 50 μg/ml milrinone resulted in the same percentage of inotropic effect as in the absence of ryanodine (not shown) but did not induce biphasic contractions. This result indicates that milrinone’s positive inotropic action does not require a functional sarcoplasmic reticulum.

Frequency and Temperature Dependence of the Inotropic Effect. Because the inotropic action of milrinone was either positive or negative in different preparations under the conditions described above, we examined the effects of frequency of stimulation and temperature separately. The inotropic effect of milrinone at 28°C was assessed in preparations driven at frequencies ranging from 0.1 to 1.5 Hz. Figure 3A shows the normalized steady-state frequency-force relationships under drug-free conditions and in the presence of milrinone. Twitch amplitude was measured at each frequency after steady-state effect was reached, normalized in each muscle to the maximum control twitch amplitude observed between 0.1 and 1.5 Hz, and then averaged. Under drug-free conditions, increased twitch tension was observed as the frequency was increased from 0.1 to about 0.9 Hz; a small decline occurred at higher frequencies. In the presence of 50 μg/ml milrinone, the relationship was almost flat from 0.1 to 0.7 Hz and showed a negative slope from 0.7 to 1.5 Hz. Crossing of the control and drug frequency-force relationships occurred at about 0.6 Hz, and at 28°C this resulted in a limitation of the positive inotropic effect of milrinone to frequencies lower than 0.6 Hz. The fact that this cross-over frequency was very close to our usual stimulation frequency (0.5 Hz) may be partly responsible for the variability in the steady-state inotropic influence of milrinone reported above.

The effect of stimulation frequency on the behavior of P2 was measured between 0.1 and 1 Hz in four additional muscles. Over this range, frequency had very little effect on P2.

The influence of temperature on the inotropic effect was evaluated between 20° and 35°C in preparations stimulated at 0.5 Hz. Figure 3B shows the relationships between the twitch amplitude (P1 component) and temperature in the absence and presence of 50 μg/ml milrinone. Twitch amplitudes were normalized in each preparation to the maximum control amplitude over the range 20°-35°C, and then averaged. Under control conditions, increasing temperature from 20° to 35°C resulted in a marked decrease (76%) in the twitch amplitude. In contrast, in the presence of 50 μg/ml milrinone, the P1 versus temperature curve was almost flat from 20° to 35°C. As shown in Figure 3B, the two curves crossed at 27°C. Consequently, for temperatures below 27°C, milrinone induced only a negative inotropic effect at the frequency used. Thus, the standard temperature for most of our experiments (28°C) was very close to this “cross-over” temperature and the temperature effect may therefore have also contributed to the variable steady-state inotropic effect of milrinone.

**Nature of the Biphasic Twitch**

**Separation of Twitch Components.** Induction of the biphasic twitch pattern shown in Figure 2 was ob-

**FIGURE 3.** Frequency and temperature dependence of the effect of 50 μg/ml milrinone on twitch amplitude. A. Normalized force-frequency relationship at 28°C. Data points represent mean values from three muscles, and vertical bars, SD. B. Normalized twitch amplitude at 0.5 Hz between 20° and 35°C. Data points represent mean values from 5 (milrinone) or 6 (control) muscles, and vertical bars, SD. In panels A and B, control (open symbols) and milrinone (filled symbols) twitch amplitudes have been normalized to the maximum tension elicited under drug-free conditions and then averaged. In both panels, the first (P1) component was measured when biphasic twitches were present.
served in 75% (30 out of 40) of the muscles tested for AP-elicited twitches. Its occurrence was not influenced (χ² test) by the final positive or negative inotropic effect of the drug, since it was seen in 70.8% (17 out of 24) and 81.2% (13 out of 16) of the two groups of muscles, respectively. The overall twitch duration, measured at 100% relaxation, was slightly but significantly decreased by the drug (from 697.7 ± 146.6 to 638.2 ± 123.2 msec; \( p < 0.01 \), paired \( t \) test, \( n = 28 \)). The times to peak were 189.9 ± 39.3 msec (\( n = 26 \)) for the first component and 340.4 ± 74.2 msec (\( n = 18 \)) for the second one. These times represented 25.6 and 56.6% of the total duration of the twitch respectively. The time to the monophasic peak under control conditions occurred at 243.5 ± 36.1 msec (\( n = 26 \)), which was 33.7% of the total duration of the monophasic twitch. Statistical analysis showed that the time to peak of the first component (189.8 msec) occurred significantly earlier than the time to the monophasic peak under drug-free conditions (243.5 msec; \( p < 0.001 \), paired \( t \) test). Action potential duration, measured at 0 and -60 mV, was not significantly increased by the drug (Table 1) although there appeared to be a relationship between the amplitude of the P2 component and AP duration. As shown in Figure 4, a small decrease in temperature prolonged the duration and caused a marked increase in the relative P2 amplitude.

Aberrant conduction can cause asynchronous contractions of portions of a muscle and result in a multiphasic twitch pattern, so it was important to rule out conduction abnormalities as a spurious cause of the biphasic pattern. We did not observe any evidence under the microscope of inhomogeneous contraction resulting from non-uniform excitation of the preparation, whether stimulation was via field electrodes or bipolar punctate wires. To completely rule out this possibility, we monitored conduction in eight experiments by impaling the muscle with two microelectrodes. The results of a typical experiment are shown in Figure 5. For these experiments, muscles were stimulated at the fixed end, and microelectrodes were impaled in the middle and at the distal end. As shown in Figure 5, milrinone induced a biphasic twitch without any significant increase in the conduction time or other evidence of conduction aberrancy.

**Effects of Rest.** In some preparations, biphasic twitches were not seen when the muscles were stimulated at 0.5 Hz, but the two components could be separated by resting the preparation for 0.5–5 minutes. In the preparation shown in Figure 6, all twitches elicited after a 1-minute rest period were monophasic, as shown in the top panel. However, after 3 minutes of rest (lower panel), the first, second, and third twitches elicited on restimulation of the preparation showed a biphasic pattern typical of the one we usually observed in the presence of milrinone.

**Effects of Milrinone Concentration on Twitch Components.** The effects of cumulative doses of milrinone, from 50 to 300 μg/ml, on the P1 and P2 components in a typical preparation are shown in Figure 7A. When milrinone concentration was increased, there was a progressive decline in P1 amplitude and an increase in P2. This resulted in an apparently monophasic twitch in the presence of 300 μg/ml milrinone, but with a much delayed time to peak (393.7 ± 29.3 vs. 277.5 ± 25.3 msec under drug-free conditions; \( n = 4 \)). Increasing the milrinone concentration from 50 to 300 μg/ml did not change the time to peak of the second
component (−2.7 ± 12.1%) but greatly increased the duration of the relaxation phase (+57.9 ± 32.6%) measured as the time from the peak of the second component to 100% relaxation. These effects of high concentrations of milrinone were reversible, as shown in the last panel of Figure 7A. The simultaneous decrease in P1 and increase in P2 induced at high milrinone concentration occurred without appreciable changes in action potential duration (not shown). Normalized cumulative dose-response curves for the effects of milrinone on P1 and P2 are given in Figure 7B. Analysis of variance indicated that the milrinone-induced decrease in P1 amplitude was statistically significant (p < 0.05) for 200 and 300 μg/ml of drug compared to 50 μg/ml. P1 amplitude reached with 300 μg/ml was also significantly less (p < 0.05) than the value at 100 μg/ml. On the other hand, P2 amplitude increased with increasing milrinone concentration although the change did not reach statistical significance (p > 0.05). In addition, the maximum effect of milrinone on P2 was achieved at 200 μg/ml whereas the effect on P1 did not appear maximal even at 300 μg/ml. These differing effects of milrinone on P1 and P2 amplitudes suggest that different mechanisms may underlie these two components of contraction.

Temperature Sensitivity of P1 and P2. If different mechanisms are responsible for P1 and P2, these mechanisms might have different temperature dependencies. Therefore, we examined the behavior of the two components of the biphasic twitch over a broad range of temperatures. Figure 8 illustrates the behavior of the control twitch and of P1 and P2 over a 10°C variation of the temperature of the perfusate. Under control conditions (Figure 8A), when the temperature was decreased from 34° to 24°C, the twitch amplitude progressively increased, as did the time to peak tension and the total duration of the twitch. In this preparation (as in most of the muscles) the maximum rates of rise and relaxation of the twitch [positive and negative (dT/dt)max (not shown)], also increased as temperature was decreased, peaking at 26°C. In the presence of 50 μg/ml milrinone (Figure 8B), when temperature was decreased from 33 to 23°C, there was progressive development of the second component. Cooling had little effect on P1 in 8 muscles (Figure 8C), increased it in 1, and decreased it in 2 (Figure 8B). In all muscles, there was slowing of the rates of rise and relaxation of the twitch in the presence of milrinone when the temperature was decreased. To quantify the temperature effect on the monophasic control twitch, P1, and P2, we determined the values of the thermal ratio R10 between 20 and 34°C for the time to peak as well as for the time to the maximum positive first derivative of the twitch.
steady stimulation can alter calcium movements in car-
twitch (Table 2).

tone and more sensitive than the control monophasic
more sensitive to changes in temperature than the first

twitch and that of PI. In contrast, the second compo-
tension, + (dT/dt)^2 decreased with an increase

time in temperature, the calculated thermal ratio is less than
unity. Note that under these conditions, the smallest
Rl0 is associated with the highest temperature sensitiv-
ity. For example, an Rl0 value equal to 0.5 would result
from a two-fold change while a value of 0.2 would
represent a five-fold change in the measured variable
over a 10° temperature range. Control twitch and P1
appeared to be only moderately sensitive to changes
in temperature, as the times to peak tension and to
+(dT/dt)max had Rl0's larger than 0.5. There was no
statistical difference between the Rl0 for the control
twitch and that of P1. In contrast, the second compo-
ent of the biphasic twitch appears to be significantly
more sensitive to changes in temperature than the first
one and more sensitive than the control monophasic
twitch (Table 2).

**Effects of Rest.** Rest intervals introduced during
steady stimulation can alter calcium movements in car-
diac muscle (e.g., Bers17). As shown in Figure 6, rest
intervals unmasked biphasic contractions that were not
apparent during steady-state stimulation. The typical
effect of varying the rest period on the two components
of the biphasic twitch is shown in Figure 9. The top
panel of Figure 9 illustrates the protocol used. Each of
the lower panels shows superimposed the steady-state
twitch (SS) just before the rest period, and the first
(B1), second (B2), and third beat (B3) elicited upon
restimulation of the preparation. When the rest period
was increased from 7.5 to 30 sec there was a rapid
decline of the twitch at B1, as compared to the steady-
state twitch. This decrease in twitch tension amplitude
at B1 was mostly due to a decrease of the first compo-
ent (P1), P2 being less affected: whereas P1 was
larger than P2 for the shortest rest period, it became
smaller than P2 for longer rests. Comparison of rate of
decline of P1 and P2 in the first postrest beat shows that
P1 decays much more rapidly than P2 as rest is pro-
longed (Figure 9). After a 10-minute rest, P1 and P2
(measured in the first beat, B1) decreased to about 18
and 44% of their steady-state amplitude. Under drug-
free conditions, B1 amplitude reached 10.5% of the
steady-state twitch amplitude after a similar rest peri-
od.

**Pharmacologic Dissection of the Two Components
of the Twitch.** All the results described thus far support
the idea that different mechanisms might underlie the
components P1 and P2 of the biphasic twitch. We
therefore tested the hypothesis that P1 might be mostly
dependent on calcium release from the sarcoplasmic
reticulum, and P2 dependent on some other process,
possibly transmembrane calcium influx.

Among the more commonly used drugs that affect
sarcoplasmic reticulum function are ryanodine and
cafeine. Ryanodine has been shown to preferentially
inhibit calcium release from the sarcoplasmic reticu-
num without inhibiting the calcium uptake. In con-
trast, caffeine appears to exert its action on the sarco-
plasmic reticulum by causing calcium release and
inhibiting its reuptake (for review, see Chapman19).

The time course of effect of these drugs on biphasic
twitches is illustrated in Figure 10. The onset of caf-
eine action was rapid, since P1 was most entirely
suppressed 3 minutes after addition of the drug. Caf-
eine also induced a marked increase in P2 amplitude
and time to P2 peak. The combined effects of caffeine
and milrinone resulted in the return to a monophasic
pattern of twitch tension, with a markedly delayed time
to peak.

The effects of 100 mM ryanodine in the presence of
50 µg/ml milrinone are shown on the bottom panel of

![Figure](image)

**FIGURE 8.** Effect of lowering the temperature of the perfusate on the shapes of the control twitch (from 34° to 24°C, panel A) and the twitch in 50 µg/ml milrinone (from 33° to 23°C, panel B). Preparation 215. Panel C shows the twitches recorded in the presence of 50 µg/ml milrinone in a different preparation (1218-2), between 34° and 24°C. Numbers near the tracings indicate the temperature in degrees Celsius. Stimulation frequency was 0.5 Hz. Note the different effect on PI in panels B and C. Twitches are redrawn from oscilloscope records.

**TABLE 2.** Values of Rl0 for Time to + (dT/dt)max and Time to Peak Tension (TTP) of the Two Components P1 and P2 and of Control Twitch Between 20° and 34° C

<table>
<thead>
<tr>
<th>Component</th>
<th>(+ dT/dt)max</th>
<th>TTP</th>
<th>n</th>
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<tr>
<td>Control</td>
<td>0.686 ± 0.069</td>
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<tr>
<td>P1</td>
<td>0.673 ± 0.062</td>
<td>0.554 ± 0.043</td>
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<tr>
<td>P2</td>
<td>0.475 ± 0.073</td>
<td>0.434 ± 0.066</td>
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Milrinone 50 µg/ml, 0.5 Hz. Mean ± SD. Rl0 values were calculated as shown in "Materials and Methods)."
**FIGURE 9.** Effect of rest period duration on the biphasic twitch induced by 50 μg/ml milrinone. Top panel shows the experimental protocol used (schematic drawing for illustrative purposes only). Rest duration is indicated near each set of superimposed tracings. Steady-state stimulation at 0.5 Hz, 28°C. Preparation 410-F.

**FIGURE 10.** Effects of caffeine and ryanodine on the biphasic twitch induced by 50 μg/ml milrinone at 28°C and 0.5 Hz. Top panel: time course of action of 10 mM caffeine. Preparation 330-F. Bottom panel: time course of action of 100 nM ryanodine. Preparation 411-F. In both panels, upper traces are twitch tensions, and lower ones their first derivatives. M corresponds to steady-state action of milrinone preceding addition of the second drug, and numbers to the time (minutes) after addition of caffeine or ryanodine to the perfusate.

Figure 10. In five muscles, ryanodine always preferentially and strongly inhibited the first component of the biphasic twitch. The inhibition of P1 (86.7%) was relatively similar to the inhibition of the monophasic twitch amplitude induced under milrinone-free conditions by 100 mM ryanodine alone (81.5%). Unlike caffeine, ryanodine had only minor effects on P2 amplitude and time to peak. The effect of caffeine on P2 could result from a larger calcium influx during the plateau of the action potential, since caffeine increases both calcium current and AP duration in ferret papillary muscle. Although high concentrations of ryanodine increase AP duration, low concentrations (1 μM or less) increase AP plateau amplitude slightly and have only minor effects on the duration (Malecot, unpublished observations, and see References 20 and 21). In contrast to caffeine, ryanodine has been shown to be without effect on calcium current or to slow its inactivation without changing its magnitude. Part of the caffeine effect on P2 could also be due to an increase in myofilament Ca sensitivity.

In some experiments, milrinone induced aftercontractions. It has been suggested that after-contractions are caused by intracellular calcium overload and oscillatory release from the sarcoplasmic reticulum. If ryanodine does indeed reduce Ca release from the SR in the presence of milrinone, it should modify milrinone-induced aftercontractions. Figure 11 illustrates the effects of 100-nM ryanodine on the aftercontractions induced by 50 μg/ml milrinone in a representative ferret trabecula. After-contractions were transiently increased, but after 20-minutes exposure to ryanodine they were completely suppressed.

**Drugs Affecting Calcium Influx.** Drugs that modify calcium influx can change contractility in several ways. Increased influx may influence the myofibrils directly (in species in which transmembrane influx of calcium directly activates the myofibrils). Alternatively, increased influx may affect calcium-induced calcium release from the sarcoplasmic reticulum (SR), either through the increase in trigger calcium, or through increased steady-state loading of the SR. Adding calcium influx-altering agents only during a 30-second quiescent period, and measuring the first post-rest beat (B1), limits the above complications in 2 ways. First, the agent can be washed in for 30 seconds in the absence of stimulation, which minimizes changes in cellular or SR calcium load that might be induced by the agent during stimulation. Second, the beat measured, B1, reflects the second component (P2), since P1 decays more rapidly during rest than P2. This protocol is illustrated at the top of Figure 12. Drugs were added at the start of the rest period and washed out after the 10th postrest stimulus. The early washout minimized cumulative effects of the drugs. Only thin preparations (diameter <0.4 mm) were used, in order to minimize diffusional delays. Results of a typical experiment are given in the bottom portion of Figure 12. Under the initial conditions, i.e., in the presence of milrinone alone, the first beat (B1) elicited after a 30-sec rest period consisted mostly of the P2 component of the biphasic twitch, although P1 was usually detectable. Addition of 2 mM CoCl, resulted in...
FIGURE 11. Effects of 100 nM ryanodine on aftercontractions induced by 50 μg/ml milrinone in a representative trabecula at 28°C. Upper tracings correspond to the lower parts of the twitches shown on the bottom of the figure, recorded at high sensitivity to emphasize the aftercontractions. a = steady-state effect of milrinone; b = 6.5 minutes, and c = 20 minutes after addition of ryanodine. Note the transient increase in aftercontraction amplitude before its suppression, the simultaneous decrease in PI and aftercontraction, and the unmasking of P2. Preparation 403-M.

A marked decrease of the twitch amplitude at B1 compared to the control B1. The decline in tension occurred mostly at P2, averaging -45%, whereas the decline at P1 averaged -20% (both effects on P1 and P2 compared to the control B1, n = 3). Increasing the calcium concentration of the perfusate to 10 mM resulted in the opposite effect, i.e., a marked increase in the amplitude of B1 compared to the control B1, mostly at P2 (+56%) and less at P1 (+42%, n = 4). Addition of 10 mM caffeine at the start of the rest period resulted, as predicted from the results illustrated in Figure 10, in a marked decrease (compared to SS, not control) of the P1 component at all beats after restimulation. Caffeine also induced a pronounced increase of the amplitude of the second component (P2) compared to that in the pre-caffeine B1 twitch (+106%, n = 4). P2 amplitude in the B1 twitch was also increased above its value in the steady-state period just before the rest period and drug exposure started.

To further test the idea that the first component of the milrinone-induced biphasic twitch is mostly dependent on the release of calcium from the SR and that the second one is more likely due to an increased calcium influx or some other process, we repeated the experiments of Figure 12 in the presence of steady-state concentrations of both 50 μg/ml milrinone and 100 nM ryanodine. The twitch we observed in the presence of both drugs consisted almost entirely of the P2 component as already mentioned (see Figure 10). Activators or inhibitors of calcium influx were added at the start of a 30-second rest period, as described for Figure 12. The results (not shown) were qualitatively similar to those shown in Figure 12 but increased in magnitude. Drugs that decreased calcium influx almost entirely suppressed the twitch at B1, whereas those which increased influx greatly increased contractility at B1.

Discussion

Fundamental Effects of Milrinone on Calcium Movements

Our voltage-clamp results (Figure 1) indicate that milrinone increases the calcium current of ferret papillary muscles. In this respect, the drug has qualitatively the same effect as its parent compound, amrinone. This increase in calcium current is predictable since it has been shown that milrinone inhibits phosphodiesterase activity and increases cAMP levels. We did not find a modification of the voltage dependence of the calcium current, and our results support the idea that milrinone, like amrinone, increases calcium current through an increase of conductance and not via an increase in driving force.

Milrinone, at the concentrations used in the present study, has been shown to dramatically decrease the ability of the SR of skinned rabbit cardiac fibers to take up calcium that is releasable by caffeine. The parent compound, amrinone, has also been shown to decrease...
calcium-induced release of SR calcium in mechanically skinned cardiac cells (A. Fabiato, personal communication). Su also reported that 0.5–1.0 mM milrinone did not alter myofilament calcium sensitivity, but did increase the maximum tension developed by 10–15%.* R.J. Solaro (personal communication) also found no effect of milrinone on the calcium sensitivity of canine cardiac myofilaments. Amrinone (0.5 mM) has also been shown not to affect calcium sensitivity of myofilaments extracted with glycerol and detergent. ** Na–Ca exchange in isolated sarcoplasmic reticulum vesicles is not affected or only slightly inhibited by 0.2–1 mM milrinone (Reference 27 and P. Mansier, D.M. Bers, and C. Malecôt, unpublished observations). Amrinone has been reported to produce modest inhibition of cardiac sarcoplasmic Na–Ca exchange.***

These actions, especially the effect of milrinone on calcium current, appear to be sufficient to explain the positive inotropic effect of the drug on monophasic twitches.

Hypothetical Basis for Biphasic Contraction

In mammalian cardiac muscle, the SR is generally accepted as a major source of "activator" calcium. It had previously been thought that the amount of calcium influx associated with the calcium current was too small to directly activate the myofilaments. However, recent results with a variety of techniques have increased estimates of single-beat calcium influx. Thus calcium influx may play a direct role in the activation of the myofilaments. However, such direct activation might be expected to be slower, owing to the longer diffusion distance involved. The relative contributions of calcium influx and SR release to normal twitches vary in different preparations and with different conditions.† Ferret ventricular muscle activation probably depends primarily on calcium release from the SR. This is supported by our finding that 100 nM ryanodine decreased twitch tension by over 81%.

The fundamental effects of milrinone discussed above support consideration of two mechanisms for milrinone's induction of biphasic twitches: increased calcium current and inhibition of SR calcium release. Thus, the net effect on tension will depend on whether the increased calcium influx dominates (increased tension) or the SR effect is primary (decreased tension). These opposing effects may be the basis for the shift from positive to negative steady-state inotropic action observed with a decrease in temperature (Figure 3B).

The fact that the early component (P1) of the twitch can be eliminated by either ryanodine or caffeine, while P2 is resistant to these agents, is a strong indication that P1 represents SR release of calcium (Figure 10). Although ryanodine may slow the inactivation of calcium current, this is probably secondary to its effects on the SR and in any case would not explain the observed decrease in tension. Similarly, caffeine has other effects in addition to the action on the SR, but these would not explain the strong reduction of P1 that we observed with this drug. We postulate that the second component, P2, represents tension activated by transsarcomembranous calcium influx. This is suggested by findings that P2 is enhanced at the first beat after exposure to either increased extracellular calcium or caffeine, and inhibited at the first beat following exposure to cobalt (Figure 12). Because these agents were added during rest, they should have had minimum effect on cellular calcium stores until the first beat tension was registered. The ability of milrinone to increase calcium influx and decrease SR sequestration would combine to enhance the effectiveness of calcium influx in activating the myofilaments.

Onset and Concentration-Dependence of Biphasic Contractions Induced by Milrinone

When a muscle is first exposed to milrinone (50 μg/ml) there is a consistent initial increase in force associated with a reduced time to peak tension and maintenance of a monophasic twitch profile (see Figure 2). We suggest that this early phase of milrinone action is attributable to increased Ca current and thus increased Ca triggering of SR Ca release or (after the first beat) an increase in the amount of releasable Ca in the SR. In this respect, the early effects of milrinone are similar to the effects of catecholamines (for review, see Scholz). During the next 5–10 minutes the twitches become biphasic. This is largely the result of a decrease in P1, which seems to unmask the P2 component. These secondary effects can best be explained by a decrease in SR calcium release. Thus, the SR effect of milrinone is manifested more slowly than the effect on calcium current. Furthermore, the effect on calcium current appears to be maximal at lower concentrations than the SR effect (Figure 7B), consistent with the hypothesis that different mechanisms are involved. These effects qualitatively resemble those of caffeine, but milrinone appears to have a greater effect on calcium influx relative to its effect on SR function.

Frequency-Force Effects

In most mammalian species, an increase in the frequency of stimulation induces an increase in the force of contraction. This is also the case for ferret papillary muscle under control conditions (Figure 3A). This has been attributed to increased sodium and calcium entering the cell during increased activity. Increased calcium could be taken up by the SR and recirculated on the next beat while increased sodium would result in increased calcium via the sodium–calcium exchange. In the presence of milrinone, only a very small positive frequency effect was seen between 0.1 and 0.5 Hz (Figure 3A). This may be attributable to the depressant effect of milrinone on SR function. In the presence of milrinone, the SR may be unable to increase its Ca loading with increasing frequency.

Temperature Effects

It has been shown that a decrease in temperature increases the sensitivity of the myofilbrils to calcium and decreases the maximum tension developed. This results in a crossing over of the tension–pCa relationships.* Therefore, the change in tension induced by a
decrease in temperature will depend on the initial pCa, i.e., on the amount of free calcium. Since Ca concentration near the myofilaments probably does not approach saturation during a twitch, the dominant effect of lowering the temperature would be the increase in myofilament Ca sensitivity. Decreased temperature also increases the intracellular sodium activity in ferret ventricle. This increase in intracellular sodium might be expected to increase SR Ca loading by a shift in sarcolemmal Na–Ca exchange. Both the increased myofilament sensitivity and increased SR Ca loading may contribute to the strong positive inotropic effect of temperature reduction in control muscles (Fig. 3B). The apparent lack of temperature sensitivity of P1 amplitude in the presence of milrinone when the temperature is decreased from 35°C to 20°C (Figure 3B) may be attributable to a higher free Ca concentration achieved during the twitch since milrinone increases the calcium current (Figure 1) and reduces the ability of the SR to increase its calcium load.

Induction of Biphasic Twitches

The second component of contraction could not be attributed to aberrant conduction or other experimental artifact since biphasic twitches occurred without changes in the conduction time (Figure 5) and were reproducible, dose-dependent (Figure 7), and reversible. The total twitch duration was not increased but rather underwent a mean decrease of approximately 60 msec (see “Results”). As noted above, several authors have reported that biphasic twitches, usually involving development of tonic tension, can be observed under a variety of conditions that prolong the depolarized state of the membrane. However, under control conditions at 28°C, voltage-clamped ferret papillary muscles show a tonic component of contraction only when the membrane is kept depolarized to 0 mV for at least 500–900 msec, from a holding potential of −60 mV (unpublished observations). This is considerably greater than the usual action potential duration at this potential and temperature (Table 1).

Thus, under the experimental conditions used in the present study, milrinone induces a partial separation of the twitch into two components. The first component, P1, is probably caused by SR Ca release, which is decreased in magnitude but faster than in control conditions. The second component, P2, is likely associated with an increased Ca influx during the action potential plateau. This increased Ca influx is mainly due to an increased Ca current, although a shift in the Na–Ca exchange may play a role. It is possible that the P2 component of contraction is also present under control conditions but is masked by a larger P1 component (owing to a larger SR Ca release).

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**KEY WORDS** • milrinone • biphasic twitches • ferret cardiac muscle • voltage clamp • calcium current • sarcoplasmic reticulum • ryanodine • caffeine • hypothermia
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