Effects of caffeine and ryanodine on depression of post-rest tension development produced by Bay K 8644 in canine ventricular muscle

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1 Post-rest inotropy in canine ventricular myocardium has proved a useful indicator of sarcoplasmic reticular calcium release. This phenomenon is converted to rest depression by the calcium channel activator (agonist), Bay K 8644 as well as other chemically diverse agents such as caffeine and ryanodine.

2 Rapid cooling contractures and post-rest contraction amplitude were used as independent measures of sarcoplasmic reticular calcium content and release. Simultaneous recordings of transmembrane action potentials and their accompanying contractions were obtained to determine the association between electrophysiological and mechanical events. The present study was designed to elucidate the mechanism by which Bay K 8644, caffeine and ryanodine alter force production after variable periods of rest.

3 Bay K 8644 (1 μ M) increased steady state contraction in response to a constant train of stimulation, caused rest-depression after 2 and 8 min rest, prolonged action potential duration and increased action potential plateau amplitude. Augmented steady state tension was not accompanied by any change in time to peak tension or rapid cooling contracture amplitude. However, the post-rest rapid cooling contracture was greatly diminished compared to that observed prior to Bay K 8644 treatment.

4 Caffeine (3 and 5 mM) caused rest-depression with an increase in steady state contraction amplitude. Along with this there was a slight decrease in action potential duration and plateau amplitude and an increase in time to peak tension. The rapid cooling contractures were virtually abolished at all conditioning intervals. The effect of caffeine on twitch tension and cooling contracture is consistent with the ability of this compound to inhibit calcium sequestration by the sarcoplasmic reticulum.

5 A combination of Bay K 8644 and caffeine caused significantly less rest-depression than that seen with Bay K 8644 alone. The augmented twitch tension was accompanied by a long time to peak tension and action potential duration. However, there was no increase in the amplitude of the rapid cooling contracture, either after a regular train of stimulation or after rest, compared to that seen after Bay K 8644.

6 Ryanodine (10 nM), produced rest-depression, reduced steady state twitch tension and augmented the rest-depression produced by Bay K 8644. The steady state rapid cooling contracture remained unchanged when both agents were present simultaneously, while the post-rest rapid cooling contracture was significantly depressed compared to that observed with Bay K 8644 alone.

7 Bay K 8644 and ryanodine appear to have similar actions with respect to promoting diastolic loss of calcium from the sarcoplasmic reticulum. Although caffeine also decreases post-rest potentiation, it antagonizes rest-depression caused by Bay K 8644. The data from these experiments

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suggest that this reversal is a result of depressed intracellular calcium buffering and enhanced myofilament sensitivity produced by caffeine in the presence of increased transmembrane calcium influx promoted by Bay K 8644.

Introduction

Previous studies of the effect of the racemic dihydropyridine Bay K 8644 on microscopic currents in cardiac tissue have shown that it facilitates the inwardly directed calcium current (Thomas *et al.*, 1985a) by increasing the open time of membrane-bound voltage-gated channels (Hess *et al.*, 1984). In mechanical studies on preparations of isolated ventricular muscle, the addition of Bay K 8644 increases peak isometric tension development in response to rhythmic electrical stimulation (Thomas *et al.*, 1985a; Hryshko & Bose, 1985; Bose *et al.*, 1987). However, in addition to increasing twitch tension in response to steady state stimulation, this inotropic agent also significantly impaired the ability of the muscle to develop tension after a period of rest (Hryshko, 1987; Bouchard *et al.*, 1988; Bose *et al.*, 1988a,b; Hryshko *et al.*, 1989).

Contraction amplitude in ventricular muscle from several mammalian species is transiently increased after brief periods of rest. This potentiation is biphasic in that a maximum is observed following some characteristic period of rest (Koch-Weser & Blinks, 1961; 1963; Schouten *et al.*, 1987). The sarcoplasmic reticulum has been implicated in rest-potentiation (Bers, 1985; Bose *et al.*, 1988a; Walsh & Tormay, 1989). In contrast to the phenomenon of post-rest inotropy which is observed with short duration of rest, extended periods of rest lead to depressed post-rest contractions in most mammalian species (Koch-Weser & Blinks, 1961; 1963), similar to the effect observed with Bay K 8644. This depression is due to a progressive loss of calcium from the sarcoplasmic reticulum (King & Bose, 1983; Bers, 1985; Hilgemann, 1986a; Bridge, 1986). In atrial preparations (Thomas *et al.*, 1985a) even after prolonged rest there is a substantial residual 'rested state' contraction, believed to be due to release of calcium from the sarcoplasmic reticulum. This is in contrast to the 'rested state' contraction in ventricular muscle from some mammalian species which is predominantly due to trans-sarcolemmal calcium influx and is characterized by a slower onset of a very small contraction (Reiter *et al.*, 1978). The inhibition of post-rest force production of the type seen in the presence of Bay K 8644 is similar to that observed during long rest intervals in control muscles, suggesting that this agent might enhance diastolic calcium loss from the sarcoplasmic reticulum leading to a reduction in the availability of activator calcium from the release site in this organelle.

Caffeine and ryanodine are commonly used to study excitation-contraction coupling in mammalian cardiac muscle as they inhibit calcium release from the sarcoplasmic reticulum or its uptake into this organelle (Weber & Herz, 1968; Blinks *et al.*, 1972; Fabiato, 1981; Bers *et al.*, 1987). The objective of the present study was to design experiments which would make use of the known properties of these compounds to reveal the mechanism by which Bay K 8644 affects post-rest force production. To achieve this, Bay K 8644 was tested alone and in combination with either caffeine or ryanodine to determine if they had a similar mechanism of action on post-rest tension development. It was reasoned that this should make the combined effect of Bay K 8644 and any of the other agents on rest potentiation additive. Rapid cooling contractures were also used to assess indirectly sarcoplasmic reticular calcium content. Simultaneous recordings of transmembrane action potentials and twitch contractions were obtained to determine the relationship between electrical and mechanical events within a given contraction. Preliminary data have been presented to the Federation of American Societies for Experimental Biology (Bouchard *et al.*, 1988).

Methods

Experimental preparation

Mongrel dogs of either sex weighing 8–12 kg were anaesthetized with sodium pentobarbitone (30 mg kg⁻¹, i.v.). Hearts were rapidly excised through a lateral thoracotomy and the coronary vasculature flushed with 50 ml cold (4°C) Krebs-Henseleit (KH) solution. KH solution had the following composition (mm): NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.4, NaHCO₃ 25 and glucose 11. Thin, free running right ventricular trabeculae (0.2–0.5 mm diameter) were tied with 8-0 silk (Matuda) and dissected free from the wall of the heart.

Tension measurements

Isolated muscles were obtained as mentioned previously and transferred to a 2 ml horizontal recircu-

Figure 1 Typical response of canine ventricular trabeculae to varying periods of rest in an untreated muscle (a) and after treatment with $1 \mu\text{M}$ Bay K 8644 (b), 5 mM caffeine (c) and 10 nM ryanodine (d). Rest durations of 2 (left) and 8 (right) min were imposed after a continuous train of stimulation at a frequency of 0.5 Hz .

lating bath containing oxygenated (95% $\text{O}_2/5\% \text{ CO}_2$) KH solution maintained at a pH of 7.4 and a temperature of 37°C . Muscles were field stimulated by pulses delivered by a computer-driven stimulus sequencer (Boyechko & Bose, 1982) at a frequency of 0.5 Hz and an amplitude 75–100% above threshold voltage and equilibrated for 45–60 min after which they were stretched to an optimal length for maximal isometric force production. Isometric contractions were recorded with a force transducer (Grass FT03C). Periods of rest ranging from 30–240 s were randomly imposed on the otherwise constant (steady state) stimulus pattern.

Rapid cooling contracture

The muscle chamber formed part of a recirculation apparatus with a single bath and two reservoirs connected in parallel with the bath. This system allowed virtually instantaneous (1 s) switching of bathing solutions. Rapid cooling contractures were elicited by cooling the preparation from 37° to $0\text{--}2^\circ\text{C}$ by changing the solution entering the muscle chamber through a parallel by-pass system. Identical rest periods to those used in the twitch contraction experiments were examined, where the rapid cooling

contractures were measured in the place of a regularly driven beat (steady state rapid cooling contracture) or after variable periods of inactivation (post-rest rapid cooling contracture).

Electrophysiology

For simultaneous recordings of transmembrane action potentials and contractions, one end of the trabecula was fixed with stainless steel insect pins to a silicone rubber base in the bath and the other end was connected to an isometric force transducer. Glass micropipettes were pulled with a Brown & Flaming micropipette puller (Model P-77; Sutter Instruments Co.) and filled with 3 M KCl (tip impedance $8\text{--}25 \text{ M}\Omega$) to record transmembrane action potentials from the surface cells of the trabeculae. Electrodes were connected to a high input impedance amplifier (Neuroprobe 1600; Transidyne General Corp.). A 50 mV calibration signal and 0 mV absolute voltage level were recorded prior to each action potential. These signals, together with simultaneous recordings of action potentials and contractions were recorded on a thermal chart recorder (Model 8500; Astro Med Inc.) and a Zenith VHS recorder (Model VP-2220; Zenith Corp.) coupled to

an analog to digital converter and PCM adapter (Model VR-10; Instrutec Corp.). Signals were monitored during experiments with a 4 channel Hewlett-Packard storage oscilloscope.

Drugs

Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) was a generous gift from Dr A. Scriabine (Miles Institute of Preclinical Research) and was dissolved in 100% ethanol. The final concentration of ethanol in the solution did not exceed 0.1% and was usually 0.01%. In the presence of 0.01% there was no decrease in steady state contractions while with 0.1% there was a 25% decrease in contractile force. However there was no decrease in rest potentiation with this entire range of concentrations. Caffeine and ryanodine were obtained from Sigma Chemicals and were dissolved in twice distilled deionized water. All chemicals for physiological solutions were reagent grade. Bay K 8644 was used at a concentration of 1 μ M. Although rest depression could be demonstrated with much lower concentrations (i.e., 0.01 μ M or more), the onset and stabilization of the effect were much slower. The same was true for ryanodine which was effective in concentrations as low as 1 nM. The concentration range of caffeine chosen (3–5 mM) was similar to the one shown by us previously to impair sarcoplasmic reticulum-mediated contractions (King & Bose, 1983; Hryshko *et al.*, 1989). Further increase in the concentrations of caffeine produced greater effects but the tissue response deteriorated.

Statistics

Statistical significance was determined with analysis of variance followed by Duncan's new multiple range test for multiple comparisons and Student's paired *t* test for self-controlled experiments (Steel & Torrie, 1960). The level of significance chosen for all experiments was $P < 0.05$. Data have been expressed as mean $\pm$ s.e.mean.

Results

Rest potentiation

Figure 1 depicts the contractile response of canine isolated right ventricular trabeculae to 2 and 8 min rest periods. The control muscle (Figure 1a) shows post-rest potentiation after both rest intervals. Addi-

Figure 2 Pooled data from 6 experiments showing the effect of combining caffeine (5 mM) or ryanodine (10 nM) with Bay K 8644 (1 μ M) on tension development of the first post-rest beat after 8 min rest. Results are expressed as a percentage of steady state tension of the contraction preceding the test interval. Statistical significance of observed differences were analyzed with an analysis of variance followed by Duncan's new multiple range test for multiple comparisons. * $P < 0.05$.

tion of Bay K 8644 (1 μ M, 1b), caffeine (3 mM, 1c) and ryanodine (10 nM, 1d) resulted in rest-depression. The amplitudes of post-rest contractions were consistently found to be smaller after 8 min rest compared to those observed after rest for 2 min in all preparations studied.

Figure 2 illustrates the effect of combining Bay K 8644 with caffeine or ryanodine on tension developed after 8 min of rest. As may have been expected if these compounds acted by a common functional mechanism, further addition of ryanodine resulted in an increase in the depression of post-rest twitch tension compared to that observed with Bay K 8644 alone. In contrast, the addition of caffeine to the Bay K 8644-treated preparation reduced the rest-depression. Both combinations of treatments produced a slight depression of tension development in response to a rhythmic train of stimulation, but this was statistically not significant. The most interesting difference between the contractile response in the presence of Bay K 8644 alone or in combination with one of the two other inotropes, was seen in contractions following a period of rest. Qualitatively similar changes were seen after rest for 2 min (this study; Hryshko, 1987; Bouchard *et al.*, 1988; Hryshko *et al.*, 1989).

Rapid cooling contractures

Rapid cooling contractures were elicited to estimate the functional capacity of the sarcoplasmic reticulum

Figure 3 Typical responses of canine ventricular trabeculae to a rapid change in temperature from 37° to 0°C in less than 1 s. The rapid cooling contractures were obtained in the place of a regularly driven beat (left), or after 2 (centre) and 8 (right) min rest. The moment of solution change is indicated by the arrow under the individual recordings: (a) represents the control trace and (b)–(d) after administration of Bay K 8644 (1 μ M), caffeine (5 mM), and ryanodine (10 nM). The stimulus frequency was 0.5 Hz for all rapid cooling contracture experiments.

Figure 4 Effect of combining Bay K 8644 (1 μ M) with caffeine (5 mM) or ryanodine (10 nM) on rapid cooling contracture amplitude. The protocol was similar to that described in Figure 3.

Figure 5 Pooled data from 6 rapid cooling contracture experiments showing the effect of combining Bay K 8644 (1 μ M) with caffeine (5 mM) or ryanodine (10 nM) on rapid cooling contractures obtained after 2 and 8 min of rest. Data are expressed as a percentage of contraction prior to initiating the rest interval. Significant differences between treated and untreated groups were determined by analysis of variance followed by Duncan's new multiple range test for multiple comparisons. * $P < 0.05$.

to store and release activator calcium (Kurihara & Sakai, 1985; Bridge, 1986).

Figure 3 illustrates the typical response of the isolated trabeculae to rapid cooling. Figure 3a shows a progressive decrease in the amplitude of the cooling contracture with progressive increase in the rest period prior to cooling. In the presence of Bay K 8644 (1 μ M; Figure 3b) there was a greater decrease in rapid cooling contracture at all the rest durations studied. On the other hand Bay K 8644 slightly increased the amplitude of rapid cooling contracture immediately following a train of regular stimuli. This may be a reflection of an inability of the sarcoplasmic reticulum to retain calcium during rest without an impairment of the calcium uptake process. Caffeine (5 mM; Figure 3c) also decreased the amplitude of the rapid cooling contracture after the various durations of rest. Unlike Bay K 8644, caffeine markedly reduced the amplitude of the rapid

Figure 6 Simultaneous recording of action potentials and accompanying contractions from isolated trabeculae after treatment with 1 μ M Bay K 8644 (top panel; a) or Bay K 8644 and 3 mM caffeine (bottom panel; b) after 2 (left) and 8 (right) min rest. In each case, the post-rest action potential and contraction (indicated by arrow) have been superimposed on the steady state beat preceding the period of rest. The stimulation frequency during all electrophysiological studies was 0.5 Hz.

cooling contracture immediately following a train of regular stimuli. Ryanodine (10 nM; Figure 3d) resembled Bay K 8644 in inhibiting post-rest rapid cooling contractures without inhibiting cooling contractures immediately after steady stimulation. Unlike Bay K 8644, ryanodine had a negative inotropic effect on the trabeculae.

The effect of combining Bay K 8644 with either caffeine (Figure 4) or ryanodine is shown in Figure 4. Responses of control muscles and those after treatment with Bay K 8644 are shown for reference (Figure 4a and b). On further addition of caffeine (5 mM) to the Bay K 8644-treated muscle, rapid cooling contractures were virtually abolished immediately following a train of stimuli or after rest (Figure 4c). Despite this, tension development in response to regular stimulation was maintained. The maintenance of tension development in response to rhythmic stimulation, in spite of the absence of rapid cooling contracture in the place of a regularly driven beat, may reflect the dependence of steady state force generation on trans-sarcolemmal calcium influx in the presence of caffeine. Ryanodine (10 nM) further decreased the post-rest (2 and 8 min) rapid cooling contracture already reduced by Bay K 8644. It should be noted that unlike caffeine, ryanodine did

Table 1 Effect of Bay K 8644, caffeine and ryanodine on twitch parameters

Treatment	n	TPT _{ss}	% change		RT _r
			RT _{ss}	TPT _r	
Bay K 8644 (1 μ M)	5	107.0 $\pm$ 6.7	116.6 $\pm$ 8.5*	112.9 $\pm$ 10.4	129.8 $\pm$ 11.5*
Caffeine (5 mM)	6	124.5 $\pm$ 1*	135.3 $\pm$ 7.1*	113.0 $\pm$ 3.5*	134.0 $\pm$ 10.6*
Ryanodine (10 nM)	6	162.5 $\pm$ 7.9*	277.5 $\pm$ 11.3*	125.0 $\pm$ 7.5*	290.5 $\pm$ 11*

Values are mean $\pm$ s.e.mean.

TPT = Time to peak tension.

RT = Relaxation time.

ss and r denote steady-state and post-rest contractions respectively.

* denotes $P < 0.05$ compared to control.

not decrease the cooling contracture immediately following a train of stimuli. The results of the rapid cooling experiment indicates that the spectrum of action of ryanodine resembles that of Bay K 8644. The pooled data from 6 experiments are shown in Figure 5.

Electrophysiological studies

Transmembrane action potentials and accompanying twitch contractions were obtained to determine if the apparent trans-sarcolemmal calcium entry associated with membrane depolarization due to caffeine was antagonizing the effect of Bay K 8644 in decreasing post-rest twitch tension. Time to peak tension can be influenced by both sarcoplasmic reticular calcium uptake and release. Thus, an increase in this measure of contraction may be a manifestation of either enhanced and/or prolonged trans-sarcolemmal calcium entry or a reduction of calcium uptake by the sarcoplasmic reticulum.

The effects of Bay K 8644 (1 μ M) alone and in combination with caffeine (3 mM) or ryanodine (10 nM) on the transmembrane action potential and accompanying contraction are illustrated in Figures 6 and 7. The recordings in each figure were made from a single trabecula. Data from 7 such experiments are summarized in Figures 8 and 9. Figure 8 represents the pooled data showing the effect of Bay K 8644, caffeine and ryanodine on the action potential duration at 50% repolarization during contractions obtained in response to regular stimulation or after 2 and 8 min rest.

As demonstrated in earlier experiments (Saha *et al.*, 1989, Hryshko *et al.*, 1989) administration of Bay K 8644 was found to prolong significantly the action potential duration at all intervals studied (see Figures 5 and 7). Similar effects on the time course of membrane depolarization were observed when caffeine was combined with Bay K 8644 (Figures 6 and

8). The addition of ryanodine to the Bay K 8644-treated muscle produced a dramatic increase in action potential duration during all test beats (Figures 7 and 8). Thus, the combination of either caffeine or ryanodine with Bay K 8644 was seen to have similar, albeit quantitatively different effects on transmembrane action potential duration. Presumably, these interventions may have similar effects on

Figure 7 Simultaneous recording of action potentials and accompanying contractions from isolated trabeculae after treatment with 1 μ M Bay K 8644 (a) or Bay K 8644 and 10 nM ryanodine (b) in response to rhythmic stimulation (left) or after 8 min rest (right).

Figure 8 Pooled data from 7 experiments showing control action potential duration for 50% repolarization (A), the effect of Bay K 8644 ($1 \mu\text{M}$; B) and a combination of Bay K 8644 with caffeine (3 mM ; C) or ryanodine (10 nM ; D). Measurements were made during steady state contractions and after 2 and 8 min rest. Significance of differences between treated and untreated groups were determined by an analysis of variance followed by Duncan's new multiple range test for multiple comparisons. * $P < 0.05$.

trans-sarcolemmal calcium influx associated with the prolonged action potentials, which would help to explain the partial protection of Bay K 8644-induced rest-depression by caffeine but not the contractile response of the trabeculae to the combination of Bay K 8644 and ryanodine.

Table 1 illustrates the effect of the three test agents on time to peak tension and relaxation time for contractions elicited in response to a regular train of stimuli and after 2 min rest. Addition of Bay K 8644 had no effect on time to peak tension. However, a small but statistically significant prolongation of the terminal portion of relaxation occurred (i.e., the final 10%). In comparison, both caffeine and ryanodine significantly prolonged the time to peak tension as well as relaxation time, ryanodine having a much greater effect than caffeine.

Pooled data from 7 experiments showing the effect of combining Bay K 8644 with caffeine or ryanodine on time to peak tension measurements obtained during steady state contractions and those elicited after 2 and 8 min rest are illustrated in Figure 9. A single dose of Bay K 8644 had a significant effect on the time to peak tension at any conditioning interval. In contrast, the combination of either caffeine or ryanodine with Bay K 8644 significantly enhanced the time course of contraction during continuous stimulation and after rest and thus was greater than

Figure 9 Pooled data from 7 experiments illustrating control time to peak tension (A), the effect of Bay K 8644 ($1 \mu\text{M}$; B) and the combination of Bay K 8644 with caffeine (5 mM ; C) or ryanodine (10 nM ; D). Measurements were made during steady state contractions and after 2 and 8 min rest. Statistical analysis of this data was similar to the tests performed in earlier figures. * $P < 0.05$.

the change produced by either agent alone. In this regard, it is important to stress that the combination of either caffeine or ryanodine with Bay K 8644 produced similar directional changes in action potential duration and time to peak tension but only caffeine appeared to protect partially against the Bay K 8644-induced rest-depression. Furthermore, these data suggest that trans-sarcolemmal calcium influx alone may not be the primary mechanism responsible for the ability of caffeine to antagonize Bay K 8644-induced rest depression.

Discussion

The new observation of this study is that although Bay K 8644, caffeine and ryanodine convert rest-potential in canine ventricular trabeculae to rest-depression, there are several fundamental differences in the spectrum of actions of these three agents. The conversion of rest-potential to rest-depression by Bay K 8644, first described by us (Bose *et al.*, 1987; Hryshko, 1987; Bouchard *et al.*, 1988; Hryshko *et al.*, 1989), is indicative of depletion of sarcoplasmic reticular calcium stores during diastole.

Considerable experimental evidence suggests that redistribution of calcium within the sarcoplasmic reticulum during diastole may be responsible for the potentiation of contraction amplitude (rest-potential) immediately following a period of rest (Koch-Weser & Blinks, 1961; 1963; King & Bose, 1983; Bers, 1985; Bers *et al.*, 1987; Hilgemann,

1986a,b; Bridge, 1986; Schouten *et al.*, 1987). From similar experiments it has also been suggested that there may be a continuous but slow loss of calcium from the sarcoplasmic reticulum during diastole. The latter, if allowed to continue for an extended period of time results ultimately in a decrease in contractility (rest-depression; Koch-Weser & Blinks, 1961; 1963; Bose *et al.*, 1988a). In the present study, the addition of Bay K 8644 to the bathing solution caused rest-depression which is similar to that seen normally with prolonged rest. This was in addition to its contrasting action of enhancing the amplitude of the steady state contraction. Based on a consideration of several models of excitation-contraction coupling in mammalian cardiac muscle proposed by various investigators (Koch-Weser & Blinks, 1963; Wood *et al.*, 1969; Morad & Goldman, 1973; Wohlfart & Noble, 1982; Schouten *et al.*, 1987; Bose *et al.*, 1988a), rest-depression due to Bay K 8644 might reflect one of two processes: (i) an acceleration of ongoing diastolic calcium loss from the sarcoplasmic reticulum passing through the sarcoplasm so as to influence the contractile apparatus or (ii) a pathway by which calcium lost from this organelle bypasses the contractile system. Experiments involving rapid cooling of the cardiac muscle to measure sarcoplasmic reticular calcium content as well as others using caffeine or ryanodine as pharmacological tools were done to obtain some insight into the ability of Bay K 8644 to perturb intracellular calcium handling by the sarcoplasmic reticulum.

Bay K 8644

We have shown previously that a racemic mixture of the dihydropyridine chemical Bay K 8644 prolongs action potential duration both during steady stimulation as well as after rest (Bose *et al.*, 1987; Hryshko *et al.*, 1989; Saha *et al.*, 1989). This is consistent with the ability of Bay K 8644 to increase transsarcolemmal calcium entry. The increase in amplitude of the steady state contraction can be explained on this basis. Of particular interest is the unusual effect of this compound not only to decrease rest-potential but to convert it to rest-depression. This is indicative of a decrease in sarcoplasmic reticular calcium release. Such an effect can be due either to increased refractoriness of the calcium release mechanism or to a depletion of the release pool. Despite this, the ability of the sarcoplasmic reticulum to take up calcium from the myoplasm is not affected, as shown by the lack of change of the time to peak tension seen in this study and others (Thomas *et al.*, 1985a). Further evidence in support of the maintained ability of the sarcoplasmic reticulum to pump calcium in the presence of Bay K 8644 is the increase in amplitude of rapid cooling

contracture following a regular train of stimuli, suggesting an increase in sarcoplasmic reticular calcium content. However, the rapid decrease in the amplitude of the rapid cooling contractures after initiation of rest indicates that the loss of calcium from the sarcoplasmic reticulum is increased by Bay K 8644. Previous investigations have failed to demonstrate any effect of this agent on sarcoplasmic reticular function in skinned cardiac muscle fibres or on calcium transport in sarcoplasmic reticular vesicles from skeletal muscle (Thomas *et al.*, 1985b; Zorzato *et al.*, 1985). One factor which may contribute to this unusual effect of Bay K 8644 on post-rest contraction, as opposed to contractions resulting from continuous stimulation, may be the functional junction between the t-tubule and sarcoplasmic reticulum which is retained only in the intact ventricular preparation. The inhibitory effect of Bay K 8644 on post-rest contraction is not seen in atrial preparations (D. Bose, unpublished observations) which are poor in t-tubules (Sommer & Johnson, 1979).

Post-rest force production has previously been shown to be strongly influenced by compounds that affect calcium handling by the sarcoplasmic reticulum (Hilgemann 1986a,b; Bers, 1987; Bers *et al.*, 1987). In such experiments, a gradual diastolic increase in the content of calcium in the extracellular space of cardiac muscle was found to be facilitated by the administration of ryanodine and was accompanied by a decrease in post-rest tension development. Thus, it appears that post-rest contraction in most mammalian species is governed to a greater extent by intracellular calcium release. It should be noted that while post-rest rapid cooling contracture amplitude decreases continuously with increasing rest durations, post-rest contractions evoked by electrical stimulation are biphasic in amplitude. Such a finding is consistent with the notion that rapid cooling contracture better represents total sarcoplasmic reticular calcium content while electrically evoked post-rest contractions better represent the calcium pool in the release site of the sarcoplasmic reticulum. This explanation, however, is incomplete as it raises the question of the route which this calcium presumably takes in moving from the sarcoplasmic reticulum, eventually to the extracellular space.

We have suggested that the the apparent 'leak' of calcium from the sarcoplasmic reticulum takes place close to the t-tubule-lateral cisternal junctions. This is based on experiments performed on skinned fibres and sarcoplasmic reticular vesicles mentioned previously (Thomas *et al.*, 1985b; Zorzato *et al.*, 1985), and those measuring asynchronous diastolic sarcomere motion mediated by sarcoplasmic reticular calcium release and measurements of time to peak tension of contractions (Bose *et al.*, 1987; present

study). Scattered light intensity fluctuation of coherent light due to wavelike myofilament motion is believed to be caused by spontaneous oscillatory calcium release from the sarcoplasmic reticulum of ventricular muscle (Lappe & Lakatta, 1980; Lakatta & Lappe, 1981). Since these oscillations are measured during diastole, their depression after the addition of Bay K 8644 (Bose *et al.*, 1987; 1988a,b) suggests that calcium present in the myoplasm during diastole is decreased by Bay K 8644. This could result from decreased release from the lateral cisternae, probably due to decreased content. Ryanodine, which is known to increase calcium 'leak' from the sarcoplasmic reticulum (Bers *et al.*, 1987) also reduces scattered light intensity fluctuation (Sutko *et al.*, 1986b; Kobayashi & Bose, unpublished observations). The time to peak tension associated with contractions produced by continuous electrical stimulation or after variable periods of rest has been consistently reported to be unaffected by treatment with Bay K 8644 (Thomas *et al.*, 1985a; Hryshko, 1987; Bouchard *et al.*, 1988). This suggests that prolongation of the duration of active state (due to alterations of sarcoplasmic reticular calcium uptake and release) do not contribute to the positive inotropy produced by this agent. Failure to slow relaxation rate throughout the twitch, as opposed to slowing of the final 10% of the relaxation phase, suggests that sarcoplasmic reticular calcium pump activity is also not markedly impaired by Bay K 8644. Diastolic 'leak' of calcium from the sarcoplasmic reticulum should be expected to result in a uniform slowing of relaxation, as occurs with caffeine or ryanodine alone. As this did not occur in the presence of Bay K 8644, it may be concluded that the pathway taken by calcium after it leaves the sarcoplasmic reticulum during diastole does not include primarily the myofilaments and this release may indeed be from regions of the sarcoplasmic reticulum closer to the sarcolemma. In this regard it should be noted that low external sodium partially reverses depression of post-rest force production by agents or procedures which cause rest-depression, including Bay K 8644 (Sutko *et al.*, 1986a; Bose *et al.*, 1987). This suggests that the Na-Ca exchange process may be mediating the 'apparent' diastolic loss of calcium in muscles, including those treated with Bay K 8644.

Bay K 8644 plus caffeine

In most species the effect of administration of caffeine on excitation-contraction coupling in heart muscle appears to be biphasic. This reflects the diversity of actions of this compound. The initial response to caffeine is an enhancement of calcium-induced release of calcium from the sarcoplasmic reticulum as measured by increased force production, increased scat-

tered light intensity fluctuation and ^{45}Ca flux (Fabiato, 1981; Stern *et al.*, 1985; Sutko *et al.*, 1986; Rasmussen *et al.*, 1987). The second action of caffeine is gradually to deplete the intracellular calcium pool(s), and in turn, calcium release from the sarcoplasmic reticulum. The small positive inotropy observed with this agent is probably a manifestation of a number of intracellular processes which favour myofilament activation, including inhibition of sarcoplasmic reticular calcium ATPase (Weber & Herz, 1968), enhanced myofilament sensitivity (McClellan & Winegrad, 1978) and inhibition of phosphodiesterase (Butcher & Sutherland, 1962).

Caffeine has been previously shown to inhibit post-rest tension development (Bers, 1983; Hryshko, 1987). The interaction between caffeine and Bay K 8644 was tested to gain insight into the depressant action of Bay K 8644 on the post-rest beat. It was hypothesized, that if these agents utilized a similar mechanism to produce rest-depression, then the effect of combining them should be additive. This effect was not observed. Instead, caffeine was found to reverse partially the depressant effect of Bay K 8644 on post-rest twitch tension. Moreover, rapid cooling contractures observed under the influence of both drugs retained the characteristics of that observed with caffeine alone, which suggests that sarcoplasmic reticular calcium stores were severely depleted during continuous stimulation under the influence of both inotropes, even though the post-rest tension was less depressed. Thus, it seems unlikely that the caffeine-induced protection against post-rest twitch tension depression was due to enhanced calcium release from the sarcoplasmic reticulum. This explanation is supported by the absence of significant cooling contractures when evoked immediately after a train of regular stimuli or after periods of rest or in studies where other indirect measures of intracellular calcium movements were found to be depressed by caffeine administration (Sutko *et al.*, 1986b; Rasmussen *et al.*, 1987; Bers, 1987).

In addition to the possibility of enhanced transsarcolemmal calcium entry associated with the effect of caffeine to prolong action potential duration, increased myofilament activation during the post-rest beat may be a likely explanation for the caffeine-induced antagonism of the effect of Bay K 8644 on post-rest tension development. It could be argued that enhanced activation of contraction may be related either to the prolongation of action potential duration and a possible increase in the calcium current, or time to peak tension under these conditions. However, as discussed below, similar electrophysiological changes were found with Bay K 8644 alone and in combination with ryanodine, both of which resulted in greater rest-depression. Further-

more both caffeine and ryanodine prolong time to peak tension when present alone or in combination with Bay K 8644 to a similar extent. Despite the similarity in their effect on the twitch profile, antagonism of Bay K 8644-induced rest depression was observed only with the combination of Bay K 8644 and caffeine. These data do not support the hypothesis that enhanced trans-sarcolemmal calcium influx alone is responsible for the reduction of Bay K 8644-induced rest-depression by caffeine. It appears that despite the characteristic rest-depression produced by either caffeine or Bay K 8644 alone, the decreased depression when the two are combined may result from the augmented calcium current due to Bay K 8644 as well as sensitization of the contractile apparatus and reduced sarcoplasmic reticular calcium sequestration due to caffeine.

Bay K 8644 plus ryanodine

Ryanodine is a negative inotrope, which reacts specifically with the calcium release channels localized on the terminal cisternae of the sarcoplasmic reticulum (Inui *et al.*, 1987). Its binding is modulated by caffeine (Pessah *et al.*, 1986), although in this study the two were never present simultaneously. This alkaloid decreases ventricular contractility during regular stimulation and after rest by decreasing the content of calcium in the sarcoplasmic reticular pool, measured by rapid cooling (Bers *et al.*, 1987; Bouchard *et al.*, 1988), increasing the passage of calcium from the intracellular stores to the extracellular space (Hilgemann, 1986a,b; Bers *et al.*, 1987), decreasing diastolic calcium-mediated myofilament motions (Sutko *et al.*, 1986b), while only slightly reducing ^{45}Ca uptake (Rasmussen *et al.*, 1987).

Addition of ryanodine in the presence of Bay K 8644 further decreased post-rest twitch tension and cooling contractures. The data are in agreement with the prediction that the two drugs share a similar mechanism of action. This is supported by the lack of change in rapid cooling contracture amplitude immediately following a train of regular beats and the further increase in action potential duration observed under the influence of both agents. Thus, although the release of calcium from the release site of the sarcoplasmic reticulum is impaired by ryanodine (Hilgemann, 1986a,b; Bers *et al.*, 1987; Rousseau *et al.*, 1987), the ability of this organelle to sequester myoplasmic free calcium appears to be unaffected. One effect of ryanodine which does not parallel that of Bay K 8644, however, is the increase in time to peak tension (162.5%) and marked increase in relaxation time (277.5%). Other effects such as those observed on the action potential duration under the influence of both drugs differ significantly from the response seen with Bay K 8644. This

suggests that even in the face of a possible large increase in the duration of trans-sarcolemmal calcium entry the sarcoplasmic reticular storage pool for calcium is unable to retain its content of calcium over the course of time, similar to that observed with Bay K 8644 alone. If one assumes that calcium pumping by the sarcoplasmic reticulum is not impaired by the combination of Bay K 8644 and ryanodine (on the basis of rapid cooling contractures not being impaired during steady stimulation), then the significant increase in the time taken for both peak tension development and relaxation with this treatment indicates that calcium may be passing through the myofilaments on its way out from the sarcoplasmic reticulum during the process of 'leak'. Caffeine which causes an intermediate amount of prolongation in time to peak contraction and relaxation time compared with ryanodine may be acting by a different mechanism, as suggested earlier. However it would be impossible to rule out increased 'leak' of calcium from the sarcoplasmic reticulum under its influence. Studies on calcium release channels in the lateral cisternae indicate that they are converted to a high conductance state by caffeine (Rousseau & Meissner, 1989). Ryanodine in contrast keeps the channels open in a low conductance state (Rousseau *et al.*, 1987) which may explain a longer duration of calcium 'leak' leading to a greater prolongation of tension development and recovery. The fact that a similar extent of change is not seen with only Bay K 8644 may be because in this case the 'leak' may be occurring from a very limited site on the sarcoplasmic reticulum.

In summary, the results of the experiments where Bay K 8644 was coadministered with either caffeine or ryanodine suggest that the latter two drugs do not share a similar mechanism of action in depressing post-rest tension development in canine ventricular muscle. Of the two agents tested in combination with Bay K 8644, ryanodine produced contractile (post-rest) and electrophysiological changes which were somewhat similar to those observed with Bay K 8644 alone. It is probable that the depression of post-rest contraction amplitude by Bay K 8644 is indicative of acceleration of ongoing diastolic calcium loss from the sarcoplasmic reticulum into the extracellular space, without influencing the myofilaments significantly during transit. This leaves less activator calcium available for release upon resumption of stimulation.

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