Br. J. Pharmac. (1985), **85**, 97–108

The effects of milrinone and piroximone on intracellular calcium handling in working myocardium from the ferret

Judith K. Gwathmey & James P. Morgan

The Charles A. Dana Research Institute and the Harvard-Thorndike Laboratory of Beth Israel Hospital, Department of Medicine (Cardiovascular Division), Beth Israel Hospital and Harvard Medical School, 330 Brookline Avenue, Boston, Massachusetts, U.S.A. 02215

1 The effects of milrinone and piroximone were compared to those of isoprenaline, dibutyryl adenosine 3′:5′-cyclic monophosphate (dibutyryl cyclic AMP), forskolin, isobutylmethylxanthine, increased extracellular calcium ($[Ca^{2+}]_o$) and caffeine in ferret right ventricular papillary muscles that were loaded intracellularly with aequorin, a bioluminescent calcium indicator that emits light when it combines with calcium.

2 The positive inotropic action of each drug, except caffeine, was associated with an increase in the peak amplitude of the aequorin light signal (i.e. intracellular Ca^{2+} transient) reflecting an increased amount of calcium available for excitation-contraction coupling; the positive inotropic effect of caffeine appears to occur by other mechanisms.

3 The time courses of the aequorin light signal and corresponding tension response were shortened by isoprenaline, forskolin, isobutylmethylxanthine, dibutyryl cyclic AMP, milrinone and piroximone; unchanged by increased $[Ca^{2+}]_o$ and prolonged by caffeine, suggesting that the rates of Ca^{2+} release and uptake by the sarcoplasmic reticulum were respectively increased, unchanged or decreased by these groups of drugs.

4 Relative to changes in $[Ca^{2+}]_o$, the ratio of the peak of the aequorin light signal to the peak of the tension response was increased by isoprenaline, milrinone and piroximone, and decreased by caffeine, indicating that the Ca^{2+}-sensitivity of the myofilaments was respectively decreased, and increased by these drugs.

5 The effects of milrinone and piroximone on the amplitude and time course of the aequorin light signal, as they relate to changes in uptake and release of calcium from the sarcoplasmic reticulum and to changes in the sensitivity of the myofilaments to Ca^{2+}, are consistent with the findings that positive inotropic doses of these agents act by increasing intracellular concentrations of cyclic AMP.

6 Higher doses of milrinone and piroximone produced negative inotropic effects that were characterized by diminution of developed tension but no change or an increase in the amplitude of the aequorin light signal, suggesting a decrease in the sensitivity of the contractile elements to Ca^{2+}.

7 Toxic doses of milrinone, piroximone and isoprenaline were associated with development of a Ca^{2+}-overload state characterized by the presence of after-glimmers, after-contractions and dysrhythmias, and by decreased amplitude of both the aequorin light signal and tension response.

8 The negative inotropic and toxic effects of milrinone and piroximone can be explained only in part by increased intracellular concentrations of cyclic AMP; we suggest that these drugs may have other cardiac actions.

Introduction

It is well-known that the calcium ion (Ca^{2+}) plays a central role in excitation-contraction coupling of the mammalian heart (Chapman, 1979). Most interventions with positive or negative inotropic effects on the heart (1), alter intracellular Ca^{2+} handling (2), change the responsiveness of the contractile apparatus to Ca^{2+}, or (3), exert both types of effects (Morgan & Blinks, 1982). The most direct way of determining

which action an inotropic intervention might have is to monitor intracellular Ca^{2+} transients and correlate them with mechanical performance. Aequorin, a bioluminescent protein that emits light when it combines with Ca^{2+}, can be used to record intracellular Ca^{2+} transients in intact and actively contracting mammalian working myocardium (Blinks *et al.*, 1982a). Changes in the amplitude and time course of the aequorin light signal correlate closely with known subcellular actions, since drugs with similar subcellular actions produce similar patterns of light and tension responses (Morgan *et al.*, 1984a). This correlation provides the basis for a functional classification scheme that can be used to investigate drugs with unknown mechanisms of action (Morgan & Morgan, 1984).

Milrinone and piroximone are newly introduced inotropic drugs that act by an unknown mechanism(s). Results from several laboratories have shown that these agents do not produce their inotropic effects via α-adrenoceptor, β-adrenoceptor or histamine receptor stimulation (Alousi *et al.*, 1983; Roebel *et al.*, 1984). Biochemical studies have demonstrated that milrinone and piroximone and closely related compounds increase intracellular cyclic adenosine 3′:5′-monophosphate (cyclic AMP) concentrations in the heart, an effect that appears to be due to inhibition of an isoenzyme of phosphodiesterase (Honerjäger *et al.*, 1981; Endoh *et al.*, 1982; Kariya *et al.*, 1982; 1984). Therefore, it has been postulated that the mechanism of inotropic action of these drugs is due, at least in part, to an increase in intracellular cyclic AMP concentrations. However, previous experiments with aequorin-loaded cat papillary muscles have shown that amrinone, a close structural analogue of milrinone, produces a pattern of light and tension responses that is distinct from inotropic drugs that act primarily to increase intracellular cyclic AMP concentrations, such as β-adrenoceptor agonists and dibutyryl cyclic AMP (Morgan *et al.*, 1980; Morgan & Morgan, 1984a). This finding suggests that amrinone and related drugs may have effects on intracellular Ca^{2+} handling that cannot be attributed to increases in cyclic AMP concentrations alone.

The purpose of the present study was to delineate further the effects of these newer inotropic agents on intracellular Ca^{2+} handling in mammalian working myocardium. Our results show that the positive inotropic effects of these drugs on papillary muscles from the ferret are related to an increase in intracellular $[Ca^{2+}]$. Moreover, the effects of milrinone and piroximone on both the amplitude and the time course of the Ca^{2+} transient are similar to those produced by other agents that increase intracellular cyclic AMP concentrations, but are different from inotropic agents that act by additional mechanisms, such as caffeine and increased extracellular calcium ($[Ca^{2+}]_o$). However, additional negative inotropic and toxic effects were observed with milrinone and piroximone that can only be partially explained by an increase in intracellular cyclic AMP concentrations.

Methods

Male ferrets, approximately 12 weeks of age and weighing between 600 and 650 g, were anaesthetized with chloroform. Their chests were then quickly incised and the hearts removed and placed in a physiological salt solution that was constantly bubbled with a gas mixture of 95% O_2 and 5% CO_2. The composition of the salt solution was as follows (mM): NaCl 120, KCl 5.9, dextrose 11.5, $NaHCO_3$ 25, $NaH_2PO_4H_2O$ 1.2, $MgCl_2$ $6H_2O$ 1.2 and $CaCl_2$ 2.5. The pH of the solution was 7.4. The right ventricle was quickly opened and a right ventricular papillary muscle of 1 mm or less in diameter was selected and dissected free. The papillary muscle was then placed in a bath containing the oxygenated physiological salt solution and the temperature was maintained at 30°C. The papillary muscle was attached to a tension transducer for recording isometric tension development. The muscle was stimulated to contract at 1 to 300 s intervals, using threshold voltage, and square-wave pulse of 5 ms duration delivered through a punctate platinum electrode located at the base of the papillary muscle. The muscle was stretched until L_{max} was obtained, which was reflected by no further increase in active tension, and allowed to equilibrate for a minimum of 1 h. It was then chemically loaded with aequorin as described in detail elsewhere (Morgan & Morgan, 1984b; Morgan *et al.*, 1984b). Light signals were recorded with a photomultiplier (EMI 9635B) using a light collecting apparatus with a design similar to that described by Blinks (1982). Because the light levels were very low and photomultiplier shot noise was prominent, it was necessary to average successive signals (from 16 to several hundred, depending on light intensity) to obtain a satisfactory signal-to-noise ratio. Signal averaging was performed only after responses had reached a steady state. The light signal was appropriately amplified and recorded using a filter with a 10 ms time constant. The light and tension responses and the stimulus artifact were recorded simultaneously on both magnetic tape and on chart strip recording paper. The light signal was recorded in nA. Values for tension are shown as $mN\,mm^{-2}$ of cross-sectional area. Cross-sectional area was approximated from the length and weight of the tissue.

Cumulative dose-response curves were performed for a variety of inotropic drugs. With the exceptions of milrinone and piroximone, each of these agents was dissolved in distilled water before being added to the bath. In order to obtain the calcium dose-response

curves, a phosphate-free salt solution was placed in the bath and calcium was added in incremental doses up to 16 mM. This process avoids calcium precipitation from the standard salt solution. Milrinone and piroximone were dissolved in hydrochloric acid and the appropriate amount of this acidic stock solution was added to the bathing medium in order to produce the desired final concentration of drug. Before allowing this solution to come into contact with the muscle, the pH was adjusted to 7.4 by the addition of small amounts of NaOH. The pH was again checked at the end of each response to be certain that it had not changed. No visible precipitate formed when the drug solutions were prepared in this manner. Similar amounts of acidic solution without milrinone were neutralized with NaOH; these solutions had no inotropic effect on the muscle.

It is possible for drugs to interact directly with aequorin and thereby alter the luminescent reaction or the sensitivity of aequorin to Ca^{2+} (Blinks *et al.*, 1982). Therefore, each of the drugs used in these experiments was tested *in vitro* using the basic method and calibration device described by Blinks *et al.* (1978). Briefly, aequorin was added to a solution containing a low concentration of Ca^{2+}; under these conditions a low level of luminescence persists for several minutes until the aequorin is gradually consumed. After initiating the luminescent reaction, drugs in concentrations equal to or greater than the doses used in these experiments were added to the reaction cuvette. In particular, milrinone and piroximone were tested in doses as high as 1.2×10^{-3} M. In no case did the drugs tested affect the intensity or time course of the luminescence indicating that none of the drugs tested interacts directly with aequorin in a way that would alter the experimental results.

Drugs

The following drugs and chemicals were used: milrinone (Sterling-Winthrop, Inc.), piroximone (MDL 19, 205) Merrell-Dow, Inc.), (−)-isoprenaline bitartrate (Sigma Chemical Co.), caffeine (Sigma Chemical Co.), forskolin (Calbiochem-Behrings, Div. American Hoeschet Corp.), $CaCl_2$ (BDH Chemicals Ltd), N^6O^{21}-dibutyryl adenosine 3′:5′-cyclic monophosphate (dibutyryl cyclic AMP; Sigma Chemical Co.) and 3-isobutylmethyixanthine (Sigma Chemical Co.). The aequorin used in these experiments was purchased from the laboratory of Dr J.R. Blinks in Rochester, Minnesota, U.S.A.

The concentration of each drug or chemical is expressed as final bath concentration.

Statistics

Student's *t* test for paired data was used for statistical analysis; *P* values < 0.05 were considered significant.

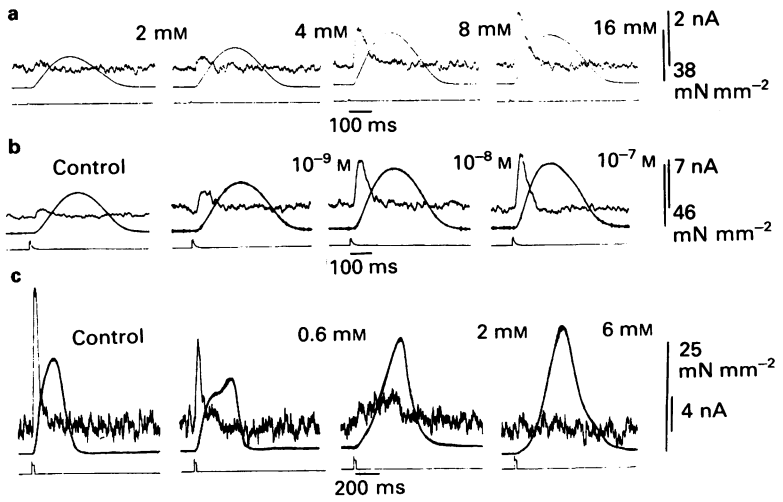

Figure 1 Effects on the aequorin light signal (upper noisy trace) and tension response (middle smooth trace) of (a) Ca^{2+}, (b) isoprenaline, (c) caffeine. Muscles were stimulated to contract at 3 s intervals; 30°C. For this and subsequent figures, lower trace in each panel is the stimulus artifact. The aequorin signal amplitude (i.e., light) is expressed as nA of current recorded from a photomultiplier; tension is expressed in mN per mm^2. Each panel represents the average of fifty to several hundred responses obtained at the steady state.

Results

Effects of drugs on the amplitude of the aequorin light signal and tension response

Figure 1 shows the effects of calcium, isoprenaline, and caffeine; Figure 2, milrinone and Figure 3, piroximone, on the aequorin light signals and tension responses of papillary muscles from the ferret. With the exception of caffeine, the positive inotropic effect of each of these drugs is associated with an increase in intracellular $[Ca^{2+}]$, as reflected in the increased amplitude of the aequorin light signal (i.e. intracellular Ca^{2+} transient). These effects were observed in 16 experiments with isoprenaline, 24 with calcium, 3 with caffeine, 7 with piroximone and 17 with milrinone.

Effects of drugs on the sensitivity of the myofilaments to calcium

In order to evaluate the relationship between intracellular $[Ca^{2+}]$ and developed tension, the peak amplitude of the aequorin light signals and tension responses under the influence of various inotropic interventions were compared to the effects of changes in $[Ca^{2+}]_o$. Only experiments that did not show signs of significant aequorin consumption between dose-response curves were used in this analysis. This sort of analysis requires comparison of the effects of an inotropic agent with the effects of changing $[Ca^{2+}]_o$ in the same papillary muscle preparation. As shown in Table 1, at similar levels of developed tension, the ratio of the amplitude of the peak light to peak tension

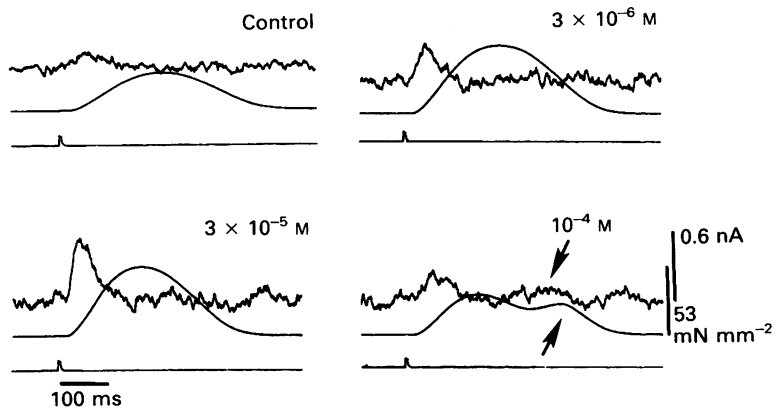

Figure 2 Effects of milrinone on a ferret papillary muscle loaded with aequorin. Arrows indicate after-contractions in tension trace and after-glimmers in light trace.

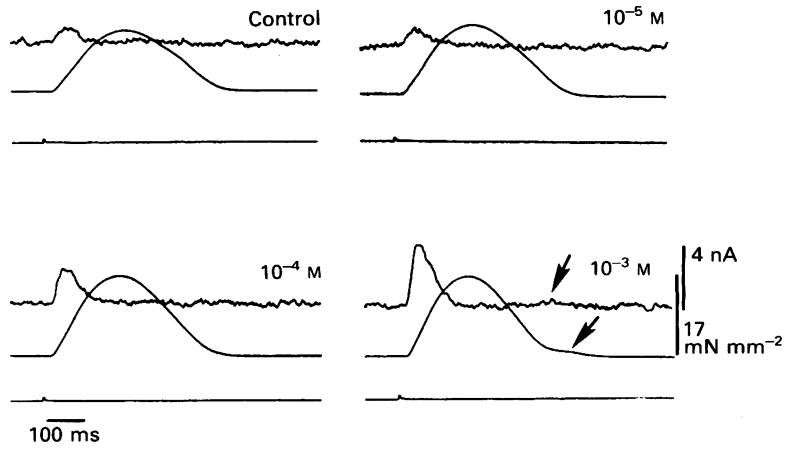

Figure 3 Effects of piroximone on a ferret papillary muscle loaded with aequorin. Arrows indicate after-contractions in tension trace and after-glimmers in light trace.

Table 1 Effects of drugs on the sensitivity of the myofilaments of the ferret papillary muscle to calcium

Drug	n	**Ratio of peak light induced by drug: peak light induced by $[Ca^{2+}]_o$*
Piroximone	3	3.7 ± 0.3
Milrinone	6	2.6 ± 0.6
Isoprenaline	7	1.9 ± 0.3
Caffeine	3	0.5 ± 0.2

*Determined at concentrations of drug and $[Ca^{2+}]_o$ producing similar levels of developed tension; see Results section. Values show means ± s.e.mean from *n* preparations.

response was increased by isoprenaline, milrinone, piroximone and decreased by caffeine. These changes suggest that, relative to increased $[Ca^{2+}]_o$, isoprenaline, milrinone, and piroximone decrease, and caffeine increases the sensitivity of the contractile apparatus to calcium.

Effects of drugs on the time course of the aequorin light signal and tension response

Figure 4 shows that isoprenaline shortens, increased $[Ca^{2+}]_o$ does not change, and caffeine prolongs the time course of the aequorin light signal. Figure 5 shows that, like isoprenaline, other drugs that increase intracellular cyclic AMP levels, including dibutyryl

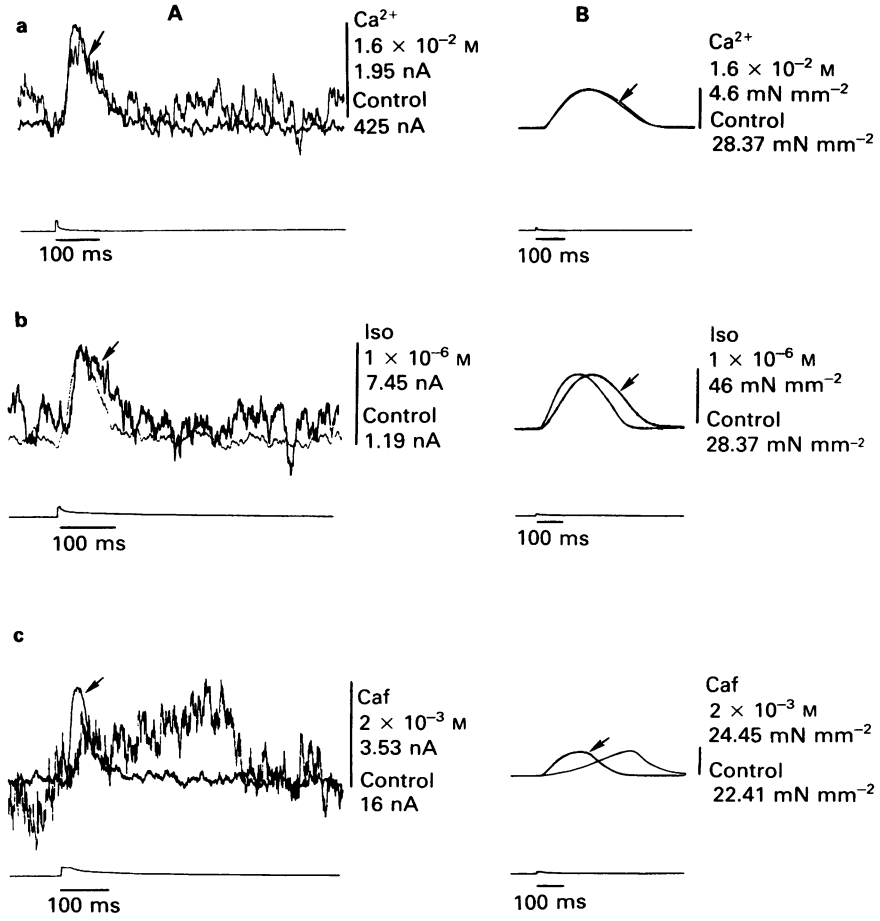

Figure 4 Effects of (a) Ca^{2+}, (b) isoprenaline (Iso) and (c) caffeine (Caf) on the time course of (A) light and (B) tension responses. Control and drug responses are superimposed with peak amplitudes adjusted so that time courses can be compared directly. Absolute light and tension responses for drug and control are indicated to the right of each panel. Arrows indicate control response.

cyclic AMP, isobutylmethylxanthine and forskolin shorten the duration of the aequorin light signal; Figure 6 shows that milrinone and piroximone have similar effects. Compared to the striking prolongation of the aequorin light signal produced by caffeine, the shortening produced by agents that increase intracellular cyclic AMP was less dramatic but statistically significant. For example, when analysed quantitatively in terms of the half-time of decline of the light signal, in eight muscles exposed to 1×10^{-5} M milrinone the half-time of decline from peak light was decreased by an average of 11.2% ($P < 0.03$). On the other hand, in contrast to the effects on the aequorin light signal, these agents produced a more striking effect on the time course of the tension response. Figure 4 shows that isoprenaline decreases, increased $[Ca^{2+}]_o$ does not change and caffeine prolongs the time course of developed tension. Figures 5 and 6 show that, like isoprenaline, other drugs that increase intracellular cyclic AMP levels, including milrinone and piroximone, decrease the duration of the tension response.

Negative inotropic effects of milrinone and piroximone

Figure 2 shows the biphasic inotropic action that was observed in 8 of the 17 experiments with milrinone. Note in Figure 2 that, although the amplitude of the aequorin light signal continues to increase in a dose-related manner, tension begins to decrease at 3×10^{-5} M milrinone. This negative inotropic effect typically occurred at doses higher than those associated with the positive inotropic actions of the drug. Similar biphasic inotropic actions were observed in 1 of the 7 experiments with piroximone. This negative inotropic effect is not due to tolerance or tachyphylaxis since the amplitude of the aequorin light signal either did not change or continued to increase in a dose-related manner as developed tension decreased, and developed tension increased again when the drug

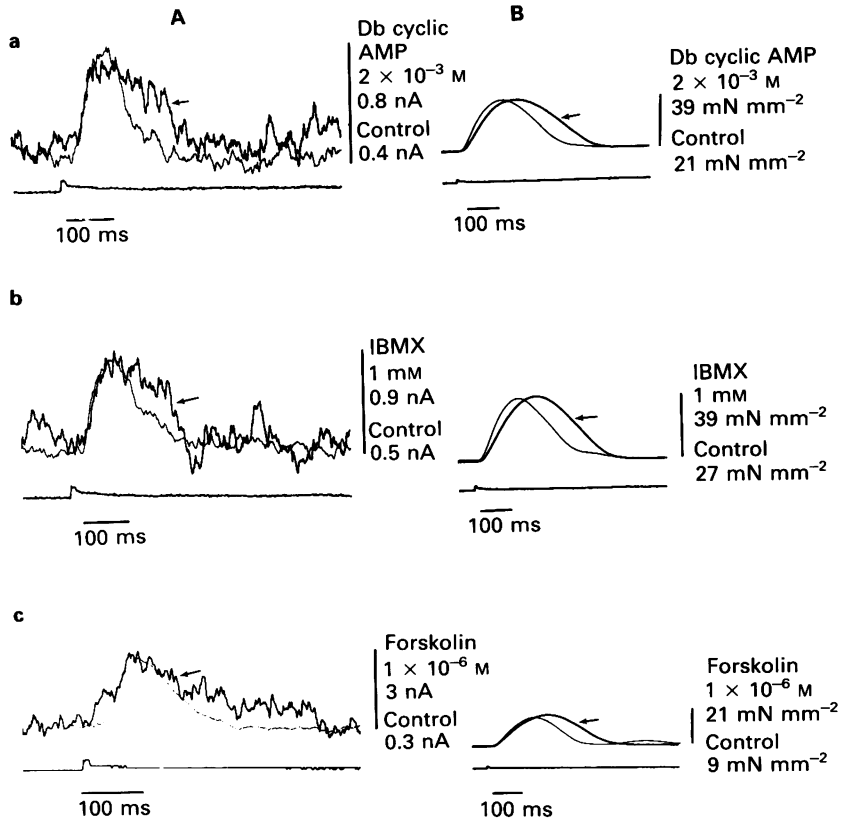

Figure 5 Effects of (a) dibutyryl cyclic AMP (Db cyclic AMP), (b) isobutylmethylxanthine (IBMX) and (c) forskolin on the time course of the (A) light and (B) tension responses. Control and drug responses are superimposed with peak amplitudes adjusted so that time courses can be compared directly. Absolute light and tension responses are indicated to the right of each panel. Arrows indicate control responses.

was washed from the bath. Under the conditions of these experiments, negative inotropic effects were not observed with isoprenaline or other drugs that increase cyclic AMP concentrations in the heart, except after prolonged exposure of a muscle to the drug, which would be expected to produce tolerance (in these cases the aequorin light signal and tension responses both decreased), or in toxic doses that produced a Ca^{2+}-overload state (see below). Moreover, this negative inotropic action was not a result of a shift of the pH in the bath since the bathing solution was carefully adjusted and measured at pH 7.4 before and after coming into contact with the muscle. Also, no effect on contractility was observed when equivalent amounts of acidic vehicle without drug were added to the bathing medium and back-titrated with base to pH 7.4 before being allowed to come into contact with the muscle.

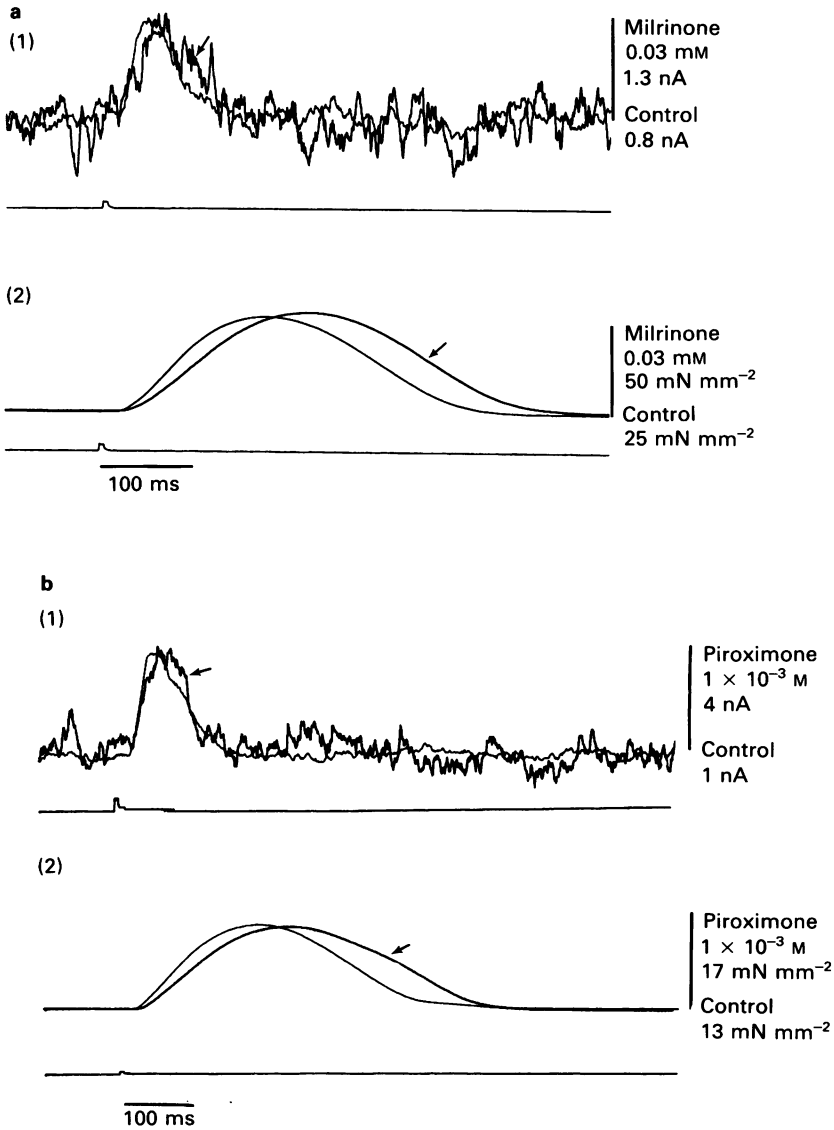

Figure 6 Effects of (a) milrinone and (b) piroximone on the time course of the (1) light and (2) tension responses. Control and drug responses are superimposed with peak amplitudes adjusted so that time courses can be compared directly. Absolute light and tension responses are indicated to the right of each panel. Arrows indicate control responses.

Toxic effects of milrinone and piroximone

Figures 2 and 3 illustrate the toxic effects that were observed in some experiments with high concentrations of milrinone (9 of 17 experiments) and piroximone (1 of 7 experiments). As indicated by the arrows in Figure 3, 10^{-3} M piroximone produced after-contractions in the tension recording that were preceded by a second distinct signal after the initial signal in the aequorin light response. Figure 2 shows a more striking example of this effect with 10^{-4} M milrinone. These second light signals have been referred to as 'after-glimmers'. In some experiments, multiple after-glimmers and after-contractions followed the initial response. In 2 experiments with milrinone, dysrhythmias were observed at doses that produced after-responses. Toxic doses of milrinone and isoprenaline were associated with a fall in both tension and in light, as shown in Figure 2. It was of interest to note that resting (i.e. diastolic) $[Ca^{2+}]$, as judged by the level of the baseline of the aequorin light signal, was not detectably increased by these doses of milrinone and piroximone. Similar toxic effects were observed with isoprenaline, and examples of after-glimmers and after-contractions are shown in Figure 5 for isobutylmethylxanthine and forskolin. These toxic effects could be reversed by washing the drug from the bath, although the amplitude of the light signal did not always return to control levels after these large doses of drugs suggesting that significant consumption of aequorin may have occurred.

Discussion

Interpretation of aequorin light signals in heart muscle

As shown in Figure 1 for ferret papillary muscles, the aequorin signals recorded from mammalian working myocardium (including also cat, kitten, rat, dog, rabbit and man) consist of a single component that temporally precedes the corresponding tension response. These findings are consistent with current models of excitation-contraction coupling which postulate that mechanical contractile events are preceded by changes in intracellular calcium concentrations (Fozzard, 1977; Chapman, 1979; Fabiato & Fabiato, 1977; 1979). The aequorin light signals recorded from mammalian working myocardium appear to reflect predominantly the release and uptake of Ca^{2+} from the sarcoplasmic reticulum (Morgan & Blinks, 1982). Therefore, the peak of the aequorin light signal can be used as a measure of the amount of Ca^{2+} released by the sarcoplasmic reticulum. The time to peak of the aequorin light signal appears to reflect the relative balance between the phase of the Ca^{2+} transient during which Ca^{2+} release predominates (ascending phase) and the phase during which resequestration of Ca^{2+} predominates (descending phase). The half-time of decline from the peak of the aequorin light signal can be used as a measure of the speed of Ca^{2+} reuptake by the sarcoplasmic reticulum. These interpretations of the components of the aequorin light signal are undoubtedly oversimplifications of the complex sequence of events that occur during excitation-contraction coupling in the heart, but are consistent with our current understanding of Ca^{2+} movements in myocardial cells, and form the basis of an empirically useful model for analysis of Ca^{2+} transients (Morgan *et al.*, 1984a; Morgan & Morgan, 1984a). Determination of the relative effects of drugs on the amplitude of the aequorin light signal at similar levels of tension development provides an index for changes in the sensitivity of the contractile apparatus to Ca^{2+}; an index that correlates well with results from experiments in skinned cardiac muscle fibres (Fabiato & Fabiato, 1979; Allen & Kurihara, 1980; McClellan & Winegrad, 1980; Mope *et al.*, 1980; Hess & Wier, 1984).

Effects of inotropic drugs on amplitude of the aequorin light signal and tension response

The amplitude of the aequorin light signal in ferret working myocardium is increased by most inotropic drugs, including isoprenaline, dibutyryl cyclic AMP, isobutylmethylxanthine, forskolin, and increased $[Ca^{2+}]_o$, as well as milrinone and piroximone. Similar findings have been noted in other mammalian species (Allen & Kurihara, 1980; Morgan & Morgan, 1984a). These results are consistent with the expectation that an increased concentration of intracellular calcium would be reflected at the level of the myofilaments and result in increased activation. The precise mechanisms by which each of these agents increases intracellular calcium vary. Isoprenaline, dibutyryl cyclic AMP, isobutylmethylxanthine and forskolin have been shown to act primarily to increase intracellular levels of cyclic AMP (Katz, 1979; Tsein, 1977; Späh, 1984); this has been shown to have a variety of subcellular actions (Katz, 1979). One of these is to increase the amount of calcium that enters the cell during the plateau phase of the action potential; this effect may be due to phosphorylation of a sarcolemmal protein that increases calcium conductance through the so-called 'slow-channels' (Nathan & Beeler, 1975). The increased amount of calcium that enters the cell during each action potential then becomes available for loading of intracellular stores and for release during subsequent action potentials via the calcium-induced calcium release process. Milrinone and piroximone have also been found to increase the intracellular cyclic AMP concentrations by phosphodiesterase inhibition, and their effects on the aequorin light signal

are consistent with this action (see references, above).

Increased $[Ca^{2+}]_o$ enhances the rate of exchange of intracellular sodium for extracellular calcium at the Na^+-Ca^{2+} exchange site on the sarcolemma (Chapman, 1979). This increased activity of the Na^+-Ca^{2+} exchange mechanism produces an increase in intracellular $[Ca^{2+}]$ which becomes available for loading of stores. Of the inotropic drugs studied, only caffeine produced a positive inotropic effect while actually lowering the peak of the calcium transient. The decrease in the amplitude of the Ca^{2+} transient, as well as its prolongation, correlate with known effects of caffeine on the sarcoplasmic reticulum where it has been shown to block the uptake (and subsequently the release) of Ca^{2+} (Blinks *et al.*, 1972; Morgan & Blinks, 1982).

The aequorin signals recorded in these experiments were not translated into exact Ca^{2+} concentrations due to the non-linearity of the light response (Cannell & Allen, 1984). However, the influence of various drugs upon the relationship between intracellular $[Ca^{2+}]$ and developed tension can be estimated by considering the ratio, in a single papillary muscle, of the peak of the aequorin signal to the peak of the developed tension response, (Allen & Kurihara, 1980; Morgan *et al.*, 1983; Morgan & Morgan, 1984a). The results of this sort of analysis are consistent with those from skinned cardiac muscle preparations which showed that the sensitivity of the myofilaments to Ca^{2+} is decreased by drugs that increase intracellular cyclic AMP levels, and increased by the methylxanthines. This observation may explain the positive inotropic action of caffeine which, although it decreases the amplitude of the Ca^{2+} transient, alters the calcium sensitivity of the myofilaments so that any given concentration of Ca^{2+} will produce a greater degree of activation (Wendt & Stephenson, 1983). Increasing extracellular Ca^{2+} has recently been shown to produce a dose-related increase in intracellular cyclic AMP concentrations (Daugherty & Woodward, 1981). As discussed above, an increase in intracellular cyclic AMP levels has been shown to decrease the sensitivity of the myofilaments to Ca^{2+} and this effect may account for the slight decrease in tension that occurred while the amplitude of the aequorin light signal continued to rise at 16 mM $[Ca^{2+}]_o$, as shown in Figure 1; alternatively, the fall may be due to Ca^{2+} overload.

Effects of inotropic drugs on the time course of the aequorin light signal and tension response

As shown in Figures 4, 5 and 6, drugs that are known to increase intracellular cyclic AMP concentrations (i.e. isoprenaline, dibutyryl cyclic AMP, forskolin, isobutylmethylxanthine, milrinone and piroximone) decrease the time course of the aequorin light signal and corresponding tension response. This finding is consistent with observations that an increase in cyclic AMP can produce an increased rate of calcium uptake by the sarcoplasmic reticulum (Katz, 1979). This effect is much more dramatic in species such as the cat which have longer activation times than the ferret (Blinks *et al.*, 1982b; Morgan & Blinks, 1982; Morgan *et al.*, 1983). Increases in $[Ca^{2+}]_o$ did not have a significant effect on the time course of the aequorin light signal nor on the tension response. This was surprising, since a Ca^{2+}-dependent re-uptake mechanism has been described in the sarcoplasmic reticulum from other mammalian species (Tada & Katz, 1982). However, similar results with Ca^{2+} were found in aequorin-loaded cat papillary muscles maintained at 37.5°C (Morgan *et al.*, 1983; Morgan & Morgan, 1984). On the other hand, Allen & Kurihara (1980), under slightly different experimental conditions, showed a significant shortening of the time course of the aequorin signal with increases in $[Ca^{2+}]_o$ in cat ventricular muscle but no change in rat ventricular muscle. Caffeine produces an effect in the opposite direction to isoprenaline; i.e. the duration of the calcium transient and corresponding tension response were both markedly prolonged compared to the control. This effect can be explained by the subcellular actions of caffeine which, in addition to phosphodiesterase inhibition with an increase in intracellular cyclic nucleotide concentrations, has been shown to block the uptake and subsequently the release of calcium from intracellular stores (Blinks *et al.*, 1972). These latter actions would be expected be prolong the duration of the calcium transient and produce a negative inotropic effect as shown in Figure 1 for 0.6 mM caffeine. In addition, as mentioned above, the methylxanthines have been shown to increase the sensitivity of the myofilaments to calcium and this effect is illustrated at the higher doses of caffeine in Figure 1, where the amplitude of the aequorin light signal falls as tension continues to rise. These caffeine data show that the duration of the calcium transient can be an important determinant of the time course of tension development. However, the time course of the calcium transient in ferret papillary muscles appears to change little during stimulation with a variety of other inotropic agents (see Figures 4, 5 and 6), although effects on the time course of the tension response are more apparent. These results suggest that in contrast to other mammalian species with longer activation times, the duration of contraction of the ferret heart is to a greater extent controlled by changes in sensitivity of the contractile apparatus compared to changes in the time course of the intracellular calcium transient itself.

Biphasic inotropic action of milrinone and piroximone

Milrinone and piroximone both demonstrated biphasic inotropic actions at higher concentrations.

Negative inotropic effects were not observed in previous studies with amrinone in aequorin-loaded cat working myocardium (Morgan *et al.*, 1980) but have been seen in some preliminary studies in cardiomyopathic human myocardium (unpublished results from this laboratory) and have been observed in canine Purkinje fibres (Rosenthal & Ferrier, 1982). The time course of the aequorin light response was not significantly altered, suggesting that changes in intracellular Ca^{2+} handling by the sarcoplasmic reticulum are not responsible for this effect. However, the ratio of peak light to peak tension was increased at some doses producing a negative inotropic effect, suggesting that it may be related in part to a decrease in the sensitivity of the myofilaments to Ca^{2+}. At lower doses, this effect on the myofilaments may be overcome by the marked increase in amplitude of the intracellular calcium transient; at higher doses, the effect on the myofilaments appears to predominate. However, an increase in intracellular cyclic AMP levels may not be the only mechanism involved in the negative inotropic action of milrinone and piroximone since single high doses of isoprenaline, and other drugs known to increase cyclic AMP concentrations, did not consistently produce a negative inotropic effect with an increase in amplitude of the aequorin light signal under the conditions of these experiments.

A decrease in developed tension while the amplitude of the Ca^{2+} transient continues to increase has also been found to occur when intracellular pH is lowered (Allen & Orchard, 1983). It is possible that large doses of milrinone and piroximone produce similar metabolic effects on the cell. As shown in Figure 2 for milrinone, the negative inotropic effects seen at toxic doses were associated with a corresponding decrease in the amplitude of the aequorin light signal. The mechanisms of this effect are not clear, but may reflect decreased release of Ca^{2+} from the sarcoplasmic reticulum in addition to a decrease in the sensitivity of the myofilaments to Ca^{2+}. The significance in man of the biphasic inotropic response to milrinone is unknown at present since, as in the case of other drugs such as caffeine and ryanodine, it may occur only in certain species of mammals (Bodem & Sonnenblick, 1975; Sutko & Willerson, 1980) or under specific experimental conditions (Blinks *et al.*, 1972). Similar biphasic inotropic effects have been described for adrenaline in amphibian (Morad *et al.*, 1978) and mammalian myocardium (Kavaler & Morad, 1966), and have been attributed to increased intracellular concentrations of cyclic AMP. Whatever the mechanism, it is possible that this effect could account for treatment failures with milrinone in some patients (Baim *et al.*, 1983). Blinks & Endoh (1984) have recently described a positive inotropic effect of sulmazol in dog trabecular muscles that was associated with a prolongation and a decreased amplitude of the aequorin signal; we did not observe this sort of effect with milrinone or piroximone in the ferret.

Toxic effects of milrinone and piroximone

The toxic effects that were noted at higher doses of milrinone and piroximone were associated with the presence of after-glimmers and after-contractions and the occurrence of dysrhythmias. Previous studies have shown that after-contractions occur with toxic doses of most positive inotropic agents that increase intracellular $[Ca^{2+}]$ (Allen & Kurihara, 1980; Morgan & Blinks, 1982; Blinks *et al.*, 1982b, Wier *et al.*, 1983; Orchard *et al.*, 1983), particularly those that increase intracellular cyclic AMP concentrations (Opie *et al.*, 1978). Experimental evidence suggests that these after-responses are produced by an oscillatory release of calcium from intracellular stores (Allen *et al.*, 1984). This oscillatory release may be caused by increased sarcoplasmic levels of calcium produced by high doses of inotropic drugs, that is, calcium-induced calcium release. The development of a calcium-overload state could account for the occurrence of dysrhythmias due to increased automaticity in Ca^{2+}-dependent pacemaker fibres, triggerable responses, or conduction abnormalities with the development of re-entrant dysrhythmias. Although the precise mechanisms of the dysrhythmogenic effects are not clear from these experiments, the occurrence of dysrhythmias is of interest since studies in other mammalian species have shown that milrinone, piroximone and related drugs are not arrhythmogenic (Rosenthal & Ferrier, 1982; Piwonka *et al.*, 1983; Roebel *et al.*, 1983). However, amrinone-induced arrhythmias have been observed in rabbit papillary muscle (Onuaguluchi & Tanz, 1981).

The authors thank Dr A. Farah of Sterling-Winthrop Laboratories for a generous supply of milrinone and Dr L. Roebel of Merrell-Dow, Inc. for a generous supply of piroximone. This work was supported in part by Grants HL 31117 and HL 07374 from the National Heart, Lung and Blood Institute, Bethesda, Maryland, U.S.A. Correspondence to J.K.G. please.

References

ALLEN, D.G., EISNER, D.A. & ORCHARD, C.H. (1984). Characterization of oscillations of intracellular calcium concentration in ferret ventricular muscle. *J. Physiol.*, **352**, 113–128.

ALLEN, D.G. & KURIHARA, S. (1980). Calcium transients in mammalian ventricular muscle. *Eur. Heart J.*, **1**, suppl A, 5–15.

ALLEN, D.G. & ORCHARD, C.H. (1983). The effects of

changes of pH on intracellular calcium transients in mammalian cardiac muscle. *J. Physiol.*, **335**, 555–567.

ALOUSI, A.A., STANKUS, G.P., STUART, J.C. & WALTON, L.H. (1983). Characterization of the cardiotonic effects of milrinone, a new and potent cardiac bipyridine, on isolated tissues from several animal species. *J. cardiovasc. Pharmac.*, **5**, 804–811.

BAIM, D.S., MCDOWELL, A.V., CHERNILES, J., MONRAD, E.S., PARKER, J.A., EDELSON, J., BRAUNWALD, E. & GROSSMAN, W. (1983). Evaluation of a new bipyridine inotropic agent-milrinone-in patients with congestive heart failure. *N. Engl. J. Med.*, **309**, 748–756.

BLINKS, J.R. (1982). The use of photoproteins as calcium indicators in cellular physiology. In *Techniques in Cellular Physiology*, ed. Baker, P.F. pp. 1–38. County Clare, Ireland: Elsevier/North Holland.

BLINKS, J.R. & ENDOH, J. (1984). Sulmazol (AR-C 115 BS) alters the relation between $[Ca^{2+}]$ and tension in living canine ventricular muscle. *J. Physiol*, **353**, 63P.

BLINKS, J.R., MATTINGLY, P.H., JEWELL, B.R. VAN LEEUWEN, M., HARRER, G.C. & ALLEN, D.G. (1978). Practical aspects of the use of aequorin as a calcium indicator: Assay, preparation, microinjection and interpretation of signals. *Methods Enzymol*, **57**, 292–328.

BLINKS, J.R., OLSON, C.B., JEWELL, B.R. & BRAVENY, P. (1972). Influence of caffeine and other methylxanthines on mechanical properties of isolated mammalian heart muscle. *Circulation Res.*, **30**, 367–392.

BLINKS, J.R., WIER, W.G., HESS, P. & PRENDERGAST, F.G. (1982a). Measurement of Ca^{2+} concentrations in living cells. *Prog. biophys. mol. Biol.*, **40**, 1–114.

BLINKS, J.R., WIER, W.G., MORGAN, J.P. & HESS, P. (1982b). Regulation of intracellular $[Ca^{++}]$ by cardiotonic drugs. In *Advances in pharmacology and toxicology II: cardiorenal and cellular pharmacology*, ed. Yoshida, H., Hagihara, Y. & Ebashi, S. pp. 205–216. Oxford: Pergamon Press.

BODEM, R. & SONNENBLICK, E.H. (1975). Mechanical activity of mammalian heart muscle: variable onset, species differences, and the effect of caffeine. *Am. J. Physiol.*, **228**, 250–261.

CANNELL, M.B. & ALLEN, D.G. (1984). Model of Calcium movements during activation in the sarcomere of frog skeletal muscle. *Biophys. J.*, **45**, 913–925.

CHAPMAN, R.A. (1979). Excitation-contraction coupling in cardiac muscle. *Prog. biophys. mol. Biol.*, **35**, 1–52.

DAUGHERTY, A. & WOODWARD, B. (1981). Calcium and calcium slow channel antagonists on cyclic nucleotide levels in the isolated rat heart. *J. mol. cell. Cardiol.*, **13**, 843–854.

ENDOH, M., YAMASHITA, S. & TAIRA, N. (1982). Positive inotropic effect of amrinone in relation to cyclic nucleotide metabolism in the canine ventricular muscle. *J. Pharmac. exp. Ther.*, **221**, 775–783.

FABIATO, A. & FABIATO, F. (1977). Calcium release from the sarcoplasmic reticulum. *Circulation Res.*, **40**, 119–129.

FABIATO, A. & FABIATO, F. (1979). Calcium and cardiac excitation-contraction coupling. *A. Rev. Physiol.*, **41**, 473–484.

FOZZARD, H.A. (1977). Heart: excitation-contraction coupling. *A. Rev. Physiol.*, **39**, 201–220.

HESS, P. & WIER, W.G. (1984). Excitation-contraction coupling in cardiac Purkinje fibers. Effects of caffeine on the intracellular $[Ca^{2+}]$ transient, membrane currents and contractions. *J. gen. Physiol.*, **83**, 413–433.

HONERJÄGER, P., SCHÄFER-KORTING, M. & REITER, M. (1981). Involvement of cyclic AMP in the direct inotropic action of amrinone. Biochemical and functional evidence. *Naunyn-Schmiedebergs Arch. Pharmac.*, **318**, 112–120.

KARIYA, T., WILLE, L J. & DAGE, R.C. (1982). Biochemical studies on the mechanism of cardiotonic activity of MDL 17, 043. *J. cardiovasc. Pharmac.*, **4**, 509–514.

KARIYA, T., WILLE, L.J. & DAGE, R.C. (1984). Studies on the mechanism of the cardiotonic activity of MDL 19205: effects on several biochemical systems. *J. cardiovasc. Pharmac.*, **6**, 650–655.

KATZ, A.M. (1979). Role of the contractile proteins and sarcoplasmic reticulum in the response of the heart to catecholamines: an historical review. *Adv. cyclic nucleotide Res.*, **11**, 303–343.

KAVALER, F. & MORAD, M. (1966). Paradoxical effects of epinephrine on excitation-contraction coupling in cardiac muscle. *Circulation Res.*, **23**, 492–501.

McCLELLAN, G.B. & WINEGRAD, S. (1980). Cyclic nucleotide regulation of the contractile protein in mammalian cardiac muscle. *J. gen. Physiol.*, **75**, 283–295.

MOPE, L., McCLELLAN, G.B. & WINEGRADS. (1980). Calcium sensitivity of the contractile system and phosphorylation of troponin in hyperpermeable cardiac cells. *J. gen. Physiol.*, **75**, 271–282.

MORAD, M., WEISS, J. & CLEEMAN, L. (1978). The inotropic action of adrenaline on cardiac muscle: does it relax or potentiate tension? *Eur. J. Cardiol.*, **7**, suppl., 53–62.

MORGAN, J.P. & BLINKS, J.R. (1982). Intracellular Ca^{++} transients in the cat papillary muscle. *Can. J. Physiol. Pharmac.*, **60**, 524–528.

MORGAN, J.P., CHESBRO, H.H., PLUTH, J.R., PUGA, F.J. & SCHAFF, H.V. (1984a). Intracellular calcium transients in human working myocardium as detected with aequorin. *J. Am. Coll. Cardiol.*, **3**, 410–418.

MORGAN, J.P., DEFEO, T.T. & MORGAN, K.G. (1984b) A chemical procedure for loading the calcium indicator aequorin into mammalian working myocardium. *Pflügers Arch.*, **400**, 338–340.

MORGAN, J.P., LEE, N.K.M. & BLINKS, J.R. (1980). Mechanism of inotropic action of amrinone: unusual pattern of Ca^{++} transients as detected with aequorin. (1980). Fedn. Proc., **39**, 854.

MORGAN, J.P. & MORGAN, K.G. (1984a). Intracellular calcium levels during contraction and relaxation of mammalian cardiac and vascular smooth muscle as detected with aequorin. *Am. J. Med.*, **77**, suppl 5A, 33–46.

MORGAN, J.P. & MORGAN, K.G. (1984b). Stimulus-specific patterns of intracellular calcium levels in smooth muscle of ferret portal vein. *J. Physiol.*, **351**, 155–167.

MORGAN, J.P., WIER, W.G., HESS, P. & BLINKS, J.R. (1983). Influence of Ca^{2+} channel blocking agents on calcium transients and tension development in isolated mammalian heart muscle. *Circulation Res.*, **52**, suppl I, 47–52.

NATHAN, D. & BEELER, G.W. (1975). Electrophysiologic correlates of the inotropic effects of isoproterenol in canine myocardium *J. mol. cell. Cardiol.*, **7**, 1–15.

ONUAGULUCHI, G. & TANZ, R.D. (1981). Cardiac effects of amrinone on rabbit papillary muscle and guinea pig Langendorff heart preparations. *J. cardiovasc. Pharmac.*,

3, 1342–1355.

OPIE, L.H., MULLER, C.A. & LUBBE, W.F. (1978). Cyclic AMP and arrhythmias revisited. *Lencet*, **ii**, 921–923.

ORCHARD, C.H., EISNER, D.A. & ALLEN, D.G. (1983). Oscillations of intracellular Ca^{2+} in mammalian cardiac muscle. *Nature*, **304**, 735–738.

PIWONKA, R.W., CANNIFF, P.C. & FARAH, A.E. (1983). *In vitro* electrophysiologic properties of amrinone in mammalian cardiac tissue. *J. cardiovasc. Pharmac.*, **5**, 1058–1067.

ROEBEL, L.E., DAGE, R.C., CHENG, H.C. & WOODWARD, J.K. (1984). *In vitro* and *in vivo* assessment of the cardiovascular effects of the cardiotonic drug MDL 19205. *J. cardiovasc. Pharmac.*, **6**, 43–49.

ROSENTHAL, J.E. & FERRIER, G.R. (1982). Inotropic and electrophysiologic effects of amrinone in untreated and digitalized ventricular tissues. *J. Pharmac. exp. Ther.*, **221**, 188–196.

SPAH, F. (1984). Forskolin, a new positive inotropic agent, and its influence on myocardial electrogenic cation movements. *J. cardiovasc. Pharmac.*, **6**, 99–106.

SUTKO, J.L. & WILLERSON, J.T. (1980). Ryanodine alteration of the contractile state of rat ventricular myocardium. Comparison with dog, cat, and rabbit ventricular tissues. *Circulation Res.*, **46**, 332–343.

TADA, M. & KATZ, A.M. (1982). Phosphorylation of the sarcoplasmic reticulum and sarcolemma. *A Rev. Physiol.*, **44**, 401–443.

TSIEN, R.W. (1977). Cyclic AMP and contractile activity in heart. *Adv. cyclic nucleotide Res.*, **8**, 363–420.

WENDT, I.R. & STEPHENSON, D.G. (1983). Effects of caffeine on Ca-activated force production in skinned cardiac and skeletal muscle fibers of the rat. *Pflügers Arch.*, **398**, 210–216.

WIER, W.G., KORT, A.A., STERN, M.D., LAKATTA, E.G. & MARBAN E. (1983). Cellular calcium fluctuations in mammalian heart; Direct evidence from noise analysis of aequorin signals in Purkinje fibers. *proc. natn. Acad. Sci. U.S.A.*, **80**, 7367–7371.

(Received August 21, 1984.
Revised November 26, 1984.
Accepted December 19, 1984.)