Excitation-Contraction Coupling in Cardiac Purkinje Fibers

Effects of Caffeine on the Intracellular [Ca^{2+}] Transient, Membrane Currents, and Contraction

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ABSTRACT The effects of caffeine on tension, membrane potential, membrane currents, and intracellular [Ca^{2+}], measured as the light emitted by the Ca^{2+}-activated photoprotein aequorin, were studied in canine cardiac Purkinje fibers. An initial, transient, positive inotropic effect of caffeine was accompanied by a transient increase in the second component of the aequorin signal (L_2) but not the first (L_1). In the steady state, 4 or 10 mM caffeine always decreased twitch tension and greatly reduced both L_1 and L_2. At a concentration of 2 mM, caffeine usually reduced but occasionally increased the steady state twitch tension. However, 2 mM caffeine always reduced both L_1 and L_2. Caffeine eliminated the diastolic oscillations of intracellular [Ca^{2+}] induced by high extracellular [Ca^{2+}]. In voltage-clamp experiments, 10 mM caffeine reduced the transient outward current and the peak tension elicited by step depolarization from a holding potential of −45 mV. In the presence of 20 mM Cs^+, 10 mM caffeine reduced slow inward current. However, the time course of this reduction was far slower than that in tension and light observed in separate experiments. The simplest explanation of the results is that caffeine inhibits the sequestration of Ca^{2+} by the sarcoplasmic reticulum. The results also suggest that in Purkinje fibers caffeine increases the sensitivity of the myofilaments to Ca^{2+}.

INTRODUCTION

Caffeine is among the most widely used drugs in the study of excitation-contraction coupling. However, its mechanism of action remains controversial. A full understanding of the actions of caffeine requires knowledge of the various simultaneous cellular Ca^{2+} movements involved in excitation-contraction coupling. These include Ca^{2+} entry via sarcolemmal Ca^{2+} channels and Na/Ca exchange, Ca^{2+} release and accumulation by the sarcoplasmic reticulum (SR),

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Ca^{2+} binding to cytoplasmic macromolecules, and extrusion of Ca^{2+} from the cell. Aequorin-injected canine Purkinje strands are particularly advantageous for a study of the mechanism of action of caffeine because their cytoplasmic [Ca^{2+}] transient has already been characterized (Wier, 1980*a*; Wier and Isenberg, 1982). It consists of two components, L_1 and L_2, which result from two different cellular mechanisms for increasing cytoplasmic [Ca^{2+}] after membrane excitation. The source for L_2 has been shown to be a Ca^{2+} store, presumably the SR. It has been suggested that the source of Ca^{2+} for L_1 is the transmembrane Ca^{2+} current (Wier and Isenberg, 1982).

On the basis of previous studies, caffeine could be expected to interfere with excitation-contraction coupling in striated muscle, primarily on the level of the SR. In cardiac preparations, inhibition of Ca^{2+} uptake by the SR has been postulated to be one of the actions of the drug (Blinks et al., 1972; Fabiato and Fabiato, 1973; Niedergerke and Page, 1981). Enhancement of Ca^{2+} release from the SR, which seems to be the primary mode of action of the drug in skeletal muscle, has also been postulated to occur in cardiac tissue by Endo (1977) and Chapman and Leoty (1976). The effects of caffeine and theophylline on the [Ca^{2+}] transient in aequorin-injected cat papillary muscle preparations are consistent with a predominant effect of these drugs on the Ca^{2+} uptake mechanism of the SR (Allen and Kurihara, 1980; Blinks et al., 1982; Morgan and Blinks, 1982).

In skinned cardiac preparations, caffeine has been reported to increase the sensitivity of the myofilaments to Ca^{2+} (Fabiato and Fabiato, 1976; Endo and Kitazawa, 1978). The results reported by Blinks et al. (1982) and Morgan and Blinks (1982) in theophylline- and caffeine-treated, aequorin-injected cat papillary muscle are consistent with this.

Since caffeine has also been reported to influence Ca^{2+} entry via the sarcolemmal Ca^{2+} current (Carmeliet and Vereecke, 1969; Eisner et al., 1979; Goto et al., 1979; Coraboeuf and Carmeliet, 1982), we also studied the effects of the drug on membrane currents in our preparation.

METHODS

Canine Purkinje strands were mounted in an experimental chamber which allowed the simultaneous recording of contraction, transmembrane potential, and the light emitted by the microinjected, calcium-activated photoprotein (aequorin). The preparation, the experimental setup, the intracellular microinjection of aequorin, and the recording and calibration of the resulting light signals have been described in detail (Allen and Blinks, 1978; Wier and Isenberg, 1982; Wier and Hess, 1984). Light signals with a satisfactory signal-to-noise ratio were obtained when 50–200 responses were averaged from fibers in which 10–20 cells had been pressure-injected with aequorin. Exponential (RC) filters were used in the recording of tension (τ = 4 ms) and aequorin luminescence (τ = 2 ms).

Solution and Chemicals

The preparations were superfused at 35°C with an oxygenated physiological salt solution of the following composition: 154 mM NaCl, 5.4 mM KCl, 1.0 mM $MgCl_2$, 2.7 mM $CaCl_2$, 12 mM Hepes, 11 mM glucose, pH 7.4. Caffeine was obtained through Calbiochem-Behring Corp. (La Jolla, CA). With the use of a Ca^{2+} electrode made with Simon's neutral

carrier (Oehme et al., 1976), it was shown that addition of caffeine (up to 100 mM) did not change the calcium activity in the physiological salt solution. Similarly, caffeine in concentrations up to 100 mM had no direct effect on either Ca^{2+}-activated or Ca^{2+}-independent aequorin luminesence.

Voltage-Clamp Experiments

A conventional two-microelectrode voltage clamp (Deck et al., 1964), with the addition of a virtual ground, was used to control membrane potential and measure membrane current. To minimize capacitative coupling between the two microelectrodes, the method of shielding described by Kass et al. (1979) was used in most experiments. The preparations used for the voltage-clamp experiments were 150–250 μm in overall diameter and were shortened, according to the method of Deck et al. (1964), to a length of 1.5–2 mm. The current-passing microelectrode was introduced into the midpoint of the muscle and the voltage-sensing microelectrode was introduced into the preparation at a distance one-half to one-third of the way from midpoint to the end. Membrane currents were recorded on FM tape (passband, DC to 650 Hz) and/or digitized on a multichannel analyzer (TN 1710; Tracor Northern, Middleton, WI) on which averaging, subtraction, and integration of current records could be performed. Membrane currents were averaged, not filtered, to achieve noise reduction. The total capacity of the preparations was obtained from the integral of the current surge elicited by a 10-mV hyperpolarization. When normalized to the strand surface area, a total apparent specific capacitance of 4.9 ± 1.1 $\mu F/cm^2$ (mean ± SEM, $n = 8$) was found, a value close to that reported for calf and rabbit Purkinje fibers (Colatsky and Tsien, 1979). The total capacity was also used to estimate the total cell surface area of the preparations, on the assumption that the true specific capacitance of cell surface membrane was 1 $\mu F/cm^2$. The maximum current density thus calculated at the peak of the slow inward current was always <10 $\mu A/cm^2$. As shown by Kass et al. (1979), current densities of that magnitude can be expected to be a reasonably accurate measure of the true membrane current.

RESULTS

Effects of Caffeine on Contraction, Membrane Potential, and [Ca^{2+}] Transient

Exposure of canine Purkinje fibers to 4 mM caffeine led initially to a transient increase in peak twitch tension and then to a sustained decrease to below the control level. Twitch tension usually reached a steady level after ~2–3 min. In some experiments, a slowly developing, slight further reduction of peak twitch tension over the next 5–10 min could be observed. In six experiments, the twitch tension initially increased to 207 ± 27% (mean ± SEM) and then decreased to 44 ± 6% of control. Lower (2 mM) or higher (10 mM) concentrations of caffeine had qualitatively similar effects on peak tension, but the negative inotropic steady state effect was less pronounced in 2 mM caffeine and more pronounced in 10 mM caffeine when compared with the effect of 4 mM drug on the same preparation. A typical example of the time course of the effect of 2 and 4 mM caffeine on peak tension of the same preparation is shown in Fig. 1. In one preparation exposed to 2 mM caffeine, peak tension after the initial increase decreased to a level slightly above control, resulting therefore in a positive inotropic steady state effect.

The time course of a single contraction was characteristically changed by

caffeine. At all drug concentrations, the duration of the twitch was prolonged, time to peak tension was increased, and the initial rate of tension development was reduced (Figs. 2 and 3). Caffeine had no effect on resting tension except in high concentrations (10–100 mM), where the resulting pronounced prolongation of the twitch sometimes led to fusion of the individual beats and prevented complete relaxation of the preparation. All effects of caffeine on contraction were readily reversible within a few minutes after washout of the drug.

Pronounced effects of caffeine on membrane potential were seen only at caffeine concentrations of 4 and 10 mM (Fig. 3). The plateau became shifted toward more negative potentials and the rate of the terminal repolarization decreased. The membrane potential during diastole became less negative by 5–10 mV. Washout of the drug completely reversed all effects on the electrical activity of the fibers.

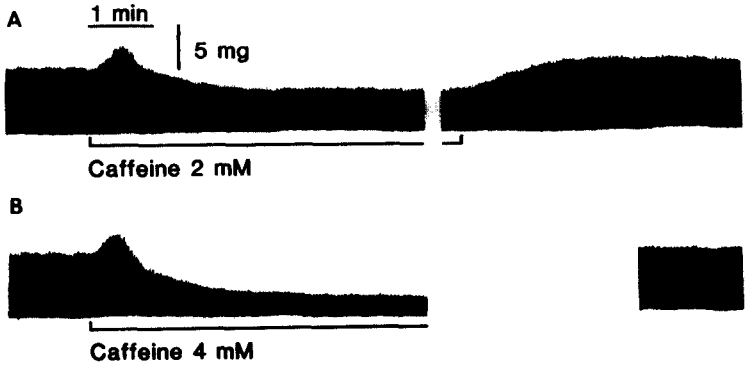

FIGURE 1. Effect of two concentrations of caffeine on contraction of a canine Purkinje strand. The continuous tension recording was interrupted for 4 min in *A* and for 10 min in *B*. The record of recovery from 4 mM caffeine was obtained 4.5 min after washout of the drug; the time course of the recovery was similar to that in *A*. Frequency of stimulation, 1 Hz; preparation D-13; cross section, 0.049 mm^2.

Figs. 2 and 3 also show the intracellular [Ca^{2+}] transients, measured as the light emitted by the Ca^{2+}-activated photoprotein aequorin and recorded simultaneously with the transmembrane potential and the force of contraction. The signals were calibrated in units of fractional luminescence (L/L_{max}) (Allen and Blinks, 1978), as described by Wier and Hess (1984). The signals under control conditions showed the two components characteristically found in Purkinje fibers: a rapidly rising first component (L_1) with a peak ~30 ms after the stimulus and a second, plateau-like component (L_2), which reached its maximum ~80 ms after the stimulus. During the transient positive inotropic effect of caffeine (Fig. 2*C*) the [Ca^{2+}] transient was increased. This increase, as visualized by the superposition of the signal with its pre-drug control in panel *E* of Fig. 2, was an increase mainly in L_2.

In the steady state, caffeine greatly reduced the [Ca^{2+}] transient. In 2 mM caffeine (Fig. 2), the aequorin signal seemed to lack L_1. Luminescence gradually rose to a peak that occurred at about the same time as that of L_2 in the control

luminescent signal. Although the rising phase of the [Ca^{2+}] transient was considerably slowed in the presence of caffeine, the duration of the [Ca^{2+}] transient was littled affected. Higher concentrations of caffeine (4–10 mM, Fig. 3) further reduced the amplitude of the transient, both L_1 and L_2 being now almost completely undetectable. The reduction of the aequorin signal occurred during the negative inotropic effect, after the initial increase in tension and light, and was essentially complete 1–2 min after the start of the exposure of a fiber to caffeine. As with the effects on tension and membrane potential, the caffeine-induced changes in the [Ca^{2+}] transient were fully reversible.

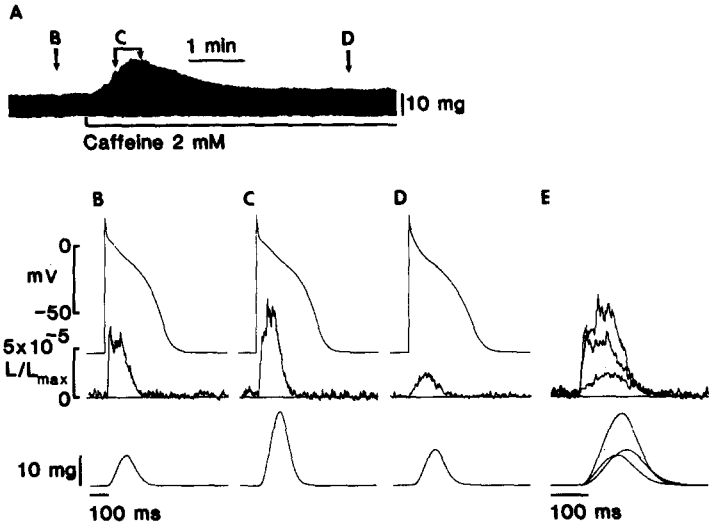

FIGURE 2. Effects of 2 mM caffeine on membrane potential, [Ca^{2+}] transient, and tension. (*A*) Continuous recording of contraction. (*B–D*) Simultaneous recordings of membrane potential (top), aequorin light (middle), and tension (bottom) made at times indicated by arrows in *A*. All the traces in *B* and *D* are averages of 50 signals under steady state conditions; the traces in *C* are the average of 30 signals obtained during a non-steady state as shown in *A*. The light signals are calibrated in units of fractional luminescence (L/L_{max}) and the baselines indicate the level of the background light. (*E*) Superposition of light and tension records shown in *B–D*. Frequency of stimulation, 1 Hz; preparation D-14; cross section, 0.11 mm^2.

Caffeine had no detectable effect on the aequorin luminescence between beats. In six experiments, the aequorin luminescence during diastole under control conditions was not different from that in the presence of caffeine (2 mM in two experiments and 4 mM in four experiments).

Caffeine Blocks Transient Elevations in Diastolic [Ca^{2+}]$_i$, Afterdepolarizations, and Aftercontractions

The transient, sometimes oscillatory, changes of diastolic intracellular calcium observed, e.g., in high extracellular calcium or in the presence of cardiac glycosides (Wier, 1980*b*), are probably caused by Ca^{2+} released by the sarco-

plasmic reticulum (Kass et al., 1978). The effect of caffeine on this mechanism was studied by addition of caffeine to a preparation that had developed such changes in diastolic [Ca^{2+}] during superfusion with a solution containing 18 mM [Ca^{2+}] (Fig. 4). The elevation of extracellular [Ca^{2+}] from 2.7 to 18 mM increased peak tension almost fivefold and peak light about fourfold. An increased rate of initial repolarization led to the appearance of a notch in the action potential. Membrane potential, aequorin luminescence, and tension showed temporally similar changes during diastole. Addition of 4 mM caffeine reduced the peak

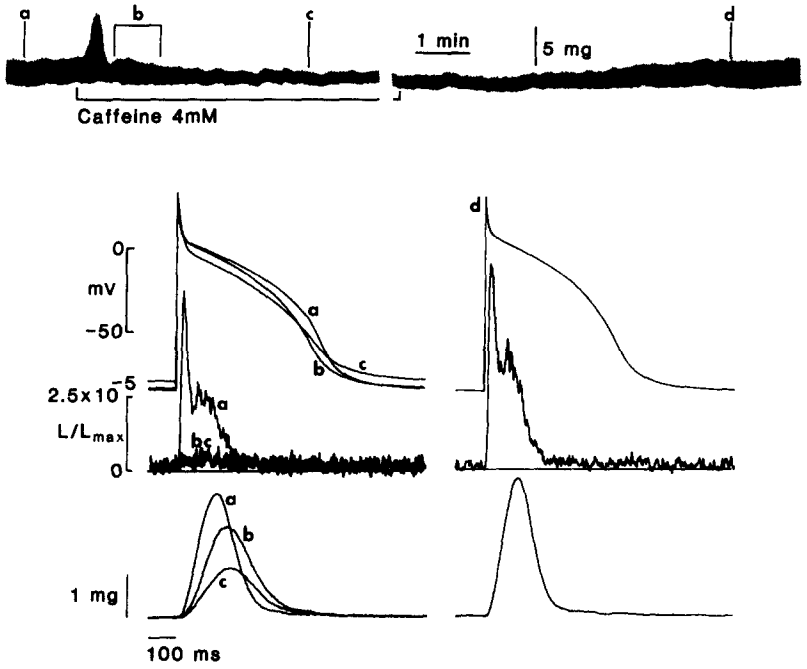

FIGURE 3. Effects of 4 mM caffeine on membrane potential, [Ca^{2+}] transient, and tension. The upper part shows the continuous recording of tension and indicates, by the lines labeled *a*, *b*, and *c*, at what times the simultaneous records of membrane potential, light, and tension shown (correspondingly labeled) in the lower part of the figure were obtained. Traces labeled *a*, *c*, or *d* are averages of 100 signals; traces labeled *b* are the averages of 50 signals. Light calibration as in Fig. 2. Frequency of stimulation, 1 Hz; preparation D-19; cross section, 0.045 mm^2.

tension to about the same level as in 2.7 mM Ca^{2+} and prolonged the twitch duration and time to peak tension. No initial transient positive inotropic effect was observed after exposure to the drug. The calcium transient was dramatically reduced and the action potential lost its notch and increased in duration because of a slower rate of repolarization. Caffeine blocked the transient elevation of diastolic [Ca^{2+}] and the concomitant afterdepolarization and aftercontraction. As can be seen from comparison of the luminescent signals at higher gain, the level of luminescence during diastole was higher in 18 mM Ca^{2+} plus caffeine than in 2.7 mM Ca^{2+}. All effects of caffeine and of 18 mM Ca^{2+} were reversible.

Effect of Caffeine on Slow Inward Current

The source of calcium for L_1 is not known, but the similarity of some of the properties of L_1 to those of the slow inward current led originally to the suggestion (Wier, 1980*a*) that L_1 may be closely related to, or even arise directly from, Ca^{2+} entering via the slow channel. Subsequent voltage-clamp studies (Wier and Isenberg, 1982) also indicated that L_1 had some of the properties expected for a signal related to Ca^{2+} entering via the slow inward current. However, a relationship between L_1 and the $[Ca^{2+}]$ transient might also be observed if a component of slow inward current is Ca^{2+}-activated, a possibility

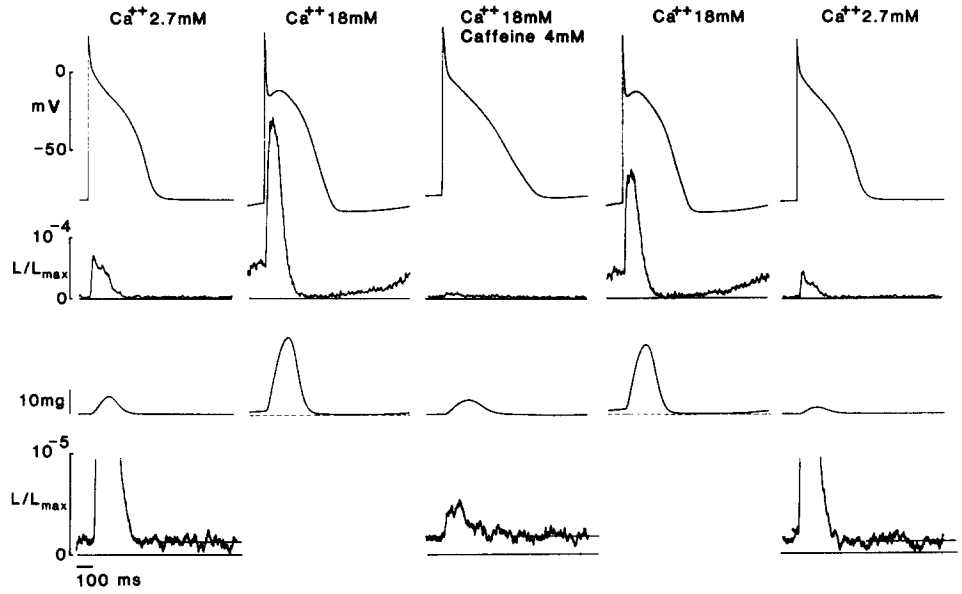

FIGURE 4. Effects of caffeine in the presence of high extracellular $[Ca^{2+}]$. Simultaneous, averaged records of membrane potential (top row), light (second row), and tension (third row) obtained in the steady state under the conditions indicated above each column. 100 sweeps were averaged in all cases. The bottom row shows the light signals on a 16 times higher gain and filtered with a time constant of 20 ms. Light calibrations as in Fig. 2. Frequency of stimulation, 1 Hz; preparation D-14; cross section, 0.11 mm^2.

noted by Clusin (1980). Since caffeine produced a rapid and very marked reduction of L_1, we further investigated the possible relationship between L_1 and the slow inward current by studying the effects of caffeine in voltage-clamp experiments.

Fig. 5*A* shows a typical record of current, voltage, and tension. The voltage-clamp pulses had a duration of 300 ms and were repeated at a frequency of 1 Hz. The holding potential was set at −45 mV in order to inactivate the fast Na^+ current. A voltage-clamp step to −5 mV elicited an inward current surge following the initial capacitative transient. The peak of the inward current occurred ~8–10 ms after the voltage change. This inward current was sensitive

to the bath [Ca^{2+}]; the current was reversibly abolished by superfusion of the fiber with Ca^{2+}-free solution.

Fig. 5*A* also shows the effect of 3 min exposure to 4 mM caffeine on the membrane current and contraction of a voltage-clamped Purkinje fiber. During this time, the peak inward current slowly increased by ~20%. The initial, transient, positive inotropic effect was much more rapid and did not seem to be associated with the change in the peak inward current. 10 min after washout of the drug, the preparation, which had completely recovered from the drug effect, was re-exposed to 4 mM caffeine, but this time under conditions of external

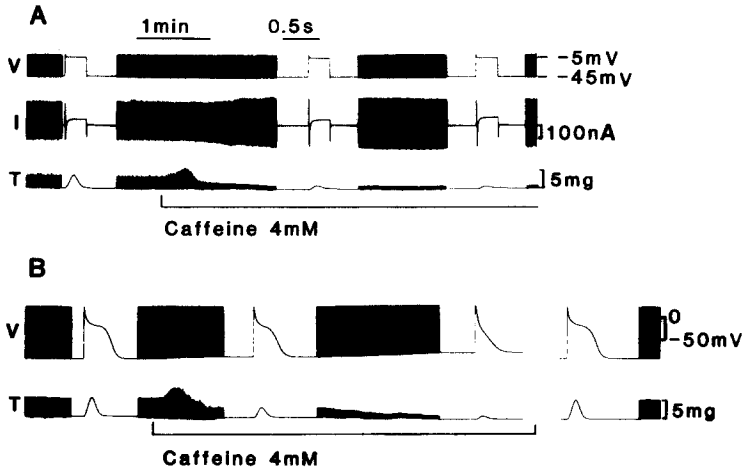

FIGURE 5. Effect of 4 mM caffeine on peak inward current and tension. Comparison between clamped and unclamped mode of stimulation. (*A*) Continuous recordings of membrane potential (*V*), current (*I*), and tension (*T*). Soon after the end of the traces in *A*, impalements were lost, and the preparation recovered from caffeine in the unclamped mode. Clamp duration, 300 ms; clamp frequency, 1 Hz. (*B*) Continuous recordings of membrane potential (*V*) and tension (*T*) during external stimulation. Traces begin 10 min after the end of the traces in *A*. The last part of the records in *B* shows the reversal of the drug effect at the end of a 7-min washout. Frequency of stimulation, 1 Hz; preparation VC-2; cross section, 0.049 mm^2; total capacity, 34 nF.

stimulation (Fig. 5*B*). The effects of caffeine on contraction and membrane potential were the same as those described earlier and the drug effects on contraction were similar to those observed under voltage-clamp conditions in the same fiber (Fig. 5*A*), which indicates that the effect of caffeine on excitation-contraction coupling was not modified by the voltage-clamp procedure.

In four experiments, 4 mM caffeine produced a maximum increase of 22 ± 9% in the peak inward current elicited by voltage-clamp steps from a holding potential of −45 to −5 mV. However, higher concentrations of the drug and longer exposure showed that the effect of caffeine on the peak inward current was, in fact, biphasic and that after the initial increase, which was complete within

2–3 min, the current slowly declined (Fig. 6). The peak inward current reached a maximum ~2 min after the start of a 17-min exposure to 10 mM caffeine and then gradually fell to a level ~15% below control within the next 15 min. Washout of the drug produced a biphasic effect in the opposite direction. Fig. 6*B* shows the current records at the times indicated in *A*. While the continuous recording was obtained with voltage-clamp steps from −45 to +5 mV, currents elicited by voltage steps to more positive potentials were also obtained during the steady state of the drug effect and after washout of the drug. As shown in Fig. 6*B*, in the absence of caffeine a transient outward deflection became

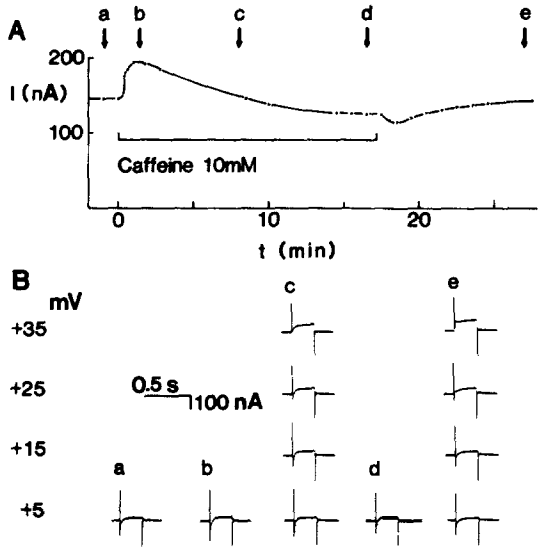

FIGURE 6. Biphasic effect of 10 mM caffeine on peak inward current. (*A*) Time course of the change in peak inward current induced by 10 mM caffeine. Holding potential, −45 mV; clamp steps to +5 mV. The amplitude of the current was measured as the difference between the peak inward current and the steady outward current at the end of the pulse. (*B*) Current records obtained by clamp steps from −45 mV to the potentials indicated at left. The signals were recorded before, during, and after the exposure to caffeine, at the times indicated in *A*. Clamp pulse duration, 300 ms; clamp pulse frequency, 1 Hz; preparation VC-5; total capacity, 39 nF.

apparent in current associated with steps to voltages higher than +5 mV and became more pronounced the larger the voltage-clamp step. Comparison of these current records with those obtained in the presence of caffeine shows that this deflection was reduced by the drug. The result of this experiment is important because it suggests that (*a*) the total current contains a significant component of transient outward current (Dudel et al., 1967; Fozzard and Hiraoka, 1973; Kenyon and Gibbons, 1979; Siegelbaum and Tsien, 1980), and (*b*) the transient outward current, or a component of it, is reduced by caffeine in this tissue, as it is in sheep Purkinje fibers (Coraboeuf and Carmeliet, 1982).

Effects of Caffeine on Peak Inward Current in Cs^+-containing Solution

The presence of transient outward current makes it difficult to assess the action of a drug on the inward calcium current (slow inward current) and we therefore attempted to separate the two current components by selective inhibition of the transient outward current. The most promising method to selectively block time-dependent outward currents so far developed is that of Tillotson and Horn (1978), modified for cardiac Purkinje fibers by Marban (1981). This method consists of the substitution of Cs^+ for intracellular K^+. We have attempted to

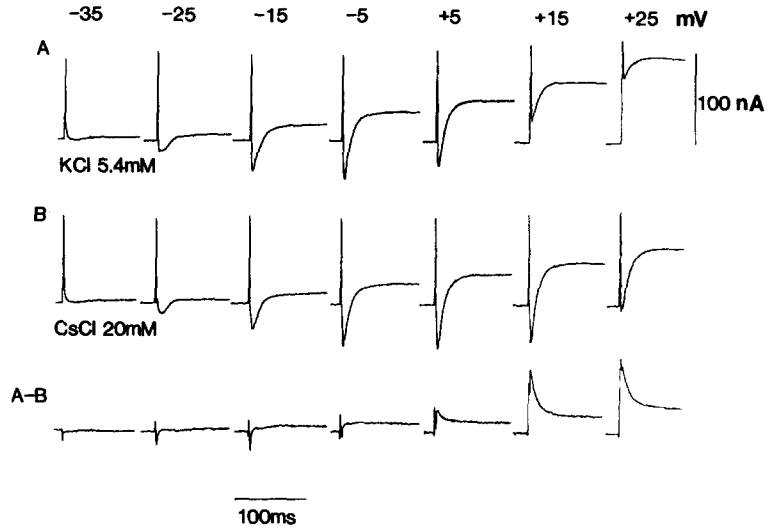

FIGURE 7. Effect of substitution of CsCl for KCl on the membrane currents. Average membrane currents elicited by eight voltage-clamp steps from a holding potential of −45 mV to the potential indicated above each column. Clamp pulse duration, 300 ms; clamp pulse frequency, 1 Hz. (*A*) Currents in normal physiological solution containing 5.4 mM KCl. (*B*) Currents after 40 min in K^+-free solution containing 20 mM CsCl. (*A* − *B*) Cs^+-sensitive currents obtained by subtraction of the currents in *B* from those in *A*. Preparation VC-8; total capacity, 34 nF.

block or reduce time-dependent outward currents simply by prolonged superfusion with a solution in which KCl was substituted by 20 mM CsCl. Cs^+ is transported into cardiac cells (Guerin and Wallon, 1979; Carmeliet, 1980), probably because Cs^+ substitutes for K^+ on the Na^+/K^+ pump (Eisner and Lederer, 1980).

Fig. 7 shows the effect of K^+-free, 20 mM Cs^+-containing solution on the membrane currents. The current records in the top row were obtained in the normal physiological salt solution (5.4 mM K^+). The middle row shows the currents recorded after a 40-min exposure to 20 mM CsCl. The records in the bottom row, obtained by electronic subtraction of the currents in CsCl from those in the control solution, represent the current blocked by Cs^+. In the potassium-containing solution, currents elicited by voltage clamp to potentials

positive to −5 mV contained a transient outward current component which was reduced after perfusion by extracellular CsCl. Cesium also reduced the steady state outward current. In four out of five experiments, substitution of KCl by 20 mM CsCl had similar effects on the membrane currents.

The effects of caffeine on the peak inward currents in preparations that had been perfused with potassium-free, 20 mM CsCl-containing solution (Fig. 8) were significantly different from those observed in the normal bathing solution. In four experiments, 10 mM caffeine reversibly reduced peak inward current by 21 ± 8% at the end of a 5-min exposure to the drug. In none of these experiments was an initial increase of the peak inward current observed.

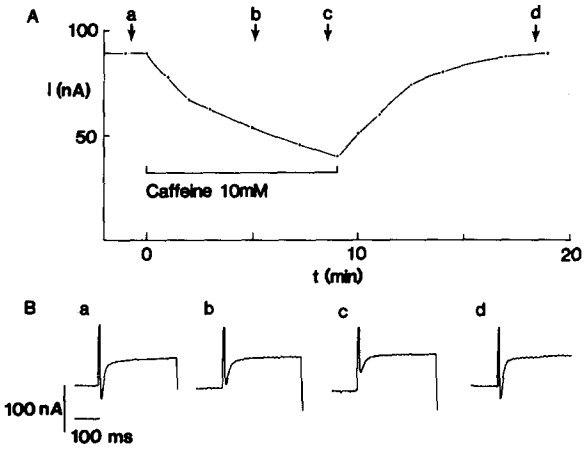

FIGURE 8. Effect of 10 mM caffeine on peak inward current in Cs^+-containing solution. (*A*) Time course of the change in peak inward current induced by 10 mM caffeine. Holding potential, −40 mV; clamp steps to 0 mV. The current was measured as in Fig. 7. (*B*) Current records obtained at times indicated in *A*. K^+-free solution containing 20 mM CsCl. The preparation had been exposed to this solution for 90 min before its exposure to caffeine. Clamp pulse duration, 300 ms; clamp pulse frequency, 1 Hz; preparation VC-10; total capacity, 150 nF.

DISCUSSION

Most of the effects of caffeine on twitch tension can be related to the effects of the drug on the intracellular [Ca^{2+}] transient. The initial, transient, positive inotropic effect is accompanied mainly by an increase in component L_2 of the [Ca^{2+}] transient. The steady state negative inotropic effect clearly is associated with a pronounced reduction of the amplitude of the [Ca^{2+}] transient. However, a steady state positive inotropic effect of caffeine, such as we occasionally observed, is also associated with a reduction of the [Ca^{2+}] transient (Fig. 2), contrary to the assumption of Niedergerke and Page (1981). Thus, our data do not support their suggestion that increased Ca^{2+} influx underlies the positive inotropic effect that may persist after depletion of the SR. In Purkinje fibers this effect seems rather to reflect an influence of caffeine on the sensitivity of the

myofilaments to Ca^{2+} (see below). Similarly, the prolonged time to peak tension and the decrease of the initial rate of the tension development may be related to the slower rise of intracellular $[Ca^{2+}]$. The reduction of the $[Ca^{2+}]$ transient by caffeine also explains the ability of the drug to reverse the positive inotropic effect produced by high extracellular $[Ca^{2+}]$, as described in this paper, as well as that produced by strophanthidin, as reported by Vassalle and Lin (1979). Similarly, the abolition of aftercontractions is the result of the abolition of the underlying changes in cytoplasmic $[Ca^{2+}]$. Vassalle and Lin (1979) explained the biphasic effect of caffeine on tension by an initial increase in $[Ca^{2+}]_i$ to an "optimal" level for tension development and a subsequent further increase to produce a "Ca^{2+} overload," which would result in a negative inotropic effect. The present results clearly show that this hypothesis is wrong. Our results emphasize the danger of speculations about $[Ca^{2+}]_i$ from tension measurements and the need for direct measurements of $[Ca^{2+}]_i$.

Does Caffeine Change the Sensitivity of the Contractile Apparatus to Calcium?

Even though under most circumstances caffeine-induced changes of the contraction were paralleled by changes of the $[Ca^{2+}]_i$ transient in the same direction, the relationship between $[Ca^{2+}]_i$ and tension may also have been affected by the drug. Fabiato and Fabiato (1976), Endo and Kitazawa (1978), and Fabiato (1981) found that caffeine increases the Ca^{2+} sensitivity of skinned cardiac muscle. In analyzing our results, the best we can do to detect possible changes in the Ca^{2+} sensitivity of the myofibrils is to observe the relation between peak light and peak tension in the presence and absence of caffeine. Conclusions are especially difficult to draw from such comparisons under conditions like those of caffeine, in which the time courses of light and tension are also changed. Nevertheless, the dissimilarity between the magnitude of the effect of caffeine on light and that on tension was so pronounced that the conclusion that caffeine increases the sensitivity of the myofilaments to calcium in intact cells seems justified. In Fig. 2, caffeine (2 mM) in the steady state nearly abolished L_1 and reduced L_2 by ~50%, while peak tension was actually slightly higher than control. In Fig. 4, comparison of the signals in 2.7 mM Ca^{2+} (panel *A*) and in 18 mM Ca^{2+} in the presence of 4 mM caffeine (panel *C*) shows that although peak tension is comparable in both situations, peak light in caffeine is only a small fraction of that in the absence of the drug.

Effects of Caffeine on Membrane Currents, and the Relation of Membrane Currents to the $[Ca^{2+}]$ Transient

From the voltage-clamp experiments and the observation of action potentials, we conclude that caffeine has the following effects on the currents studied in this paper: (*a*) caffeine, at all concentrations tested, reduces the transient outward current; (*b*) at least in the higher concentrations tested, caffeine also reduces slow inward current. This conclusion is based primarily on the observation that, in preparations in which transient outward current had been blocked with Cs^+, caffeine always monophasically reduced the inward current. We therefore suggest that in K^+-containing solutions, the initial increase in inward current caused

by caffeine is not due to an increase in slow inward current, but to the decrease in transient outward current. Only thereafter, in high concentrations of caffeine, is the decrease in slow inward current reflected by a decrease in total inward current. Our present results on the effect of caffeine on slow inward current are in qualitative agreement with those of Eisner et al. (1979) in sheep Purkinje tissue, but differ from those of Goto et al. (1979), who reported that caffeine produced an increase in slow inward current in bullfrog atrial muscle.

Siegelbaum et al. (1977) have suggested that at least part of the transient outward current in calf Purkinje tissue is Ca^{2+} activated. The present results seem to directly support this idea, in that caffeine reduces both the intracellular [Ca^{2+}] transient and the transient outward current. However, if the current is Ca^{2+} activated, it probably depends on the early Ca^{2+} accumulation associated with L_1.

Caffeine reduced both slow inward current and the [Ca^{2+}] transient, particularly L_1. However, there seemed to be marked differences in the time course, extent, and dose dependence of these effects. The reduction in inward current occurred more slowly, to a lesser extent, and required higher drug concentrations than did the reduction in L_1. For example, 10 mM caffeine reduced slow inward current by only 21% (on average) in 5 min, while L_1 was always essentially abolished by 4 mM caffeine within 2 min (Fig. 3). Furthermore, in the presence of 4 mM caffeine and 18 mM Ca^{2+}, L_1 was very greatly reduced. It seems probable that the slow inward current under these conditions would be substantially larger than that under control conditions. These observations are strong evidence against the idea that L_1 arises directly from transmembrane Ca^{2+} current. However, L_1 will tend to be reduced by caffeine, even in the absence of any decreased Ca^{2+} current, simply because of caffeine's enhancement of Ca^{2+} binding to myofilaments. Thus, because of the complex nature of the signals involved and the lack of simultaneous measurements of [Ca^{2+}] transients and Ca^{2+} currents, we do not believe that a definite conclusion about the relationship of L_1 and the Ca^{2+} current is yet warranted. Nevertheless, the present data clearly raise the possibility that entering Ca^{2+} does not directly generate L_1, and we will now discuss a possible alternative source of Ca^{2+} for L_1. Since the other possible route of Ca^{2+} entry, Na/Ca exchange, has been excluded as a source of Ca^{2+} for L_1 (Wier and Isenberg, 1982), L_1 would have to arise from Ca^{2+} released from an intracellular store. However, the stores for L_1 and L_2 would have to have different properties, since under a variety of experimental conditions the amplitudes of L_1 and L_2 are affected in quite different ways. Since we conclude (see next paragraph) that caffeine blocks the Ca^{2+} uptake of the SR, and since the drug very effectively and rapidly reduces L_1, it seems possible that the source of Ca^{2+} for L_1 could also be the SR. The store for L_1 would be expected to be small, functionally separate from that for L_2, easily saturated, quickly repleted, and quickly depleted by caffeine. Anatomically, one candidate for such a store would be the regions of the SR that make peripheral couplings with the surface membrane. This location of the storage might account for the early peaking of L_1 after membrane excitation. It might also be suggested that Ca^{2+} entering the cell through the Ca^{2+} channel is a graded trigger for release of Ca^{2+} from this

store as well as its major source of replenishment. Such a store could account for the experimentally observed similarity of some of the properties of L_1 to those expected for a signal arising from Ca^{2+} entering via the slow inward current.

Mechanism by Which Caffeine Influences the Intracellular [Ca^{2+}] Transient in Purkinje Fibers

Fabiato and Fabiato (1972, 1973) concluded from their work on skinned cardiac cells that caffeine blocks the uptake of Ca^{2+} into the SR, thereby depleting its stores. In our study on intact Purkinje strands as well, inhibition of Ca^{2+} sequestration by the SR is the simplest mechanism that can explain all the observed effects of caffeine on the [Ca^{2+}] transient. The inhibition of Ca^{2+} sequestration leads to an increase of the amplitude of component L_2 of the [Ca^{2+}] transient during the first few beats following application of the drug. Subsequently, depletion of the SR greatly reduces the releasable amount of Ca^{2+}, such that in the steady state the amplitude of the [Ca^{2+}] transient is very much reduced. Blinks et al. (1972) have very convincingly shown on a simple model how inhibition of Ca^{2+} sequestration could (at least transiently) increase the amplitude of the cytoplasmic Ca^{2+} transient, while at the same time prolonging it and delaying the time of its peak. A prolongation and delayed peak are not readily observable in the aequorin signals obtained during the transient inotropic effect. This could be due to the relatively poor signal-to-noise ratio obtained when only a few signals are averaged. As shown in Fig. 4, caffeine also completely abolished the diastolic oscillations of Ca^{2+} produced by high extracellular Ca^{2+} and dramatically reduced the amplitude of the [Ca^{2+}] transient. Again, this result is most simply explained on the basis that caffeine depletes the SR by inhibition of its Ca^{2+} uptake mechanism. Since inhibition of Ca^{2+} uptake by the SR can explain all of the observed effects of caffeine on the intracellular [Ca^{2+}] transient, no extra assumptions about a possible enhancement of Ca^{2+} release are needed. In skeletal muscle, enhancement of Ca^{2+} release seems to be the predominant mode of action of the drug (Endo, 1977), and this mechanism, although never unequivocally demonstrated in cardiac muscle, has also been postulated to occur in this tissue (Chapman and Leoty, 1976; Endo, 1977). The transient contractures that caffeine causes in resting cardiac preparations (Chapman and Leoty, 1976) can also be explained by block of Ca^{2+} uptake alone if it is assumed that at rest there is some "basal" turnover of Ca^{2+} between the cytoplasm and the SR as a result of continuous Ca^{2+} release and uptake.

Comparison with the Effects of Caffeine on Other Cardiac Preparations

Our results on the effects of caffeine on tension are identical with those of Vassalle and Lin (1979) on the same preparation and are similar to those described by Chapman and Leoty (1976) for ventricular preparations and atria from ferret and other mammalian hearts. The only difference between our results and those of Chapman and Leoty is that in their experiments caffeine produced a short initial contracture as opposed to the transient positive inotropic effect seen in our study. It seems likely that this difference could be accounted for by the experimental conditions used by Chapman and Leoty. Their very

small preparations and fast solution change would promote a very abrupt onset of the drug action by minimizing slow mixing of solutions in the experimental chamber and delayed equilibration of the drug in the extracellular space.

A negative inotropic effect of caffeine has also been described in dog papillary muscle and in rat heart (Ohba, 1973; Henderson et al., 1974), but generally caffeine has been found to potentiate the heartbeat in preparations other than Purkinje fibers (Nayler, 1963; de Gubareff and Sleator, 1965; Blinks et al., 1972; Kimoto, 1972; Clark and Olson, 1973; Allen and Kurihara, 1980; Morgan and Blinks, 1982). The effect of caffeine on intracellular calcium transients was studied with the use of aequorin by Allen and Kurihara (1980) and Blinks et al. (1982) and that of theophylline by Morgan and Blinks (1982) in cat papillary muscle. In both cases, the drug had a positive inotropic effect. The main effect of caffeine and theophylline on the aequorin signal was to slow down the initial rate of rise and prolong the signal, whereas its amplitude was not consistently changed.

It seems likely that positive inotropic effects result from a predominance of the action of caffeine to increase the sensitivity of the contractile apparatus to Ca^{2+}, while negative inotropic effects result from a predominance of the action of the drug to reduce the intracellular Ca^{2+} transient.

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